Mechanisms of Ageing and Development 129 (2008) 341–348

Contents lists available at ScienceDirect

Mechanisms of Ageing and Development

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

Dietary lipoic acid supplementation can mimic or block the effect of dietary restriction on life span

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ARTICLE INFO

Article history:
Received 30 January 2008
Received in revised form 31 March 2008
Accepted 12 April 2008
Available online 22 April 2008

Keywords:
Lipoic acid
Dietary restriction
Rats
Longevity
Epigenetic
Antioxidant

ABSTRACT

Dietary restriction feeding extends survival in a range of species but a detailed understanding of the underlying mechanism is lacking. There is interest therefore in identifying a more targeted approach to replicate this effect on survival. We report that in rats dietary supplementation with alpha-lipoic acid, has markedly differing effects on lifetime survival depending upon the dietary history of the animal. When animals are switched from DR feeding to *ad libitum* feeding with a diet supplemented with alpha-lipoic acid, the extended survival characteristic of DR feeding is maintained, even though the animals show accelerated growth. Conversely, switching from *ad libitum* feeding a diet supplemented with alpha-lipoic acid to DR feeding of the non-supplemented diet, blocks the normal effect of DR to extend survival, even after cessation of lipoic acid supplementation. Unlike the dynamic effect of switching between DR and *ad libitum* feeding with a non-supplemented diet where the subsequent survival trajectory is determined by the new feeding regime, lipoic acid fixes the survival trajectory to that established by the initial feeding regime. *Ad libitum* feeding a diet supplemented with lipoic acid can therefore act as mimetic of DR to extend survival.

1. Introduction

Since the initial reports of McCay et al. (1935), dietary restriction feeding (DR) regimes have been used routinely in ageing research to extend longevity and in some mammalian species such as the rat, to slow the rate of ageing. Although this is a robust and reproducible methodology to manipulate lifespan, a detailed understanding of the mechanism underlying this effect remains uncertain and controversial. The effect of DR feeding (30–50% of control food intake) on extending survival has been demonstrated in a range of invertebrate and vertebrate species that include yeast, *C. elegans*, *Drosophila*, spiders, fish and rodents (Weindruch and Walford, 1988; Austad, 1989). Dietary restricted feeding has been the primary experimental approach used in mammals to investigate the underlying biochemical mechanisms of ageing and the link to the incidence and prevalence of age-related morbidity (McCay et al., 1935; Weindruch and Walford, 1988; Masoro, 1992; Merry, 1999). Studies in the Rhesus and squirrel monkey have been continuing for over 20 years to determine if life span can be similarly extended in primate species (Roth et al., 1999; Mattison et al., 2003). Demographic data from the island of Okinawa, where the population is exceptionally long-lived, suggest that restricted food intake may also be effective in prolonging human life span (Willcox et al., 2006, 2007). A detailed molecular understanding however, of how DR feeding induces this extension in life span has still to be achieved though a reduction of reactive oxygen species (ROS) production is generally thought to be a key response (Merry, 2002, 2004). ROS production has been implicated both in the accumulation of damage to macromolecules (Merry, 2002) and also in the regulation of redox-sensitive transcription factors (Salehi et al., 1999; Chung et al., 2001; Kim et al., 2002), and through them to altered gene expression profiles (Goyns et al., 1998; Lee et al., 1999, 2002). Given this thinking it has been postulated that dietary antioxidant supplements might be equally effective in extending the lifespan in mammals and considerable effort has been directed in identifying such compounds that may mimic the effect of DR to enhance survival, but with little success (Harris et al., 1990; Lee et al., 2004).

Recently the mitochondrial cofactor α-lipoic acid, has been the focus of intensive research in diseases where damage by ROS has been postulated, such as diabetes and neurological degeneration (Bliska and Wlodek, 2005; Cakatay, 2006). Lipoic acid is a disulfide

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doi:10.1016/j.mad.2008.04.004

derivative of octanoic acid that forms an intra-molecular disulphide bond in its oxidised form, but which is readily reduced to dihydrolipoic acid (DHLA). This redox couplet (redox potential ($E'_o = -0.32$ V)) has the potential to act as a powerful antioxidant under certain cellular conditions because the low negative redox potential makes the LA/DHLA couplet a strong reductant, but it can also exhibit pro-oxidant effects (Moini et al., 2002). The in vivo balance between these anti- and pro-oxidant effects of lipoic acid and DHLA remains uncertain and controversial. Under normal physiological conditions both α-lipoic acid and DHLA appear to be tightly bound to cell protein but after dietary supplementation both forms are present in tissues in the free form (Moini et al., 2002). The *R*-enantiomer of α-lipoic acid, an essential cofactor, is present in the diet as a lipoamide in vegetables such as spinach, broccoli and tomatoes, (3.15 ± 1.11, 0.94 ± 0.25, and 0.56 ± 0.23 μg lipoyllysine/g dry wt, respectively) and in animal tissues such as kidney, heart, and liver, (2.64 ± 1.23, 1.51 ± 0.75, and 0.86 ± 0.33 μg lipoyllysine/g dry wt, respectively), and is synthesised de novo from fatty acids and cysteine (Moini et al., 2002). In all tissues DHLA as well as α-lipoic acid is reported to be present and the reduced form represents 21–45% of the total tissue content.

During mammalian ageing, acute dietary supplementation with lipoic acid has been shown to oppose detrimental changes in the rat myocardium, such as increased oxidant generation, and oxidative damage to DNA and lipids (Hagen et al., 2002). Furthermore in old animals, acute supplementation with α-lipoic acid has been shown to improve memory function and to induce partial reversal of oxidative damage to RNA in the hippocampus (Arivazhagan et al., 2000, 2002; Liu et al., 2002a,b). However, in spite of such reports of attenuation of age-related indices of oxidative stress and oxidant generation by acute dietary supplementation with the (*R*)-enantiomer, or the (*R*,*S*) racemic mixture, chronic dietary supplementation with α-lipoic acid is reported to have no effect on prolonging survival in mice (Lee et al., 2004). While a partial reversal of oxidative stress biomarkers can be observed in old rodents fed a diet supplemented with α-lipoic acid, a clear demonstration that feeding this compound on a chronic basis will mimic the effect of DR feeding to extend survival has not been reported.

