Biomarkers of oxidative stress, antioxidant defence and inflammation are altered in the senescence-accelerated mouse prone 8

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Abstract In this study we compared biomarkers of oxidative stress, stress response, antioxidant defence and inflammation between mice ($n=10$ per group, female, 7 months old) with an accelerated (SAMP8) and a normal ageing phenotype (SAMR1). As compared to SAMR1 mice, SAMP8 mice exhibited higher levels of lipid peroxides and protein carbonyls as well as a lower activity of the proteasomal subunit β -5. Furthermore, heme oxygenase-1 and paraoxonase-1 (PON-1) status was lower in SAMP8 mice indicating

impaired stress response. Biomarkers of inflammation such as C-reactive protein and serum amyloid P were elevated in SAMP8 mice. Interestingly, impaired stress response and increased inflammation in SAMP8 mice were associated with elevated concentrations of ascorbic acid and α -tocopherol in the liver. An age-dependent increase in hepatic vitamin E and a decline in PON-1 gene expression were also observed in aged compared to young C57BL/6 mice.

Keywords SAMP8 · Accelerated ageing · Stress response · Proteasomal activity · Ascorbic acid · Tocopherol

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Introduction

Ageing is associated with progressive loss of function of various tissues including the liver (Hoare et al. 2010). Liver ageing may be mediated by increased oxidative stress (Radák et al. 2004; Mármol et al. 2010), partly due to impaired stress response (Kourtis and Tavernarakis 2011), as well as chronic inflammatory processes (Chung et al. 2009).

The senescence-accelerated mouse (SAM) model is a rodent model of ageing widely used in experimental ageing research (Takeda 1999). Among the two strains of SAM mice, senescence-accelerated mouse-resistant 1 (SAMR1) serves as a control exhibiting a normal ageing phenotype. Senescence-accelerated mouse prone 8 (SAMP8), on the other hand, exhibits an

accelerated ageing phenotype with elevated biomarkers of oxidative stress (Sato et al. 1996; Rebrin et al. 2005; Álvarez-García et al. 2006; Petursdottir et al. 2007), inflammation (Tha et al. 2000), mitochondrial dysfunction (Carretero et al. 2009), impaired antioxidant defence (Álvarez-García et al. 2006; Gong et al. 2008), insulin resistance (Cuesta et al. 2012), atherogenesis (Fenton et al. 2004) and liver dysfunction (Liu et al. 2008) which may ultimately lead to a shorter life span.

Antioxidant defence comprises enzymatic as well as non-enzymatic mechanisms. Both heme oxygenase-1 (HO-1) and paraoxonase-1 (PON-1) are important antioxidant enzymes of the liver, and their activities may change with age. HO-1 is an inducible enzyme that catalyzes the rate-limiting step in the oxidative degradation of cellular heme that liberates iron, carbon monoxide and biliverdin (Idriss et al. 2008). Besides antioxidant activity, HO-1 exhibits anti-inflammatory and other cytoprotective functions (Wagner et al. 2011). PON-1 is a HDL-associated serum enzyme that is mainly synthesized in the liver. PON-1 mediates its activity by hydrolyzing oxidized lipids of the LDL particle. PON-1 status may be impaired by oxidative stress and pro-inflammatory cytokines (Schrader and Rimbach 2011). Both HO-1 and PON-1 status may be affected by dietary factors including vitamin C (Kunes et al. 2009) and vitamin E (Tsakiris et al. 2009).

Vitamin C is a cytosolic antioxidant and cofactor of many enzymatic reactions. Unlike humans, primates, and guinea pigs, rodents, including laboratory mice, express the rate-limiting enzyme of vitamin C biosynthesis, L-gulonolactone oxidase (Gulo), and thus produce ascorbic acid endogenously (Chatterjee et al. 1961). Vitamin C uptake from the plasma into the liver is mediated by sodium ascorbate co-transporters, such as the sodium-dependent vitamin C transporter type 1 (SVCT1) and type 2 (SVCT2) (Savini et al. 2008).

