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ORIGINAL CONTRIBUTION

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Diet restriction in mice causes differential tissue responses in total reducing power and antioxidant compounds

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Summary *Background:* Diet restriction (DR) has been shown to extend the life spans of various laboratory animals, the mechanism may involve a decrease in oxidative stress. When determining if the total tissue defense has been altered, it is important to observe the overall direct antioxidant capacity, which consists of low molecular weight antioxidants (LMWA) and enzymes.

Aim: To determine DR induced changes in total reducing power and overall direct antioxidant capacity of various mouse tissues.

Methods: Young female Sabra mice were fed a 60 % food restricted diet for 40 days (DR group). Organs of the DR group and of ad libitum (AL) fed controls were then dissected and examined. A cyclic voltammetry method was used to quantify the total reducing power, which correlates with the overall LMWA activity. Specific LMWA were identified by HPLC-ECD. Superoxide dismutase activity and H_2O_2 degrading ability were measured in order to include the enzymatic antioxidant component.

Results: Short-term DR caused alterations in the total reducing power of various mouse tissues, indicating changes in the total scavenging ability of these tissues. Overall direct antioxidant capacity of heart, kidney and muscle was enhanced; liver and small intestine deteriorated; brain did not differ between DR and AL groups; lung and spleen exhibited a mixed response.

Conclusions: We have shown for the first time that DR causes changes in the total reducing power of different mouse tissues, thus, affecting the overall direct antioxidant capacity. These findings support the suggestion that there may be a biological regulation of the antioxidant system.

Key words Mouse – diet restriction – cyclic voltammetry – reducing power – antioxidants

Introduction

Diet restriction (DR) has been shown to extend the life span of various laboratory animals, including rodents [1,2]. One of the possible reasons suggested is a decrease in oxidative stress, inflicted by the ongoing generation of reactive oxygen species (ROS) [3]. Many researchers have measured a lower oxidative stress in various organs in diet restricted animals, as opposed to ad libitum (AL) food consuming controls [4–7]. A variety of changes have been observed regarding specific components of the antioxidant system in different tissues [4,5,8]. However, when trying to determine if the total tissue defense has altered, it is important to observe the overall direct antioxidant capacity, meaning all molecules capable of scavenging and neutralizing ROS. This is due to the way in which the antioxidant system combats ROS. The low molecular weight antioxidants (LMWA), namely ascorbate, uric acid, glutathione, vitamin E, coenzyme Q_{10}, lipoic acid and many more, act synergistically by regenerating each other [9–12] and by

working in concert against a wide array of ROS in various forms [10,13,14]. Additionally, there are numerous substances with antioxidative ability, that are usually not measured in relation to DR, such as NADH, lipoic acid, carnosine, bilirubin and others. Because all of these LMWA act as a group, it is necessary to examine the total antioxidant status when looking for any manipulation-inflicted changes in the antioxidant defense system.

A direct and convenient method for this purpose is by use of cyclic voltammetry (CV). The CV profile allows for the detection of various reducing agents present in a biological sample [15,16]. It can provide information concerning the type and overall concentration of LMWA. Since most LMWA are reducing agents, the amount of these substances reflects the tissue's overall antioxidative ability. Because CV cannot identify specific materials, additional identification was achieved by use of HPLC-ECD. In order to complete the picture and include the enzymatic component of the antioxidant system, superoxide-eliminating ability and H_2O_2-degrading ability were also measured.

The purpose of this study was to address the question whether short-term DR causes changes in the total antioxidant capacity of various tissues. The total reducing power combined with the enzymatic assays allow for this overview.

Materials and methods

Animals, diet and tissue preparation

Four-month-old female Sabra mice were assigned to a DR group or an AL group, 12 animals in each group. Both cages were placed in a temperature-controlled room at 22 °C with a 12-h light/dark cycle (lights on at 0700). The food provided was Purina Chow. DR animals received a measured amount of 2.16 g/(day · mouse), equivalent to 57 kcal/(week · mouse), which is about 60 % of the daily requirement for their initial weight [17]. AL diet animals had free access to food at all times. Both groups could drink water at all times. After 40 days, animals were killed by decapitation. Blood was collected into heparin coated testtubes (Monoject, USA), and brain, heart, lungs, spleen, liver, small intestine, kidneys and quadriceps muscle were immediately dissected and frozen in liquid nitrogen. Organs were stored at –70 °C until homogenization. Blood was centrifuged at 1000g for 10 minutes, and the separated plasma was kept at –70 °C until analysis. Tissue samples were homogenized in 3 ml of PBS, pH of 7.4, at 4 °C. This was the minimal volume of buffer which was sufficient for all tests. Two mL was used for water-soluble cyclic voltammetry, and for lipophilic LMWA extraction. The remaining part of the homogenate served for HPLC-ECD analyses, enzymatic analyses and protein determination. The lipophilic extraction was performed by adding 2 mL of sample to 7.5 mL of methanol:hexane (1:4), mixing thoroughly, and centrifugation at 1000g for 10 minutes. The upper and lower layers were separated, and the organic solvents were removed by evaporation. The residue was dissolved in acetonitrile:methanol (1:1), containing 1 % tetrabutyl ammomium perchlorate as an ion pairing agent. Samples were then subjected to lipophilic CV and HPLC-ECD analyses.

All materials were purchased from Sigma Chemical Co. (St. Louis, MO), all solvents were analytical grade. The experimental protocol was approved by the animal facility review board of the Hebrew University, Hadassah medical school.

Cyclic voltammetry (CV)

These analyses were carried out using a BAS model CV–1B cyclic voltammeter (West Lafayette, IN). An example of a typical cyclic voltammogram, of mouse small intestine, is shown in Fig. 1. Cyclic voltammograms were recorded at a range of 0–1.3 V, at a rate of 100 mV/s, versus an Ag:AgCl reference electrode. The working electrode was a glassy carbon disk (BAS MF–2012), 3.2 mm in diameter, which had been polished prior to each measurement with a polishing kit (BAS PK–1). A platinum wire served as the counter electrode. CV tracings were analyzed to determine the peak potential (Ep(a)) and the anodic current (Ia). The peak potential used for describing the anodic waves is half the increase in the current of the peak ($E_{1/2}$). This potential is determined by its LMWA components and represents their ability to donate electrons to the working electrode. Differences between anodic waves (>50 mV difference in $E_{1/2}$) result from different LMWA oxidation potentials consisting of these waves. The anodic current correlates with the concentration of the LMWA constituents. A decrease in the amplitude of a wave is mostly due to a decrease in the concentration of its components, and an increase in amplitude implies an increase in their levels. PBS itself does not show any anodic waves up to 1.3 mV. At least three recordings were performed for each sample, verifying that repeated scans showed similar CV tracings. The minimum concentration of a reducing substance that can be identified by CV is 1μM. This enables us to measure only LMWA at their physiological levels and not other reductants such as free metal ions, which are present in much lower concentrations.