2. Materials and methods

2.1. Diets

The non-supplemented CRM diet was supplied by Dietex International, Witham, UK. The lipoic acid supplemented diet was the CRM diet enriched with a racemic mixture of DL-thioctic acid (α-lipoic acid) obtained from Fisher Scientific U.K., Limited, at 1.5 g/kg by the Special Diet Systems Division of Dietex International.

2.2. Animals

All animal husbandry procedures involved were carried out in accordance with the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986. Male BN rats (Substrain BN/SsNOlaHSD) were obtained from Harlan UK at 21–28 days of age and maintained under barrier conditions on a 12-h light:12-h dark cycle (08:00–20:00) at 22 ± 1 °C. Sentinel animals were routinely screened to ensure a consistent health status throughout the study and across all dietary groups. All animals were caged in groups of four and fed the non-supplemented CRM diet *ad libitum* until 2 months of age, when they were transferred to single housing and assigned randomly to one of 12 dietary groups as summarised in Table 1. DR fed animals were fed either the CRM diet, or the lipoic acid supplemented CRM diet at 55% the daily food intake of control rats (Group 1), to maintain their body weight at approximately 55% that of age-matched *ad libitum* fed animals (Fig. 2). The diet of animals maintained on a DR regime was supplied daily as pre-weighed ratios between 10:30 and 11:00 h. The daily food ration supplied to DR animals was the same for age-matched animals irrespective of whether they were fed the CRM diet, or the lipoic acid supplemented CRM diet. Twelve dietary feeding combinations were studied to compare the effect of *ad libitum* or DR feeding with, and without lipoic acid supplementation, on lifetime survival. Survival trajectories were also determined subsequent to a dietary switch at either 6 or 12 months of age. All groups were run simultaneously and so experienced identical husbandry and housing conditions.

2.3. Statistics

Survival data from Groups 1 to 12 were compared for statistically significant differences by the log–rank Peto test using StatsDirect Statistical software, Version 2.6.2, that allowed for right-censored data. Where animals were right-censored from experimental groups to provide tissue, rats were selected randomly from the surviving animals. The rats in Group 1 were *ad libitum* fed the non-supplemented CRM diet and served as the control, reference group for survival comparisons. An exact conditional maximum likelihood estimate of the hazard ratio was calculated to contrast the death rate between each group and the reference control group. A

Table 1
Dietary groups with the associated median survival and 95% confidence limits, mean survival ± S.E.M. and the hazard ratio

Group number	*n*	Dietary group description	Median survival (95% CI) (days)	Mean survival (days)	S.E. (days)	Hazard ratio[a]
1	102	Control animals fed *ad libitum* the CRM diet throughout life	926 (909–943)	854	22	1.000
2	75	Fed a restricted intake of the CRM diet from 2 months to maintain body weight at 55% age-matched control animals	1047 (930–1163)	1025	25	0.259
3	75	Animals fed *ad libitum* the CRM diet supplemented with R/S racemic mixture of α-lipoic acid from 2 months of age	900 (839–961)	858	27	0.983
4	24	DR fed the CRM diet until 12 months, then DR fed the α-lipoic acid supplemented diet	1125 (1078–1172)	1068	38	0.178
5	25	*Ad libitum* fed CRM diet, animals switched to DR feeding at 12 months	1031 (1007–1055)	1000	33	0.235
6	25	DR fed the CRM diet from 2 to 12 months, then switched to *ad libitum* feeding	975 (935–1015)	914	44	0.620
7	25	*Ad libitum* fed the CRM diet, animals switched to DR feeding at 6 months	1078 (1048–1108)	1021	45	0.213
8	25	DR fed the CRM diet 2–6 months, then switched to *ad libitum* feeding	928 (858–998)	909	28	0.954
9	25	Animals fed *ad libitum* α-lipoic acid supplemented diet 2–12 months, then switched to DR feeding the CRM diet (no α-lipoic acid supplementation after 12 months)	934 (874–994)	859	57	0.865
10	25	Animals fed *ad libitum* lipoic acid supplemented diet 2–6 months, then switched to DR feeding the CRM diet (no α-lipoic acid supplementation after 6 months)	1086 (1059–1113)	1021	51	0.201
11	25	DR fed the CRM diet from 2 until 12 months, then switched to *ad libitum* feeding the α-lipoic acid supplement CRM diet	1041 (895–1187)	1009	34	0.353
12	24	DR fed the CRM diet 2 until 6 months, then fed *ad libitum* the α-lipoic acid supplement CRM diet	996 (927-1065)	947	43	0.432

[a] The hazard ratio is the decreased life-time risk of death of the dietary groups when compared with that of the *ad libitum*, control (Group 1) that was fed *ad libitum*, the CRM non-supplemented diet. The hazard ratio was determined by the Peto log–rank analysis with the Group 1 set to 1.0. Values lower than 1.0 represent a slower rate of death than observed in the control animals in Group 1.