α -Tocopherol, the major form of vitamin E (tocopherols and tocotrienols) in humans, is the most important lipid antioxidant protecting membranes from oxidative damage (Burton et al. 1982; Traber and Stevens 2011) and known to possess gene-regulatory activities (Rimbach et al. 2002; Azzi et al. 2003; Han et al. 2004; Rimbach et al. 2010). When acting as an antioxidant, tocopherols are converted to their corresponding tocopheroxyl radical forms, which can be regenerated back to their parent tocopherols by

direct interaction with vitamin C (Scarpa et al. 1984). The vitamins C and E are part of an interlinking set of redox antioxidant cycles (Constantinescu et al. 1993), which has been termed the “antioxidant network” (Packer et al. 2001).

We have previously shown that ascorbic acid may modulate antioxidant defence mechanisms in cultured hepatocytes (Wagner et al. 2011). However, little is known about the interaction between enzymatic and non-enzymatic antioxidants in SAMP8 mice. Therefore, we investigated whether accelerated ageing is associated with changes in hepatic HO-1 and PON-1 expression, proteasomal activity, concentrations of antioxidant vitamins and peptides and, consequently, biomarkers of lipid and protein oxidation, inflammation and stress response.

Materials and methods

Chemicals and reagents

HPLC-grade methanol and acetonitrile were obtained from VWR-International (Darmstadt, Germany). For the protein carbonyl quantification, potassium chloride, monopotassium phosphate, sodium chloride, sodium phosphate dibasic dihydrate, disodium phosphate, monosodium phosphate and guanidine hydrochloride, citric acid, sulphuric acid, hydrogen chloride, sodium hydroxide, bovine serum albumin and Roti Quant® were purchased from Carl Roth GmbH (Karlsruhe, Germany). Ascorbic acid, Tween 20, 2,4 dinitrophenylhydrazine, biotin-conjugated rabbit IgG polyclonal antibody raised against a DNP conjugate of keyhole limpet haemocyanin (anti-DNP), streptavidin biotinylated horseradish peroxidase and o-phenylenediamine were from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Hydrogen peroxide was obtained from Merck KGaA (Darmstadt, Germany).

Animals and study design

Animal experiments were performed according to German animal welfare laws and regulations and with permission of the appropriate authorities. Study 1: Ten female SAMR1 and ten female SAMP8, aged 9–10 weeks, were obtained from Harlan Winkelmann GmbH (Borchen, Germany). The mice were housed

in groups of three to four in macrolon cages equipped with softwood bedding, a water bottle, a mouse house and a table tennis ball. Mice were fed a Western-type diet (Altromin Spezialfutter GmbH & Co. KG, Lage, Germany) with 0.15 % cholesterol and 20 % fat, in which half of the fat was from olive oil, for 4.5 months. Diet contained 150 mg/kg vitamin E as RRR α -tocopheryl acetate and 20 mg/kg ascorbic acid. Study 2: Thirty male ($n=6$ per group each) C57BL/6 mice were purchased from Janvier SAS (St. Berthevin Cedex, France). Mice were housed individually in macrolon cages and were fed a Western-type diet (Ssniff, Soest, Germany) containing 21 % butterfat, 1.25 % cholesterol and 20 mg/kg vitamin E (as RRR- α -tocopheryl acetate) for 8 weeks. Final age of mice was 4, 10, 14, 18 and 22 months. In both studies mice were kept in a climate-controlled room (temperature, 22 ± 2 °C; humidity, 55 ± 5 %) with a 12-h light/dark cycle and had free access to feed and water. Food intake was controlled daily and body weight weekly. Food intake and final body weight were not statistically different between groups (data not shown). At the end of the experiment, the mice were fasted for 12 h before anaesthesia by CO₂ and decapitation. Blood samples were collected in tubes and serum was separated by centrifugation (Eppendorf 5804 R, Rotor F34-6-38, Wesseling-Berzdorf, Germany). Tissues (liver, lung, brain) were excised, snap frozen in liquid nitrogen and stored at -80 °C until analysed.

PON-1 arylesterase activity in serum

Arylesterase activity was measured by using phenylacetate as an artificial substrate for PON-1. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. The assay mixture included 4 mM of phenylacetate and 1 mM of CaCl₂ in 20 mM of Tris-HCl, pH 8.0. Non-enzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. One unit of arylesterase activity is equal to 1 μ mol of phenylacetate hydrolyzed per minute per millilitre (Fuhrman et al. 2006).

Liver tissue preparation

Liver homogenates were prepared by homogenizing one volume of liver in ten volumes of ice-cold phosphate-buffered saline (pH 7.4, *w/v*) and centrifugation at $4,800\times g$ at 4 °C for 10 min (Eppendorf 5804

R, Rotor F34-6-38, Wesseling-Berzdorf, Germany). The supernatant was stored at -80 °C until further use.