HPLC-ECD

Identification and measurement of specific LMWA was carried out, in order to explain the changes in CV waves and to allow for assessment of some specific agents [18]. The HPLC system (Kontron Instrument 320, Zurich, Switzerland) was equipped with an electrochemical de-

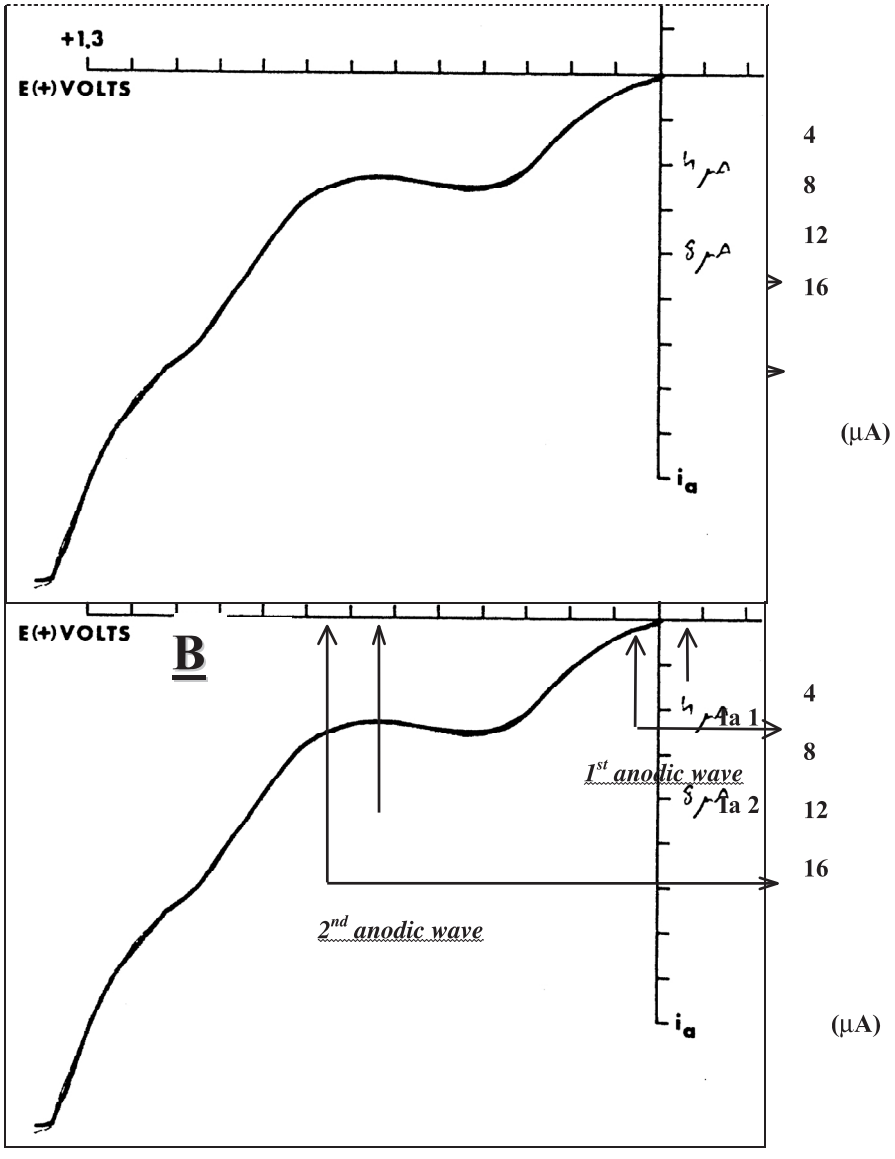

Fig. 1 A typical cyclic voltammogram of mouse small intestine homogenate. **A** hydrophilic LMWA, **B** lipophilic LMWA. Two anodic waves are seen for each medium. $Ep_{(a)}$ is the peak potential, and $E_{1/2}$ is the potential at half the increment of the wave. Both are determined from the *x*-axis and correlate with the type of LMWA present in the wave. Ia is the anodic current, which correlates with the concentration of the LMWA in the wave, and is determined from the *y*-axis.

tector (BAS CC–5, West Lafayette, IN). Water-soluble samples had been treated with 25 % TCA to precipitate out proteins, and a 20μL deproteinized sample was injected into the system. The ECD potential was set to 500 mV to allow detection of ascorbate and uric acid, the major constituents of the first water-soluble anodic wave detected by the CV [19]. The ECD potential of the lipophilic assay was also set to 500 mV. The hydrophilic mobile phase contained 40 mmol/L sodium acetate buffer, pH 4.75, 0.54 mmol/L Na_2EDTA and 1.5 mmol/L tetrabutyl ammonium hydroxide. The lipophilic mobile phase contained 20 mmol/L lithium perchlorate in methanol:ethanol:isopropanol (22.5:73.6:3.9) and enabled identification of α-tocopherol and coenzyme Q_{10}. Separations were performed at a flow rate of 1 mL/min, with a sensitivity of 50 nA, and recorded on a PC-based data acquisition and processing system. Ascorbate, uric acid and tocopherol peaks were identified and quantified by standard curves. Coenzyme Q_{10} results are expressed as the area under the curve (AUC).

SOD activity

Superoxide dismutase activity was measured by the method of McCord [20]. Accumulation of reduced cytochrome was recorded for 3 minutes using a Uvikon 933 spectrophotometer by absorption at a wavelength of 550 nm. The slope obtained was translated into SOD activity by standard SOD curves prepared each day. One unit of

SOD activity is defined as the amount that will inhibit the oxidation of cytochrome C by 50 % in a hypoxanthine/xanthine oxidase coupled system.

Catalase-like activity

Total H_2O_2 removing ability was measured using the method of Aebi [21]. This method has been selected because it measures the total ability of a tissue to remove H_2O_2. Measuring one enzyme alone, GPx for example, could not predict this ability [22], as it may sometimes prove less important. H_2O_2 degradation was measured spectrophotometerically (Uvikon 933) by monitoring absorption at 240 nm for 3 minutes. A sample (20–40µL; varying upon tissue) was added to a cuvette containing 14 mmol/L H_2O_2. Activity was calculated by comparison with a catalase standard curve, and therefore the results are expressed as catalase activity, even though additional peroxidases are present in the tissue samples. One unit of catalase activity is defined as the amount of enzyme required to decompose 1 µmol of H_2O_2 in 1 minute.

Protein determination

Performed by the method of Bradford [23], the protein amounts measured were used for expressing enzymatic activity or LMWA concentrations per amount of tissue present in the samples. 30µL sample was added to 1 mL Bradford reagent. Absorption at 595 nm was recorded by a spectrophotometer (Uvikon 933) and was translated into mg of protein using bovine serum albumin standard curves.

Statistical analysis

Results are expressed as mean ± SD of 8–12 animals per group. For display purposes, error bars on graphs are presented as mean ± SEM. A two-tailed *t*-test was used for comparison between DR and AL groups, with a level of significance $P<0.05$. Two ratios were calculated, which represent systems working in synergism. An ascorbate:tocopherol ratio represents the ability of ascorbate to regenerate tocopherol [9]. The higher the ratio, the more ascorbate molecules present per α-tocopherol, and hence the chance of regeneration is increased. A catalase-like activity:SOD units ratio assesses the ability to remove H_2O_2 which also originates from the action of SOD. The higher the ratio, the more H_2O_2 produced by SOD will be eliminated. This is of importance, as these two systems should work together for better antioxidant defense [24–26]. Even though SOD is considered to be an antioxidant enzyme, an increase in SOD activity alone produces a higher flux of H_2O_2 [26] and could prove lethal to a living cell [25].

