Chapter 5

Chronic inhibition of the respiratory chain in human fibroblast cultures: Differential responses related to subject chronological and biological age

Pim Dekker, Laurens M. van Baalen, Roeland W. Dirks, P. Slagboom , Diana van Heemst, Hans J. Tanke, Rudi G.J. Westendorp, Andrea B. Maier

J Gerontol A Biol Sci Med Sci 2011 Nov [Epub ahead of print]

Summary

Respiratory chain function becomes less efficient with age resulting in increased levels of damaging reactive oxygen species (ROS). We compared rotenone-exposed fibroblast strains from young and old subjects, and from offspring of nonagenarian siblings and the partners of the offspring. Rotenone increased ROS levels, inhibited growth rate and increased telomere shortening (all $p<0.05$). Non-stressed strains from young subjects showed lower ROS levels ($p=0.031$) and higher growth rates ($p=0.002$) than strains from old subjects. Stressed strains from young subjects showed smaller increases in ROS levels ($p=0.014$) and larger decreases in growth rate ($p<0.001$) than strains from old subjects. Telomere shortening rates were not different between groups. Stress-induced decreases in growth rate were larger in strains from offspring than from partners ($p=0.05$). Strains from young and old subjects are differentially affected by chronic inhibition of the respiratory chain. Changed growth rates in strains from offspring resemble those from strains from young subjects.

Introduction

Aging is determined by accumulation of molecular damage and a paucity of well functioning differentiated cells that lead to the loss of bodily functions and various age-related diseases (1). During the lifespan of an organism, damage to cellular components accumulates. Depending on the type of cell and adverse conditions, cells can repair the damage, arrest proliferation irreversibly (senescence), undergo programmed cell death (apoptosis) or die in an uncontrolled manner (necrosis). Senescent cells affect the integrity of tissues by altering their morphology and by excreting factors that promote tumour invasiveness (2). Furthermore, when cells are removed by apoptosis or necrosis, tissues are compromised if cells are not replaced by the proliferative pool of progenitor cells. A decreased proliferative capacity of progenitor cells is thought to be one of the hallmarks of *in vivo* aging (3).

There is much debate about the factors that determine if cells go into senescence. Telomere shortening has long been considered as a cellular clock (4). When telomeres are critically short, cells go into replicative senescence or, in case of loss of p53 function, die by necrosis after crisis (5). However, critically short telomeres are not a prerequisite for senescence since this state can also be induced by oxidative stress causing DNA-damage and changes in chromatin structure involving p53/Rb and p16/21 pathways. Furthermore, expression of certain oncogenes, mainly from the RAS pathways, can lead to senescence. Before cells undergo senescence, they show a decreased rate of proliferation. The fact that oxidative stress both accelerates telomere shortening (6) and can drive cells into senescence would imply that telomere length plays an important role in this stress-induced deceleration of proliferation. As early as 1956 Harman described that mitochondria become increasingly dysfunctional with age, leading to increased levels of reactive oxygen species (ROS) (7). It has been suggested that these increased ROS levels could accelerate telomere shortening (8). Conversely, it does not seem to be telomere length per se which determines the rate of proliferation, but rather the protective status of shortened telomeres (9) which inhibits proliferation.

Earlier we described that skin fibroblast strains derived from chronologically young subjects, when compared with fibroblast strains from very old subjects, are less prone to go into senescence and more prone to go into apoptosis, both under non-stressed and stressed conditions (10). These differences were mirrored by similar differences under stress-induced conditions between fibroblast strains derived from offspring of nonagenarian siblings within families enriched for longevity and their partners from the general population (11). Though of the same chronological age, these offspring were previously observed to be of a younger biological age than their partners as reflected by their beneficial glucose and lipid metabolism, preservation of insulin sensitivity and resistance to cellular stress (10;12;13). The fibroblast strains from these offspring resembled the responses of fibroblast strains from young subjects, i.e. were biologically younger. Here we study if chronic low-level inhibition of the respiratory chain induces increased rates of telomere shortening in human fibroblast strains and if this is related to growth rate, senescence and ROS levels. To allow telomere shortening, multiple population doublings are necessary and therefore we decided to culture fibroblasts for seven weeks rather than one week as described earlier (10). Our hypothesis is that chronic inhibition of the respiratory chain function by exposure to a low dose of rotenone, which decreases but does not arrest proliferation, leads to increased ROS levels, Senescence Associated-β-galactosidase (SA-β-gal) activity, decreased growth rates and concomitant acceleration of telomere shortening of fibroblasts depending on the chronological and biological age of the subject. For this study we compare the fibroblast strains from young versus old subjects and from offspring of nonagenarian siblings versus their partners.

