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Methionine restriction decreases visceral fat mass and preserves insulin action in aging male Fischer 344 rats independent of energy restriction

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Summary

Reduced dietary methionine intake (0.17% methionine, MR) and calorie restriction (CR) prolong lifespan in male Fischer 344 rats. Although the mechanisms are unclear, both regimens feature lower body weight and reductions in adiposity. Reduced fat deposition in CR is linked to preservation of insulin responsiveness in older animals. These studies examine the relationship between insulin responsiveness and visceral fat in MR and test whether, despite lower food intake observed in MR animals, decreased visceral fat accretion and preservation of insulin sensitivity is not secondary to CR. Accordingly, rats pair fed (pf) control diet (0.86% methinone, CF) to match the food intake of MR for 80 weeks exhibit insulin, glucose, and leptin levels similar to control-fed animals and comparable amounts of visceral fat. Conversely, MR rats show significantly reduced visceral fat compared to CF and PF with concomitant decreases in basal insulin, glucose, and leptin, and increased adiponectin and triiodothyronine. Daily energy expenditure in MR animals significantly exceeds that of both PF and CF. In a separate cohort, insulin responses of older MR animals as measured by oral glucose challenge are similar to young animals. Longitudinal assessments of MR and CF through 112 weeks of age reveal that MR prevents age-associated increases in serum lipids. By 16 weeks, MR animals show a 40% reduction in insulin-like growth factor-1 (IGF-1) that is sustained throughout life; CF IGF-1 levels decline much later, beginning at 112 weeks. Collectively, the results indicate that MR reduces visceral fat and preserves insulin activity in aging rats independent of energy restriction.

Key words: adipose; aging; energy expenditure; insulin; methionine restriction.

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Introduction

Increased median and maximum lifespans have been reported in male Fischer 344 (F344) rats maintained on a diet restricted in the essential amino acid methionine (Orentreich *et al*., 1993). Recently, methionine restriction was reported to extend the lifespan of mice as well (Miller *et al*., 2005). Characteristically, methionine-restricted (MR, 0.17% methionine) rats have significantly reduced body weights and consume less food per animal but more on a per-gram body weight basis when compared to control-fed animals (CF, 0.86% methionine) (Orentreich *et al*., 1993; Zimmerman *et al*., 2003). However, growth is unimpaired in animals consuming 0.86% methionine while being pair-fed (PF) to the lower food intake of MR animals. Also, increasing the energy content of the MR diet does not result in body weight gain. Taken together, these results suggest that the decrease in energy intake is not a factor in life extension (Orentreich *et al*., 1993).

Rats, like human beings, become increasingly insulin resistant with age due primarily to increased adiposity (Barzilai & Rossetti, 1995). Longevity in MR rodents might result from sustained insulin sensitivity through a reduction in fat accumulation, which should be reflected by levels of the adipocytokines leptin and adiponectin. Rodents and human beings are also similar in that plasma leptin levels correlate positively with body weight, adipose tissue, and body mass index (Frederich *et al*., 1995; Considine *et al*., 1996). Calorie-restricted (CR) rodents exhibit reduced fat mass, reflected in lower leptin levels (Greenberg & Boozer, 1999). In contrast, adiponectin levels correlate negatively with adiposity; adiponectin levels are reduced in obesity and type II diabetes (Arita *et al*., 1999; Hotta *et al*., 2000) and increased in CR animals (Zhu *et al*., 2004). Important exceptions include the long-lived growth hormone receptor knockout (GHRKO) mouse, which has both increased adiposity and adiponectin (Bartke *et al*., 2004; Al-Regaiey *et al*., 2005) and PPAR-γ (peroxisome proliferator-activated receptor gamma) agonist (i.e. thiazolidinedione) induced adipogenesis with concomitant increases in adiponectin in type II diabetics (Yang *et al*., 2002). However, thiazolidinedione administration to obese Zucker rats results in remodeling of fat depots with a greater number of small adipocytes in the white adipose tissue coupled with a reduction in the population of large adipocytes (Okuno *et al*., 1998); in human beings, it results in a relative shift of adipose tissue from visceral to subcutaneous depots (Kelly *et al*., 1999; Akazawa *et al*., 2000; Nakamura *et al*., 2001). Thus, morphology changes and differentiation might account for these observations.

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Both leptin and adiponectin play major roles in energy homeostasis, fat metabolism, and insulin regulation. Administering leptin to leptin-deficient obese (ob/ob) mice decreases food intake, reduces body weight, and improves insulin action through centrally mediated mechanisms (Campfield *et al.*, 1995; Pelleymounter *et al.*, 1995). Initially, adiponectin was thought to improve insulin sensitivity by lowering skeletal muscle triglyceride levels through increased β-oxidation of free fatty acids (Fruebis *et al.*, 2001; Yamauchi *et al.*, 2001); however, central actions of this peptide are now also recognized. Intracerebroventricular administration of adiponectin to both normal and *ob/ob* mice reduces body weight. Unlike leptin, adiponectin treatment does not suppress food intake, indicating that weight loss results from increased energy expenditure (Qi *et al.*, 2004).

The present study examines the relationship between reduced adiposity and preservation of insulin sensitivity in aging MR rats and tests the hypothesis that, despite modest reductions in food intake, the observed consequences of MR are not secondary to CR. Visceral fat weight, energy expenditure, and key plasma markers [insulin, leptin, adiponectin, glucose, insulin-like growth factor-1 (IGF-1), and thyroid hormone] associated with aging and/or body composition were measured in long-term MR animals and in animals fed control diet to match the lower food intake of MR animals (i.e. PF). Separate experiments were also conducted in MR animals to assess responses to oral glucose challenge and to evaluate longitudinal changes in levels of IGF-1, triglyceride, and cholesterol.

