Experimental Gerontology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Experimental Gerontology

journal homepage: www.elsevier.com/locate/expgero

The imbalanced redox status in senescent endothelial cells is due to dysregulated Thioredoxin-1 and NADPH oxidase 4

Christine Goy [b,1], Philip Czypiorski [b,1], Joachim Altschmied [b,1], Sascha Jakob [b], Lothar L. Rabanter [b], Alison C. Brewer [c], Niloofar Ale-Agha [b], Nadine Dyballa-Rukes [b], Ajay M. Shah [c], Judith Haendeler [a,b,*]

[a] Central Institute of Clinical Chemistry and Laboratory Medicine, University of Duesseldorf, 40225 Duesseldorf, Germany
[b] IUF — Leibniz Research Institute for Environmental Medicine at the University of Duesseldorf gGmbH, 40225 Duesseldorf, Germany
[c] King's College London BHF Centre of Excellence, The James Black Centre, London SE5 9NU, UK

ARTICLE INFO

Article history:
Received 9 December 2013
Received in revised form 28 February 2014
Accepted 4 March 2014
Available online xxxx

Section Editor: Natascia Ventura

Keywords:
Cathepsin D
Endothelial cells
Imbalanced redox homeostasis
NADPH oxidase 4
Senescence
Thioredoxin-1

ABSTRACT

Environmental stressors as well as genetic modifications are known to enhance oxidative stress and aging processes. Mitochondrial and nuclear dysfunctions contribute to the onset of aging. One of the most important redox regulators in primary human endothelial cells is Thioredoxin-1 (Trx-1), a 12 kD protein with additional anti-apoptotic properties. Cellular generators of reactive oxygen species are NADPH oxidases (NOXs), of which NOX4 shows highest expression levels in endothelial cells. Therefore, the aim of the study was to investigate how Trx-1 and NOX4 are regulated during stress-induced premature senescence in endothelial cells. We treated primary human endothelial cells for two weeks with H_2O_2 to generate stress-induced premature senescence in these cells. In this model senescence-associated β-Galactosidase and nuclear p21 as senescence markers are increased. Moreover, total and mitochondrial reactive oxygen species formation is enhanced. An imbalanced redox homeostasis is detected by elevated NOX4 and decreased Trx-1 levels. This can be rescued by lentiviral expression of Trx-1. Moreover, the lysosomal protease Cathepsin D is over-activated, which results in reduced Trx-1 protein levels. Inhibition of "over-active" Cathepsin D by the specific, cell-permeable inhibitor pepstatin A abolishes the increase in nuclear p21 protein, ROS formation and degradation of Trx-1 protein, thus leading to blockade of stress-induced premature senescence by stabilizing the cellular redox homeostasis. Aortic Trx-1 levels are decreased and Cathepsin D activity is increased in NOX4 transgenic mice exclusively expressing NOX4 in the endothelium when compared to their wildtype littermates.
Thus, loss of Trx-1 and upregulation of NOX4 importantly contribute to the imbalance in the redox-status of senescent endothelial cells *ex vivo* and *in vivo*.

1. Introduction

Cardiovascular diseases and the process of aging have been described as inseparably tied together, since aging is one major risk factor for cardiovascular diseases. However, several studies recently showed that physiological aging could also be independent of the development of cardiovascular diseases (North and Sinclair, 2012; Strait and Lakatta, 2012) because of the characteristic physiological decline in organ functions during aging of organisms including human beings. There is a long lasting debate whether reactive oxygen species (ROS) are causally involved or only bystanders in the process of aging. It has become clear that adaptive reactions occur within cells and that physiological concentrations of ROS, which differ between cell types, are needed for intracellular signaling events. Therefore a balanced redox homeostasis is required. For this purpose oxidative and anti-oxidative systems exist in cells. Key molecules in the endothelium to balance the cellular redox status are the NADPH oxidases (NOXs), which exclusively produce ROS, and the Thioredoxin-1 system — consisting of Thioredoxin-1 (Trx-1) and Thioredoxin-1 reductase, which reduces oxidized proteins.

The NOX family contains up to now seven members, the 'classical' NADPH oxidases NOX1–NOX5, and the dual oxidases Duox1 and Duox2 (Bedard and Krause, 2007). These enzymes were first identified in white blood cells where they induce the respiratory burst in host defense (Babior et al., 1973). NOXs are protein complexes primarily distinguishable by their membrane spanning catalytic 'NOX' subunit. The predominant NADPH oxidase in endothelial cells is NOX4.

