



Research paper

Knock out of the NADPH oxidase Nox4 has no impact on life span in mice



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ARTICLE INFO

Keywords:
Nox4
Aging

ABSTRACT

The free radical theory of aging suggests reactive oxygen species as a main reason for accumulation of damage events eventually leading to aging. Nox4, a member of the family of NADPH oxidases constitutively produces ROS and therefore has the potential to be a main driver of aging. Herein we analyzed the life span of Nox4 deficient mice and found no difference when compared to their wildtype littermates. Accordingly neither Tert expression nor telomere length was different in cells isolated from those animals. In fact, Nox4 mRNA expression in lungs of wildtype mice dropped with age. We conclude that Nox4 has no influence on lifespan of healthy mice.

1. Main body

Reactive oxygen species (ROS) are linked to aging and are thought to promote and accelerate the process of physiological aging. The free radical theory of aging postulates that an increased damage by accidental ROS production is one of the reasons for aging [4]. This has been attributed to an increased formation of ROS by mitochondria and enzymes such as NADPH oxidases.

The family of NADPH oxidases consists of 7 members: Nox1-5 and DUOX1 and 2. Those members can be categorized into two groups. Nox1-3 and Nox5, DUOX1 and DUOX2 are acutely activated. Thus, these NADPH oxidases contribute to acute signaling. In contrast to all other members of the NADPH oxidase family the enzyme Nox4 is constitutively active and produces relative small amounts of ROS, as compared to the other members of the family. Thereby Nox4 maintains the cellular redox state [2]. As Nox4 is upregulated in senescent cells it has been identified as a potential mediator of aging [7].

These cell culture data, so far, have not been tested in vivo and so far no evidence has been provided that Nox4 triggers aging in the intact organism. Several publications including our own work suggest that Nox4-dependent ROS formation might have beneficial functions [6]. We therefore question the concept that Nox4 reduces life span and assume that the enzyme has no effect on life expectancy or even extends the life span. This aspect was addressed in Nox4 knock out

mice. Importantly, no evidence was obtained that Nox4 impacts on murine life span. We compared lifespan in wildtype (n=27) and Nox4^{-/-} (n=20) with male and female mice mixed (Fig. 1A). Differences in median and 90% lifespan were evaluated. Median and 90% lifespan was 800 days and 1020 days in wildtype and 839 and 922 days in Nox4^{-/-}, respectively. Difference in median lifespan (Nox4^{-/-} minus wildtype) was 41 days (95% confidence interval -72 days to 67 days, p=0.41). Therefore, we can conclude that median lifespan in Nox4^{-/-} mice was shorter or equal to that in wild-type mice or at most increased by 8.4%. Difference in 90% lifespan (Nox4^{-/-} minus wild-type) was -18 days (95% confidence interval -176 days to 155 days, p=0.85). Therefore, we can conclude that 90% lifespan in Nox4^{-/-} mice was shorter or equal to that in wild-type mice or at most increased by 15.2%.

As Nox4 expression is high in endothelial cells we decided to use lungs as model organ-system which contains a large number of endothelial cells for further analysis. With increasing age Nox4 expression in lungs was drastically reduced (Fig. 1B). The telomerase reverse transcriptase Tert is a ribonucleoprotein enzyme essential for the replication of chromosome termini and therefore is involved in cellular senescence [8]. We isolated lung endothelial cells from mice at the age of three months where still Nox4 is highly expressed and analyzed for Tert expression. Tert expression was significant but only slightly increased in the absence of Nox4 and accordingly Nox4 knock