Lipid peroxidation

Lipid peroxidation was assayed fluorometrically as thiobarbituric acid reactive substances (TBARS) in heart homogenates after protein precipitation with TCA and extraction in 1-butanol. Excitation and emission wavelengths were 520 and 560 nm, respectively. Calibration curve was prepared with TEP (1,1,3,3-tetraethoxypropane) as an external standard (Morel et al. 1983).

Quantification of protein carbonyls

Protein carbonyl content was determined in the homogenized liver tissue supernatant according to the ELISA method by Buss et al. (1997) with required modifications. The detection system used an anti-dinitrophenyl rabbit IgG antiserum (Sigma-Aldrich, Steinheim, Germany) as the primary antibody and a monoclonal antirabbit IgG antibody peroxidase conjugate (Sigma-Aldrich, Steinheim, Germany) as the secondary antibody. Colour change was induced with *o*-phenylenediamine and H₂O₂.

Proteasomal activity

Twenty to 40 mg of tissue was homogenized in lysis buffer (250 mM sucrose, 25 mM HEPES, 10 mM magnesium chloride, 1 mM EDTA and 1.7 mM DTT) using an Ultra-Turrax® and centrifuged at $14,000\times g$ for 30 min. The supernatant was used for determination of protein content using the Bradford assay and for measurement of the proteasomal activity. For proteasomal activity, samples were incubated in 225 mM Tris buffer (pH 7.8), 45 mM potassium chloride, 7.5 mM magnesium acetate, 7.5 mM magnesium chloride and 1 mM DTT. For the peptidyl-glutamyl-like (β 1), trypsin-like (β 2) and chymotrypsin-like (β 5) activity, the substrates Z-Leu-Leu-Glu-AMC (Biochem, Boston, USA), Ac-Arg-Leu-Arg-AMC (Biochem, Boston, USA) and *N*-succinyl-Leu-Leu-Val-Tyr-AMC liberation of the substrates were measured with a fluorescence reader at 360 nm excitation and 460 nm emission. Free AMC was utilized as a standard (Breusing et al. 2009).

α -Tocopherol analysis

Tissue samples (50 mg) were placed in a glass tubes with a screw cap and 2 mL ethanol (EtOH) containing 1 % ascorbic acid (*w/v*) and 700 μ L H₂O. Three-hundred microlitres of saturated potassium hydroxide solution was added, closed, vortexed for 10 s and incubated for 30 min at 70 °C in a shaking water bath. Thereafter, samples were immediately chilled on ice, and 50 μ L butylated hydroxytoluene (BHT) solution (0.1 % BHT in EtOH, *w/v*) and 2 mL n-hexane were added. Test tubes were mixed per hand and centrifuged for 5 min at 1,500 rpm. Five-hundred microlitres of the supernatant was transferred to a clean test tube and dried at room temperature (Savant SpeedVac; Thermo, Langenselbold, Germany). Samples were reconstituted with 150 μ L MeOH/H₂O (98:2, *v/v*) and stored at 20 °C until analysis. For quantification of the tocopherols, 40 μ L of the sample was injected into an HPLC system (Jasco, Gross-Umstadt, Germany; pump PU2080Plus, autosampler AS2057Plus, detector FP2020Plus) and separated on a Waters Spherisorb ODS-2 column (3 μ m, 100 $\times$ 4.6 mm) by isocratic elution at a flow rate of 1.2 mL/min with MeOH/H₂O (98:2, *v/v*) as mobile phase. The fluorescence detector was set to an excitation wavelength of 290 nm and an emission wavelength of 325 nm. Peaks were recorded and integrated using the chromatography software Jasco ChromPass I. The concentrations of α -tocopherol were quantified using an external standard curve (Tocopherol Set, Calbiochem®, Schwalbach, Germany).