Abbreviations: ROS, reactive oxygen species; NOXs, NADPH oxidases; Trx-1, Thioredoxin-1; SA, senescence associated.

* Corresponding author at: IUF — Leibniz Research Institute for Environmental Medicine, Auf'm Hennekamp 50, 40225 Düsseldorf, Germany. Tel.: +49 211 3389 291; fax: +49 211 3389 331.
E-mail addresses: christinegoy100@gmail.com (C. Goy), czypiorski@gmail.com (P. Czypiorski), Joachim.Altschmied@uni-duesseldorf.de (J. Altschmied), Sascha.Jakob@uni-duesseldorf.de (S. Jakob), Lothar.Rabanter@uni-duesseldorf.de (L.L. Rabanter), alison.brewer@kcl.ac.uk (A.C. Brewer), Niloofar.Ale-Agha@uni-duesseldorf.de (N. Ale-Agha), Nadine.Dyballa@uni-duesseldorf.de (N. Dyballa-Rukes), ajay.shah@kcl.ac.uk (A.M. Shah), juhae001@uni-duesseldorf.de (J. Haendeler).
[1] Authors contributed equally to the work.

http://dx.doi.org/10.1016/j.exger.2014.03.005
0531-5565/

The role of NOXs in aging processes has been greatly overlooked, since the overproduction of ROS in aging and diseases has mainly been attributed to the leakiness of the respiratory chain in mitochondria (Krause, 2007). A recent study by Lener et al. demonstrated that knockdown of NOX4 in human endothelial cells leads to delayed replicative senescence (Lener et al., 2009). Thus, an upregulation of NOX4 in the aged endothelium could be envisioned, but has not been investigated so far.

The Thioredoxin-1 system consists of two antioxidant oxidoreductase enzymes, Trx-1 and Thioredoxin-1 Reductase. Trx-1 is ubiquitously expressed in mammalian cells (Holmgren, 2000). Its importance is underpinned by the early lethality of Trx-1 deficient knockout mice (Matsui et al., 1996). Besides its enzymatic activity as an oxidoreductase, Trx-1 directly interacts with other proteins in the cytosol, nucleus and at the plasma membrane and thereby modulates their functions (Zschauer et al., 2013). Therefore, Trx-1 regulates a huge number of different cellular processes like differentiation, proliferation, and apoptosis. The physiological functions of Trx-1 in different types of organisms have evolved from a fundamental reaction – the reduction of oxidized proteins – to numerous, different specialized functions. In the endothelium, Trx-1 is one of the most important anti-oxidative enzymes (Haendeler, 2006).

A role of Trx-1 in aging processes has been suggested by findings in *Caenorhabditis elegans* (*C. elegans*), where Trx-1 knockout animals have a reduced life span (Fierro-Gonzalez et al., 2011). In the human endothelium, high ROS levels induce degradation of Trx-1 protein (Haendeler et al., 2005). Moreover, in a model of replicative senescence Trx-1 protein levels are reduced and are rescued by re-introduction of Trx-1 (Altschmied and Haendeler, 2009). Thus, an important role for Trx-1 in aging processes is plausible and routes to protect its loss are of potential clinical interest.

Thus, we determined in the present study whether the anti-oxidative protein Trx-1 and the oxidative protein NOX4 are regulated during stress-induced premature senescence of primary human endothelial cells. We found that Trx-1 protein levels are decreased and NOX4 levels are increased in stress-induced premature senescence. This can be rescued by lentiviral expression of Trx-1. Moreover, the lysosomal protein Cathepsin D is "over-activated", which explains the diminished Trx-1 protein levels. Inhibition of this "over-activation" completely inhibited the loss in Trx-1 protein levels and the induction of stress-induced premature senescence. Moreover, Cathepsin D is also "over-activated" and Trx-1 protein levels are decreased in transgenic mice overexpressing NOX4 in the endothelium. Thus, NOX4 expression and Trx-1 protein levels negatively correlate *in vivo* in the endothelium, suggesting that an imbalance in the redox homeostasis indeed contributes to endothelial cell senescence and thereby endothelial dysfunction.