Results and discussion

Animal weight

At the beginning of the experiment, AL and DR group weights did not differ significantly (DR:39 ± 2.2 g; AL:40 ± 3.9 g). At the end of 40 days of dietary restriction to the DR group, weights were 29 ± 2.9 g for DR group and 44 ± 5.8 g for AL group ($P<0.00001$). AL weights did not differ significantly between the beginning and end of the experiment (40 ± 3.9 g vs. 44 ± 5.8 g, $P=0.08$).

Brain

Brain weights did not differ significantly between groups (DR:458 ± 28 mg; AL:480 ± 46 mg). CV analysis of whole brain homogenates revealed two water-soluble LMWA groups ($E_{1/2}$ of 340 mV and 810 mV) and two lipid-soluble LMWA groups ($E_{1/2}$ of 235 mV and 895 mV). HPLC-ECD demonstrated the presence of ascorbate, uric acid, α-tocopherol and coenzyme Q_{10}. SOD and catalase-like activities were present in the homogenate. None of the measured parameters or ratios differed between the DR and AL groups.

These results show that the brain is a very conservative organ regarding the antioxidant system. It is not easily affected by short-term DR. A previous study employing cyclic voltammetry has shown the LMWA levels to remain stable throughout the aging process [15]. We can therefore anticipate that DR, which is believed to slow the aging process, will not affect the overall antioxidant status in the brain. Sohal et al have also shown that brain enzymatic activities remain stable following DR, at this same age of mice [5]. Furthermore, ROS production was shown to be lower in these samples, following DR. It could therefore be assumed that short-term DR affects the mouse brain by lowering ROS production without changing the total direct antioxidant capacity.

Lung

Lung weights did not differ between groups (DR:214 ± 58 mg for both lungs; AL:246 ± 67 mg). CV analysis of whole lung homogenates revealed two water-soluble LMWA groups ($E_{1/2}$ of 367 mV and 817 mV) and one lipid-soluble LMWA group ($E_{1/2}$ of 790 mV), HPLC-ECD demonstrated the presence of uric acid and α-tocopherol (Fig. 2). Ascorbate and coenzyme Q_{10} levels were low and detectable in only a few samples, not allowing for statistical analysis. All results are shown in Tables 1 and 2. The first water-soluble anodic wave was 40 % smaller in the DR group than in the AL group. Uric acid levels tended to be lower in the DR group than in the AL group. The lipid-soluble anodic wave was 40 % larger in the DR group, whereas α-tocopherol levels were 50 % smaller. SOD and

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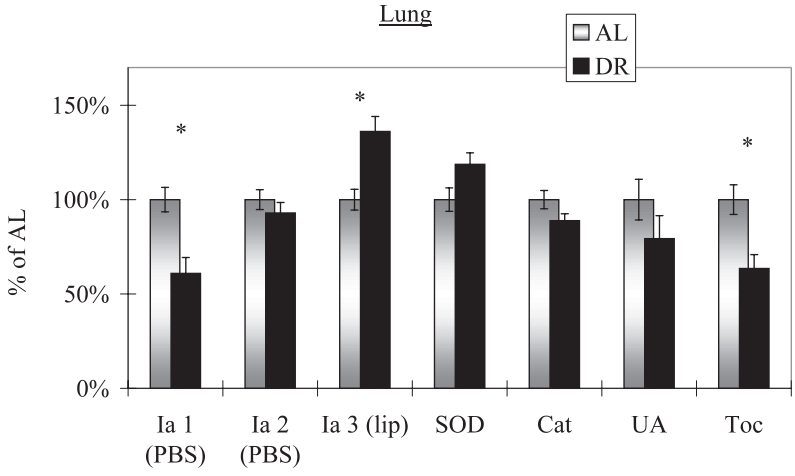

Fig. 2 Differences in antioxidant parameters in DR versus AL lungs: values are percent of AL control, and are expressed as mean ± SEM. Significant differences ($P< 0.05$, for exact value see text) are marked by an asterisk. The single lipophilic anodic wave is termed 'Ia 3' due to its relatively high $E_{1/2}$ (~790 mV). (*DR* diet restricted; *AL* ad libitum feeding controls; *CV* cyclic voltammeter; *LMWA* low molecular weight antioxidants; *Ia 1* first anodic CV wave; *Ia 2* second anodic CV wave; *Ia 3* third anodic CV wave; *SOD* superoxide dismutase activity; *Cat* catalase-like activity; *Asc* ascorbate; *UA* uric acid; *Toc* α-tocopherol; Q_{10} Coenzyme Q_{10}).

catalase-like activities did not differ between groups in a statistically significant matter. However, the catalase:SOD ratio was 25 % lower in the DR group (16 ± 4 vs. 21 ± 4, $P<0.01$).

Most differences observed between groups in the lungs occurred in the LMWA system. The slight decline in uric acid levels could be reflected in the reduction of the first anodic wave, of which uric acid and ascorbate are major constituents [16]. In this regard, it seems that antioxidant activity in the hydrophilic medium of the lung tissue has weakened. On the other hand, the lipid-soluble anodic wave was larger in the DR group, mostly indicating an increase in its LMWA constituents. The decline in α-tocopherol levels in the DR group could be due to the smaller dietary intake of the vitamin by DR animals. It could also imply lower tissue needs due to DR, which leads to a lowering of oxidative stress. The rise in the reducing agents as revealed by CV is probably more important than the decline in a single LMWA mλ α-tocopherol in this instance.

In general, it seems that short term DR lowered the antioxidant capacity in the hydrophilic medium of the mouse lung, but enhanced that of the lipophilic medium.

Heart

Hearts were smaller in the DR group (125 ± 22 mg vs. 167 ± 46 mg for AL, $P<0.015$). CV analysis of whole heart homogenates revealed three water-soluble LMWA groups ($E_{1/2}$ of 430 mV, 710 mV and 880 mV) and three lipid-soluble LMWA groups ($E_{1/2}$ of 210 mV, 340 mV and 755 mV), HPLC-ECD demonstrated the presence of ascorbate, uric acid, α-tocopherol and coenzyme Q_{10} (Fig. 3). All results are shown in Tables 1 and 2. The first water-soluble anodic wave was 25 % smaller in the DR group as opposed to the AL group. Ascorbate levels were 25 % higher in this group, with a tendency for uric acid levels to be higher. α-Tocopherol levels did not differ between groups, and when ascorbate levels were taken into account, the ascorbate: α-tocopherol ratio was found to be 15 % higher in the DR group (4.02 ± 0.31 vs. 3.44 ± 0.56, $P<0.008$).