Material and methods

Study designs

The Leiden 85-plus Study (14) is a prospective population-based study in which all inhabitants aged 85 years or older of the city of Leiden, the Netherlands, were invited to take part. Between September 1997 and September 1999, 599 out of 705 eligible subjects (85%) were enrolled. All participants were followed for mortality and 275 subjects survived to the

age of 90 years. During the period December 2003 up to May 2004, a biobank was established from fibroblasts cultivated from skin biopsies from 68 of the 275 surviving 90-year-old participants (15). During the period August to November 2006, we also established a biobank of fibroblast strains established from biopsies taken from 27 young subjects (23-29 years old).

The Leiden Longevity Study (LLS) (11) was set up to investigate the contribution of genetic factors to healthy longevity by establishing a cohort enriched for familial longevity. From July 2002 to May 2006, 421 families were recruited consisting of 944 long-lived Caucasian siblings together with their 1671 of their offspring and 744 of the partners thereof. There were no selection criteria on health or demographic characteristics. Compared with their partners, the offspring were shown to have a 30% lower mortality rate and a lower prevalence of cardio-metabolic diseases (11;12). During the period November 2006 and May 2008, a biobank was established from fibroblasts cultivated from skin biopsies from 150 offspring-partner couples.

Fibroblast Cultures

Three-mm (Leiden 85-plus Study) and 4-mm skin biopsies (LLS) were taken from the sun unexposed medial side of the upper arm. Fibroblasts were grown in D-MEM:F-12 (1:1) medium supplemented with 10% fetal calf serum (FCS), 1 mM MEM sodium pyruvate, 10 mM HEPES, 2 mM glutamax I, and antibiotics (100 Units/mL penicillin, 100 µg/mL streptomycin, and 0.25–2.5 µg/mL amphotericin B), all obtained from Gibco, Breda, the Netherlands. Different FCS batches were used for fibroblasts from the Leiden 85-plus Study (Gibco, batch no. 40G4932F) and for fibroblasts from the LLS (Bodinco, Alkmaar, the Netherlands, batch no. 162229). This medium will be referred to as standard medium. Fibroblasts were incubated at 37℃ with 5% CO2, under conditions of ambient oxygen and 100% humidity. All cultures that are used in the present study were grown under predefined, highly standardized conditions as published earlier (15). Trypsin (Sigma, St Louis, MO, USA) was used to split fibroblasts using a 1:4 ratio each time they reached 80-100% confluence to make sure that each strain had undergone the same number of population doublings.

Experimental set-up

Fibroblasts were thawed from frozen stocks on day zero. Passage 11 fibroblasts were used from young and old subjects and passage seven fibroblast strains from offspring and partners. On day four, seven and eleven fibroblasts were further passaged in order to multiply fibroblasts. On day 18 the experiments were started. Fibroblast strains were seeded at 2000 and 3300 cells/cm^2 in 25 cm^2 culture flasks for non-stressed and rotenone-stressed cultures respective-ly, in batches of eight strains per condition. To chronically stress fibroblast strains, medium was supplemented with rotenone (Sigma, St Louis, MO, USA), known to induce an increase in the intracellular production of ROS at the mitochondrial level (16). Since cell division was necessary for telomere shortening to occur, a low concentration of 20 nM was used in order to prevent complete arrest of proliferation. Because rotenone has a half-life of one to three days (17), medium was changed twice a week throughout a culture period of seven weeks. Fibroblast strains were counted every week and passaged at 2000 and 3300 cells/cm^2 again in new culture flasks. At day 49 fibroblast strains were seeded for assays measuring ROS levels, SA-β-gal activity and telomere length as described below.