Results

Growth curves: longitudinal studies

Growth was dramatically reduced in male F344 rats exposed to MR prior to puberty (Fig. 1). In this cohort, significant differences ($P < 0.0001$) between the CF and MR regimens were detected after 6 weeks of MR. During this time, the weight of CF rats increased by nearly 80%, whereas MR animals gained only 31% from the baseline. CF animals continued to grow during their first year, attaining an average weight of 445 g at 60 weeks of age. In contrast, age-matched MR rats averaged 225 g. CF body weight plateaued at 65 weeks of age and MR at 89 weeks. Although ancillary growth parameters were not determined in the present study, data from previous experiments indicate that tail length – a marker of somatic growth – is significantly reduced by nearly 15% in mature (greater than 1 year of age) MR rats compared to CF animals.

Survival at 112 weeks of age (length of the longitudinal study period) was 65% for CF and 89% for MR animals. The overall median survival time for the MR animals (including those from the longitudinal study) was 30% greater than for CF animals, which agrees with our previous data (Orentreich *et al.*, 1993). The maximum survival of the MR rat improved by 23 weeks to 179 weeks of age (data not shown), about the same difference in time between the groups for the body weight to plateau.

Pair-feeding studies

Methionine-restricted animals had reduced food intake; however, the reported consequences of MR appear to be unrelated to the modest reduction in energy consumption, as control animals grew when pair fed to the daily intake of MR animals (Orentreich *et al.*, 1993; Zimmerman *et al.*, 2003). To extend these observations and to examine associations between insulin responsiveness and visceral fat deposition, CF, MR, and PF animals were evaluated following 80 weeks of feeding.

Growth, assessed by weight gain, was markedly reduced in MR animals (Table 1). After 80 weeks of MR, the body weight of these animals had increased by 147 g over baseline. Despite reduced food consumption ($P < 0.005$ vs. CF; not significant vs.

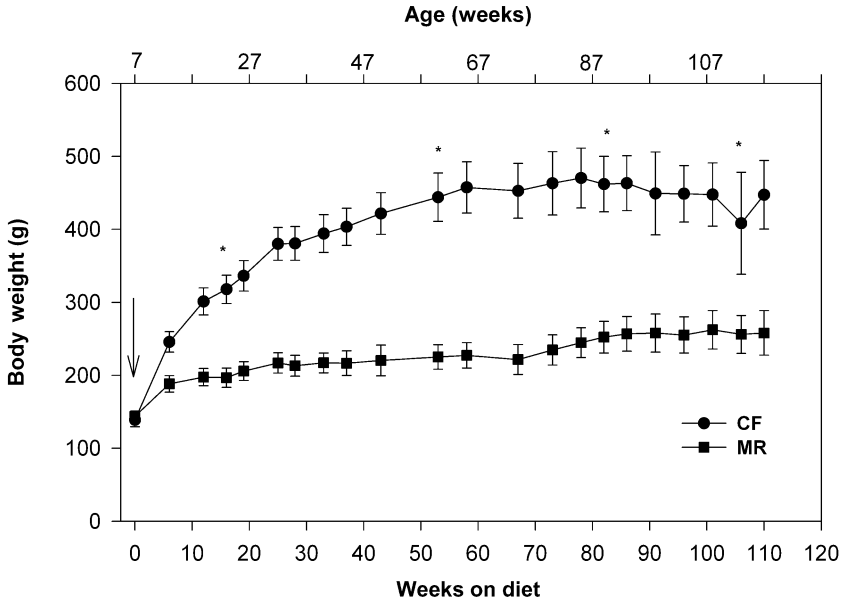

Fig. 1 Lower body weights of male F344 rats fed methionine-restricted (MR) diet vs. control-fed (CF) diet beginning at 7 weeks of age. Upper horizontal axis represents the age of the animals. Arrow corresponds to time of MR initiation. Asterisks correspond to blood collection time points. Values are mean ± SD, $n = 20$ for CF (●) and $n = 19$ for MR (■).

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Table 1 Pair-feeding (PF) studies demonstrate that the effects of long-term (80 weeks) methionine restriction (MR) on body weight, visceral fat (VF) accumulation, and endocrine indices are independent of energy restriction

		CF	PF	MR	*P*-value
Body weight (g)	7 week	122 ± 12.1	125 ± 19.4	124 ± 13.0	NS
Body weight (g)	87 week	519 ± 51.5	429 ± 26.6	271 ± 29.7	**
Food consumption	(g/day)	21.0 ± 2.5	14.7 ± 0.8	16.6 ± 1.7	NS[b]
VF	(g)	72.1 ± 15.8	55.6 ± 10.2	20.1 ± 4.0	**
VF	(% BW)	13.7 ± 1.8	13.0 ± 2.4	7.4 ± 0.8	**
Leptin	(pmol L^{-1})	1880 ± 25	1650 ± 244	444 ± 100	**
Adiponectin	(μg mL^{-1})	2.9 ± 0.3	3.3 ± 0.3	6.1 ± 0.9	**
Insulin	(pmol L^{-1})	735 ± 315	435 ± 135	105 ± 30	**
Glucose	(mmol L^{-1})	8.2 ± 0.4	7.8 ± 0.5	7.2 ± 0.5	*
IGF-1[a]	(ng mL^{-1})	875 ± 159	638 ± 22.7	297 ± 62.7	*
Total T_4	(nmol L^{-1})	46.5 ± 6.5	45.9 ± 5.5	44.1 ± 3.7	NS
Total T_3	(nmol L^{-1})	1.3 ± 0.2	1.5 ± 0.3	3.6 ± 1.0	**

NS, nonsignificant.
[a]To convert to nmol L^{-1}, multiply by 0.13; [b]NS MR vs. PF only.
*$P < 0.05$; **$P < 0.005$ (MR vs. PF)
MR is similarly significant vs. CF.
All chemistries obtained from nonfasting specimens. Data shown reflect food consumption calculated as a running average of the intake during the last 4 weeks of the study. VF represents the sum of the epididymal, retroperitoneal (including perinephric), and inguinal fat pad weights.

