Experimental Gerontology 45 (2010) 586–595

Contents lists available at ScienceDirect

Experimental Gerontology

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

Do UCP2 and mild uncoupling improve longevity?

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ARTICLE INFO

Article history:
Received 1 December 2009
Received in revised form 4 March 2010
Accepted 16 March 2010
Available online 21 March 2010

Keywords:
Uncoupler
Longevity
ROS
UCP2/3
Fatty acids
Mitochondrial membrane potential
HUVEC

ABSTRACT

Mild uncoupling of mitochondrial respiration is considered to prolong life span of organisms by reducing the production of reactive oxygen species (ROS). Experimental evidence against this hypothesis has been brought forward by premature senescence in cell cultures treated with uncouplers. Exposing HUVEC to a mixture of nutritionally important fatty acids (oil extract of chicken yolk) mild uncoupling with "naturally acting substances" was performed. This treatment also resulted in premature senescence although ROS production did not increase. Fatty acids activate uncoupling proteins (UCP) in the inner mitochondrial membrane. UCP2 expression proved to be sensitive to the presence of fatty acids but remains unchanged during the ageing process. UCP3 expression in senescent HUVEC and avUCP expression in senescent CEF were considerably less than in young cultures. No indication for protonophoric reduction of mitochondrial membrane potential was found in UCP2 overexpressing HeLa cells and only little in HUVEC. ROS levels increased instead of being reduced in these cells. Stable transfection with UCP2-GFP was possible only in chick embryo fibroblasts and HeLa cells and resulted in decreased proliferation. Stable transfection of HUVEC with UCP2-GFP resulted in death of cultures within one or two weeks. The reason for this behaviour most probably is apoptosis preceded by mitochondrial fragmentation and loss of membrane potential.

1. Introduction

Ageing can be considered to result from the accumulation of dysfunctional organelles and DNA caused by an imbalance of the production of harmful molecules and their elimination by protecting reactions. The hypothesis that ageing is a consequence of free radicals derived from mitochondria (Harman, 1956, 1972) proved to be one of the most fruitful hypotheses in driving ageing research (Sanz et al., 2006). Reactive oxygen species (ROS) formation is an inevitable by-product of the electron transport chain, and despite their role as signalling molecules or stimulators of cell proliferation, elevated ROS levels are supposed to induce oxidation of lipids, proteins and DNA (Jendrach et al., 2005, 2008; Unterluggauer et al., 2007). Accumulation of these impairments render cells dysfunctional and are thus limiting life span (Passos et al., 2007; Jendrach et al., 2008). Mild uncoupling has been proposed as a possibility to reduce ROS formation and probably also to retard ageing (Brand, 2000). Among the natural components, which may exert this function are the mitochondrial uncoupling proteins (UCP). UCP are wide spread among animals and exhibit some tissue specificity. Five types have been described for mammals. The best investigated is UCP1 which primarily is found in brown adipose tissue and involved in non-shivering thermogenesis. UCP2 and UCP3 are very similar and are about 50% homologue to UCP1 and about 70% to each other. UCP2 was found in many tissues as are white adipose tissue, spleen, kidney, lung, islets of Langerhans and brain (for review see Erlanson-Albertsson, 2004). No immediate role of UCP2 in thermogenesis could be found, but reduction of ROS production by interfering with ubisemiquinone and correction of the energy balance during high-fat feeding has been demonstrated (Surwit et al., 1998; Rippe et al., 2000). Inversely ROS can activate UCP2 from the matrix side of isolated rat kidney mitochondria (Echtay et al., 2002). Control of insulin secretion by insulinoma cells (Azzu et al., 2008) represents a further function related to energy metabolism. The closely related UCP3 was primarily found in skeletal muscle, its exact physiological role is

Abbreviations: mtΔψ, mitochondrial inner membrane potential; CEF, chick embryo fibroblasts; DASPMI, di-aminostyryl-methyl-pyrimidinium iodine; DHE, dihydro-ethidium; FCCP, carbonyl cyanid-*p*-triflouromethoxyphenylhydrazin; HUVEC, human umbilical vein endothelial cells; ROS, reactive oxygen species; TMRE, tetramethyl-rhodamine-ethylester.

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doi:10.1016/j.exger.2010.03.011

not known. The same applies to UCP 4 and UCP 5 described recently to be located in brain mitochondria.

The main sites of ROS generation are complex I and complex III as was shown by experiments blocking the respiratory chain and thus increasing ROS production. The physiological conditions supporting production of ROS at these two sites differ considerably (O'Rourke et al., 2005). Therefore the influence of uncoupling electron transport from oxidative phosphorylation on ROS production cannot be predicted straightforward, it might either reduce ROS production via complex I (Fridell et al., 2005; Abe et al., 2006) or it may increase ROS production via complex III (Schönfeld and Wojtczak, 2007; for review see O'Rourke et al., 2005). These considerations formed the theoretical basis of experiments on the effect of mild uncoupling on ageing of cells in culture (Stöckl et al., 2007). Uncoupling was achieved by chronic exposure of mammalian cell cultures as well as yeast cells to low concentrations of the uncoupler FCCP. However, this treatment reduced the life span of the cultures by inducing premature senescence and increased ROS production (Stöckl et al., 2007). These observations are in line with findings of lowered mt$\Delta\psi$ (mitochondrial inner membrane potential) in post-mitotic HUVEC which also produce more ROS than young HUVEC with mitochondria having high membrane potential (Unterluggauer et al., 2007). Also exposure of HepG2 cells to 50 μM dinitrophenol, another uncoupler of oxidative phosphorylation, retarded proliferation, but cells adapted to the decreased membrane potential by increasing their respiratory capacity (Desquirets et al., 2006). Finally the question remains, whether lowering of mt$\Delta\psi$ is cause or consequence of high ROS production and retarded growth.