The presence of three LMWA groups in each medium, reflected by cyclic voltammetry as three anodic waves, allows the heart to inactivate a wide range of ROS effectively. In a previous cyclic voltammetry study, the first anodic wave was also seen in rat hearts, the third anodic wave was present only in old rat hearts, and the second wave was not observed [15]. This shows the advantage of CV methodology, which measures all physiologic LMWA at once. Measuring only specific LMWA would not have revealed this inter-species variation.

All changes observed in the heart occurred in the hydrophilic LMWA system, as the lipophilic and enzymatic systems remained unchanged. The first water-soluble anodic wave weakened, despite increases in its major components – ascorbate and uric acid. This can be explained by an interference of uric acid in the ascorbate electron delivery to the electrode, as uric acid is a weaker electron donor [19]. A high uric acid concentration is reflected in the $E_{1/2}$ of the first anodic wave (430 mV), which is very close to uric acid standards tested in our lab (420 ± 25 mV). In vivo, however, uric acid aids ascorbate by keeping it in the reduced state [14]. Therefore, the rise in ascorbate and uric

Table 1 Results for hydrophilic anodic currents (*Ia 1, Ia 2, Ia 3*), ascorbate (*Asc*), uric acid (*UA*), catalase-like activity (*Cat*) and SOD activity (*SOD*). Statistically significant differences have their *P* value below each result. *AL* ad libitum feeding controls, *DR* diet restricted group.

Tissue		Ia (1) (μM/mg prot)	Ia (2) (μM/mg prot)	Ia (3) (μM/mg prot)	Asc (μM/mg prot)	UA (μM/mg prot)	Cat (U/ ml)* mg prot)	SOD (U/ ml)* mg prot)
Lung	AL	1.04 ± 0.23	1.98 ± 0.36	–	–	11.7 ± 3.04	29 ± 5	1.37 ± 0.27
	DR	0.64 ± 0.19 (P< 0.0002)	1.84 ± 0.36	–	–	9.31 ± 2.37 (P= 0.06)	26 ± 3	1.62 ± 0.34
Heart	AL	0.26 ± 0.08	0.57 ± 0.10	1.17 ± 0.19	8.34 ± 1.29	21.7 ± 3.68	25 ± 5.07	4.55 ± 1.28
	DR	0.20 ± 0.04 (P<0.03)	0.60 ± 0.12	1.27 ± 0.21	10.4 ± 2.24 (P<0.015)	27.2 ± 7.81 (P=0.2)	24 ± 6.31	3.55 ± 1.13
Spleen	AL	0.55 ± 0.06	0.82 ± 0.10	1.40±0.20	7.65 ± 2.01	-	17.0 ± 4.51	0.19±0.04
	DR	0.38 ± 0.07 (P<0.0001)	0.65 ± 0.10 (P<0.0001)	1.47±0.32	4.05 ± 1.53 (P<0.0001)	–	10.0 ± 2.70 (P<0.0005)	0.18±0.04
Liver	AL	5.92 ± 0.97	–	20.0 ± 2.72	146 ± 38	15.3±4.53	582 ± 33	48 ± 10
	DR	1.70 ± 0.39 (P<0.0001)	–	13.6 ± 1.07 (P<0.0001)	45 ± 13 (P<0.0001)	15.6±2.71	536 ± 54 (P<0.04)	70 ± 30 (P<0.0004)
Intestine	AL	1.30 ± 0.16	–	2.28 ± 0.35	27 ± 6.84	22 ± 6.33	7.29 ± 0.99	0.47 ± 0.11
	DR	0.94 ± 0.21 (P<0.0007)	–	2.37 ± 0.56	13.2 ± 3.4 (P<0.0001)	20 ± 5.26	5.06 ± 1.42 (P<0.004)	0.59 ± 0.17
Kidney	AL	1.03 ± 0.20	9.93 ± 2.19	–	4.92 ± 1.31	–	55 ± 14	3.16 ± 0.91
	DR	1.01 ± 0.33	10.1 ± 2.97	–	7.65 ± 2.21 (P<0.006)	–	82 ± 21 (P<0.004)	3.51 ± 0.59
Muscle	AL	–	0.15 ± 0.04	0.36 ± 0.10	0.68 ± 0.05	–	3.31 ± 0.90	0.29 ± 0.05
	DR	–	0.15 ± 0.02	0.36 ± 0.06	0.74 ± 0.07 (P=0.2)	–	4.44 ± 1.39 (P=0.06)	0.29 ± 0.04

Table 2 Results for lipophilic anodic waves (*Ia1, Ia 2, Ia 3*), α- tocopherol (α-toc) and coenzyme Q_{10} (Q_{10}). Statistically significant differences have their *P* value below each result. *AL* ad libitum feeding controls, *DR* diet restricted group.

Tissue		Ia (1) (μA/mg prot)	Ia (2) (μA/mg prot)	Ia (3) (μA/mg prot)	α- toc (μM/mg prot)	Q_{10} (μM/mg prot)
Lung	AL	-	-	2.09 ± 0.38	2.95 ± 0.78	-
	DR	-	-	2.85 ± 0.72 (P<0.02)	1.87 ± 0.66 (P<0.002)	-
Heart	AL	0.49 ± 0.12	0.66 ± 0.14	1.28 ± 0.46	2.45 ± 0.35	2.93 ± 0.84
	DR	0.63 ± 0.23	0.73 ± 0.10	1.49 ± 0.42	2.63 ± 0.68	2.48 ± 0.75
Spleen	AL	0.19 ± 0.07	0.08 ± 0.04	1.44 ± 0.34	1.44 ± 0.26	3.7 ± 0.86
	DR	0.11 ± 0.04 (P<0.01)	0.12 ± 0.02 (P<0.02)	1.47 ± 0.33 (P<0.04)	1.42 ± 0.32	4.81 ± 1.10
Liver	AL	2.03 ± 0.50	-	23 ± 6.47	17.4 ± 4.22	-
	DR	0.95 ± 0.18 (P<0.0001)	-	25 ± 5.72	8.09 ± 2.36 (P<0.0004)	-
Intestine	AL	0.64 ± 0.11	0.47 ± 0.13	1.54 ± 0.81	1.80 ± 0.15	7.03 ± 2.32
	DR	0.23 ± 0.06 (P<0.0001)	0.24 ± 0.03 (P<0.001)	1.81 ± 0.66	1.50 ± 0.18 (P<0.006)	8.20 ± 2.30
Kidney	AL	0.38 ± 0.10	-	5.60 ± 1.49	5.76 ± 1.60	14.7 ± 4.15
	DR	0.30 ± 0.12	-	6.27 ± 1.56	7.44 ± 1.92 (P<0.05)	21 ± 8.46 (P<0.03)
Muscle	AL	0.21 ± 0.07	-	0.76 ± 0.19	1.26 ± 0.19	-
	DR	0.25 ± 0.05	-	0.83 ± 0.10	1.47 ± 0.20 (P<0.02)	-