Flow cytometric measurement of ROS

Levels of ROS were measured using the intracellular redox dye dihydrorhodamine 123 as described earlier (18). In short, fibroblasts were seeded at 2000 cells/cm^2 in 25-cm^2 flasks. After three days in culture fibroblasts were incubated in medium supplemented with 30 µM dihydrorhodamine 123 (Invitrogen, Breda, The Netherlands). One stock of dihydrorhodamine 123 was prepared and aliquotted before the experiments to prevent changes in fluorescence intensity due to probe oxidation, leading to results that are independent of ROS generation (19). To reduce quenching of the fluorescent probe, ambient light in the laboratory was reduced as much as possible. After incubation with dihydrorhodamine 123, fibroblasts were trypsinized, washed in ice-cold PBS, pelleted and resupended in 200 µl ice-cold PBS. Fibroblasts were kept on ice before measurement of median fluorescence intensity (MdFI) in the FITC-channel.

Flow cytometric measurement of SA-β-galactosidase activity

Fibroblasts were seeded at 2000 cells/cm^2 in 25-cm^2 flasks. Fibroblasts were prepared as described earlier (20). In short, to change the lysosomal pH to pH 6, fibroblasts were incubated with medium containing 100 nM bafilomycin A1 (VWR, Amsterdam, the Netherlands) for one hour. Fibroblasts were then incubated with 33 µM of the β-galactosidase substrate 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C12FDG, Invitrogen, Breda, The Netherlands), in the presence of 100 nM bafilomycin. After trypsinisation, fibroblasts were washed once and resuspended in 200 µl ice cold PBS. Fibroblasts were measured in the FITC-channel and analysis was performed on the MdFI values.

Telomere length analysis

To measure telomere length, a flow-FISH kit was used (DAKO, Heverlee, Belgium) and fibroblasts were treated according to the manufacturer protocol. In short, fibroblasts were trypsinised, mixed 1:1 with the reference cell line (line 1301, Banca Biologica e Cell Factory, Genoa, Italy), hybridised without and with Cy3-labeled PNA-probe. After labeling with propidium iodide (PI) for DNA-content, samples were measured on a LSRII flow cytometer (Becton Dickinson, Franklin Lakes, USA). The probe-signal was measured in the FITC-channel and the PI signal in the Cherry-Red channel. Results were calculated according to the manufacturer's protocol and telomere length was expressed as percentage of the reference cell line. To determine telomere shortening rates, telomere length was also measured at the beginning of the experiment at day 18. The shortening rate was calculated by dividing the difference in telomere length after seven weeks of culturing by the number of cumulative population doublings and was expressed as percentage per PD.

Statistics

All analyses were performed with the software package SPSS 16.0.01 (SPSS Inc., Chicago, IL). To study the effect of rotenone during seven weeks culturing, results were first analyzed regardless of young/old and offspring/partner comparisons. Since the FCS used for fibroblast strains from the 85-plus Study was a different batch than the batch used for the LLS, fibroblast strains from the former (young and old) were analyzed separately from the latter (offspring and partner). Differences in ROS levels, SA-β-gal activity, telomere length and

telomere shortening rate between groups (young/old, offspring/partner) in non-stressed conditions were analyzed using linear mixed models. In strains from the 85-plus Study, results were adjusted for gender only and in strains from the LLS for gender and age. The same analyses were applied to rotenone-stressed differences (non-stressed subtracted from rotenone-stressed) in these parameters.

Growth curves were produced by plotting cumulative population doublings against time. To analyze differences between groups, the slopes of the lines were compared by calculating the interaction terms time*young/old and time*offspring/partner in a linear mixed model, adjusting for gender in strains from the 85-plus Study and for gender and age in strains from the LLS.

Results

Baseline characteristics of subjects of different chronological and biological ages from whom fibroblast strains were established are shown in Table 1. Middle aged offspring from nonagenarian siblings and their partners were of similar age, height and weight.

Effect of chronic exposure to rotenone on ROS levels, SA-β-gal activity, growth rate and rate of telomere shortening

When compared with non-stressed fibroblast strains, levels of ROS and SA-β-gal activity were significantly increased in fibroblast strains stressed with rotenone for seven weeks (Table 2). Compared with non-stressed strains, growth rate was significantly decreased at all time points for rotenone-stressed fibroblast strains (Table 2, Figure 1A and B). For the strains from young and old subjects, telomere length decreased during seven weeks from 22.2±1.2% to 16.9±0.7% (±SEM) in non-stressed fibroblasts and from 22.2±1.2% to 17.1±0.6% in stressed fibroblasts. For the strains from offspring and partners telomere length decreased during seven weeks, from 23.7±1.3% to 18.8± 0.7% in non-stressed fibroblasts and from 23.7±1.3% to 19.2±0.6% in stressed fibroblasts. In all cases $p<0.001$ for the difference between 0 weeks and 7 weeks. Telomere length was not different between non-stressed fibroblast strains and rotenone-stressed fibroblasts after seven weeks of serial culturing.

