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A tumor preventive effect of dietary restriction is antagonized by a high housing temperature through deprivation of torpor

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Abstract

Energy restriction (ER) has proven to be the only effective means of retarding aging in mice. The mechanisms of multiplicity of effects of ER on aging remain, however, fragmentary. ER induces daily torpor, the induction of which is reduced by increasing the ambient temperature to 30°C. The effects of preventing hypothermia in ER animals were studied in terms of the expected consequences of ER on survival, disease pattern and a number of physiological parameters in autoimmune prone MRL/lpr mice and lymphoma prone C57BL/6 mice. The results demonstrate that torpor plays a crucial role in the prevention of lymphoma development but does not have an affect on other aspects of ER, such as prevention of autoimmune diseases. Copyright © 1996 Elsevier Science Ireland Ltd.

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1. Introduction

Caloric restriction, or selective restriction of energy intake (ER), has proven to be the only effective means of retarding physiological aging and extending the maximum life span in rodents and other animals [1]. Additionally, ER decreases the incidence and delays the time of onset of most age-related diseases [1].

Several hypotheses have been advanced on the mechanisms whereby ER exerts these numerous effects, at least five of which are amenable to investigation using the chronic energy restriction model [1,2]: (1) ER has been shown to strengthen the defense system against oxidative stress and to reduce lipid peroxidation [3–7] and thereby decreasing free radicals which have been theorized to be a cause of aging; (2) ER reduces levels of blood glucose and glycolated haemoglobin [8,9] which, according to the glycation theory, would retard aging [10]; (3) It has been suggested by several investigators [11,12] that adrenocortical steroids play a key role in mediating the cancer-preventive and age-retarding effects of food restriction in rodents; (4) The health-enhancing effects of ER may have an immunologic component since ER inhibits immunological perturbations in all strains of autoimmune-prone mice so far studied, including NZB, (NZB × NZW)F1, MRL/Mp-lpr/lpr, BXSB and kd/kd mice [13–16]. Additionally, ER's influence on a possibly crucial lymphocyte subpopulation has been associated with both delayed onset of disease and greatly prolonged life-span [13–16]; and (5) ER inhibits cellular proliferation [17–19] and enhances programmed cell death (apoptosis) [20,21] which could explain ER's inhibitory effect on cancer [22].

Although ER has been shown to induce torpor [23–25], the role of hypothermia in the various effects of ER has remained unclear. Recently, ER is also shown to induce remarkable hypothermia in rats and primates after long-term ER [26,27]. Thus so far hypothermia after long-term ER is considered to be a universal phenomenon observed in wide varieties of species. These are some pieces of evidence that hypothermia plays crucial roles in the action of ER. For example, it is noteworthy that the inhibitory effects of ER on cellular proliferation may be in part reversed by preventing torpor and/or attenuating hypothermia in ER animals [24–26]. Recent studies have demonstrated that ER induces hepatic carbamyl phosphate synthetase I, suggesting a metabolic similarity ('cross-adaptation') to hibernation [28]. These findings suggest that induction of hypothermia by ER may explain all or at least some of the effects of ER.

The present study examines to what extent the prevention of hypothermia in ER animals affects the expected outcome of ER in terms of survival, disease pattern and a number of physiologic parameters in autoimmune prone MRL/lpr (MRL) mice and lymphoma prone C57BL/6 (B6) mice. As previously demonstrated [24–26], torpor and hypothermia can be prevented by maintaining the animals at

30°C. We compared life spans and incidence of diseases, glucose tolerance, levels of several antioxidant enzymes, lipid peroxidation, proportions of lymphocyte subsets and expression of Fas antigen on the cell surface, which mediates apoptotic signals [29], circadian glucocorticoid profiles and levels of T3 in ER and control populations maintained at room temperature and at 30°C.

We report here that the prevention of hypothermia attenuates the preventive effects of ER on the incidence and age of onset of lymphoma in B6 mice, but does not affect the decrease in autoimmune disease mortality in MRL mice. Furthermore, comparison of the effects of ER at the two temperatures indicated that none of the ER affected parameters measured were specific to the anti-lymphoma effects of ER. This suggests that unknown physiological adaptation(s), possibly associated with hypothermia, are crucial for the anti-lymphoma effects of ER in mice.