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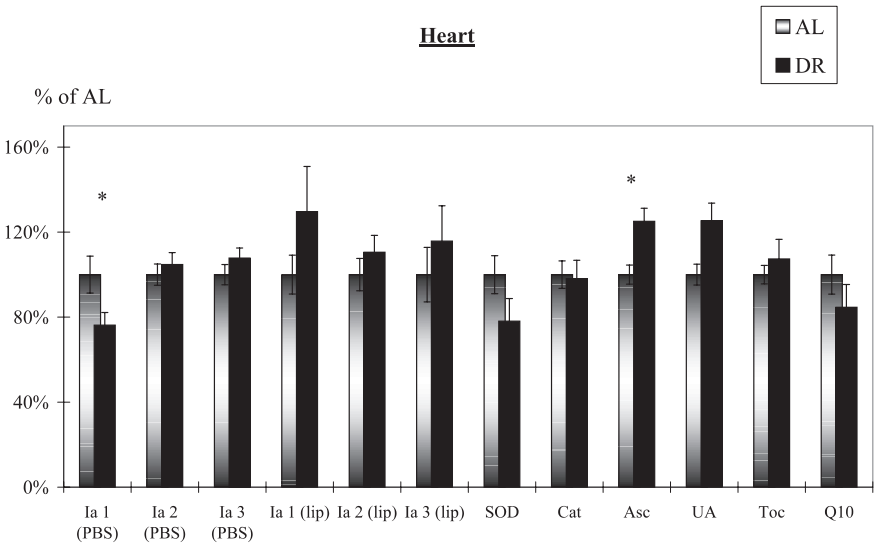

Fig. 3 Differences in antioxidant parameters in DR versus AL hearts. Values are percent of AL controls and are expressed as mean ± SEM. Significant differences ($P< 0.05$, for exact value see text) are marked by an asterisk. For abbreviations see Fig. 2.

acid concentrations, despite a lower CV wave, is beneficial in terms of antioxidant defense. Another aspect of the higher ascorbate level in the DR group is the increased ability to recycle membrane α-tocopherol. This is shown by the higher ascorbate to λ : α-tocopherol ratio in the DR group. Therefore α-tocopherol is somewhat aided by a better recycling of the tocopheryl radicals by ascorbate. No significant changes were observed for the enzymatic system. Previous studies regarding the antioxidant enzymes in heart have shown various changes in DR versus AL animals. Xia et al. described a lower SOD activity and increased catalase activity [4], Sohal et al. measured an unchanged SOD activity and a lower catalase activity [5], whereas Kim et al. showed an increased cytosolic SOD activity, an increased GPx activity and unchanged catalase activity [27]. These different results are probably due to different research protocols (e. g., different age, diet composition, or type of animal). The SOD and catalase activities in heart require further investigation.

In conclusion for the changes in mouse heart antioxidant capacity following short-term DR, it seems that the hydrophilic medium features a better direct antioxidant defense. There is no significant change in lipophilic antioxidative ability, which is probably partially enhanced by the rise in hydrophilic LMWA.

Spleen

Spleens were much smaller in the DR group (86 ± 14 mg, 156 ± 38 mg for AL, $P<0.00002$). CV analysis of whole spleen homogenates revealed three water-soluble LMWA groups ($E_{1/2}$ of 345 mV, 670 mV and 820 mV) and three lipid-soluble LMWA groups ($E_{1/2}$ of 150 mV, 370 mV and 790 mV), HPLC-ECD demonstrated the presence of ascorbate, α-tocopherol and coenzyme Q_{10} (Figure 4). Uric acid concentration was undetectable. All results are shown in Tables 1 and 2. The first water-soluble anodic wave was 30 % smaller in the DR group as opposed to the AL group, while ascorbate levels were 50 % smaller. The second water-soluble anodic wave was 20 % smaller in the DR group as opposed to the AL group. The lipid-soluble anodic waves also exhibited significant changes. The first wave was 40 % smaller in the DR group, while the second was 50 % larger than AL controls. α-Tocopherol levels were unchanged, but the ascorbate:tocopherol ratio was lower by 55 % in the DR group (2.64±0.75 vs. 5.88±1.85, $P<0.001$). Coenzyme Q10 levels were 30 % larger in the DR group. Catalase-like activity was 40 % lower in the DR group, and the catalase:SOD ratio was consequently 50 % lower in this group (48 ± 9 vs. 104 ± 32, $P<0.0002$).

The first water-soluble anodic wave was weaker in the DR group this time due to a lower ascorbate level. Combining the weaker second anodic wave and the lower catalase-like activity in DR spleens, it appears that the hydrophilic medium has a reduced direct antioxidant capacity as opposed to AL controls. Ascorbate, catalase and other peroxidases are extremely important in scavenging and neutralizing ROS. In the lipophilic medium, the decline in the first lipid-soluble anodic wave seems to be compensated by an increase in the second anodic wave and Q_{10} levels. Previous researchers have shown interactions between various lipid-soluble antioxidants, where the addition or subtraction of one substance influences another [28–30].

In conclusion for the changes brought about by short term-DR, it appears that the hydrophilic medium has a lower direct antioxidant defense in DR spleens, while the lipophilic medium features a mixed response. It is possible that some of the lipophilic changes are compensatory to

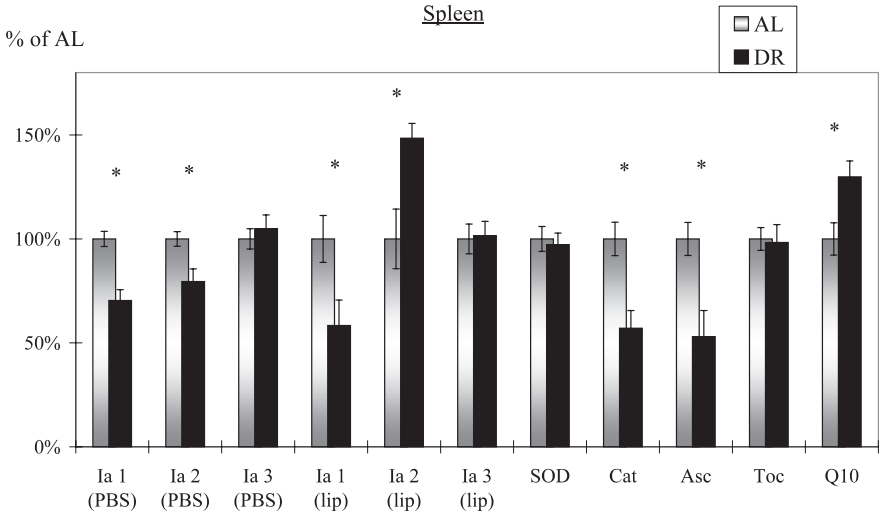

Fig. 4 Differences in antioxidant parameters in DR versus AL spleens. Values are percent of AL controls and are expressed as mean ± SEM. Significant differences ($P< 0.05$, for exact value see text) are marked by an asterisk. For abbreviations see Fig 2.

others, by using endogenously synthesized substances, such as coenzyme Q_{10}.