Analysis of α -CEHC in liver tissue

Liver tissue (200 mg) was homogenized with a Micra D-8 homogenizer (ART Prozess- & Labortechnik GmbH & Co. KG, Mullheim, Germany) in 400 μ L PBS containing 1 % ascorbic acid (*w/v*). Twenty-five-microlitre β -glucuronidase/sulphatase (3 mg dissolved in 100 μ L acetate buffer, pH 4.5) was added, and the samples incubated for 2 h at 37 °C in a Thermomixer (Eppendorf AG, Hamburg, Germany). Subsequently, samples were cooled on ice and acidified with 50 μ L glacial acetic acid. Carboxyethyl hydroxychromanol (CEHC) was extracted with 2 mL hexane/dichloromethane (1:1, *v/v*) by hand inversion for 1 min. A volume (1.5 mL) of the supernatant was transferred

to a fresh test tube and dried under vacuum in a centrifugal evaporator (Savant SpeedVac). Samples were resuspended in 100 μ L H₂O/acetonitrile (60:40, *v/v*) and transferred to an HPLC vial, and 70 μ L was injected into the HPLC system. Tissue α -CEHC was quantified on a Jasco X-LC HPLC system (autosampler, 3159-AS; two pumps, 3185-PU; solvent mixer, 3180-MX; degasser, 3080-DX; Jasco, Groß-Umstadt, Germany) and detected on an ESA 5600A four-channel coulometric electrochemical array detector (Dionex, Idstein, Germany). Separation was performed on a Reprosil C18 column (5 μ m, 250 $\times$ 4.6 mm; Trentec-Analysentechnik, Rutesheim, Germany) using acetonitrile/50 mM ammonium acetate solution (40:60, *v/v*) as mobile phase at a flow rate of 1.1 mL/min. Electrodes were set to the following potentials: 250, 325, 400 and 475 mV. Peaks were recorded and integrated with the chromatographic software CoulArray 3.10 (ESA). Concentrations of α -CEHC were quantified against authentic external standards (purity $\geq$ 98 %; Cayman Chemicals).

Quantification of glutathione and ascorbic acid by HPLC with coulometric electrochemical detection

To measure the glutathione (GSH) and ascorbic acid content, liver tissue homogenates were prepared as described by Rebrin et al. (2005). The chromatographic separation was carried out with an autosampler 851-AS (Jasco GmbH Deutschland, Gross-Umstadt, Germany) and a pump 510 (Waters, Milford, MA, USA) on a Trentec Reprosil-Pur 120 C18 AQ column (150 $\times$ 4.6 mm, 3 μ m) from Trentec-Analysentechnik (Rutesheim, Germany) at a flow rate of 1 mL/min. The mobile phase for isocratic elution consisted of 50 mM sodium phosphate monobasic and 0.25 % (*v/v*) acetonitrile, the pH was adjusted to 2.5 with 85 % o-phosphoric acid, and the eluent was vacuum-filtered through a 0.2- μ m hydrophilic polypropylene membrane filter (Pall Corporation, MI, USA). The column temperature was maintained at 40 °C, and the analytes were quantified with an eight-channel ESA Model 5600A CoulArray Detector (ESA Inc., Chelmsford, MA, USA). For analyte detection, increasing potentials of 50, 175, 350, 600, 750 and 825 mV were applied on channels. Serial dilutions were prepared from stock solutions in PBS/10 % m-phosphoric acid. The injection volume of samples and calibration

standards was 10 µL. Each sample was analysed in duplicate.

Determination of cholesterol and triacylglycerols in liver

Liver samples (50 mg) were homogenized with 0.9 % NaCl, and lipids were extracted with hexane–isopropanol (60:40 v/v). The mixture was stored in the dark for 45 min and centrifuged at 4,000×g for 15 min. The organic phase was extracted again with hexane–isopropanol. Cholesterol and triacylglycerol concentrations in liver were determined spectrophotometrically at 500 nm using test kits (Fluitest® Chol Biocon® Diagnostic, Voehl-Marienhagen, Germany).

RNA isolation and real-time quantitative RT-PCR

RNA was isolated from liver samples (20–30 mg) using TRIsure lysis reagent (Bioline, Luckenwalde, Germany). Real-time quantitative PCR was performed as a one-step procedure (SensiMix One-Step Kit; Quantace, Berlin, Germany) with SybrGreen detection, using the Rotorgene 6000 cycler (Corbett Life Science, Sydney, Australia). Relative mRNA concentrations of genes were quantified by the use of a standard curve. Target gene mRNA concentration was related to the mRNA concentration of the house-keeping gene GAPDH. Primers were designed by standard tools (Spidey, Primer3 and NCBI BLAST) and purchased from MWG (Ebersberg, Germany).