However, since the rotenone-stressed fibroblast strains had undergone less population doublings (PDs), the rate of telomere shortening per PD was significantly higher in rotenone-stressed fibroblast strains (Table 2).

Table 1. Clinical characteristics of young and old subjects from the Leiden 85-plus Study representing a difference in chronological age and of offspring and partners from the Leiden Longevity Study who differ in biological age.

	Leiden 85-plus Study		Leiden Longevity Study	
	Young	Old	Offspring	Partners
	n=10	n=10	n=10	n=10
Demographic data				
Female	7	5	5	5
Age, years (mean±SD)	25.5 (1.8)	90.2 (0.3)	57.3 (7.6)	57.4 (8.7)
Anthropometric data				
Height, cm (mean±SD)	177 (10)	164 (7)	174 (12)	174 (7)
Weight, kg (mean±SD)	69.9 (11.1)	69.0 (9.3)	76.3 (19.2)	74.5 (13.5)
Current smoking - no./total known	1/8	1/10	2/10	1/10
Diseases				
Myocardial infarction, no.	0	1	0	0
Stroke, no.	0	1	0	1
Hypertension, no.	0	6	1	2
Diabetes mellitus, no.	0	1	0	3
Malignancies, no.	0	0	1	0
Chronic obstructive pulmonary disease, no.	0	1	0	1
Rheumatoid arthritis, no.	0	3	0	0

Table 2. Effect of chronic exposure to rotenone on levels of reactive oxygen species (ROS), Senescence Associated-β-galactosidase (SA-β-gal) activity, population doublings (PDs) and telomere length, measured after 7 weeks in human fibroblasts from the Leiden 85-plus Study and Leiden Longevity Study. Average rate of telomere shortening during 7 weeks was also calculated. Values are given as mean (SE).

	Leiden 85-plus Study			Leiden Longevity Study		
	Non-stressed n=20	Rotenone n=20	p	Non-stressed n=20	Rotenone n=20	p
ROS (MdFI)	1427 (109)	1571 (115)	<0.001	1380 (78)	1551 (88)	<0.001
SA-β-gal (MdFI)	4116 (398)	4743 (348)	<0.001	3964 (264)	4442 (284)	<0.001
PDs (no.)	16.13 (0.53)	10.85 (0.40)	<0.001	15.12 (0.48)	9.83 (0.39)	<0.001
Telomere length (% of ref. cell line)	16.9 (0.7)	17.1 (0.6)	0.82	18.8 (0.8)	19.2 (0.8)	0.67
Telomere shortening rate (%/PD)	-0.32 (0.07)	-0.49 (0.13)	0.002	-0.32 (0.08)	-0.43 (0.16)	0.042

MdFI: median fluorescence intensity. Data were analyzed with linear mixed models, adjusting for gender, batches of experiments, repeat measures and age (for offspring/partner comparison).

Differential effect of chronic exposure to rotenone on ROS levels, SA-β-gal activity, growth rate, telomere length and rate of telomere shortening dependent on chronological and biological age

Serial culturing under non-stressed conditions for seven weeks

ROS levels were significantly lower in fibroblast strains from young subjects compared with old subjects, but no differences were found in SA-β-gal activity. Fibroblast strains from young subjects showed a significantly higher growth rate when compared with fibroblast strains from oldest old subjects (Figure 1C). Telomere length was not different between strains from young and old subjects at 0 weeks (24.0±2.6% and 23.6±2.6% respectively, p=0.54) or at 7 weeks (17.0±0.9% and 17.3±0.9% respectively, p=0.86). Shortening rates were also not different between strains from young and old subjects (Table 3).

No statistically significant differences in ROS, SA-β-gal activity and growth rate were found between fibroblast strains from offspring and from their partners in non-stressed conditions, although ROS levels were consistently lower in fibroblast strains from offspring when compared with strains from partners (Table 3 and Figure 1D). Telomere length was not different between strains from offspring and partners at 0 weeks (25.2±2.5% and 25.1±2.5% respectively, p=0.93) or at seven weeks (19.2±0.8% and 18.7±0.8% respectively, p=0.61). Shortening rates were also not different between strains from offspring and partners (Table 3).