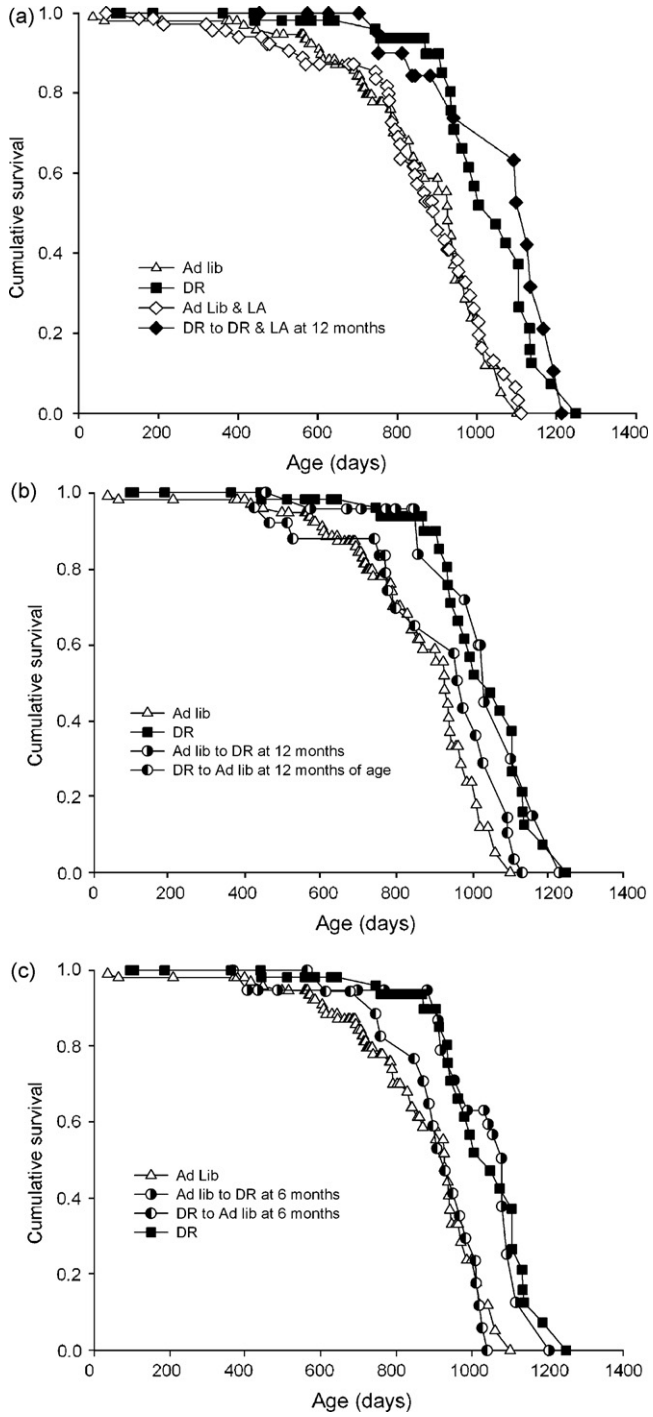

Fig. 1. Survival profiles for *ad libitum* and DR rats fed the non-supplemented and α-lipoic acid supplemented diets. (a) Survival profiles based on a Kaplan–Meier analysis for *ad libitum* and DR rats fed the non-supplemented CRM diet, or the lipoic acid supplemented CRM diet. There is a highly significantly difference in death rates between *ad libitum* (Group 1) and DR (Group 2) animals fed the non-supplemented diet ($P < 0.0001$, hazard ratio 0.259). When animals were fed either *ad libitum* (Group 3) or DR (Group 4) with the α-lipoic acid supplemented diet from 2 months, and 12 months of age respectively, DR feeding again induced a significantly slower death rate ($P < 0.0001$, hazard ratio 0.178). No significant effect of lipoic acid supplementation of the diet was seen on survival. No significant differences were seen in survival between the two groups of animals fed *ad libitum* (Groups 1 and 3), or between the two DR fed groups (Groups 2 and 4). (b) Survival profiles generated by a Kaplan–Meier analysis are shown for animals fed the non-supplemented diet but switched from *ad libitum* to DR feeding (Group 5), or from DR to *ad libitum* feeding (Group 6), at 12 months of age. Switching the feeding regime from *ad libitum* feeding to DR induced a significantly slower death rate in comparison with the life-time *ad libitum* fed control group (Group 1), ($P = 0.0017$, hazard ratio 0.235). The reverse switch from DR to *ad libitum* feeding showed a residual effect of DR feeding on survival, but this did not reach statistical significance in comparison with the survival profile of the *ad libitum* fed control rats (Group 1), ($P = 0.151$, hazard ratio 0.620). (c) This panel shows a repeat of the study shown in panel b, but here the dietary switch was made at 6 months of age, (Groups 7 and 8). Again switching to DR feeding induced a significantly slower death rate in comparison with life-time *ad libitum* feeding (Group 1) ($P < 0.001$, hazard ratio 0.213). When animals were switched at 6 months of age from DR to *ad libitum* feeding (Group 8), no significant extension in life-time survival was seen in comparison with the *ad libitum* fed, reference group (Group 1) ($P = 0.877$, hazard ratio 0.954).

Kaplan–Meier analysis was completed for survival for each dietary group and was used to plot the survival curves shown in Figs. 1, 4 and 5. The power of group comparisons for a subsequent log–rank analysis of the survival data with a two-sided probability error of $P < 0.05$ was calculated according to the method of Dupont (1990). In those group comparisons where a statistical difference in survival compared to Group 1 animals is reported, the power of the comparison ranged from a 65% probability of detecting a true difference in median survival times at $P < 0.05$ for Group 12, 90% probability for Groups 4, 7, 10 and 11, and >95% probability for Groups 2, and 5.

The statistical difference in group survival identified by the log–rank analysis were confirmed by multiple group comparisons for right-censored data using the 'Survival' programme, SPSS Version 14.0, based on the Wilcoxon [Gehan] statistic.

3. Results

3.1. Effect of α-lipoic acid supplementation on survival

Table 1 summarises for all twelve dietary groups the median survival with associated 95% confidence interval, mean survival ± S.E.M. and the hazard ratio.