Primer sequences for analysed genes are summarized in Table 1.

Western blot analysis

Liver tissue (20 mg) was homogenized in radioimmunoprecipitation assay buffer (50 mM Tris–HCl, 150 mM NaCl, 0.5 % deoxycholate, 0.1 % SDS and 1 % NP-40; pH 7.4 with protease-inhibitor cocktail, 1:100; Sigma, St. Louis, MO, USA). Lysates were purified by centrifugation (12,000 rpm, 4 °C, 20 min) after incubation on ice for 30 min. Total protein concentrations in each lysate were quantified using a BCA Protein Assay kit (Pierce, Rockford, USA). Total protein of the lysates (40 µg per lane) was mixed with loading buffer, denatured at 95 °C for 5 min and separated by 12 % SDS gel electrophoresis followed by transferring the proteins to a PVDF membrane, which was then blocked for 2 h in blocking buffer (3 % non-fat milk in Tris-buffered saline, pH 7.4, with 0.05 % Tween-20 (TBS/T)). Primary antibodies were diluted in 3 % non-fat milk (HO-1, 1:1,000; α-tubulin, 1:10,000), and the blots were incubated overnight at 4 °C. The blots were washed and incubated with the respective IgG secondary antibodies (Santa Cruz Biotechnology, Heidelberg, Germany) in blocking buffer with gentle agitation for 1 h at room temperature. The blots were washed, developed with Immun-Star Western Chemiluminescent kit (Bio-Rad Laboratories, Hercules, CA, USA) and scanned with a ChemiDoc XRS system (Bio-Rad, Munich, Germany).

Table 1 Forward (F) and reverse (R) primers used in real-time PCR experiments

Primer	Sequence	Annealing temperature (°C)
GAPDH	F: GACAGGATGCAGAAGAGATTACT R: TGATCCACATCTGCTGGAAGGT	55
Gulo	F: CCAAGATCACACCAACAAGG R: GTCTTGGACATGCTGCTTGA	57
CRP	F: AGATCCCAGCAGCATCCATA R: CAGTGGCTTCTTTGACTCTGC	59
HO-1	F: GAGCCTGAATCGAGCAGAAC R: AGCCTTCTCTGGACACCTGA	59
SAP	F: AAGCTGCTGCTTTGGATGTT R: CATTGTCTCTGCCCTTGACA	55
SVCT1	F: GGGAAAGCCCTCTTCTTTTC R: TGAAGCACGTCAGGTAATGC	57
SVCT2	F: TATTCCTGGGATTGCAGCAC R: AAAGCACTGGCCTGAAACAG	57

Digital images were captured and quantified by densitometry using the Quantity-One system (Bio-Rad). Relative concentration of the proteins was quantified as the ratio between the amount of target proteins and the amount of the housekeeping protein α -tubulin.

Statistical analysis

Statistical analyses were performed using PASW Statistics 18 (IBM, Chicago, IL, USA). Data were tested for normal distribution (Kolmogorov–Smirnov and Shapiro–Wilk tests) and equality of variance (Levene's test) and analysed by *t* test or, in the case of non-parametric data, a Mann–Whitney *U* test (study 1). For the comparison of group means in study 2, one-way ANOVA was performed with the Tukey test as post hoc test. Results are expressed as mean values with SEM, and differences were considered significant when the *p* value was ≤ 0.05 .

Results

Accelerated ageing may be accompanied by an increase in lipid and protein oxidation. Therefore, we measured TBARS as well protein carbonyl concentrations in the liver of our mice. SAMP8 mice exhibited significantly higher TBARS as compared to SAMR1 mice. Furthermore, hepatic protein carbonyl concentration was significantly higher in SAMP8 mice (Table 2). Since we found significant differences in protein carbonyl concentration between both mouse strains, we determined hepatic proteasomal activity. Proteasomal activity of the β -1 and β -2 subunit was similar between both groups. However, proteasomal

activity of the β -5 subunit was significantly lower in SAMP8 in comparison to SAMR1 mice.

Antioxidant defence mechanisms may be altered during the ageing process. We therefore determined both PON-1 activity and HO-1 gene expression and protein levels. PON-1 activity in serum (Fig. 1a) and HO-1 mRNA (Fig. 1b) and protein expression (Fig. 1c) in the liver were significantly lower in SAMP8 compared to SAMR1 mice.