2. Materials and methods

2.1. Mice

Four-week old female B6 and male MRL mice were purchased from Nippon Clea (Tokyo, Japan). The mice were randomly assigned to a control (Ct/B6) group, an energy-restricted (ER/B6) group, both maintained at room temperature or to an energy-restricted group maintained at 30°C (ERI/B6). The MRL mice were assigned randomly to control (Ct/MRL) or energy-restricted (ER/MRL) groups at room temperature or to control (CI/MRL) and energy-restricted (ERI/MRL) groups at 30°C. The mice were individually housed in plastic cages with wood shavings at 20–22°C for Ct and ER groups and at 30°C for CI and ERI mice, with a relative humidity of 50% and a 12 h light (07:00–19:00) and 12 h dark (19:00–07:00) photocycle. The mice were maintained under pathogen-free conditions throughout the experiments. Body weights were determined weekly until 9 weeks of age and monthly thereafter.

2.2. Diet

The make-up of the diets for the Ct, ER and ERI groups were as previously described [30]. Mice were fed daily between 16:30 and 17:00 h. The Ct/B6 mice received 27 g (407 kJ) of the control diet per week, which averaged 20% less than the amount consumed by B6 female mice given free access to the diet. The ER/B6 mice received 17 g (235 kJ) of the ER diet per week and the ERI/B6 mice 13 g (175 kJ) of the ERI diet. The Ct/MRL mice received 25 g (377 kJ) of the control diet per week and the CI/MRL mice were fed 15 g of control diet and 5 g of ERI diet per week (total of 293 kJ/week). The ER/MRL mice received 15.5 g (214 kJ) of ER diet per week and the ERI/MRL mice 13.5 g (180 kJ) of ERI diet per week. Energy intake for CI and ERI mice were set at levels sufficient to keep their body weights comparable to the counterpart Ct and ER groups maintained

at the lower temperature. CI, ER and ERI mice consumed about the same amounts of protein, fat, vitamins and minerals but less carbohydrate than the corresponding controls.

2.3. Experimental design

Two sets of experiments were performed. First, we observed the natural course of survival of cohorts of Ct/B6, ER/B6, ERI/B6, Ct/MRL, CI/MRL, ER/MRL and ERI/MRL mice. Gross autopsy examinations were performed on all mice of these cohorts as soon after death as possible. Tissues were fixed in 10% buffered formalin and histologic examinations performed on H and E-stained paraffin embedded sections. Second, B6 animals from additional cohorts were sacrificed for flow cytometry and enzyme and hormone assays (see below). Mice for this purpose were euthanaized with pentobarbital (50 mg/kg) at 3 months, 10 months and 16 months of age. Blood was collected by cardiac puncture and liver, brain, spleen and bone marrow were collected at time of sacrifice. The tissues were perfused through the abdominal aorta with 1 ml of isotonic saline.

2.4. Flow cytometry

Spleen and bone marrow were processed into single cell suspensions. Briefly, the spleens were placed in cold media (RPMI 1640) and gently pressed between surfaces of frasted glasses and passed through a sterile nylon 100 μm mesh screen. The bone marrow was dispersed into cold media by gentle pumping with a syringe. Cell suspensions were washed once and red blood cells lysed with 0.9% ammonium chloride. The cells were washed, counted and then incubated on ice with anti-mouse antibodies labeled with fluorescein isothiocyanate (FITC) for the identification of several lymphocyte subsets. Antibody concentrations used were those suggested by the manufacturer. Anti-CD4+, CD8+, fas and anti-mouse antibodies were obtained from Becton-Dickinson (Mountain View, CA). Flow cytometry was done with the EPICS Elite (Coulter, Tokyo Japan).

2.5. Biochemical assays

After the body weights were recorded, the mice were sacrificed and their livers and brains removed. The organs were washed with ice-cold isotonic saline solution and homogenized with a Potter-Elvehjem homogenizer in ice-cold 20 mM Tris–HCl buffer, pH 7.4. Homogenates were centrifuged at $600 \times g$ at 4°C for 10 min and the supernatants preserved in liquid nitrogen until assay. All enzyme assays were carried out within a protein concentration and time range which gave a linear enzymatic rate. The activity of superoxide dismutase (SOD) in $600 \times g$ supernatants was determined by the SOD-525 method using commercially available kits (BIOXYTECH SOD-525, OXIS International, France). The SOD-525 assay is based on the SOD-mediated increase in the rate of autooxidation of 5, 6, 6a, 11b- tetrahydro- 3, 9, 10-trihydroxybenzo(c)fluorocein in aqueous alkaline

solution, to yield a chromophore with maximum absorbance at 525 nm. One SOD-525 activity unit is defined as the activity that doubles the background autooxidation (Technical note for SOD 525). Catalase activity was measured in $600 \times g$ supernatants using a standard reaction protocol [31].

Lipid peroxidation was determined by the LPO-586 method using a commercially available kit (OXIS International, France). The LPO-586 method is based on the reaction of *N*-methyl-2-phenylindole with malondialdehyde (MDA) and 4-hydroxy-alkenals. Protein concentration was measured by the method of Sedmak and Grossberg [32].