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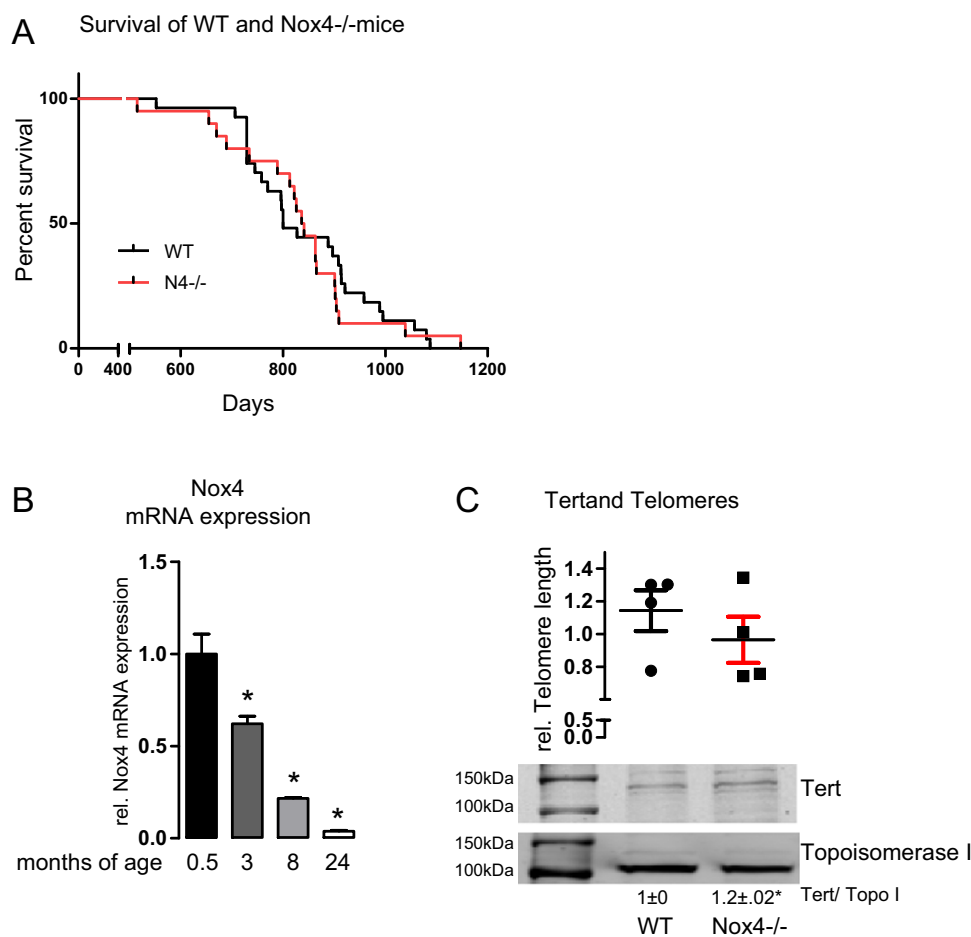


Fig. 1. : (A) **Survival of mice is independent from Nox4 expression.** Nox4^{-/-} mice and their littermates were bred in a 12 h day night cycle with free access to standard chow and drinking water until they died naturally. n=20–27 (mixed male and female mice); (B) **Expression of Nox4 is reduced in aging mice.** Lungs were isolated at the age indicated and analyzed for the expression of NADPH oxidase 4 mRNAs. n=4–5; *p < 0.05; 0.5 month of age vs. 3, 8, or 24 month of age; (C) **Tert expression and telomere length are slightly elevated in the absence of Nox4.** Lung endothelial cells were isolated from Nox4^{-/-} mice and their littermates at the age of 3 months and analyzed for Tert expression and telomere length. (A) Western blot for Tert and Telomerase with statistics. (B) Telomere length as measured with the PNA-FISH probe according to the manufactures instruction. n=4–5; *p < 0.05 WT vs. Nox4.

out had no effect on telomere length (Fig. 1C). We conclude that Nox4 does not contribute to aging in whole organisms. It rather appears that Nox4 is upregulated in cultured senescent cells as a stress response, which does not reflect the situation in vivo. In summary, knock out of Nox4 does not increase the life span of mice.

2. Methods and material

2.1. Animals and animal procedures

All animal experiments were performed in accordance with the animal protection guidelines. Nox4^{-/-} mice were generated by targeted deletion of the translation initiation site and of exons 1 and 2 of the Nox4 gene [5] and backcrossed into C57Bl/6J for more than 10 generations. Mice were housed in a specified pathogen-free facility with 12/12 h day and night cycle and free access to water and chow every time. We compared lifespan in wildtype (n=27) and Nox4^{-/-} (n=20) with male and female mice mixed.