2. Materials and methods

2.1. Cell culture

Primary human endothelial cells were cultured in endothelial basal medium supplemented with hydrocortisone (1 μg/ml), bovine brain extract (12 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 10% fetal calf serum as described previously (Haendeler et al., 2002) and used latest in passage 3. Repetitive treatment with 50 μM H_2O_2 every second day for two weeks led to stress-induced premature senescence as described previously (Haendeler et al., 2004a).

2.2. Cloning of a lentiviral Trx-1 expression vector, lentivirus production and transduction of endothelial cells

To generate a lentiviral expression vector the human U6 promoter in pLKO.1 puro (Moffat et al., 2006) was replaced with the cytomegalovirus (CMV) immediate early promoter. The coding sequence for the FLAG-Trx-1 fusion was inserted into this vector using the Gibson assembly cloning kit (New England Biolabs, Frankfurt, Germany) according to manufacturer's specifications to create pLenti-FLAG-Trx-1. Virus particles were produced by cotransfection of HEK293FT cells with pMD2.G, pCMVΔR8.91 (both http://tronolab.epfl.ch/lentivectors) and pLenti-FLAG-Trx-1, in a 3.5:6.5:10 ratio using the Calcium Phosphate Transfection Kit (Sigma, Deisenhofen, Germany). The vector without the CMV/FLAG-Trx-1 cassette was used to produce control virus particles. Virus containing culture supernatants were collected for 6 days with several medium changes, filtered through a 0.45 μm PVDF membrane and concentrated by ultrafiltration using Centricon Plus-70 centrifugal filter devices with a molecular weight cut off of 100,000 (Merck Millipore, Schwalbach, Germany). Viral titers were determined with the QuickTiter™ Lentivirus Titer Kit (Lentivirus-Associated HIV p24) (Cell Biolabs, San Diego, USA). Endothelial cells were transduced with amultiplicity of infection of approximately 3. The day after transduction the cells were washed three times and the medium replaced; 6 h later the H_2O_2 treatment was started.

2.3. Animal experiments

C57BL/6J mice expressing human NOX4 exclusively in the endothelium, which do not harbor the nicotinamide nucleotide transhydrogenase gene mutation (Ronchi et al., 2013), were a gift from Ajay Shah. Animals were killed by cervical dislocation under pentobarbital anesthesia. After flushing the circulation with PBS thoracic aortas were prepared and livers were removed, shock frozen, and stored at −80 °C until further use. All animal experiments were performed after obtaining relevant permission according to German animal protection laws.

2.4. ROS measurements

Living cells were incubated with 5 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate ($DCFH_2$-DA), 5 μM dihydroethidium (DHE) or 5 μM MitoSox for 30 min at 37 °C (Invitrogen, Karlsruhe, Germany). Cells were trypsinized and the reaction was stopped with PBS containing 10% FCS. Cells were pelleted by centrifugation, resuspended in PBS and flow cytometric analyses were performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

2.5. Immunoblots

Cells were lysed and proteins were resolved on a SDS-PAGE. Immunoblotting was performed with antibodies directed against NOX4 (1:500 (Anilkumar et al., 2008)), Thioredoxin-1 and TIM23 (both 1:500, BD Biosciences, Heidelberg, Germany), and ERK1/2 and p21 (both 1:500, Cell Signaling/NEB, Frankfurt, Germany). Blots were incubated with primary antibodies overnight at 4 °C and with secondary HRP-coupled antibodies 2 h at room temperature. Detection was performed with the enhanced chemiluminescence system (GE Healthcare, Muenchen, Germany). Semi-quantitative analyses were performed on scanned immunoblots using ImageJ (Abramoff et al., 2004).

2.6. Preparation of mitochondria

Cytosolic and mitochondrial fractions were isolated from endothelial cells as previously described (Haendeler et al., 2009).

2.7. Analysis of senescence parameters

Senescence of endothelial cells was analyzed by staining for senescence associated (SA)-β-Galactosidase activity (pH = 6) and immunostaining for the cell cycle inhibitor p21. Cells stained for senescence associated (SA)-β-Galactosidase were counted by two independent

Please cite this article as: Goy, C., et al., The imbalanced redox status in senescent endothelial cells is due to dysregulated Thioredoxin-1 and NADPH oxidase 4, Exp. Gerontol. (2014), http://dx.doi.org/10.1016/j.exger.2014.03.005