Chronic exposure to rotenone for seven weeks

Stress-induced ROS levels and SA-β-gal activity were lower in fibroblast strains from young subjects when compared with strains from old subjects. Stress-induced decreases (rotenone-stressed – non-stressed) in growth rate were larger for fibroblast strains from young subjects when compared with strains from old subjects (Figure 1E). No differences in telomere shortening rates were found between strains from young and old subjects after seven weeks culturing under stressed conditions (Table 3).

Differences in stress-induced ROS levels between fibroblast strains from offspring of nonagenarian siblings and partners were not statistically significant but were consistently lower in fibroblast strains from offspring. SA-β-gal activity was not different between fibroblast strains from offspring and strains from partners. When fibroblast strains from offspring and their partners were compared, stress-induced decreases in growth rate were larger for fibroblast strains from offspring when compared with strains from partners (Figure 1F). Telomere shortening rates showed no difference between fibroblast strains from offspring and strains from partners after seven weeks under stressed conditions.

Table 3. Effect of chronic exposure to rotenone on ROS levels (ROS), Senescence Associated-β-gal (SA-β-gal) activity and rate of telomere shortening measured in human fibroblasts from young and old subjects from the Leiden 85-plus Study, representing a difference in chronological age, and of offspring and partners from the Leiden Longevity Study who differ in biological age.

	Leiden 85-plus Study			Leiden Longevity Study		
	Young	Old		Offspring	Partner	
	n=10	n=10	p	n=10	n=10	p
ROS (MdFI)						
Non-stressed	1282 (108)	1573 (183)	**0.031**	1329 (87)	1431 (132)	0.39
Rotenone – Non-stressed	+81 (59)	+234 (35)	**0.014**	+146 (68)	+207 (65)	0.43
Bgal (MdFI)						
Non-stressed	3756 (319)	3814 (360)	0.47	4120 (423)	3790 (315)	0.67
Rotenone – Non-stressed	+472 (238)	+960 (238)	0.076	+598 (166)	+660 (120)	0.90
Telomere shortening rate (%/PD)						
Non-stressed	-0.43 (0.13)	-0.41 (0.13)	0.85	-0.39 (0.16)	-0.42 (0.16)	0.79
Rotenone	-0.68 (0.25)	-0.64 (0.25)	0.72	-0.60 (0.30)	-0.59 (0.30)	0.93

MdFI: median fluorescence intensity. Data were analyzed with linear mixed models, adjusting for gender, batches of experiments, repeat measures and age (for the offspring/partner comparison). Values in bold are statistically significant at $p<0.05$