2.6. Hormone assays

The free T3 in the serum was determined using a DCP free T3 kit (Nippon DPC Corporation, Tokyo, Japan). Corticosterone in the plasma was measured using an ^{125}I RSL corticosterone kit (Radioassay Systems Laboratory, Carson, CA).

2.7. Blood glucose determination

Glucose concentrations were determined periodically in blood collected from the tail vein. The glucose tolerance test was performed by intraperitoneal injection of a 15% glucose solution (1.5 g of glucose/kg of body weight). Blood was collected from the tail vein 0, 30, 60 and 120 min after injection.

2.8. Statistics

Values are presented as means $\pm$ S.D. and compared using analysis of variance (ANOVA). Two way ANOVA was used to analyze the effects of age, cohort groups and their interactions and to evaluate statistical contributions of individual factors. If a significant effect of age was found, one way ANOVA was applied to the analysis of effects of groups, followed by Duncan's multiple range test. Gross survival curves were generated by the Kaplan-Meier method and compared among groups within the same mouse strain. The effect of diet on natural death rate were evaluated by log-rank test [33,34]. A value of $P < 0.05$ was considered significant throughout this study.

3. Results

3.1. Body weight growth for cohort mice

Body weight growth curves for Ct/B6, ER/B6, ERI/B6, Ct/MRL, CI/MRL, ER/MRL, and ERI/MRL are shown in Fig. 1. The Ct/B6 and Ct/MRL mice gained weight rapidly, reaching 30–40 g by 300–400 and 100–200 days of age, respectively, while all ER and ERI cohorts remained around 20 g. The Ct/B6 mice, in particular, lost weight rapidly after reaching their peak weight at around 420 days. In contrast, the ER and ERI groups remained at around 20 g for most of their lives.

3.2. Survival curves

Data is summarized in Table 1. Survival time of ER and ERI mice exceeded that of Ct or CI animals for MRL mice and of Ct animals for B6 mice. Survival curves are shown in Fig. 2. There was no difference in survival between Ct/MRL and CI/MRL mice ($P = 0.444$, log-rank test). These data indicate that maintaining mice at a temperature of 30°C does not adversely influence survival in this mouse strain. Similarly there was no significant difference in the survival rates of ER/MRL and ERI/MRL ($P = 0.419$, log-rank test) mice although the 25% survival time of the

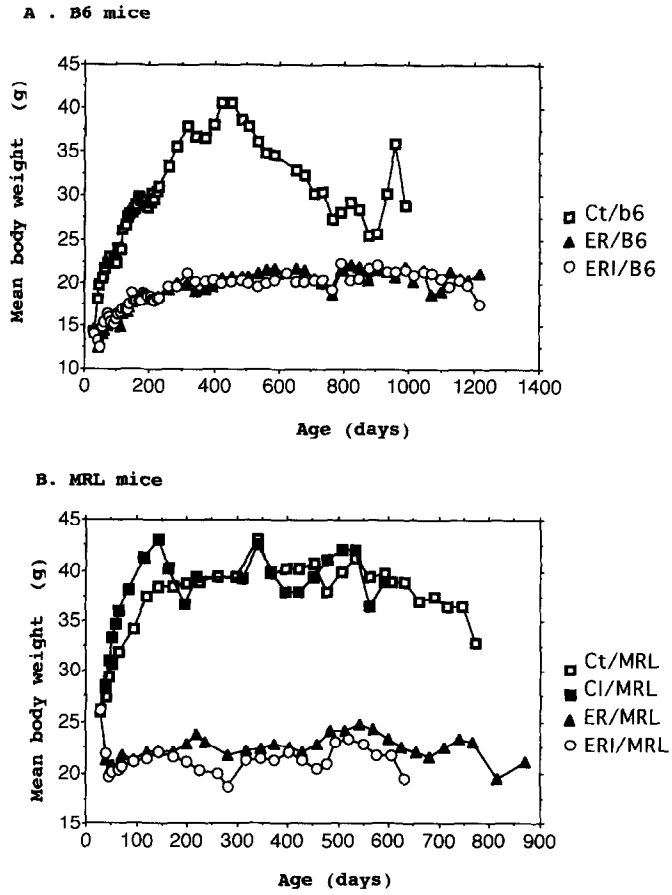

Fig. 1. Body weight growth. Means of body weight for B6 and MRL mice are shown. For clarity, S.D. bars were omitted. Coefficients of variations (100 × S.D./Mean) were less than 15% in all data points. The initial numbers of mice were: 25 for Ct/MRL, 15 for CI/MRL, 25 for ER/MRL and ERI/MRL, respectively. The initial numbers of B6 mice were: 41 for Ct/B6, 42 for ER/B6 and 39 for ERI/B6, respectively. A, means for Ct/B6, ER/B6, and ERI/B6 mice; B, means for Ct/MRL, CI/MRL, ER/MRL and ERI/MRL respectively.