Table 2 Energy expenditure in 63-week-old methionine-restricted (MR) rats exceeds control-fed (CF) or pair-fed (PF) animals when corrected for body weight (mean ± SD, $n = 6–7$)

		CF	PF	MR	*P*-value
Body weight	(g)	493 ± 32.2	351 ± 15.5	238 ± 24.0	†
RER	(VCO_2/VO_2)	0.82 ± 0.06	0.84 ± 0.05	0.85 ± 0.02	NS
Heat	(kcal h^{-1})	2.52 ± 0.13	2.10 ± 0.20	1.67 ± 0.11	*
Energy	(kcal h^{-1} kg^{-1} BW)	5.12 ± 0.33	5.97 ± 0.45	7.02 ± 0.59	†

*$P < 0.05$; †$P < 0.005$ (MR vs. CF). NS, nonsignificant; RER, respiratory exchange ratio.

MR), body weight of PF animals increased an average of 304 g over 80 weeks. CF animals gained more weight (397 g), and by the end of the study body weights in these animals had increased by 21% ($P < 0.01$) relative to PF animals. Although plasma IGF-1 values in PF were 27% lower than in CF animals, several values obtained for the PF animals were similar to the CF group (data not shown). IGF-1 values of MR animals were significantly reduced by more than 65% compared to CF and by 53% compared to PF animals (Table 1).

As with CR (Barzilai *et al.*, 1998), reduction of visceral fat is an important outcome of MR. Indeed, weight of visceral fat in chronically MR animals was reduced by 72% compared to CF animals and by 64% relative to PF animals. Visceral fat was modestly (23%) but not significantly reduced in PF compared to CF animals. When measurements were adjusted to reflect body weight differences, visceral fat for both PF and CF were similar (PF: 13.0% ± 2.4; CF: 13.7% ± 1.8), whereas a greater than 40% reduction in visceral fat was observed in MR animals. Plasma leptin levels in MR animals were suppressed more than 70% compared to PF and CF. Interestingly, the lower food intake of PF animals did not produce statistically significant reductions in leptin; differences of only 12% were observed between this group and CF. As expected, a strong correlation between leptin levels and adipose tissue weight ($r = 0.93$, $P < 0.0001$) was detected. Taken together, these results indicate that, despite a 21% reduction in food intake, the effects of MR on visceral fat and leptin appear to be specific to lower dietary methionine content.

Plasma insulin and glucose levels were higher in CF animals relative to PF; these differences were not significant. In contrast, plasma insulin levels decreased by 70% on average in long-term MR animals when compared to CF and PF. A small but statistically significant decrease ($P < 0.05$) in glucose levels was detected between MR and PF animals. Adiponectin, a plasma marker associated with improved insulin sensitivity, was markedly ($P < 0.005$ vs. PF; $P < 0.001$ vs. CF) elevated in MR. At the conclusion of the 80-week study, adiponectin levels in MR animals were higher by 85% and 110% compared to PF and CF animals, respectively.

Measurement of energy expenditure

Indirect calorimetry measurements confirmed that MR rats had increased daily energy expenditure (DEE) compared to CF and PF rats (Table 2) consistent with higher food consumption and suppressed weight gain. The 24-h energy expenditure of MR rats calculated on a per-hour basis and normalized to body weight significantly exceeded that of PF and CF (7.02 ± 0.59, 5.97 ± 0.45, 5.12 ± 0.33 kcal h^{-1} kg^{-1} BW, respectively). The increased surface area-to-mass ratio of the smaller animals could account for increased energy usage due to necessary thermogenesis; however, preliminary data comparing similarly sized rats

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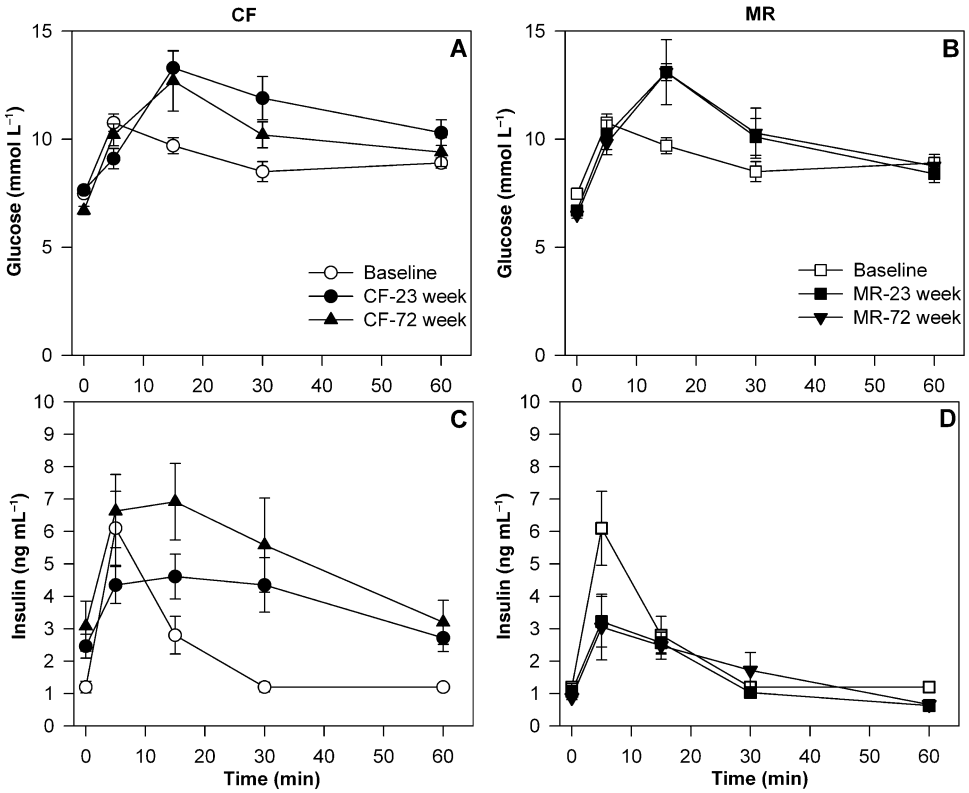