Liver

CV analysis of whole liver homogenates revealed two water-soluble LMWA groups ($E_{1/2}$ of 405 mV (DR) / 340 mV (AL), and 885 mV) and two lipid-soluble LMWA groups ($E_{1/2}$ of 240 mV and 905 mV), HPLC-ECD demonstrated the presence of ascorbate, uric acid and α-tocopherol (Fig. 5). Coenzyme Q_{10} levels were low and detectable in only a few samples, not allowing for statistical analysis. All results are shown in Tables 1 and 2. The first water-soluble anodic wave was 70 % smaller in the DR group, with ascorbate levels also measuring 70 % smaller than AL controls. Uric acid levels did not differ between groups. There is also a difference in the $E_{1/2}$ of this first wave between the two groups (405 ± 41 mV vs. 339 ± 14 mV, $P<0.0001$). The second water-soluble anodic wave was 30 % smaller in the DR group. The first lipid-soluble anodic wave was 50 % smaller in the DR group, and α-tocopherol levels were also 50 % smaller. SOD activity was 50 % higher in the DR group, and catalase-like activity was 10 % lower; therefore the catalase:SOD ratio was 45 % lower in the DR group (70 ± 22 vs. 124 ± 29, $P<0.002$).

An overview of the changes that occurred in the liver discloses that most LMWA levels declined. The first water-soluble anodic wave weakened sharply, probably due to the decline in ascorbate concentrations. Lower ascorbate levels following short-term DR have been recorded previously [31]. The decline in ascorbate concentration, in the face of a steady level of uric acid, caused a change in the composition of the first anodic wave. Hence, the wave has an $E_{1/2}$ closer to the uric acid standard. In comparison to rat liver homogenate [15], it seems that the mouse liver voltammogram is similar in shape, but somewhat “shifted" to the right (i. e., the potentials of both waves in mice are lower by about 100 mV). This indicates a difference in the relative composition of these waves. It is interesting that a LMWA group seen in many organs ($E_{1/2}$ of 700–800 mV) is missing in all liver and small intestine samples. This is not due to insufficient sample size, as these were the largest of all organ samples. Regarding coenzyme Q_{10}, a previous study showed no change in liver Q_{10} levels following DR, and also showed an increase in the coenzyme levels following vitamin E deficiency [30]. This could be another example of compensation by an endogenous antioxidant to a dietetic deficiency. The increase in SOD activity lowered the catalase:SOD ratio to half the value of the AL controls. An increase in SOD activity alone produces a higher flux of H_2O_2 [26]. In the presence of a 10 % decline in peroxidative ability, we cannot say under these experimental conditions whether this ability was sufficient to deal with the increased flux of H_2O_2. For if it is not, then this is a not a better antioxidant system, as the excess H_2O_2 will not be eliminated. Other researchers have shown unchanged activities for SOD, catalase or GPx following short-term DR [4,31,32], but lower levels of gene transcription [33]. Most of these nutritional protocols included vitamin supplementation to the DR animals, which could influence the enzyme gene expression, translation or activity. Long-term DR has been shown to maintain higher enzymatic gene transcription levels throughout aging as opposed to AL controls [34].

We therefore conclude that short-term DR could lower the antioxidative capacity of the mouse liver by reducing LMWA levels and possibly enhancing H_2O_2 production by SOD without sufficient peroxidative ability. This does not necessarily mean that the liver suffers from oxidative

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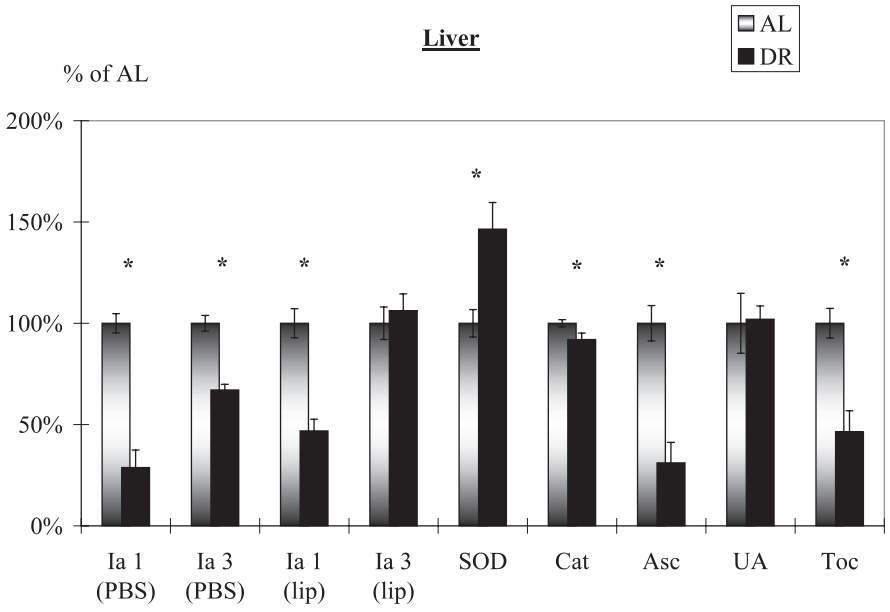

Fig. 5 Differences in antioxidant parameters in DR versus AL livers. Values are percent of AL controls and are expressed as mean ± SEM. The second anodic waves are termed 'Ia 3' due to their relatively high $E_{1/2}$ (~900 mV for both hydrophilic and lipophilic waves). Significant differences ($P< 0.05$, for exact value see text) are marked by an asterisk. For abbreviations see Fig. 2.

stress. A lower rate of ROS production has been recorded previously [4].

Small intestine

CV analysis of small intestine homogenates revealed two water-soluble LMWA groups ($E_{1/2}$ of 495 mV and 940 mV) and three lipid-soluble LMWA groups ($E_{1/2}$ of 210 mV, 409 mV and 905 mV); HPLC-ECD demonstrated the presence of ascorbate, uric acid and α-tocopherol (Fig. 6). All results are shown in Tables 1 and 2. The first water-soluble anodic wave was 30 % smaller in the DR group, with ascorbate levels being 50 % smaller. The first lipid-soluble anodic wave was 65 % smaller in the DR group, the second was 50 % smaller and the third remained unchanged. α-Tocopherol levels were 20 % lower, and the ascorbate:tocopherol ratio was 40 % lower (9.01 ± 2.38 for DR, vs. 15.34 ± 4.65 for AL, $P<0.005$). Catalase-like activity decreased by 30 % in the DR group, therefore decreasing the catalase:SOD ratio by 40 % in this group (9 ± 2 vs. 15 ± 3, $P<0.001$).

The intestinal response to short-term DR is somewhat similar to the liver. A lower first water-soluble anodic current is associated with and probably due to lower ascorbate levels. As seen in the liver, a group of LMWA that constructs the "second" water-soluble anodic wave ($E_{1/2}$ of 700–800 mV) in other tissues tested is missing. The smaller lipid-soluble anodic waves could imply lower levels of their lipid-soluble constituents. The second lipid-soluble anodic wave, which showed a strong decline, was not detected in the liver tissue. Smaller α-tocopherol levels would add to the reduction of the lipophilic antioxidant defense. There is a lower catalase-like activity, which contributes to the reduction of the antioxidative capacity in the small intestine. The catalase:SOD ratio is similar to that in the liver, even though of a different major cause – the attenuation of peroxidative ability.