It was determined initially if chronic supplementation of the diet with α-lipoic acid (1.5 g/kg) when fed either *ad libitum*, or on a restricted basis (55% *ad libitum* intake), had any effect on the subsequent survival profile associated with the two feeding regimes (Fig. 1a). In agreement with many previous reports (Yu et al., 1982; Weindruch and Walford, 1988; Merry, 1999), DR feeding (Group 2) resulted in a highly significant ($P < 0.0001$) increase in survival with a median survival time of 1047 days compared to 926 days for animals fed *ad libitum*, the non-supplemented diet (Group 1). Feeding a diet supplemented with α-lipoic acid throughout life appeared to have neither a detrimental or advantageous effect on survival for rats fed either *ad libitum* (Group 3) or on a dietary restricted basis (Group 4). The hazard ratios were 0.983 and 0.178 respectively when compared with the control, reference group. The median survival time of 900 days for rats fed *ad libitum* the supplemented diet was not significantly different to that recorded for rats fed *ad libitum* the non-supplemented diet (928 days). Similarly the median survival of 1125 days for the extended survival induced by restricted feeding with the lipoic acid supplemented diet was not significantly different to that observed in DR rats fed the non-supplemented diet (1047 days), although for this group, lipoic acid was included in the diet only from 12 months of age.

Because of the known anti-obesity effect of α-lipoic acid to inhibit food intake and retard growth when supplied at 0.25%, 0.5% and 1% in the diet (Kim et al., 2004), it was important that lipoic acid was supplied at a dose that did not inhibit the growth rate and induce an indirect dietary restriction effect (Fig. 2). The daily intake of food for the *ad libitum* and DR fed animals was not significantly different when animals were maintained on either the lipoic acid supplemented or non-supplemented CRM diets (data not shown).

Thus supplementation of the diet with α-lipoic acid at a dose that did not inhibit the growth rate appeared to have no effect on the subsequent survival profile under either *ad libitum* or DR feeding regimes.

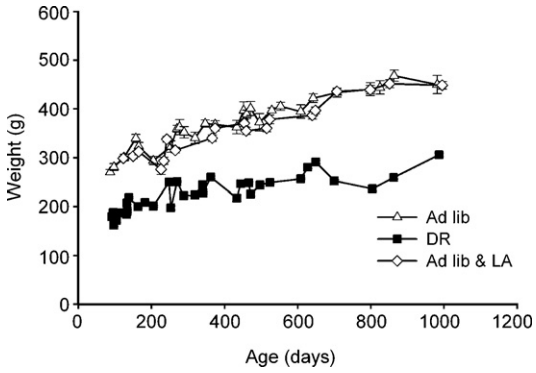

Fig. 2. Growth curves are shown for rats fed *ad libitum* and DR with the diet that was not supplemented with α-lipoic acid, and for rats fed *ad libitum* with the α-lipoic acid supplemented CRM diet. Data points are the mean ± S.E.M. (where this exceeds the width of the symbol) for 20 animals chosen randomly from the respective groups. When animals are fed *ad libitum*, supplementing the diet at 1.5 g/kg did not inhibit the growth rate.

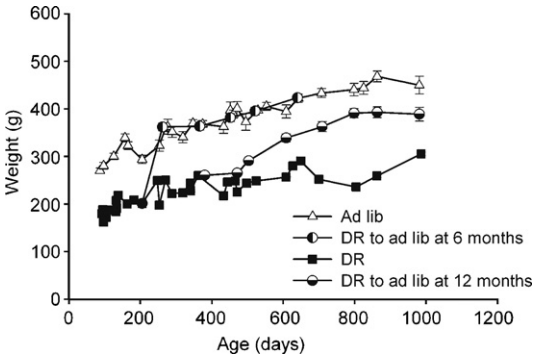

Fig. 3. Growth curves are shown for rats fed *ad libitum* and DR the diet that was not supplemented with α-lipoic acid, and for animals switched from DR to *ad libitum* feeding at 6 and 12 months of age. Data points show the mean ± S.E.M. (where this exceeds the width of the symbol) for 20 animals chosen randomly from the *ad libitum* and DR groups fed the non-supplemented diet, and for the total surviving animals in the re-fed groups at the ages shown. While complete recovery to age-matched control body weights occurs on re-feeding at 6 months of age, when DR rats are re-fed at 12 months, although accelerated growth is seen, full recovery to the control growth curve does not occur.

3.2. α-Lipoic acid can block the DR effect on survival

The effect on the subsequent survival trajectory was determined for rats fed the non-supplemented and α-lipoic acid supplemented diets when switched between *ad libitum* and DR feeding, and *vice versa* at 6 and 12 months of age. When animals were fed the non-supplemented diet the survival profile obtained confirmed how malleable the subsequent survival trajectory was to the new feeding regime (Fig. 1b and c). *Ad libitum* fed animals switched to the DR feeding regime at 12 months of age (Group 5) displayed a similar extended survival trajectory (Fig. 1b) to animals that had been maintained on DR feeding from 2 months of age (Group 2) (median survival times of 1031 and 1047 days, hazard ratios of 0.235 and 0.259 respectively). A residual effect on survival of the initial 10 months of DR feeding (between 2 and 12 months of age) was observed for rats that were switched from DR to *ad libitum* feeding at 12 months of age (Group 6). The survival profile obtained however, was not significantly different to rats fed *ad libitum* throughout life ($P = 0.151$, hazard ratio 0.620). This is in agreement with a previous report for restricted feeding with the CFY strain of rat (Merry, 1987), where a residual 10% increase in maximum survival was observed subsequent to re-feeding at 12 months of age. Repeating the study, but switching between *ad libitum* and DR feeding the non-supplemented diet at 6 months of age (Groups 7 and 8) completely reversed the survival trajectories (Fig. 1c, Table 1). The switch from *ad libitum* feeding to DR feeding at 6 months of age resulted in a hazard ratio of 0.213, while the converse switch from DR feeding to *ad libitum* feeding gave a relative hazard ratio to the control, (Group 1) animals of 0.954. No persistent effect was observed on the subsequent survival profile that resulted from either 6 months of *ad libitum* feeding, or 4 months of DR feeding with the non-supplemented diet, prior to the dietary switch.