Fig. 2 Insulin response to an oral glucose challenge is improved in methionine-restricted (MR) animals (B and D) vs. control-fed (CF) (A and C). A significant main effect of diet ($F_{(1,18)} = 21.5$ $P < 0.0002$), as well as a diet × time interaction ($F_{(4,72)} = 2.76$ $P < 0.034$), was detected for insulin. Animals were evaluated at 7 weeks of age (baseline, □) and at 23 (■) and 72 (▼) weeks on MR. The same group of animals were used as the baseline comparisons for both CF and MR. Values are mean ± SEM, $n = 5$–6.

showed increased energy utilization in MR vs. CR animals (T. Gettys, personal communication), indicating that body temperature maintenance cannot be solely responsible for the phenomenon.

Thyroid hormone indices were obtained after 80 weeks of MR and show significantly elevated ($P < 0.001$) T_3 levels (Table 1). T_3 levels increased by approximately 15% in PF animals compared to CF. Higher circulating T_3 levels are generally associated with increased energy expenditure and, although these two parameters were not measured in parallel, the increased DEE observed in mature (63-week-old) MR animals relative to PF and CF probably resulted from increased peripheral conversion of T_4 to T_3, since total T_4 levels were equivalent in all groups.

Oral glucose tolerance test

The results of the oral glucose tolerance test (OGTT) at 0, 23, and 72 weeks of feeding are presented in Figs 2 and 3. Irrespective of age, glycemic responses were similar for both CF and MR animals (Figs 2A,B, and 3A) during the 1-h OGTT. However, the mean insulin response of MR animals at 23 and 72 weeks to the oral glucose load was significantly reduced compared to CF animals (Fig. 2C,D). A significant main effect of diet ($F_{(1,18)} = 21.5$ $P < 0.0002$) as well as a diet × time interaction ($F_{(4,72)} = 2.76$ $P < 0.034$) was detected. Compared to CF, MR animals at 23 weeks showed a 61% reduction in the insulin area under the curve (Fig. 3B). A difference of 67% was detected at the 72-week time point.

Longitudinal cholesterol and triglyceride levels

Methionine restriction attenuated age-associated rises in lipid levels (Table 3). Analysis of cholesterol values yielded strong main effects of both diet ($F_{(1,13)} = 93.4$, $P < 0.0001$) and time ($F_{(3,13)} = 18.6$, $P < 0.0001$). This pattern of change over time differed significantly between CF and MR ($F_{(3,39)} = 8.2$, $P < 0.002$). Differences in cholesterol levels between MR and CF were apparent after 16 weeks. MR cholesterol levels remained unchanged throughout the study period, while CF's continued to rise. As the CF animals matured, they exhibited an 80% average increase in cholesterol, despite a 17% decrease (nonsignificant) occurring between weeks 81 and 105.

The effect of MR on triglyceride levels was more pronounced than on cholesterol throughout the study. Triglyceride levels in MR were reduced by more than 50% at 16 weeks. Although triglyceride levels continued to rise with age in CF animals, the increase was not significant when compared to levels obtained at 16 weeks. In contrast, MR levels remained lower and similar to values observed in younger animals. It is not known if this pattern was sustained in older MR animals. Triglyceride levels could not be determined after 81 weeks due to lack of plasma.

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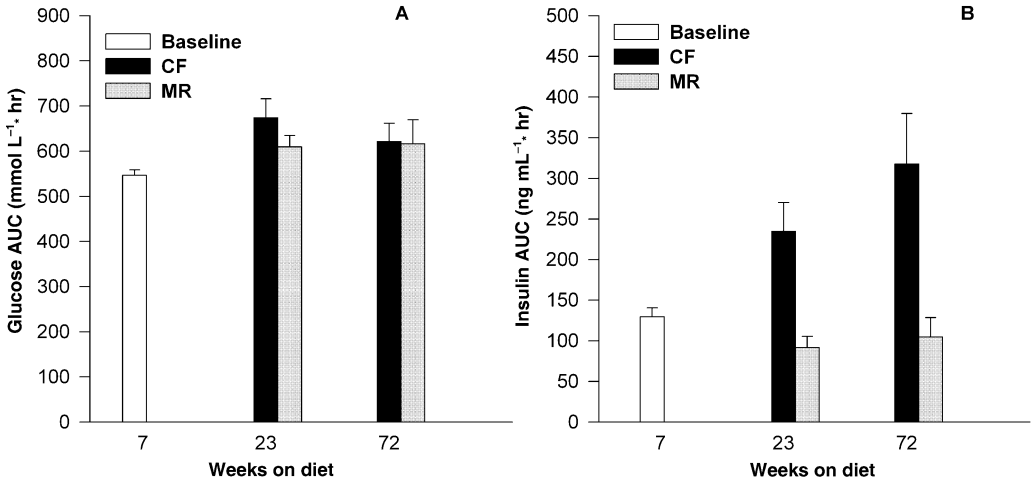

Fig. 3 Area under the curve (AUC) for glucose (A) and insulin (B) following a 60-min oral glucose tolerance test (OGTT). Methionine-restriction reduces insulin AUC at 23 weeks and 72 weeks of feeding. Using a two-factor ANOVA, a significant main effect of diet was observed ($F_{1,18}$ = 23.39, P < 0.0001). Values are mean ± SEM, n = 5–6.