The small intestine is an organ that is directly affected by the reduction of food quantity, as is the liver, for these organs need to perform less work. As other body organs, they are also exposed to the smaller caloric accessibility. With aging, the small intestine becomes less efficient in means of absorption and transportation of nutrients [35,36], and long-term DR attenuates this loss. DR also causes morphologic changes in the mucosa [36], which might suggest for a direct effect of the smaller food amount.

The overall picture of the small intestine response to short-term DR seems to be a reduction in the direct antioxidative capacity, both enzymatic and LMWA systems being affected. Once again, this does not necessarily mean that the small intestine is prone to oxidative stress following DR. The smaller workload needed by the small intestine will require less metabolism by the cells and may therefore generate less ROS.

Kidney

Kidneys were smaller in the DR group (342 ± 30 mg for both kidneys, 443 ± 59 mg for AL, $P<0.00005$). CV analysis of whole kidney homogenates showed two water-soluble LMWA groups ($E_{1/2}$ of 315 mV and 765 mV) and two lipid-soluble LMWA groups ($E_{1/2}$ of 150 mV and 845 mV); HPLC-ECD demonstrated the presence of ascorbate, α-to-

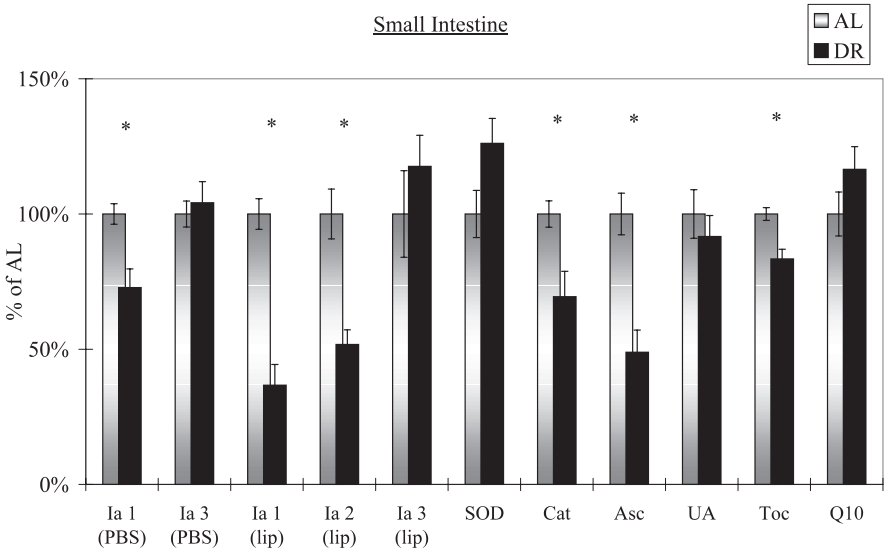

Fig. 6 Differences in antioxidant parameters in DR versus AL small intestine. Values are percent of AL controls and are expressed as mean ± SEM. The second hydrophilic anodic wave is termed 'Ia 3' due to its relatively high $E_{1/2}$ (~940 mV). Significant differences ($P< 0.05$, for exact value see text) are marked by an asterisk. For abbreviations see Fig. 2.

copherol and coenzyme Q_{10} (Fig. 7). All results are shown in Tables 1 and 2. Ascorbate levels were 55 % higher, and α-tocopherol levels were 30 % higher in the DR group. Combining these two increases raised the ascorbate:tocopherol ratio only by 10 % in the DR group (0.44 ± 0.05 vs. 0.40 ± 0.05, $P<0.05$). Coenzyme Q_{10} levels were 50 % higher in the DR group. Catalase-like activity was 50 % greater in the DR group, but calculation of the catalase:SOD ratio showed no statistically significant change, due to a slight increase in SOD activity.

The changes observed in the kidney all point to a better antioxidative capacity, but without altering the total reducing power. As the total reducing power reflects the overall LMWA activity, it may be that, while there is a need for specific LMWA, the total reducing power remains unchanged. The higher ascorbate levels found in DR kidneys could enhance ROS scavenging ability, and also affect the cell membranes by better recycling of vitamin E. Cadenas et al. have shown a strong decline in ascorbate levels following DR [8]. However, the supplementation of vitamins to the DR animals could mitigate the endogenous production of ascorbate and eventually result in lower levels. There is no change in the first water-soluble anodic wave, despite the strong decline in ascorbate levels. This could imply another important constituent of this wave, other than ascorbate or uric acid. The $E_{1/2}$ of this wave is 310–320 mV, which is close to ascorbate standards, therefore suggesting low uric acid levels. Catalase-like activity was higher in the DR group, and when adding the higher ascorbate, α-tocopherol and coenzyme Q_{10} levels, we conclude that short-term DR increased the direct antioxidative capacity of the kidney tissue. It is interesting to review the changes following long-term DR, with GPx as an example. Adding the data of three previous studies, at the age of 4 months GPx activity is higher in a DR group [8], at 6 months the activity is similar to AL controls [4], and at 9 months it is lower [5]. Only at 9 months of age has the overall peroxidative ability risen (as seen by H_2O_2 removal in [5]), and perhaps the specific activity of GPx was no longer needed. We therefore see that the exact age of the animal is of crucial importance when comparing data.

Muscle

CV analysis of quadricep muscle homogenates revealed two water-soluble LMWA groups ($E_{1/2}$ of 710 mV and 870 mV); two lipid-soluble LMWA groups ($E_{1/2}$ of 150 mV and 800 mV) were measured (Fig. 8). HPLC-ECD demonstrated the presence of ascorbate and uric acid in only a few samples, probably due to small sample size and low concentrations. This is also reflected in the CV tracing, which shows a first water-soluble anodic wave only in AL samples ($E_{1/2}$ of 455 mV). All results are shown in Tables 1 and 2. α-Tocopherol levels were 15 % higher in the DR group, but the ascorbate:tocopherol ratio remained unchanged, due to a slight increase in ascorbate concentrations. Catalase-like activity was 35 % higher in the DR group.

In the quadricep muscle, there are two interesting changes. One is the disappearance of the first water-soluble anodic wave in the DR group, despite a possible increase in ascorbate concentrations. This wave appeared in almost all AL samples, so it is possible that the amount of DR tissue samples was too small in order to obtain sufficient LMWA concentrations. It is interesting that in a previous CV study of rat muscle, a different pattern appeared – a single water-soluble anodic wave at a much higher $E_{1/2}$ (~1050 mV, which could account for a histidine related compound – carnosine or anserine) [15], thus indicating another inter-species difference. In general, we can con-