2.2. RNA isolation, cDNA synthesis and real-time polymerase chain reaction

Total mRNA was extracted from frozen murine lung tissue using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription polymerase chain reaction

(PCR) (1 µg of RNA per reaction) was performed with iScript cDNA Synthesis Kits (BioRad, Munich, Germany). The conditions for the reverse transcription were as follows: 1 cycle at 25 °C for 5 min; 1 cycle at 42 °C for 30 min; and 1 cycle at 85 °C for 5 min.

Real-time PCR was performed with an Mx3000 P (Stratagene, Heidelberg, Germany) under the following conditions: 1 cycle at 95 °C for 10 min, then 40 cycles at 95 °C for 10 s, 59 °C for 10 s, and 72 °C for 10 s, followed by a dissociation curve. Each 25 µl reaction mixture contained 12.5 µl iQ SYBR Green Supermix (BioRad, Munich, Germany), 0.5 µl forward and reverse primer, 9.5 µl sterile water, and 2 µl 1:5 diluted cDNA template. A negative control (non-template control) was included for each run. Intron-spanning primers (see below) were designed using sequence information from the bioinformatic harvester portal. Ct values were normalized to an endogenous control (porphobilinogen deaminase [PBGD]). Primers used were: NOX4forward: 5'-CCGGACAGTCTCTGGCTTATC-3'; reverse: 5'-TTG AGG GCA TTC ACC AAG TG-3'; PBGD forward: 5'-GGG AAC CAG CTC TCT GAG GA-3'; reverse: 5'-GAA TTC CTG CAG CTC ATC CA-3'.

2.3. Western blot for tert and telomerase length analysis

Lung endothelial cells were isolated from freshly prepared murine lungs as described before [3]. In brief, the tissue was minced and dispase digested at 37 °C. After several washing steps, LECs were separated magnetically using CD144 coated Dynabeads (MACS). LECs

were used between passages 5–9. To analyze proliferation cells were seeded in a density of 2×10^6 per cm^2 trypsinized and subsequently counted by an automated cell counter (Casy, Schärfe) after 1 d of culture.

For protein isolation cells were lysed in a buffer containing 20 mM TRIS/cl pH 7.5, 150 mM NaCl, 10 mM NaPPi, 20 mM NaF, 1% Triton, 10 mM Okadaic acid (OA), 2 mM Orthovanadate (OV), protein-inhibitor mix (PIM) and 40 $\mu\text{g}/\text{ml}$ phenylmethylsulfonylfluorid (PMSF). Samples were heated to 95 °C in sample buffer and were transferred on SDS-PAGE followed by Western Blotting. Analysis was performed with an infrared-based detection system using fluorescent-dye-conjugated secondary antibodies from LI-COR biosciences. Tert antibody was purchased from Santa Cruz (sc-7212). Telomere length as measured was performed a previously described [1] with adaptation to immunohistochemistry. The PNA-FISH probe TelC probe for a leading strand (repeats of TAACC) was purchased from PNA Bio Inc (F1001). Experiments were performed according to the manufactures instruction.

2.4. Statistical analysis

Statistical analyses were performed with the aid of prism. Comparison of lifespan was analyzed using the Log-Rank (Mantel-Cox) test and the Gehan-Breslow-Wilcoxon-Test. All other data are given as means \pm standard error of mean (SEM). Statistical analysis for multiple groups was performed by analysis of variance (ANOVA) followed by Bonferroni LSD-post-test and for two group comparisons by two-tailed T-test for normally distributed values. A probability value of < 0.05 was considered significant.

Conflict of interest and ethic statement

The authors of this manuscript have no conflict of interest. All animal experiments in the present study were performed according to the NIH guidelines.

Funding

This work was supported by Grants from the Deutsche Forschungsgemeinschaft (DFG) (to KS SCHR1241/1-1 and SFB815/TP1) and the ECCPS (Excellence Cluster Cardiovascular and Pulmonary System).

Acknowledgement

We thank Volker Lohberger for helpful discussion.

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