Table 1
Survival data on the cohort mice

Strain	Age (days) of survival				Number of mice		Log-rank test
	Group	75%	50%	25%	Dead	Alive	
MRL	Ct	167	205	255	25	0	
	CI	170	213	230	15	0	NS
	ER	217	269	519	23	2	*
	ERI	201	284	340	25	0	*
B6	Ct	559	778	880	41	0	
	ER	941	1143	1264	34	8	*, **
	ERI	739	810	1049	39	0	*

Observation period of 918 days of age for MRL mice and 1300 days of age for B6 mice.
NS, not significant when compared with Ct.
For MRL mice, there was no significant difference of survival rates between ER and ERI groups.
* Significantly longer than Ct (log-rank test: $P<0.01$).
** Significantly longer than ERI (log-rank test: $P<0.01$).

ER/MRL group was 1.5 times longer than that of the ERI/MRL group. Significant differences were observed between the survival times of Ct/MRL and ER/MRL ($P=0.009$, log-rank test) mice and CI/MRL and ERI/MRL mice ($P=0.009$, log-rank test). In striking contrast, ER/B6 mice lived significantly longer than ERI/B6 mice ($p<0.001$, log-rank test), although ERI/B6 mice lived longer than Ct/B6 mice ($P=0.003$, log-rank test).

3.3. Autopsy findings

Major causes of deaths of MRL mice included cerebral hemorrhage, subarachnoid hemorrhage and rupture of aortic aneurysms. Histologically, renal lesions characteristic of autoimmunity were observed in all MRL animals. Since the MRL mice died earlier because of autoimmune disease, comparisons of neoplastic death rates among groups were not meaningful. Thus, statistical analysis of tumor incidence was limited to the B6 strain (Table 2). By 1300 days, 31 out of 41 Ct mice (76%), 17 out of 42 ER mice (40%) and 26 out of 39 ERI mice (67%) had died of lymphoma. Thus, the mortality rate of ER mice due to lymphoma was significantly less than those of Ct and ERI mice (Fischers exact test, $P=0.0018$ and $P=0.026$, respectively). In addition, Ct mice died of lymphoma significantly earlier than ER or ERI mice (log-rank test: $P=0.0005$ and 0.008, respectively, Table 2). It is particularly interesting that ERI mice also died significantly earlier than ER mice due to lymphoma (log-rank test: $P=0.005$).

3.4. Effects of ER and housing temperature on physiological and biochemical parameters

Results for free T3 levels, peripheral white blood cell counts (WBC) and relative lymphocyte populations, activities of catalase and SOD and concentration of MDA in liver and brain are shown in Table 3. Free T3, catalase, SOD and MDA are parameters which are indicators of oxidative stress. Free T3 levels were significantly lower in ERI mice than the other two groups at three different ages. WBC counts tended to be lower in ER than ERI mice at all ages, although the difference was not statistically significant. Hepatic catalase activity was elevated by energy restriction as previously reported [3]. However, ER had no effect on cerebral catalase activity. Activities of SOD were not altered in either liver or brain by energy restriction or housing temperature. MDA levels in liver tended to increase between 3 and 10 months of age in all experimental groups of B6 mice. However, the degree of