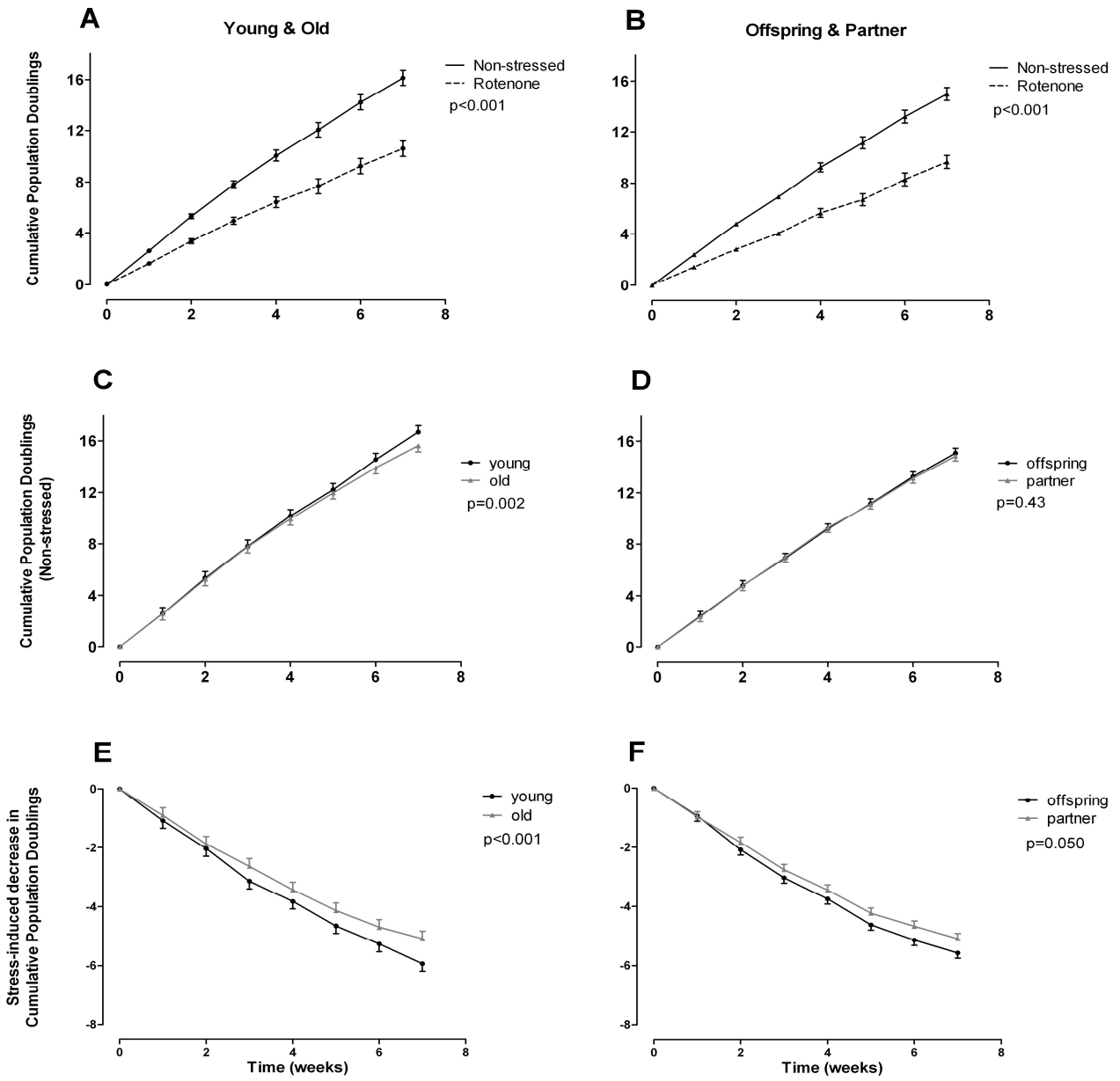

Figure 1. Cumulative population doublings under non-stressed conditions and chronic exposure to rotenone for 7 weeks comparing young/old subjects and off-spring/partner subjects (stress-induced decreases: rotenone stressed and non-stressed). A, rotenone effect on young and old subjects. B, rotenone effect on offspring and partners. C, young and old subjects compared under non-stressed conditions. D, offspring and partners compared under non-stressed conditions. E, rotenone-induced decrease compared between young and old subjects. F, rotenone-induced decrease compared between offspring and partners. Data were analyzed using linear mixed models adjusting for gender and batches of experiments. Values are given as mean (SE). p Values indicate differences between the slopes of the curves.

Discussion

The main findings of our study are that chronic inhibition of the respiratory chain with rotenone differentially affects ROS levels, SA-β-gal activity and growth rate, but not telomere shortening rate, when fibroblast strains from young subjects are compared with strains from old subjects. Even more importantly, the stress-induced changes in growth rate of fibroblast strains from offspring of nonagenarian siblings resemble the changes in growth rate of strains from young subjects.

According to the free radical theory of aging the respiratory chain function decreases with age, resulting in increased levels of ROS which will damage proteins and DNA (7). Indeed, the efficiency of oxidative phosphorylation in human skin fibroblasts becomes less efficient with chronological age (21). We aimed to simulate this process *in vitro* by inhibiting mitochondrial activity with the mitochondrial complex I inhibitor rotenone, which is known to decrease cellular respiration, paralleled by increased ROS levels (16). A low concentration of rotenone was used in order not to completely arrest proliferation. Also, it is known that long-term inhibition of mitochondrial complex I with concentrations of rotenone inducing 100% inhibition, leads to secondary toxicity (22) resulting in massive cell death (16).

As expected, fibroblast strains exposed to rotenone showed increased levels of ROS and increased SA-β-gal activity, indicating senescence (23), albeit not much is known about long-term culturing conditions of fibroblasts under chronic oxidative stress. Koopman *et al*. showed that human fibroblast exposed to low concentrations of rotenone (100nM) for up to three days show depolarisation of the $\Delta\psi$ paralleled by increased levels of ROS (16). They also reported that despite unchanging numbers of mitochondria, rotenone-treatment did induce changes in the shape of mitochondria and the extent and speed of movement of mitochondria.