Because of the possibilities of adaptive processes the influence of natural uncouplers being present for prolonged time might differ from exposure to artificial uncouplers. UCPs are the most likely regulators, and they are also involved in transport of long chain fatty acids (Hirabara et al., 2006) which by themselves have been shown to act as uncouplers and thus provide a "natural" source for mild uncoupling and thus seem to represent an ideal group of substances for research on the role of physiological uncoupling in ROS production and longevity. Indirect influence of UCP2 and 3 on mt$\Delta\psi$ is related to its action as a Ca^{2+} uniport (Trenker et al., 2007).

We used two different methods for uncoupling (an oily total extract of chicken egg yolk and UCP2 overexpression) for this purpose and analysed the effects on cellular and mitochondrial fitness as well as the consequence for the ageing process.

2. Material and methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVEC) in passage 0 were purchased from Promocell (#C-12200) and cells were cultivated in Endothelial Cell Growth Medium (Promocell #C-22010). Flasks and coverslips were coated with 0.02% gelatine for 30 min to increase adhesion and proliferation (Unterluggauer et al., 2007). Growth curves and doubling time were achieved from daily cell counts. To exclude the influence of genetic factors, HUVEC from at least three different isolations were used. The senescent status of HUVEC populations was verified by senescence-activated (SA)-ß-galactosidase staining (Dimri et al., 1995) with the Senescence Cells Histochemical Staining Kit (Sigma) according to the manufacturers instructions. Cultures that had a doubling above 120 h and contained more than 80% SA-beta-galactosidase-positive cells were classified as senescent cells.

Chick embryo fibroblasts (CEF): chicken (white leghorn) eggs (virus-free) were obtained from Charles River. Leg tissue of 10 day old embryos was homogenized and cells were separated by incubation with 0.1% trypsin for 20 min at 37 °C. A homogenous cell population was obtained after filtration (filter with 60 μm pores). Cells were cultivated for two passages until a fibroblast culture was obtained. Each culture contained cells from three different animals to minimize the influence of genetic factors. For maintenance cells were kept at 41 °C with 5% CO_2 and 95% air in IMDM (Invitrogen) with 5% FCS (Invitrogen), 5% chicken serum (Invitrogen) and 1% penicillin/streptomycin (Invitrogen) in flasks that had been coated with 0.2% gelatin (Sigma).

HeLa cells were cultured at 37 °C in Minimal Essential Medium with Earle's salts (Invitrogen) containing 10% FCS and 1% MEM Non essential amino acids (Invitrogen).

Population doublings (PD) were determined using the following equation: $PD = 3.32 * (\log10\ UCY - \log10\ I) + X$ (where UCY is the number of cells at the end of the passage; I the number of cells that were seeded at the beginning of the passage and X the previous PD number.

Chicken egg yolk oil was a generous gift by Dr. Nawrocki (Frankfurt am Main). It was composed of myristic acid 0.61%, stearic acid 6.18%, palmitic acid 26.4%, oleic acid 47.5%, linolic acid 18.5%, linoleic acid 0.73%, arachinic acid 0.03%, cholesterol 2.2%, carotinoids 33.8 mg/kg, tocopherol 42.4 mg/kg. The appropriate amount of this oil was pipetted into culture medium (vol/vol) and dissolved by at least 5 min sonication in a Branson sonifier while cooling to avoid heating of the medium.

2.2. Confocal laser scanning microscopy

Cells were observed with a Leica TCS SP5 confocal laser scanning microscope (LSM) using a HCX PL APO lambda blue 63.0×, 1.40 OIL UV or HCX PL APO 63.0×, 1.30 GLYC 37 °C UV objective with the appropriate wavelengths settings that were controlled by the LAS AF scan software (version 1.8.2) (Leica, Germany). Live cell experiments were performed at 37 °C and 5% CO_2 in a humidified chamber. Pictures were visualized with IMARIS 6.0.0 (BITPLANE Scientific solutions).

2.3. Quantification of the fluorescence intensity (TMRE-, DHE- and DASPMI staining)

The determination of ROS was achieved by staining cells with dihydro-ethidium (DHE; Molecular Probes; final concentration 5 μM) for 30 min. Four slides were used per conditions and six frames of each were analysed using ImageJ. Pictures of the fluorescent samples were taken with constant microscopical settings and the micrographs were analyzed using the program ImageJ, to evaluate the integrated fluorescence of ethidium. To determine the relative fluorescence intensity for each micrograph, each grey value was multiplied with the respective amount of pixels. The total sum of these products was divided by the total sum of pixels. The highest relative fluorescence intensity was always set to 100%. The same evaluation procedure was applied to DASPMI and TMRE stainings which served as a measure for mt$\Delta\psi$.

In transiently transfected cultures (HUVEC and HeLa cells) single cell level based determination of ROS production was required. After incubation ethidium fluorescence was quantified in digital images taken at constant illumination and exposure time. GFP-UCP2 positive cells have been encircled and measured separately from other cells, thus non-transfected cells within the same culture could be used for control.

2.4. High-resolution respirometry

Mitochondrial respiratory parameters of endothelial cells were measured by high-resolution respirometry using the Oroboros® Oxygraph 2 k (for details see e.g. Hütter et al., 2006). $3 * 10^6$ cells