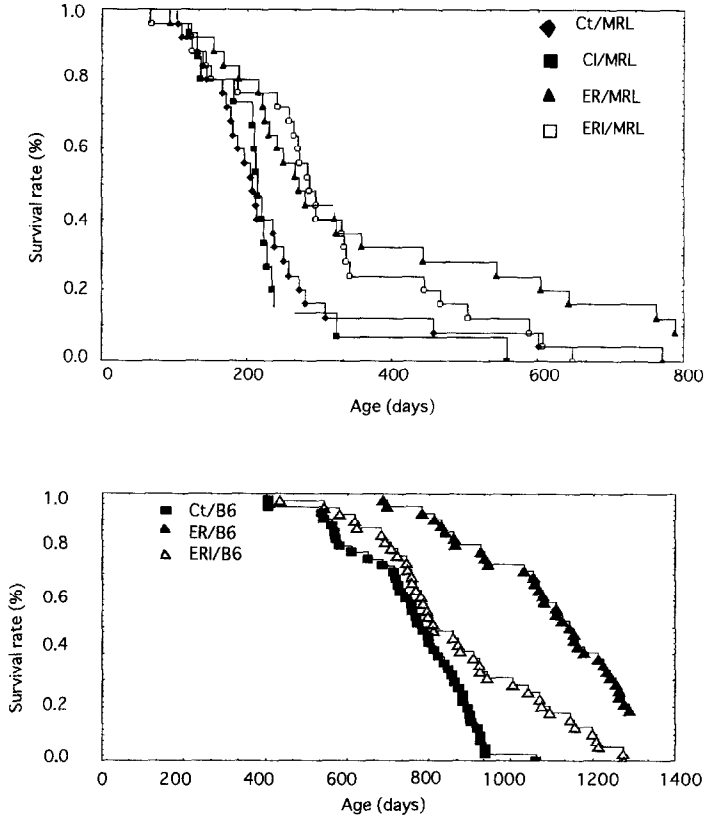

Fig. 2. Survival curves for B6 and MRL mice. B6 mice were observed for 1300 days and MRL mice for 945 days. The initial numbers of mice were the same as in Table 1.

Table 2
Inhibition of tumor growth in ER mice compared with ERI and Ct B6 mice in a 1300-day observation period

	Group		
	Ct	ER	ERI
Number of mice	41	41	39
Number of dead mice	41	34	39
Non tumor causes of death	10	14	12
Tumors other than lymphoma[a]	0	3	1
Lymphoma	31	17	26
Fischer's	Ct— $P = 0.0018$ ——— ER		
Exact test	Ct——— $P = 0.461$ ———		ERI
		ER— $P = 0.026$	ERI
Survival rate (%)	Ages of surviving rates for mice who died of lymphoma (days)		
75	677	934	752
50	785	1148	810
25	888	1241	1037
Log-rank test		*, **	*

[a] All of them were osteofibroma of the maxillary bone.
* Significantly different from Ct groups ($P < 0.01$).
** Significantly different from ERI group ($P < 0.01$).

elevation was less in ERI mice than in the other groups. In contrast, MDA levels in the brain remained at a constant level in Ct mice at all ages but decreased with aging in both ER and ERI mice. These results collectively indicate that oxidative stress appears lowest in ERI mice and lower in ER than in Ct mice.

3.5. Circadian profiles of corticosterone secretion

Plasma concentration of corticosterone together with blood glucose levels in the morning (09:00), evening (17:00) and late night (01:00) are shown for Ct, ER and ERI mice in Table 4. Corticosterone levels were higher in the evening and at midnight in ER mice and at midnight in ERI mice than in Ct mice, while they were essentially the same in the morning. Elevation or decrease in blood glucose levels were negatively correlated to corticosterone concentration. A significant negative correlation existed between glucose concentrations (X mM) and corticosterone levels (Y ng/ml): $Y = -0.0546X + 12.05$ $r = 0.51$, $P < 0.05$ ($n = 45$). We, thus, tentatively concluded that plasma corticosterone levels changed to counter-regulate blood glucose levels. Glucose homeostasis was evaluated in three groups of mice by determining glucose load and blood glucose levels in the morning (Table 5). ER increased glucose tolerance both at room temperature and at 30°C.

Table 3
Effects of energy restriction and housing temperature on physiological and biochemical parameters in B6 mice