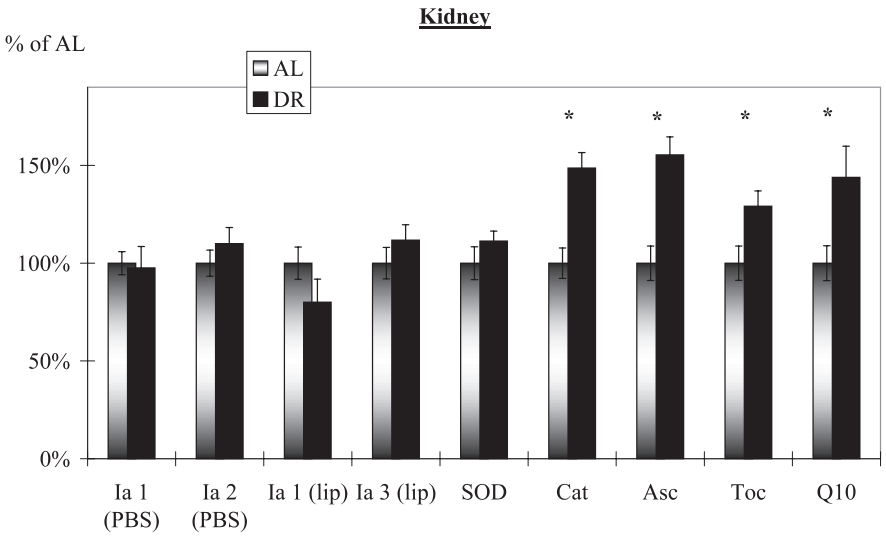

Fig. 7 Differences in antioxidant parameters in DR versus AL kidneys. Values are percent of AL controls and are expressed as mean ± SEM. The second lipophilic anodic wave is termed 'Ia 3' due to its relatively high $E_{1/2}$ (~845 mV). Significant differences ($P< 0.05$, for exact value see text) are marked by an asterisk. For abbreviations see Fig. 2.

clude that in the face of no other lipid-soluble changes, the rise in α-tocopherol levels enhanced the direct antioxidative defense of the lipophilic tissue components. As for the water-soluble components of the antioxidant system, we feel that the non-significant rises could imply a trend for better defense. The decrease in the first anodic wave in the DR group should be further examined.

Plasma

CV analysis could not be performed, due to the small amounts of blood collected from these small mice. HPLC-ECD could only reveal ascorbate in the plasma samples, and the levels did not differ between groups. When checked for correlation with ascorbate levels in other tissues, a few significant correlations were found. In the AL group, plasma ascorbate levels correlated positively with ascorbate levels in spleen ($r= 0.54$, $P<0.05$) and muscle ($r= 0.87$, $P<0.005$), and negatively with small intestine ($r= –0.79$, $P<0.005$). DR abolished these correlations, and new negative correlations were found in ascorbate levels between DR plasma and lung ($r= –0.62$, $P<0.05$) and also between DR plasma and kidney ($r= –0.62$, $P<0.025$). No correlations were found between plasma ascorbate and the appropriate diet group for brain, heart, AL lung, DR spleen, liver, DR intestine, AL kidney and DR muscle.

We cannot explain these correlations at the moment, but we clearly show how plasma ascorbate levels cannot serve as indicators for assessment of ascorbate levels in other tissues.

Conclusions

Measuring the total reducing power of tissues allows for an overview of LMWA status. The reaction of the tissues varies greatly following DR, as can be seen in Table 3. Taken together, it seems that the antioxidant defense of the liver and small intestine has deteriorated, while that of the kidney, heart and quadricep muscle was enhanced following 40 days of DR. The digestive organs could be reacting appropriately to a smaller workload. The enhancement of the kidney defense system could in part account for the diminished nephropathy described in DR animals [3]. Brain antioxidant status remained unchanged, whereas the response of the lung and spleen was ambivalent – the hy-

Table 3 A summary of the changes in total antioxidative capacity following short-term DR in each tissue examined. This capacity includes both the LMWA component and the enzymatic component of the antioxidant system. ⇑ – greater overall antioxidative capacity; ⇓ – lower overall antioxidative capacity; *NC* – no difference in antioxidative capacity. * – The first lipophilic anodic wave of the spleen was larger in the DR group, whereas the second lipophilic anodic wave was smaller.

Tissue	Hydrophilic antioxidative capacity	Lipophilic antioxidative capacity
Brain	*NC*	*NC*
Lung	⇓	⇑
Heart	⇑	*NC*
Spleen	⇓	*
Liver	⇓	⇓
Small intestine	⇓	⇓
Kidney	⇑	⇑
Muscle	⇑ / *NC*	⇑

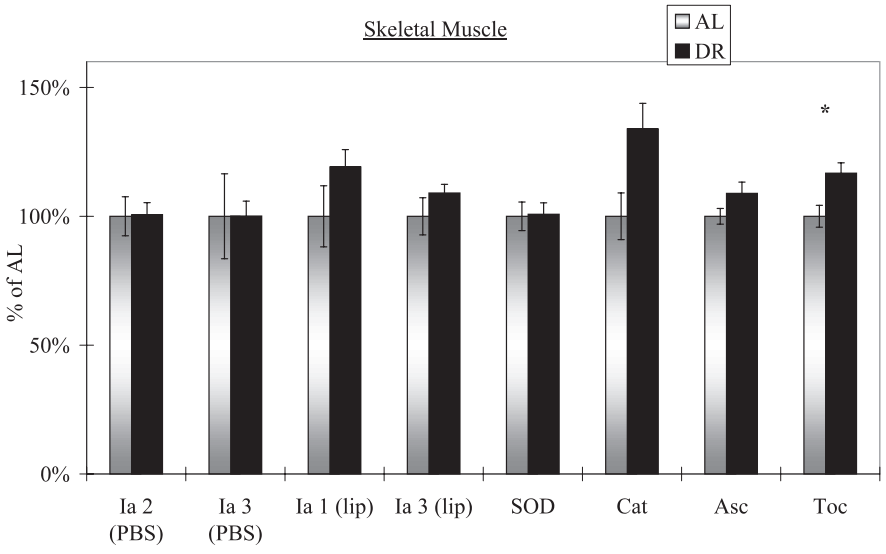

Fig. 8 Differences in antioxidant parameters in DR versus AL quadriceps muscle. Values are percent of AL controls and are expressed as mean ± SEM. The hydrophilic anodic waves are termed 'Ia 2' and 'Ia 3' due to their relatively high $E_{1/2}$ s (~710 mV and ~870mV, respectively). The second lipophilic anodic wave is termed 'Ia 3' due to its relatively high $E_{1/2}$ (~800 mV). Significant differences ($P< 0.05$, for exact value see text) are marked by an asterisk. For abbreviations see Fig. 2.

drophilic medium featured a lower reducing ability, whereas the lipophilic medium showed a mixed response.

The biological meaning of these reactions is hard to interpret. It should be noted that the whole antioxidant reaction to DR could be secondary or tertiary to the initial reduction in oxidative stress. We may be still seeing the process of adaptation, with different parts of the system reacting with varying time courses. That could account for the different responses. Additionally, we may be seeing secondary responses to primary antioxidant changes, which may even lead to overshooting. For example, there could be a synthesis of endogenous LMWA to compensate for lower dietary vitamin E intakes. It has been shown that specific LMWA can affect other LMWA concentrations or enzyme activities [28–30].

After showing that DR causes changes in the total reducing power of various tissues, this issue must be further pursued. A measurement of the ratio between all oxidants and reductants ("redox potential") may serve as a more delicate method to determine oxidative and antioxidative changes in biological systems [37]. Future work remains to show the relevance of these findings to long-term effects of DR in enhancing longevity. Also, our results may contribute to the understanding of the biology of the antioxidant system and the interactions between its several components.

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