Decreased growth rate due to oxidative stress was also found by von Zglinicki *et al*. who showed that hyperoxic condition markedly slowed proliferation (24). With the flow-FISH method we were able to measure that the telomere length shortened significantly after seven weeks of culturing. There were no differences in telomere length between non-stressed and

rotenone-stressed fibroblast strains after seven weeks of culturing, but taking into account the lower number of population doublings of the rotenone-stressed fibroblast strains, rotenone did induce a significantly faster telomere shortening rate per PD. This is consistent with the current consensus that increased oxidative stress accelerates telomere shortening (6;24).

In line with our hypothesis, strains from young subjects showed lower ROS levels when compared with fibroblast strains from old subjects, both under non-stressed and stressed conditions. For non-stressed conditions these results are supported by recent findings from Koziel *et al*. who showed higher levels of ROS, coinciding with lower mitochondrial membrane potential (Δψ), in fibroblast strains from old subjects (25). We used the fluorescent probe dihydrorhodamine 123 for the quantification of ROS. This assay is sensitive to such factors as plasma membrane efflux pumps (ABC transporters) (26), so it should be realized that any differences in these pumps between fibroblast strains from young and from old subjects may attribute to changes in the fluorescent signal. *In vivo*, ROS levels have been reported to increase with age in various tissues from animal models (27). We also found consistently lower ROS levels in strains from offspring when compared with strains from partners, albeit not significantly so. This is most probably due to the relatively low number of strains tested. Despite the lack of significance for the offspring-partner comparison, the results are support our earlier findings (10). Future studies with higher numbers of strains will have to confirm if fibroblast strains from offspring indeed show lower levels of rotenone-induced ROS when chronically exposed. Additionally, to study differences in mitochondrial functionality, it would be insightful to compare Δψ and the number, shape and (speed of) movement of mitochondria and the effect of rotenone on ROS generation by mitochondria respiring on different substrates. In model organisms it has indeed been shown that isolated mitochondria respiring on different substrates will differentially represent *in vivo* the longevity of the organisms they were derived from (28). Furthermore, with aging the binding affinities of mitochondrial proteins in the electron transport chain decrease, causing increased ROS leakage (29).

We did not find differences in SA-β-gal activity under non-stressed conditions between fibroblast strains from young subjects and from old subjects, whereas earlier we showed

lower numbers of senescent fibroblasts for strains from young subjects (10). It should be noted that in the experiments described here, fibroblasts were cultured for seven weeks whereas in the experiments described earlier fibroblasts were cultured for one week. Since it is well known that the number of senescence fibroblasts increases with increased duration of culturing (30), higher background levels of senescence after seven weeks of culturing might mask the difference in SA-β-gal activity between strains from young and old subjects. Also we did not find differences in SA-β-gal activity between strains from offspring and strains from partners under non-stressed conditions. When chronically stressed with rotenone, increases in SA-β-gal activity in strains from young subjects were lower when compared with fibroblast strains from old subjects, but no differences in stress-induced increases in SA-β-gal activity were found between fibroblast strains from offspring and partners, probably due to the relatively low number of strains tested.

Under non-stressed conditions the growth rate of fibroblast strains from young subjects was higher when compared with strains from old subjects. Similar *in vitro* results were described already in 1976 by Schneider *et al*. (31) and comparable differences were thought to exist *in vivo* (32). No differences in non-stressed growth rate were found between fibroblast strains from offspring of nonagenarian siblings when compared with strains from partners. It should be realized that we cultured the fibroblast strains under ambient oxygen conditions. The oxygen concentration in most tissues is much lower than the atmospheric oxygen concentration which is often used for cell culture. Human fibroblasts grow considerably slower in 20% oxygen when compared with 5% oxygen (33). Relatively large decreases in growth rate caused by the ambient oxygen conditions might mask the much smaller differences between fibroblast strains from offspring and partners.

Stress-induced decreases in growth rate were larger for fibroblast strains from young subjects and offspring when compared with strains from old subjects and partners, respectively, so in this respect fibroblast strains from offspring resemble the responses of strains from young subjects, both being more responsive to oxidative stress when compared with strains from old subjects and partners, respectively. Again these results confirm our earlier findings that fibroblast strains from offspring seem to be biologically younger than strains from their partners (10).