Parameters	Age			Among[a] age	Among[b] group × age
Group	3 months ($n = 5$)	10 months ($n = 5$)	16 months ($n = 5$)		
Free T3 (pg/ml)					
Ct	0.682 ± 0.343^{a}	0.794 ± 0.233^{a}	0.917 ± 0.194^{a}		
ER	0.536 ± 0.160^{a}	0.687 ± 0.128^{a}	0.905 ± 0.131^{a}	NS	NS
ERI	0.251 ± 0.146^{b}	0.374 ± 0.110^{b}	0.331 ± 0.180^{b}		
WBC (per μl of blood)					
Ct	7675 ± 928^{a}	6560 ± 950^{a}	6510 ± 1286^{a}		
ER	4025 ± 1394^{b}	3560 ± 451^{b}	4240 ± 1411^{b}	NS	NS
ERI	5033 ± 1350^{b}	3844 ± 485^{b}	5120 ± 455^{b}		
% lymphocyte					
Ct	83 ± 8^{a}	79 ± 10^{a}	80 ± 4^{a}		
ER	79 ± 7^{a}	89 ± 5^{a}	76 ± 10^{a}	NS	NS
ERI	78 ± 6^{a}	88 ± 5^{a}	78 ± 4^{a}		
Catalase (mmol/mg protein/min)					
Liver					
Ct	62.8 ± 11.4^{a}	69.1 ± 7.3^{a}	62.0 ± 11.5^{a}		
ER	$72.1 \pm 10.0^{a, b}$	$82.9 \pm 12.3^{a, b}$	78.4 ± 9.9^{b}	NS	*
ERI	83.1 ± 10.8^{b}	87.4 ± 9.9^{b}	97.3 ± 7.4^{c}		
Brain					
Ct	2.27 ± 0.84^{a}	3.14 ± 0.55^{a}	3.25 ± 0.57^{a}		
ER	3.37 ± 0.73^{a}	2.71 ± 0.70^{a}	2.31 ± 0.57^{a}	NS	*
ERI	3.26 ± 0.86^{a}	3.67 ± 0.44^{a}	2.93 ± 0.52^{a}		
SOD (Unit/mg protein)					
Liver					
Ct	505 ± 114^{a}	410 ± 30^{a}	409 ± 23^{a}		
ER	411 ± 45^{a}	493 ± 137^{a}	408 ± 26^{a}	**	NS
ERI	491 ± 63^{a}	488 ± 95^{a}	343 ± 72^{a}		
Brain					
Ct	5.3 ± 0.7^{a}	4.6 ± 0.3^{a}	5.4 ± 0.6^{a}		
ER	5.4 ± 0.9^{a}	5.1 ± 0.6^{a}	5.2 ± 0.8^{a}	NS	NS
ERI	5.5 ± 0.8^{a}	4.9 ± 0.5^{a}	5.1 ± 0.5^{a}		
MDA (mmol/g pro)					
Liver					
Ct	0.18 ± 0.08^{a}	0.67 ± 0.19^{a}	0.68 ± 0.14^{a}		
ER	0.22 ± 0.04^{a}	0.56 ± 0.14^{a}	0.53 ± 0.26^{a}	**	NS
ERI	0.20 ± 0.06^{a}	0.54 ± 0.17^{a}	0.38 ± 0.03^{b}		
Brain					
Ct	0.18 ± 0.08^{a}	0.19 ± 0.02^{a}	0.15 ± 0.04^{a}		
ER	0.17 ± 0.04^{a}	0.14 ± 0.06^{a}	0.09 ± 0.03^{b}	**	NS
ERI	0.15 ± 0.04^{a}	0.14 ± 0.07^{a}	0.07 ± 0.02^{b}		

Values are means $\pm$ S.D.

[a] The superscript letters indicate the results of comparisons among groups with the same age by one-way ANOVA. When values share the same letters, [a] and [ab], they are not significantly different ($P > 0.05$). Otherwise, they are significantly different ($P > 0.05$).

[b] Two factors, age and group and their interactions were evaluated by two-way ANOVA.

* $P < 0.05$, ** $P < 0.01$. NS, not significant.

Table 4
Effects of energy restriction and housing temperature on circadian profile of serum corticosterone and glucose concentration

Parameter	Group	Morning (09:00)	Evening (17:00)	Midnight (01:00)
Corticosterone				
(ng/ml)	Ct	59 ± 4^{a}	66 ± 11^{a}	71 ± 7^{a}
	ER	61 ± 8^{a}	106 ± 8^{b}	94 ± 18^{b}
	ERI	62 ± 9^{a}	67 ± 13^{a}	95 ± 18^{b}
Glucose				
(mM)	Ct	10.6 ± 1.7	9.5 ± 1.7^{a}	8.6 ± 1.6^{a}
	ER	9.6 ± 1.3^{a}	5.7 ± 1.1^{b}	6.4 ± 1.1^{b}
	ERI	8.0 ± 0.4^{b}	6.0 ± 1.2^{b}	6.6 ± 0.6^{b}

Values are means ± S.D.
Superscript letters indicate the results of one-way ANOVA. When values share the same letters, ^{a} and ^{ab}, they are not significantly different ($P > 0.05$). Otherwise, they are significantly different ($P < 0.05$).

3.6. Immunological parameters

Lymphocyte subsets and expression of fas antigen as determined by flow cytometry are shown in Fig. 3. Splenocytes and bone marrow cells from disease-free Ct, ER and ERI mice were analyzed at 3 ($n = 5$), 10 ($n = 5$) and 16 ($n = 5$) months of age. At no age did energy restriction or housing temperature change the percentages of CD4+ or CD8+ cells, CD4+/CD8+ ratios, the percentage of Fas positive cells, the percentage of Fas positive CD4+ or the percentage of Fas positive CD8+ cells in bone marrow and spleen.