Since PON-1 and HO-1 may be affected by inflammatory mediators, we determined CRP and SAP mRNA levels in the liver. SAMP8 mice had significantly higher CRP and SAP mRNA expression (Fig. 2), indicating a higher inflammatory state in SAMP8 than in SAMR1 mice.

Both PON-1 and HO-1 activity may be affected by tissue vitamin C and vitamin E status (Gaedicke et al. 2008; Tsakiris et al. 2009; Kunes et al. 2009; Schrader and Rimbach 2011). Interestingly, liver ascorbic acid concentrations were almost twofold higher in SAMP8 compared to SAMR1 mice (Fig. 3a). As hepatic ascorbic acid concentrations were different between both groups, we measured also the expression of the ascorbic acid transporters SVCT1 and SVCT2. Hepatic SVCT1 steady-state mRNA levels were significantly higher in SAMP8 vs SAMR1 mice (Fig. 3b), which may explain the differences in liver ascorbic acid concentrations. However, we did not find differences in SVCT2 mRNA between both groups (Fig. 3c). Furthermore, mRNA levels of Gulo, the rate-limiting enzyme of vitamin C synthesis, were comparable (Fig. 3d).

Similar to the ascorbic acid concentration in our mice, the hepatic α -tocopherol concentration was also significantly higher in SAMP8 as compared to SAMR1 mice (Fig. 4a). However, liver GSH

Table 2 Concentrations of TBARS and protein carbonyls as well as proteasomal activities in liver homogenates from 7-months-old SAMR1 and SAMP8 mice

	SAMR1	SAMP8	<i>p</i> value
TBARS (nmol/g tissue)	442 $\pm$ 35.5	700 $\pm$ 78.4	0.002
Protein carbonyls (nmol/g protein)	775 $\pm$ 35.8	874 $\pm$ 35.8	0.016
Proteasomal activity (μ mol/mg/min)			
β -1 subunit	32.8 $\pm$ 2.13	31.1 $\pm$ 2.80	n.s.
β -2 subunit	6.12 $\pm$ 0.24	6.16 $\pm$ 0.14	n.s.
β -5 subunit	56.6 $\pm$ 5.58	40.9 $\pm$ 3.05	0.020

Values are expressed as mean $\pm$ SEM (*n*=10). Means are significantly different at the given *p* value

n.s. not significant

Fig. 1 PON-1 activity in serum of SAMR1 and SAMP8 mice (**a**). Arylesterase activity was measured by using phenylacetate as an artificial substrate for PON-1. Initial rates of hydrolysis were determined spectrophotometrically at 270 nm. Non-enzymatic hydrolysis of phenylacetate was subtracted from the total rate of hydrolysis. Values are expressed as mean±SEM ($n=6-10$). mRNA expression of HO-1 in livers of SAMR1 and SAMP8 mice (**b**). Total RNA isolated from liver was subjected to RT-PCR. The amount of each mRNA was normalized to GAPDH mRNA. Values are expressed as mean±SEM ($n=10$ per group). Protein expression of HO-1 in livers of SAMR1 and SAMP8 mice (**c**). Protein levels were determined by Western blotting and a representative blot is shown. Densitometry refers to 10 animals per group

(SAMR1, 29.0 ± 5.15 nmol/mg protein, vs SAMP8, 31.9 ± 2.97 nmol/mg protein), cholesterol (SAMR1, 17.3 ± 1.22 mg/g, vs SAMP8, 16.0 ± 1.19 mg/g) and triacylglycerol concentrations (SAMR1, 39.2 ± 1.37 mg/g, vs SAMP8, 39.7 ± 1.52 mg/g) were similar in both groups.

Since we found differences in hepatic vitamin E concentrations between SAMR1 and SAMP8 mice, we determined also hepatic α -CEHC levels in our mice. α -CEHC is a major liver metabolite of α -tocopherol. Under the conditions investigated, there were no significant differences in α -CEHC concentrations between SAMR1 and SAMP8 mice (Fig. 4b).