These two switch-over studies confirmed that the effect of DR feeding to modify survival is dynamic in rats, does not induce an irreversible effect on survival so that at 12 months of age, the survival trajectories could be transposed by switching the feeding regime. It is apparent that even with group sizes as low as 25 animals, the effect of the DR feeding regime on the hazard ratio is so marked, that the power to detect a significant effect on survival at $P < 0.05$ in comparison with the control reference group is greater than 90%.

DR fed animals that were returned to *ad libitum* feeding at 6 months of age showed a rapid increase in growth rate with the body weight and growth profile rapidly matching that of the *ad libitum* fed animals (Fig. 3). However, when animals were switched between feeding regimes at 12 months of age, although the growth rate was accelerated, full recovery to the age-matched, control body weight did not occur (Fig. 3).

When however, *ad libitum* fed animals were maintained on a diet supplemented with α-lipoic acid for 10 months prior to the switch to DR feeding of the non-supplemented diet at 12 months of age (Group 9), the extended survival profile observed previously was now not seen (Fig. 4a). Prior exposure to α-lipoic acid supplementation for 10 months in combination with *ad libitum* feeding completely inhibited the transition to the DR extended survival trajectory, even though α-lipoic acid supplementation of the diet was not continued beyond 12 months of age. Even though these rats were now on the DR feeding regime with the non-supplemented diet, the death rate as shown by a hazard ratio of 0.865 was not statistically different to that of the control, *ad libitum* fed (Group 1) animals ($P = 0.695$).

When the exposure to α-lipoic acid supplementation was limited to 4 months only, from 2 to 6 months of age (Group 10), a gradual release from the effect of α-lipoic acid to block the transition to the extended survival trajectory induced by DR feeding was observed (Fig. 4b). By approximately 30 months of age, the cumulative survival profile overlaid that recorded for DR animals fed the non-supplemented diet from 2 months of age (Group 2), generating a hazard ratio of 0.201. This is similar to the hazard ratio of 0.213 for animals fed the non-supplemented diet and undergoing the same switch of feeding regime at 6 months of age (Group 7). The resultant life-time survival curve was therefore intermediate in profile between the *ad libitum* and DR survival curves, but overall the death rate was significantly reduced in comparison with the reference animals (Group 1) as shown by the log–rank analysis ($P < 0.0008$).

3.3. α-Lipoic acid can mimic the DR effect on survival

Rats were also switched from DR feeding the non-supplemented diet to *ad libitum* feeding with the lipoic acid supplemented diet at the two ages of 12 months (Group 11) and 6 months (Group 12) (Fig. 5a). *Ad libitum* feeding with α-lipoic acid supplementation, subsequent to DR feeding a non-supplemented diet for 10 months, resulted in the extended survival profile that is characteristic of DR animals fed the non-supplemented diet, even though the rats were fed *ad libitum* from 12 months of age and

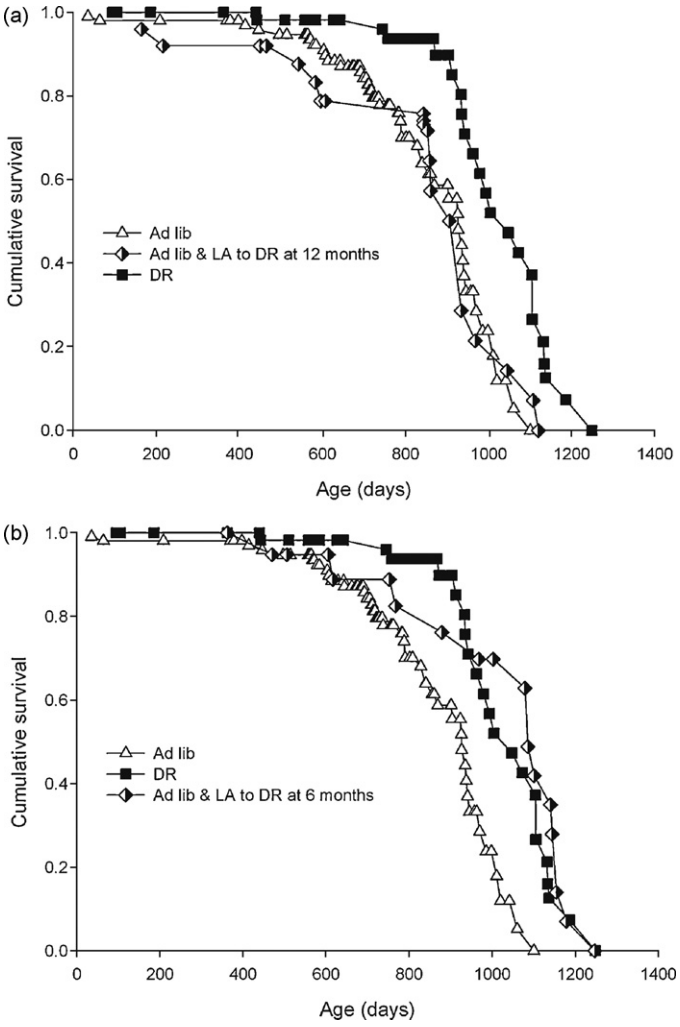

Fig. 4. Survival profiles for animals fed the DR regime subsequent to *ad libitum* feeding the α-lipoic acid supplemented diet. (a) The survival profile is shown following a Kaplan–Meier analysis for *ad libitum* feeding of the α-lipoic acid supplemented diet prior to DR feeding the non-supplemented diet from 12 months of age (Group 9). Following prior exposure to lipoic acid during the *ad libitum* feeding phase only, DR feeding failed to induce any increase in survival in comparison with the *ad libitum* fed animals fed the non-supplemented diet (Group1) ($P = 0.659$, hazard ratio 0.865). This is in contrast to the response on the survival trajectory for feeding the non-supplemented diet shown in Fig. 1b. (b) This panel shows a repeat of the study shown in panel a. Here the dietary switch was made at the earlier age of 6 months (Group 10). Prior exposure to *ad libitum* feeding with a diet supplemented with α-lipoic acid again initially inhibited the effect of the DR feeding regime to increase survival and slow the death rate. After approximately 27 months of age a significant increase in survival could be demonstrated in comparison with the *ad libitum* animals fed the non-supplemented diet (Group 1) ($P < 0.0008$, hazard ratio 0.201).