4. Discussion

The most important finding of the present study is that housing temperature at 30°C, which is known to attenuate daily hypothermia [24–26], reversed the life

Table 5
Effects of dietary restriction and housing temperature on glucose tolerance

Age	Group	B.W.(g)	Glucose concentration during IpGTT(mM)			
			0 min	30 min	60 min	120 min
12 months	Ct ($n = 5$)	31 ± 3^{a}	8.9 ± 0.9^{a}	18.1 ± 5.7^{a}	16.2 ± 6.3^{a}	10.1 ± 2.3^{a}
	ER ($n = 5$)	21 ± 1^{b}	6.6 ± 0.8^{b}	13.8 ± 3.4^{b}	10.9 ± 1.7^{b}	8.1 ± 1.7^{b}
	ERI ($n = 5$)	21 ± 1^{b}	6.3 ± 0.6^{b}	11.0 ± 1.7^{b}	9.5 ± 0.9^{b}	6.4 ± 0.7^{c}
6 months	Ct ($n = 5$)	31 ± 1^{a}	8.6 ± 1.0^{a}	13.5 ± 2.3^{a}	13.6 ± 1.2^{a}	8.8 ± 1.1^{a}
	ER ($n = 5$)	19 ± 1^{b}	7.1 ± 0.7^{b}	11.2 ± 2.3^{b}	9.1 ± 1.4^{b}	6.0 ± 0.8^{b}
	ERI ($n = 5$)	20 ± 1^{b}	7.0 ± 0.5^{b}	13.1 ± 1.8^{a}	8.7 ± 0.8^{b}	7.0 ± 0.6^{b}

Superscript letters indicate the results of one-way ANOVA. When the values share the same letters, ^{a} and ^{ab}, they are not different ($P < 0.05$). Otherwise, they are significantly different ($P < 0.05$).