Table 3 Chronic methionine restriction (0.17% methionine) blunts the age-associated increases in cholesterol and triglyceride (TG) levels in male F344 rats (mean ± SD, cholesterol n = 7, TG n = 5). Significant differences between methionine-restricted (MR) and control-fed (CF) are seen as early as 16 weeks and at all time points thereafter. See text for analysis and explanation

	Weeks on diet			
	16	54	81	105
Cholesterol (mmol L^{-1})				
CF	2.20 ± 0.15	3.96 ± 0.62	4.40 ± 0.60	3.64 ± 0.87
MR	1.80 ± 0.14	1.85 ± 0.18	2.34 ± 0.63	2.09 ± 0.68
TG (mmol L^{-1})				
CF	0.13 ± 0.03	0.19 ± 0.05	0.22 ± 0.10	*qns*
MR	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	*qns*

qns, quantity not sufficient.

Longitudinal plasma IGF-1-values

Comparison of IGF-1 levels in CF and MR rats indicated a significant main effect of diet ($F_{(1,16)}$ = 112, P < 0.0001). Lower plasma IGF-1 levels were evident after 16 weeks of MR (Fig. 4), IGF-1 levels being approximately 40% less than in the CF group at all time points. IGF-1 levels in the MR group had declined further at 54 weeks, but at 81 weeks were similar to those observed at 16 weeks. A significant correlation between IGF-1 levels and body weight (r = 0.75, P < 0.01) was detected only in MR rats. CF rats showed a gradual decline in IGF-1 levels with age; there were no significant differences noted between 88 and 112 weeks of age (81 and 105 weeks on diet). Sampling beyond 112 weeks detected diminished IGF-1 levels; however, due to the small size of the group (greater than 50% mortality), no statistical comparisons were performed. There were no correlations between IGF-1 levels and survival.

Discussion

The aging process in both rats and human beings is associated with reduced insulin sensitivity (Barzilai & Rossetti, 1995) and increased cholesterol and triglyceride levels (Liepa *et al*., 1980). The present study demonstrates that these processes are blunted in the F344 rat by a novel paradigm: reduction of intake of the essential amino acid methionine. Furthermore, PF experiments and DEE measurements rule out energy restriction as a factor in the MR effect.

The possibility that the effects of MR are secondary to CR was addressed in this and in previous studies (Orentreich *et al*., 1993; Zimmerman *et al*., 2003). Although MR animals consumed less food on a per-animal basis than did age-matched controls (Orentreich *et al*., 1993; Zimmerman *et al*., 2003), when adjusted for either lower body weight or lean body mass (g $kg^{-0.75}$), MR animals actually ate more food. CF animals restricted to the lower food intake of MR (i.e. PF) generally consumed their daily food allotment; however, the reduced food intake and consequently lower methionine intake (but not to MR levels) yielded modest reductions in body weight. Despite this weight reduction, our data indicate that, in PF rats, key plasma markers of adiposity and growth are similar or comparable to CF but not to MR.

At 80 weeks on diet, insulin and leptin levels in PF animals equaled CF values, whereas age-matched MR rats exhibited markedly lower insulin and leptin levels, indicating that the reductions resulted from low dietary methionine intake and substantially reduced and sustained plasma methionine levels (~35%). These results cannot be attributed to diminished protein intake; MR rats consumed more protein than their CF counterparts when corrected for either body weight (MR: 0.87 g day^{-1} vs. CF: 0.58 g day^{-1}) or lean body mass (MR: 6.2 g day^{-1} vs. CF: 4.9 g day^{-1}). Conventional CR, where carbohydrate not protein content is sacrificed, is neither protein restriction nor MR. In a preliminary comparison of weight-matched

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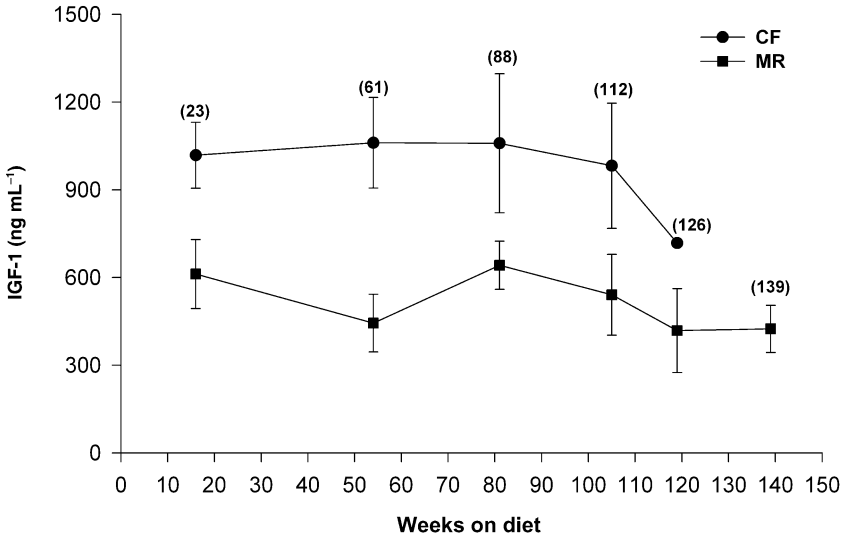

Fig. 4 Insulin-like growth factor-1 (IGF-1) levels are lower in methionine-restricted (MR) male F344 rats. Values in parentheses represent the age of the animal at the time of blood collection. Comparison of MR (■) and CF (●) indicated a significant main effect of diet ($F_{(1,16)} = 112$, $P < 0.0001$). Values are mean ± SD, $n = 8$ for CF, $n = 10$ for MR (except after 105 weeks). To convert to nmol L^{-1}, multiply values by 0.13.

MR and 40% CR rats, CR rats consumed more methionine (CR: 0.16 ± 0.03 g day^{-1} vs. MR: 0.02 ± 0.003 g day^{-1}) than MR and amounts comparable to CF (0.18 g day^{-1}). Protein restriction, in the absence of CR, prolongs longevity, potentially a benefit of modestly reduced methionine intake, but is much less effective than a similar level of protein restriction coupled with CR (Yu *et al.*, 1985).