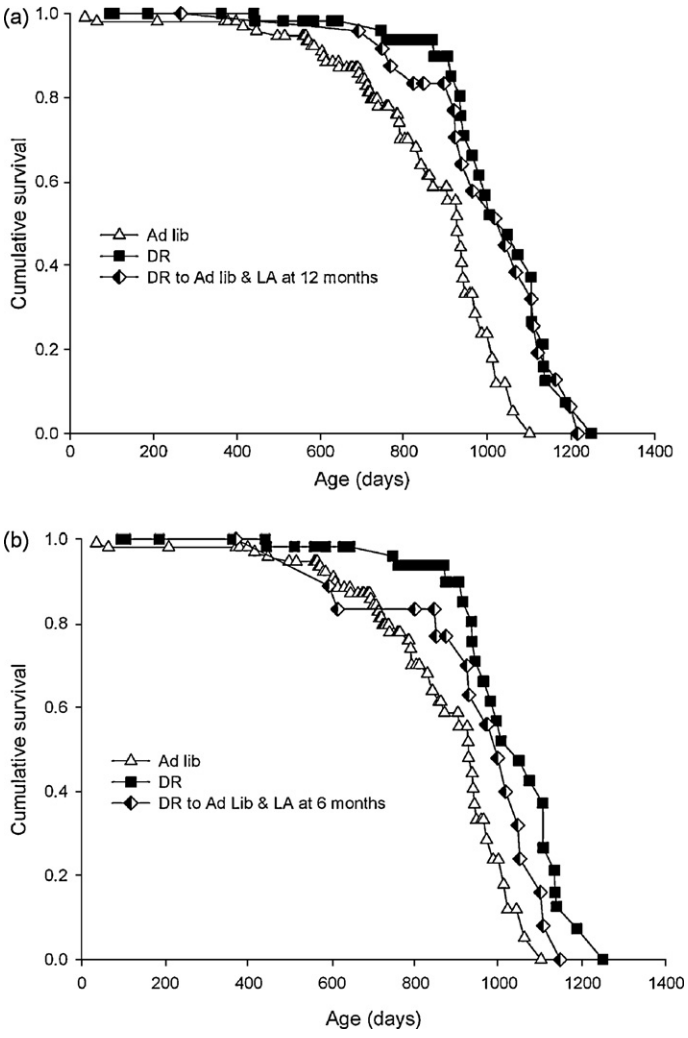

Fig. 5. Survival for rats fed DR the non-supplemented diet then switched to *ad libitum* feeding with the α-lipoic acid supplemented diet. (a) The survival profile is shown following a Kaplan–Meier analysis for animals DR fed the non-supplemented diet until 12 months of age, then fed *ad libitum* the α-lipoic acid supplemented diet (Group 11). Here, in spite of *ad libitum* feeding and accelerated growth, a significant effect to slow the death rate and extend survival is seen in comparison with animals fed *ad libitum* throughout life with the non-supplemented diet (Group 1) ($P < 0.0044$, hazard ratio 0.353). (b) This panel shows a repeat of the study shown in panel a, but here the dietary switch was made at the earlier age of 6 months (Group 12). Again the effect of *ad libitum* feeding a diet supplemented with lipoic acid induces a significant extension in survival, but the effect on slowing the death rate was only partial in comparison with animals where the dietary switch was made at 12 months of age. The effect on survival and slowing the death rate in comparison with the control, reference group however was significant, ($P < 0.0286$, hazard ratio 0.432).

showed accelerated growth (Fig. 6). The median survival time was 1041 days (Group 11) compared to 1047 days for rats maintained on DR feeding the non-supplemented diet from 2 months of age (Group 2). This effect is in contrast to the effect of switching from DR to *ad libitum* feeding with the non-supplemented diet (Fig. 1b) where the survival trajectory quickly reverted to that characteristic of the *ad libitum* fed control rats (Group 1). A significant increase in survival ($P < 0.0044$, hazard ratio 0.353) was observed in comparison with rats fed *ad libitum* the non-supplemented diet though out life.

When animals were switched at 6 months of age from DR feeding the non-supplemented diet, to one fed *ad libitum* but supplemented with α-lipoic acid (Group 12), again an increase in survival was observed even though the growth rate was accelerated (Fig. 6). The survival trajectory however, did not overlay the DR curve as seen when the dietary switch occurred at 12 months of age, but was intermediate in position between the control survival curve (Group 1) and that of DR animals fed the non-supplemented diet (Group 2) (Fig. 5b). Survival in these rats was significantly increased compared to the control reference group ($P < 0.0286$, hazard ratio 0.432) with a median survival of 996 days compared with 926 days for the control, *ad libitum* fed animals (Group 1).