Contrary to our hypothesis, telomere shortening rates were not different between fibroblast strains from young subjects and fibroblast strains from old subjects. These results suggest that higher ROS levels in fibroblast strains from old subjects do not lead to measurably higher telomere shortening rates compared with young subjects. It is important to differentiate between *in vivo* age (chronological age of donors of fibroblast strains) and *in vitro* age (number of cumulative population doublings *in vitro* of those strains). After seven weeks of culturing we found stress-induced acceleration of telomere shortening which coincided with increased ROS levels, suggesting that with increasing *in vitro* age, ROS levels are related to accelerated telomere shortening. Indeed, it has already been described that ROS levels increase in human fibroblasts in later proliferation stages *in vitro* (34). However, there seems to be an uncoupling of ROS and telomere shortening when fibroblasts strains are compared based on *in vivo* age of the subjects. The results described here suggest that increased ROS levels affect growth rate independently of telomere shortening rate. The differences in growth rate dependent on chronological age and biological age under stressed conditions were not accompanied by differences in telomere length, suggesting that changes in growth rate with increasing *in vivo* age of subjects do not depend on changes in telomere length. This finding is in line with the results of Karlseder *et al*. who suggested that it is not telomere length per se which induces senescence but rather the protected status of shortened telomeres (9). More recently these results were supported by Sahin *et al*. (35) who reported results supporting a link between telomere and mitochondrial biology. The flow-FISH method for measuring telomere length has been developed for blood cells. When applied to other cell types, for instance to epithelial cells with a large cytoplasm, the method is known to be less accurate (36). Although the flow-FISH method was found sensitive enough in this study to measure telomere shortening in human fibroblasts during seven weeks of culturing, it is important to realize that this method might not be sensitive enough to measure subtle differences between fibroblast strains from young and old subjects and from offspring and partners.

Differences between cellular phenotypes of fibroblast are dependent on differences in gene expression. Fibroblast strains from old subjects were shown to express lower levels of mRNA for genes related to oxidative stress, growth and differentiation, cell cycle or metabolic

enzymes whereas higher expression was seen for genes related to protein processing and docking, extracellular matrix, immune response, EGF-signaling and transcription (37;38). Similar findings were reported *in vivo* (39). Stress-induced differences in mRNA expression of these genes are also dependent on the age of the subject, showing blunted responses in fibroblast strains from old subjects (40). Age-dependent differences in transcription are reflected in differences in translation, as suggested by considerable age-dependent differences in cytoplasmic levels of proteins involved in metabolism and stress response (e.g. heat shock and anti-oxidant proteins) (41). Taken together, these findings support the theory that aging is the result of accumulation of random damage (42) to the (mitochondrial) genome and to synthetic and degradative pathways, modulating the whole cell machinery.

An important strength of this study is the long-term character of the experiments, mimicking age-related decreases in mitochondrial function by inhibiting mitochondrial respiratory chain function. Furthermore, the tested strains were obtained from subjects of various chronological and biological ages, collected and stored in a highly standardized manner. We chose to study stress-induced senescence rather than replicative senescence, since it has been shown that the maximum number of population doublings does not reflect human life history trajectories (43). This suggests that stress-induced senescence might better differentiate between cellular phenotypes of individual subjects. A weakness of the study is the relatively low number of strains tested.

In conclusion we report that chronic inhibition of the respiratory chain differentially affects ROS levels, SA-β-gal activity and growth rate, but not telomere shortening when fibroblast strains from young subjects are compared with strains from old subjects. Even more importantly, the stress-induced changes in growth rate of fibroblast strains from offspring of nonagenarian siblings resemble the changes in growth rate of strains from young subjects, indicating that these offspring are biologically younger when compared with the general population. When fibroblast strains from offspring are compared with strains from partners, the effects of the biological age difference are likely to be smaller than those resulting from the 70 years difference between strains from young and old subjects. This might partly explain the lack of differences in ROS-levels and SA-β-gal acitivity between fibroblast strains

from offspring and partners. On the other hand the differences between the chronological and biological age contrasts could involve different biological phenomena. Future work will have to elucidate which signaling pathways are responsible for these differences.

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