Seven-months-old SAMP mice may be in an intermediate situation as far as age-related changes in

biomarkers of antioxidant defence and oxidative stress are concerned (Schiborr et al. 2010). Therefore, we have conducted additional studies in 4-, 10-, 14-, 18- and 22-months-old C57BL/6 mice. Importantly, we have confirmed the age-dependent increase in liver vitamin E in old as compared to young C57BL/6 mice (Fig. 5a). In addition, we determined hepatic PON-1 mRNA in our C57BL/6 mice. Similar to our findings in SAMP8 vs SAMR1 mice, we observed an age-dependent decrease in PON1 gene expression (Fig. 5b).

In order to answer the question whether age-dependent changes in liver vitamin E were also reflected in other tissues, we measured vitamin E in the brain of our SAMP8 vs SAMR1 as well as in old

Fig. 2 mRNA expression of CRP (a) and SAP (b) in livers of 7-months-old SAMR1 and SAMP8 mice. Total RNA isolated from liver was subjected to RT-PCR. The amount of each mRNA was related to GAPDH mRNA. Values are expressed as mean+SEM ($n=10$ per group)

Fig. 3 Ascorbic acid concentrations (in micromoles per gram) in livers of 7-months-old SAMR1 and SAMP8 mice (a). Values are expressed as mean+SEM ($n=10$ per group) (b–d): mRNA expression of ascorbic acid-relevant genes in livers of 7-months-old SAMR1 and SAMP8 mice. Total RNA isolated from liver was subjected to RT-PCR. The amount of each mRNA was related to GAPDH mRNA levels. Values are expressed as mean+SEM ($n=10$ per group). mRNA expression of SVCT1 (b), SVCT2 (c) and Gulo (d) in livers of SAMR1 and SAMP8 mice

Fig. 4 α -Tocopherol (in nanomoles per gram) (a) and α -CEHC (in picomoles per gram) (b) concentrations in livers of 7-months-old SAMR1 and SAMP8 mice. Values are expressed as mean+SEM ($n=10$ per group)

vs young C57BL/6 mice. Unlike in the liver, we did not observe an age-dependent increase in brain vitamin E, neither in C57BL/6 mice (6.37–8.43 nmol/g) nor in SAMP8 vs SAMR1 (4.38 vs 4.44 nmol/g), suggesting that under the conditions investigated, vitamin E concentrations remain unchanged in the aged murine brain. It may be possible that in older SAMP8 mice (Petursdottir et al. 2007), age-dependent differences in brain vitamin E concentration may become more apparent. Age-related and organ-specific

changes in the concentrations of antioxidant vitamins warrant further investigations.

Discussion

Since reactive oxygen species seem to be involved in the ageing process, we determined lipid peroxide and protein carbonyl concentrations in the livers of our mice. Under the conditions investigated, TBARS,

Fig. 5 α -Tocopherol concentration (in nanomoles per gram) (a) and mRNA expression of PON1 (b) in livers of 4-, 10-, 14-, 18- and 22-months-old C57BL/6 mice. Values are expressed as mean+SEM ($n=6$ per group)

although a rather unspecific biomarker of lipid oxidation, were significantly elevated in SAMP8 mice. This is in accordance with previous studies in SAMP8 mice suggesting increased lipid oxidation in mice with an accelerated ageing phenotype (Matsugo et al. 2000; Farr et al. 2003; Gong et al. 2008). In addition to TBARS, hepatic protein carbonyls were increased in SAMP8 mice. This could be not only due to increased ROS formation, but also partly to the observed decline in proteasomal activity of the β -5 subunit. Functional studies have demonstrated that β 5-overexpressing cell lines confer enhanced survival following treatment with various oxidants (Chondrogianni et al. 2005).

Increased biomarkers of oxidative stress, as observed in our SAMP8 mice, may also be related to impaired activity of antioxidant enzymes including PON-1 and HO-1. PON-1 activity was impaired in our SAMP8 mice. Although human data are partly controversial (Caliebe et al. 2010), PON-1 has been suggested as a candidate gene for longevity due to its modulation of cardiovascular disease risk by preventing oxidation of atherogenic low-density lipoprotein (Tan et al. 2006). The underlying mechanism by which PON-1 activity is diminished in our accelerated ageing mice is unclear. It has been suggested that inflammatory molecules may decrease PON-1 activity (Dullaart et al. 2009). In the current study, both CRP and SAP, surrogate biomarkers of inflammation, were elevated in SAMP8 mice. Thus, impaired PON-1 activity in SAMP8 mice could be related to increased inflammation in this mouse strain. Accordingly, Cuesta and colleagues observed an increase in biomarkers of inflammation (e.g. TNF- α , IL1 β , iNOS) in SAMR1 vs SAMP8 mice (Cuesta et al. 2010).