After a return to *ad libitum* feeding, accelerated growth was seen at both 6- and 12 months of age for animals fed either diet (Figs. 3 and 6). Switching feeding regimes at 12 months of age

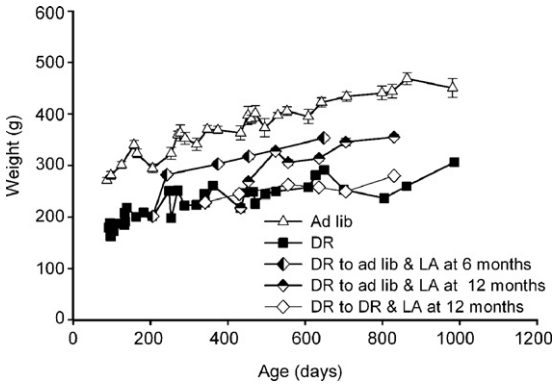

Fig. 6. Growth curves are shown for rats fed *ad libitum* and DR with the non-supplemented diet, and for animals switched from DR feeding the non-supplemented diet to *ad libitum* feeding the α-lipoic supplemented diet at 6 and 12 months of age. The growth curve for rats switched from DR feeding the non-supplemented diet to the α-lipoic supplemented diet at 12 months of age is also shown. Data points show the mean ± S.E.M. (where this exceeds the width of the symbol) for 20 animals chosen randomly from the *ad libitum* and DR groups fed the non-supplemented diet, and for the total surviving animals in the re-fed or dietary switch groups at the ages shown. Re-feeding with the lipoic acid supplemented diet had no significant effect on the accelerated growth profile at 12 months of age in comparison with that shown by animals re-fed with the non-supplemented diet. At 6 months of age however, although accelerated growth was seen, lipoic acid supplementation partially inhibited the animals from returning to the control growth curve as seen with the re-feeding of the non-supplemented diet (Fig. 3).

failed to reveal any significant difference in the accelerated growth curves achieved, irrespective of whether or not the diet was supplemented with α-lipoic acid. When the diets were switched at 6 months of age, the animals with lipoic acid supplementation failed to achieve the control body weight recorded for *ad libitum* fed animals on the non-supplemented diet (Fig. 6). When lipoic acid was included in the diet, the same rate of accelerated growth was seen at both 6 and 12 months of age on return from DR to *ad libitum* feeding.

4. Discussion

Attempts to replicate the effect of DR feeding on survival by simply feeding a diet supplemented with α-lipoic acid have not previously been successful in mice (Lee et al., 2004) and this observation is confirmed here in the rat. This observation is puzzling given the reported beneficial effects of this compound on cellular redox and behavioural biomarkers of ageing in older rats (Arivazhagan et al., 2000, 2002; Hagen et al., 2002; Liu et al., 2002a,b). Here we report that *ad libitum* feeding a diet supplemented with α-lipoic acid can induce the extended survival profile characteristic of restricted feeding regimes, if it is supplied in the diet subsequent to a period of restricted feeding a non-supplemented diet. It has been shown previously that the extended survival induced by DR feeding is a dynamic effect requiring the continuous constraint of restricted feeding to maintain the extended survival profile (Merry, 1987), and this is confirmed here for animals fed the non-supplemented diet. When animals are switched between *ad libitum* feeding and DR when maintained on the CRM diet, the subsequent survival trajectory immediately changes to that characteristic of the new feeding regime. This dynamic response of the subsequent survival trajectory to the dietary change is blocked, either fully at 12 months of age, or partially when the switch is made earlier at 6 months of age, if the diet during the *ad libitum* feeding phase is supplemented with lipoic acid.

The mechanism by which α-lipoic acid increases survival is unclear for when α-lipoic acid supplementation is combined with DR feeding from 12 months of age, no additional increase in lifespan is observed. Thus the increase in survival observed subsequent to DR feeding appears to act through an ability of α-lipoic acid to fix the subsequent survival trajectory to that established by the initial DR feeding regime. This would explain the failure to observe any additional effect on survival when the diet is supplemented with α-lipoic acid, but fed either on an *ad libitum*, or dietary restricted basis. The extended survival observed is, unlike the effect induced by DR feeding the non-supplemented diet, not associated with a retarded rate of growth. On return to full feeding with the lipoic acid supplemented diet, the growth rate is accelerated and at 12 months of age, no significant difference can be demonstrated between diets in the rate of re-growth achieved (Fig. 6). Curiously, although lipoic acid supplementation did not affect the growth rate in animals fed *ad libitum* from 2 months age (Fig. 2), it did partially retard the accelerated rate of growth induced by a return to full feeding at 6 months (Fig. 6). Why this effect on the re-feeding growth rate should be seen at 6 months, but not at 12 months of age, is unclear.

This ability to fix the original survival trajectory is also demonstrated for animals *ad libitum* fed with the α-lipoic acid supplemented diet, prior to the switch to DR feeding. Here the normal transition to the extended survival trajectory induced by DR feeding is prevented, even though at the two ages studied there is a similar loss in body mass as is observed in animals switched from *ad libitum* to DR feeding the non-supplemented diet (data not shown). Therefore the malleability in the survival trajectory that is seen in response to changes in the feeding regime between *ad libitum* and DR feeding with the non-supplemented diet, and which has also been reported in *Drosophila* (Mair et al., 2003) and mice (Spindler, 2005), is lost in the presence of α-lipoic acid supplementation. Although DR feeding regimes have previously been considered to slow the accumulation of tissue damage, probably as a result of oxidative stress, the survival data from this study with that of Mair et al. (2003) and Spindler (2005) suggests strongly that DR feeding induces a state change in the organism that is associated with extended survival, rather than inducing a slower rate of damage accrual. Little or no memory effect on survival is seen when switching from a DR feeding regime to control feeding.

An intriguing observation from this study is that α-lipoic acid can continue to block the effect of DR feeding to extend survival for over a year after its removal from the diet. Thus α-lipoic acid, a small molecule (Mwt. 206) with 57% of an intra-peritoneal dose (0.5 mg/100 g body weight) being excreted in the urine in the rat within 24 h (Harrison and McCormick, 1974), and a plasma half-life of only 30 min in the human (Cakatay, 2006), can exert a persistent effect on survival for over a year after ceasing to supplement the diet.