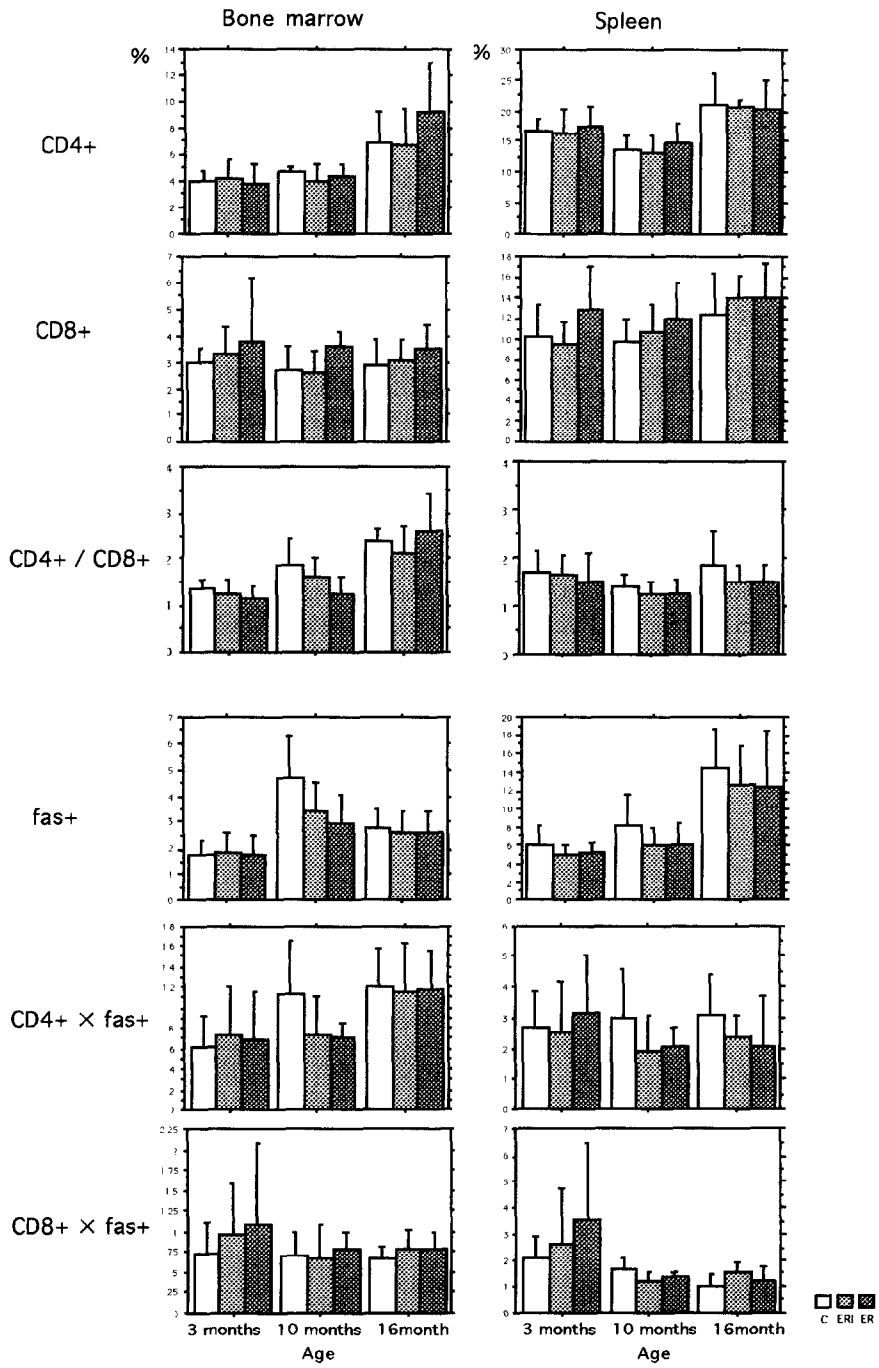

Fig. 3. Immunological investigation on lymphocyte subsets. Values are mean (box) and S.D. (bar). 5 mice of each age were analyzed from each group.

extending effects of ER in B6 mice, largely by decreasing the anti-lymphoma action of ER. In contrast, in MRL mice, the higher temperature failed to attenuate ER's effect in delaying the progress of autoimmune disease. It seems unlikely that the higher housing temperature would cause physical stress to the mice and thereby shorten life span, as 30°C is known to be the thermoneutral zone for mice [35], at which mice can minimize heat production and probably energy metabolism, as suggested by the low levels of serum T3. Furthermore, the survival of CI/MRL mice was shown to be comparable to that of Ct/MRL mice at room temperature. In addition, survival of ERI/MRL and ER/MRL mice were also comparable. Finally, 67% of ERI/B6 mice died of lymphoma, indicating that housing temperature at 30°C does not induce specific lesions but rather, modifies the tumor promotion rates. These lines of evidence suggest that a higher housing temperature may reverse anti-lymphoma prevention of ER, but not other effects (autoimmunity).

With regard to physiologic parameters, the higher housing temperature partially attenuated ER's effect on the white blood cell count, but not levels of hepatic catalase, hepatic MDA, plasma corticosterone, blood glucose or glucose tolerance. In contrast, free T3 in serum was significantly lowered by the higher housing temperature. Thus, one may argue that the lower energy intake in ERI mice may be confounded by the temperature effect in a complex manner so that ERI mice died due to lymphoma before they could have enjoy anti-aging effects of ER. ERI mice had the lowest levels of oxidative stress when compared with the other two groups, suggesting that oxidative damages might have progressed at much slower rates than in other groups. This anti-aging effect, however, could not have won an advantage over the surge of lymphoma. We thus tentatively derive a hypothesis from the present results. the hypothesis that anti-lymphoma action of ER could be dissected from anti-aging effects of ER. This possibility, however, cannot be proven at present and opens up a new research interest.

ER is known to suppress cellular proliferation and enhance apoptosis [17–22], which may in part explain its effect on neoplastic diseases. While the mechanism for these effects might involve stimulation of expression of the p53 tumor suppressor gene [36], which inhibits cellular proliferation and promotes apoptosis [37], other mechanisms must also be involved since ER also inhibits cellular proliferation and delays tumor progression in p53-knockout transgenic mice [38]. In any case, a 30°C housing temperature attenuates the effect of ER on cellular proliferation rates and mitotic activities in mice and rats [24–26]. It may be that the hypothermia induced by ER inhibits cellular proliferation and thereby extends the life span by inhibiting tumor progression.

In separate experiments we have observed that resting membrane potentials of cardiac papillary muscles from ER mice are well preserved even at lower temperatures, such as 20°C but not from Ct mice (Wada et al., submitted for publication). Such evidence strongly indicates that ER induces cold tolerance.

The present investigation failed to demonstrate significant changes in lymphocyte subsets or levels of the cell surface protein, fas, which triggers apoptosis of T cells [29] in long life B6 mice. Because both ER and ERI extend

longevities of MRL mice to a comparable degree, but differ in their effects on longevity in B6 mice, there is a possibility that different mechanisms are responsible for delaying autoimmune diseases in MRL mice and aging in B6 mice.

In conclusion, the present results demonstrate that a physiological adaptation tightly associated with hypothermia plays a role in some but not all of the effects of ER. The present study demonstrates that hypothermia can be used to isolate some of the varied effects of energy restriction, especially ER's suppressive effect on lymphoma promotion. The present experimental protocol may provide a new mechanistic insight into roles of ER's actions.

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