That both body weight and IGF-1 levels were affected by PF rats' lower dietary methionine (CF: 0.18 g day^{-1} vs. PF: 0.12 g day^{-1}) intake suggests that these parameters are sensitive to moderate reductions in methionine intake, whereas a more severe restriction is required to produce significant decreases in insulin and leptin levels. Plasma IGF-1 levels were 27% lower in the PF group compared with CF; however, values obtained in this and in previous PF cohorts overlapped with CF values. Lower IGF-1 levels have been reported in long-lived animals such as CR rodents and Ames dwarf mice (Breese *et al.*, 1991; Bartke *et al.*, 2001). The IGF-1 levels in those animals were sharply reduced (more than 40%), whereas a more modest decline was observed in our PF animals. Whether decreases in insulin, leptin, and IGF-1 levels occur parallel to changes in methionine levels is not known.

Visceral fat in rodents increases with advancing age (Barzilai & Rossetti, 1995), and accretion of visceral fat is associated with increased fasting and postprandial insulin levels, impaired glucose tolerance, and impaired insulin action in both liver and skeletal muscle (Bjorntorp, 1991; Kissebah, 1991; Belfiore & Iannello, 1998; Bergman & Ader, 2000). Indeed, in the rodent, diminished visceral fat resulting from CR, surgical ablation, or leptin treatment reverses hepatic insulin resistance (Barzilai *et al.*, 1997, 1998, 1999). In the present study, lower leptin levels observed with long-term MR corresponded to a 72% reduction in the weight of visceral fat depots compared to CF animals and a 64% reduction when compared to PF animals. Thus, the mild hyperphagia associated with MR (food consumption by MR: 6 g per 100 g BW vs. CF: 4 g per 100 g BW) did not increase fat mass relative to body weight; rather, these animals showed a substantial reduction in fat mass-to-body weight ratio relative to CF animals (Table 1). In contrast, the fat-to-body weight ratio in PF was nearly equivalent to CF despite modestly greater DEE. Further, these animals consumed less food and consequently less methionine than CF.

MR rats exhibited higher DEE than either their CF or PF counterparts (Table 2). This contrasts with CR studies, some finding no change in DEE, others a decrease (Blanc *et al.*, 2003; Gallagher *et al.*, 2003; Poehlman, 2003). Whether a reduction in DEE plays a role in the life-extending properties of CR remains an open question, due in part to a lack of consensus on the proper statistical approach to normalize metabolic rate for changes in body composition. Thus, a decline in DEE in CR, if it occurs, might or might not be proportionally greater than the loss of fat and fat-free mass. MR rats had greater DEE despite significant changes in body size and composition. This remains true whether the data are expressed on a body-weight basis or adjusted for metabolically active tissue mass. MR rats experienced greater longevity despite increased energy expenditure.

Animals fed low protein and cafeteria diets are hyperphagic and resistant to weight gain due to the ability to convert excess food to heat (Rothwell & Stock, 1979; Rothwell *et al.*, 1983). This enhanced thermogenic response is due to sympathetic activation of uncoupling proteins, such as UCP-1, that are found in the inner mitochondrial membrane of brown adipose tissue (BAT) and that disrupt the proton gradient, generating heat rather than ATP. Elevated tissue concentrations of T_3 are necessary for recruitment of BAT and up-regulation of UCP-1 (Bianco & Silva, 1987). In rats, the thyroid is the source of all circulating T_4, whereas over half of the more biologically active hormone T_3 is generated extrathyroidally via deiodination of T_4 by type II thyroxine 5′-deiodinase (5′D-II) (Bianco *et al.*, 2002). BAT contains high amounts of 5′D-II (Silva & Larsen, 1985). Long-term MR resulted in a nearly twofold increase in plasma T_3 levels compared to CF animals; this was accompanied by a nearly 70% increase in BAT weight (MR: 0.22 ± 0.02 g per 100 g BW vs. CF: 0.13 ± 0.1 g per 100 g BW). Based on these observations, T_3

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production might be augmented in BAT of MR animals, leading to increased expression of UCP-1, explaining in part the ability of the MR animal to maintain a lower body weight. T_4 levels in MR animals were not significantly different from CF, indicating that thyroid function is not altered in long-term MR.

That aging MR animals maintain lower basal insulin and glucose levels compared to age-matched CF and PF animals suggests that chronic MR prevents or at least attenuates development of insulin resistance. Indeed, insulin responses of older MR animals to an oral glucose challenge (Figs 2, 3) were similar to those at baseline. Similar reductions (> 60%) in the MR insulin area under the curve compared to CF were observed at 23 and 72 weeks on diet (30 and 79 weeks of age, respectively). Although we did not perform insulin clamp studies, lower lifelong leptin levels and diminished fat mass suggests that MR reduced hepatic glucose output.

Adiponectin improves insulin sensitivity by lowering skeletal muscle triglyceride levels through increased β-oxidation of free fatty acids (Fruebis *et al*., 2001; Yang *et al*., 2002). The resulting lower serum free fatty acids and triglyceride levels lead to decreased hepatic triglyceride content. Increases in tissue triglyceride have been reported to interfere with insulin signaling pathways (Shulman, 2000), ultimately leading to the development of insulin resistance. Our data reflect the known correlations of serum adiponectin levels to insulin sensitivity (Hotta *et al*., 2000) and the resultant benefits, i.e. reduced circulating triglyceride (Table 3). Long-term MR resulted in more than a 100% increase in adiponectin levels compared to aged-matched CF.