The selection of the ages of 6 and 12 months for the dietary switches was made in order to identify acute and chronic effects of DR feeding on the redox chemistry of tissues from these animals (data not reported here). These tissue responses were contrasted with those induced by supplementation of the diet with lipoic acid in an attempt to ascertain the importance of the redox status of tissues during ageing. It is recognised however, that α-lipoic acid and DHLA can exert both anti- and pro-oxidants effects therefore the response to α-lipoic acid supplementation on cellular redox chemistry can be expected to be complex and tissue specific (Cakatay, 2006). Data on the redox chemistry and redox sensitive transcription factors in tissues from rats fed a diet supplemented with lipoic acid, clearly demonstrate that lipoic acid can act as a pro-oxidant *in vivo*. Although a number of acute effects on the cellular redox status have been reported for α-lipoic acid (Packer et al., 1997; Packer, 1998; Suh et al., 2004b), given the persistent

effect on survival after ceasing to supplement the diet, it is difficult to envisage how these redox, or any free radical scavenging effects, can persist for so long considering the reported rapid clearance rate of this compound. It is possible that plasma concentrations and clearance rates do not reflect tissue sequestration of this compound through intracellular protein binding.

It is recognised that orally administered α-lipoic acid is absorbed, transported to tissues and reduced by two different pathways dependent upon the enantiomeric form. The R-isomer is reduced within the mitochondrial matrix where dihydrolipoamide dehydrogenase (the E3, FAD-containing enzyme of the α-keto acid dehydrogenase complexes, E.C. 1.8.1.4) reduces α-lipoic acid in a NADH dependent process. In the cytosol, and in cells lacking mitochondria such as erythrocytes, cytosolic glutathione reductase (E.C. 1.6.4.2) will reduce α-lipoic acid in a NADPH-dependent reaction with a marked preference for the S-isomer. The R-lipoate is the naturally occurring form while the S-lipoate is the synthetic form. A significant relationship exists between the mitochondrial content of tissues and the relative balance of these two reduction pathways. The ratio of NADH- to NADPH-dependent reduction of exogenous racemic α-lipoic acid is determined in each tissue by the balance between glutathione reductase activity in the cytosol, and the mitochondrial content and hence dihydrolipoamide dehydrogenase activity. In the liver there is an equal balance between these two pathways whereas in the heart, over 90% of α-lipoic acid is reduced within the mitochondria, hence in this tissue there is a marked preference for the R-isomer. Thus the reduction of exogenous α-lipoic acid is tissue specific and complex (Haramaki et al., 1997).

Long-term changes in cells often relate to the differentiated state and it is of note that α-lipoic acid has been reported to block differentiation (Lin et al., 2002), with the implication that it can modify gene expression. Specifically, α-lipoic acid can block increased expression of the connective tissue growth factor gene induced by cyclosporine (Louhelainen et al., 2006) and it can block the decreased expression of the GLUT4 gene induced by oxidative stress (Pressler-Cohen et al., 2006). Further, it is recognised that α-lipoic acid regulates a number of transcription factors (Mizuno and Packer, 1994; Suzuki et al., 1992) and recently it has been reported that it can exert this effect by directly binding to DNA (Lee and Hughes, 2002; Suh et al., 2004a). Acute i.p. administration of lipoic acid in rats increased hepatic nuclear Nrf2 concentrations by Nrf2 activation and an enhancement of its binding to the antioxidant response element (ARE) (Suh et al., 2004a). A downstream consequence of this is an up-regulation of γ-glutamylcyteine ligase, the rate-limiting enzyme of GSH synthesis and of Phase II detoxification. ARE is the *cis*-acting enhancer sequence that transcriptionally regulates Phase II detoxification that controls more than 200 antioxidant and detoxification enzymes through the Keap1–Nrf2 pathway (Kwak et al., 2003). Keap-1, the inhibitor of INrf2 in rats, has abundant free cysteine residues and is therefore a hypothetical target for lipoic acid inactivation. Lipoic acid is recognised to induce hyperacetylation of histones by inhibiting bulk histone deacetylation, thus modifying gene silencing (Stevens et al., 2006; van de Mark et al., 2003). Thus alternative mechanisms can be identified whereby lipoic acid can exert long-term effects on cellular function without the requirement for it to be continually present in tissues at concentrations whereby it can act directly to modify the redox status.

Diet can have a profound effect on genomic imprinting. The loss of imprinting with age at the insulin-like growth factor 2 (*Igf2*) locus is implicated in human cancer and developmental disease (Waterland et al., 2006). Mice weaned on methyl donor deficient diets, such as those deficient in methionine, choline, vitamin B_{12} and folic acid, exhibit a dramatic post-weaning loss of paternal allelic expression at the *Igf2* locus that persists for a least 100-days on return to control feeding (Waterland et al., 2006). This suggests a possible explanation for the effects on survival observed in which α-lipoic acid forms stable transcription-regulation complexes that block diet-induced changes in expression of genes central to the DR induced extension in longevity. It is also recognised that because of its unusual chemistry, α-lipoic acid can alter protein function by reacting with sulphur-containing amino acid residues (Cakatay, 2006).

Ad libitum feeding of the α-lipoic acid supplemented diet subsequent to a period of DR feeding can clearly mimic the effect of DR feeding to extend the lifespan in rats but the mechanism by which this is achieved is uncertain and appears unlikely to be through its recognised redox properties. The optimum period of DR feeding to achieve the maximum effect on survival feeding prior to a switch to *ad libitum* feeding with the α-lipoic acid supplemented diet would appear to lie between 4 and 10 months. Whether this compound would induce similar effects on survival in other species used as model organisms to study ageing is not known.

Acknowledgements

The authors acknowledge the financial support of the Biotechnology and Biology Research Council under the ERA Initiative (ERA16417). We also thank A. Houghton for the expert animal husbandry services provided.

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