Furthermore, HO-1 gene and protein expression were significantly diminished in our female SAMP8 mice. Interestingly, long-lived Ames dwarf mice have significantly higher expression of HO-1 and impaired stress response compared with non-mutant littermate controls (Sun et al. 2011). In male SAMP8, on the other hand, HO-1 mRNA levels were higher than in SAMR1 mice (Cuesta et al. 2010; Forman et al. 2010). Differences between our findings and findings in the literature may hence be due to gender-related differences. It has been shown that estrogen levels, which partly control HO-1 gene expression (Yu et al. 2011), are lower in SAMR1 vs SAMP8 mice (Yuan et al. 2005). HO-1 gene expression is also controlled by the redox-regulated transcription factor Nrf2 (Satoh et al.

2006). We have previously shown that ascorbic acid downregulates Nrf2-dependent HO-1 gene expression in cultured hepatocytes (Wagner et al. 2011). Thus, the decrease in HO-1 gene expression in our SAMP8 mice could be partly due to the elevated ascorbic acid levels in the liver of these mice. Present data suggest that SAMP8 mice may increase hepatic ascorbic acid uptake via SVCT1 possibly as an adaptive response mechanism in order to combat oxidative stress. Accordingly, Amano et al. (2010) showed that hepatic SVCT1 gene expression is upregulated in SMP30 mice which was accompanied by increased liver ascorbic acid concentrations, suggesting an enhanced ascorbic acid uptake by hepatic cells. Interestingly, in mice that selectively overexpress lenticular vitamin C transporters, an increased tissue vitamin C concentration was evident, which was associated with increased protein damage (Fan et al. 2006). Increased tissue concentrations of ascorbic acid, as observed in the present study, may in turn affect Nrf2-dependent gene expression thereby impairing stress response via enzymatic antioxidant defence mechanisms. Ristow et al. (2009) have recently demonstrated that ascorbic acid may interfere with physical exercise-related induction of the Nrf2 target genes superoxide dismutase and glutathione peroxidase in humans.

In this study we observed higher α -tocopherol concentrations in the livers of SAMP8 than SAMR1 mice. ESR studies suggest that the tocopheroxyl radical can be regenerated by ascorbic acid by a proton transfer mechanism (Scarpa et al. 1984; Frank et al. 2006). Thus, the higher vitamin E levels in SAMP8 mice, as observed in this and other studies, may be explained by a vitamin E-sparing effect facilitated by the elevated ascorbic acid levels. Despite higher dietary vitamin E supply, SAM mice (150 mg tocopherol/kg diet) exhibited lower liver vitamin E concentrations as compared to C57BL/6 mice (20 mg tocopherol/kg diet). Differences in liver vitamin E concentrations between SAM and C57BL/6 mice may be partly related to differences in dietary cholesterol intake (0.15 % in SAM mice vs 1.25 % in C57BL/6 mice) known to modulate vitamin E bioavailability (Jula et al. 2002).

A decrease in the metabolism and urinary excretion of the vitamin would be another potential explanation for the increased hepatic vitamin E concentrations

(Mueller et al. 2005). α -Tocopherol is metabolized to α -CEHC in the liver and then excreted via urine (Brigelius-Flohé and Traber 1999). Since both SAMP8 and SAMR1 mice exhibited similar hepatic α -CEHC concentrations, differences in liver α -tocopherol concentrations between SAMP8 and SAMR1 mice do not seem to be related to differences in α -tocopherol metabolism. In accordance to our mouse data, Brigelius-Flohé et al. (2004) also did not observe an effect of age on vitamin E metabolism, as determined by plasma α -CEHC concentration in humans.

Conclusion

In summary, the present data suggest that SAMP8 mice exhibit elevated hepatic lipid peroxidation and protein oxidation, lower proteasomal and PON-1 activity and HO-1 expression compared to SAMR1 mice. SAMP8 mice are further characterized by increased biomarkers of oxidative stress and chronic inflammation, and an impaired stress response. SAMP8 as compared to SAMR1 mice exhibit elevated hepatic ascorbic acid and α -tocopherol concentrations. Our data in SAMP8 vs SAMR1 mice could be partly confirmed in aged vs young C57BL/6 mice.

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