Effects of adiponectin may also have been mediated by central mechanisms and energy expenditure. Recent reports have shown that central administration of this adipocytokine to normal and obese mice produces body weight reduction through increased energy expenditure, not through diminished food intake (Qi *et al*., 2004). Compared to CF, MR animals consumed more food on a body-weight basis, did not gain weight, and exhibited higher DEE (Table 2). Interestingly, in our studies, PF animals accrued more body weight and had decreased DEE relative to MR animals despite consuming relatively similar quantities of food.

Methionine-restricted rats had lower lifelong serum IGF-1 levels than controls (Fig. 4). Several studies link the GH/IGF-1 axis to lifespan determination and/or the rate of aging (Bartke *et al*., 2001). IGF-1 levels are reduced in mice having altered GH signaling pathways due to disruption of the GHR/GH binding protein gene. These animals are smaller than control animals, resistant to diabetes-induced end-organ damage, and live significantly longer (Bellush *et al*., 2000; Coschigano *et al*., 2000). Ames dwarf mice, which have a primary pituitary deficiency resulting in extremely low levels of IGF-1 (Bartke *et al*., 2001), have delayed neoplastic disease (Ikeno *et al*., 2003), significantly prolonged survival (Brown-Borg *et al*., 1996), and altered methionine metabolism (Uthus & Brown-Borg, 2003). The possible regulatory role of the IGF-1 receptor (IGF-1R) in longevity and resistance to age-associated oxidative stress has also been described (Holzenberger *et al*., 2003). Mice possessing one inactive *IGF-1R* gene (*Igf1r* $^{+/-}$ heterozygotes) have increased lifespan but, unlike other IGF-1 mutants, do not develop dwarfism and, as with MR, fertility is not compromised.

GH and IGF-1 are also critical for modulation of glucose homeostasis, and the effects of fasting and dietary restriction on serum IGF-1 are well known (Thissen *et al*., 1994). In the liver, insulin requires the GH receptor to stimulate release of IGF-1. Insulin also decreases hepatic production of IGF binding proteins (IGFBP), notably IGFBP-1, a key antagonist of IGF-1 action (Ritvos *et al*., 1988; Lewitt *et al*., 1991). In Wistar rats, short-term dietary restriction of single essential amino acids reduces plasma IGF-1 but does not increase IGFBP-1 levels except in those animals that are methionine restricted or feeding on diets devoid of amino acids (Tanenaka *et al*., 2000). Thus, it is plausible in MR that the observed lower insulin levels contributed to the reduced IGF-1 levels and that increased IGFBP-1 might have been a consequence of this action.

In rodents, both CR and MR prolong life and attenuate age-associated dysregulation of insulin action. Although these two dietary interventions are relatively easy to apply in laboratory conditions, in human clinical situations compliance issues could hinder the success of these potentially lifelong regimens. MR might be the more practical diet to implement because, unlike CR, a specific component, not quantity, of diet is limited. Soy protein, which is low in methionine content, is a major component of vegetarian diets. While clinical studies detail the lipid-lowering and anti-atherosclerotic actions of soy protein (Iritani *et al*., 1996; Nevala *et al*., 2000), animal studies have begun to explore the effect of soy protein on adipose tissue function. Rats subjected to short-term feeding of a diet containing soy protein showed higher plasma levels and increased expression of adiponectin in adipose tissue compared to casein-fed animals. Soy-fed animals also exhibited lower lipid levels, lower triglyceride content, and decreased expression of fatty acid synthase in both liver and adipose tissue (Nagasawa *et al*., 2003).

Recently, MR has been shown to prolong lifespan in female (BALB/cJ × C57BL/6 J) F_1 hybrid mice (Miller *et al*., 2005). Consistent with the MR rat, observations of lower insulin, glucose, and IGF-1 levels were reported, as well as resistance to oxidative stress. In contrast to MR in the rat, mice consumed similar amounts of food when compared to CF. Our laboratory is conducting MR studies in a different hybrid mouse, testing both males and females.

Reduction of calorie intake is a well-established approach to extending lifespan in mammals. MR, on the other hand, is a relatively new approach that has been documented only in rodents and in a limited number of reports. While CR and MR share common elements (i.e. decreased adiposity and improved insulin sensitivity) the critical issue of whether these interventions exert their effects through common mechanisms has not been addressed. Complementary studies using MR and CR in the F344 rat are currently underway in our laboratory. These studies explore the biochemical changes and gene expression patterns in adipose, liver, and skeletal muscle.

We show that an important consequence of MR is decreased visceral fat deposition and preservation of insulin sensitivity independent of dietary restriction. The cascade of beneficial effects

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resulting from lower lifelong levels of insulin provides plausible explanations for the prolonged survival of these animals.

Experimental procedures

Animals and experimental diets

All studies were reviewed and approved by the Institutional Animal Care and Use Committee of the Orentreich Foundation for the Advancement of Science, Inc.

Male Fischer 344 (F344) rats (Taconic Farms, Germantown, NY, USA) were used in all studies. The animals were housed individually (PF and glucose tolerance studies) or in groups (longitudinal studies, 2–3 per unit) and maintained on a 12-h : 12-h (07:00 hours : 19:00 hours) light–dark cycle. Food and water were provided *ad libitum* except for the group that was PF. Sentinel animals housed with experimental animals were tested every 4–6 months for the presence of antibodies to rodent viruses and *Mycoplasma pulmonis*. All tests were negative.

The animals were fed on a purified diet containing either 0.86% methionine (CF) or 0.17% methionine (MR) (Orentreich *et al.*, 1993). The diets were devoid of L-cystine; hence methionine was the sole source of sulfur amino acid. The glutamic acid content of the MR diet was raised on an equal gram basis to compensate for lowered methionine content. The choline content of both diets was 0.2%. Beginning at 7 weeks of age, the animals were assigned to the MR or CF diets on which they stayed until death. Body weight and food consumption were monitored in all studies.

Blood collection protocol: longitudinal studies and oral glucose tolerance tests

Animals were lightly restrained with Metofane™ (Pitman Moore, Mundelin, IL, USA) and blood was drawn from the retro-orbital sinus plexus into a serum separator tube (BD Vacutainer® SST™, Becton Dickinson and Company, Franklin Lakes, NJ, USA) using a heparinized micropipette. The tubes were kept on ice, spun at 1500 ***g*** for 15 min at 4 °C, and the plasma stored at –40 °C.

Pair-feeding studies

A cohort was established to extend previous observations (Orentreich *et al.*, 1993; Zimmerman *et al.*, 2003) demonstrating that growth was unimpaired in animals fed at the lower food intake of MR. Two groups of animals were fed CF or MR beginning at 7 weeks of age, while a third group was pair-fed to the average daily food intake of the MR group. Body weights of all animals were measured each week. The PF group was provided with new food at the same time every morning (11:00 hours, including weekends); leftover food from the previous day was collected from the food cup and weighed. The cage bedding was searched for small pieces of food that spilled from the food cup; this was measured and deducted from the daily weight. The amount of food provided to these animals was adjusted weekly to reflect changes in MR intake (food intake for CF was also measured weekly). The data presented in Table 1 represent a running average of the last 4 weeks of the study. As expected, food intake was significantly different in MR compared to CF animals. Food intake of the PF animals was modestly but not significantly reduced compared to MR because some PF animals failed to consume the entire daily food portion. PF animals consumed on average greater than 85% of the allotment.

At the end of 80 weeks (87 weeks of age), the animals were sacrificed by decapitation. Visceral fat, represented by the sum of epididymal, retroperitoneal (including perinephric), and inguinal fat pads, was excised and weighed. Trunk blood (nonfasting) was collected into cold heparinized tubes for measurement of insulin, glucose, leptin, adiponectin, IGF-1, T_4, and T_3 levels. The plasma was stored at –40 °C until analysis. The animals were sacrificed between 10:00 and 14:00 hours. To minimize potential diurnal variations, the order of sacrifice was arranged so that representatives from each dietary regimen were sacrificed both in the morning and in the afternoon. PF animals were not given additional food on the day of sacrifice.

Energy expenditure measurement in CF, MR, and PF animals

Total daily energy expenditure (DEE) was determined at 63 weeks of age using a computer-controlled, single-chamber, indirect calorimeter (Eco-Oxymax, Columbus Instruments International Corp., Columbus, OH, USA). The open circuit respiration chamber was used to measure average oxygen consumption and carbon dioxide production in mL^{-1} kg^{-1} h^{-1}. Respiratory exchange ratio (RER, VCO_2/VO_2), heat in kcal h^{-1} (internal method), and energy expenditure in kcal h^{-1} kg^{-1} were calculated using the Oxymax System software. Briefly, CF, MR, and PF at 56 weeks on diet (n = 6–7/group) were each monitored for 24 h (two rats from each group per week) over a 3-week period. The cohort used to obtain visceral fat and metabolic indices at 80 weeks was used to obtain these measurements.

Oral glucose tolerance testing

Glucose tolerance was assessed in a group of MR and CF animals at 0, 23, and 72 weeks on diet. Following a 4-h fast (beginning at 7:30–8:00 hours), the animals were intubated orally with 1.8 g kg^{-1} of glucose (Brancho-Romero & Reaven, 1977). Blood was collected before and at 5, 15, 30, and 60 min following administration of the glucose load. The plasma was stored at –40 °C until analysis. Insulin and glucose levels were determined as described below.

Longitudinal measurement of cholesterol, triglyceride, and IGF-1 levels

Following an overnight fast plasma was collected for cholesterol, triglyceride, and IGF-1 measurements every 3 months, beginning at 16 weeks on diet. Four time points – 16, 54, 81, and 105 weeks (corresponding to 23, 61, 88, and 112 weeks of age) – were chosen for analysis. In this cohort, the 105-week point reflects the last

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sampling before 50% mortality was seen in the controls. Due to limited quantities of blood, the triglyceride data were not collected longitudinally from the same animal but from randomly chosen MR or CF rats.

Assay methods

Insulin, leptin, and adiponectin levels were determined by RIA (Linco Research, Inc., St. Charles, MO, USA). The inter- and intra-assay coefficients of variation (CV) for insulin, adiponectin, and leptin were 7.4%, 6.1%, and 5.1%, respectively. IGF-1 levels were also measured by RIA (DSL Rat IGF-1 2900 RIA Kit, Diagnostics Scientific Laboratories, Webster TX, USA); the interassay CV was 11.6%. Total T_4 levels were quantified by RIA and total T_3 levels by chemiluminescent enzyme immunoassay (Diagnostic Products Corporation, Los Angeles, CA, USA); the intra-assay CVs were 7.9% and 11.7%, respectively. Glucose, cholesterol, and triglyceride were measured enzymatically using commercial kits (Sigma Chemical Company, St. Louis, MO, USA, and Randox Laboratories distributed by Equal Diagnostics, Exton, PA, USA), with interassay CVs of 2.5%, 6.2%, and 14.2%, respectively.

Statistical analysis

Except where noted, all data are expressed as mean ± SD. Group differences (i.e. by type of diet) in continuous variables were compared using the two-sample *t*-test and adjusted for unequal variances where required. Associations between continuous variables, such as body weight, visceral fat, IGF-1, and leptin values, were determined by linear regression analysis. Group differences in blood chemistries over time were analyzed using repeated measures analysis of variance (ANOVA). *F*-tests for time and diet-by-time interactions are reported using the Greenhouse-Geisser adjustment to control for lack of sphericity. When the diet-by-time interaction was significant in the ANOVA, Bonferroni's adjustment was applied to subsequent tests of group differences within a time period to control for multiple testing. The statistical packages used in these studies were obtained from True Epistat Services (Richardson, TX, USA) and Stata (College Station, TX, USA). Statistical tests achieving critical values less than 0.05 were considered significant.

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