Hunger Does Not Diminish Over Time in Mice Under Protracted Caloric Restriction

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ABSTRACT

Caloric restriction (CR) is the only nongenetic manipulation known to reliably prolong lifespan. Modeling suggests that humans would need to restrict their intake for many years to reap any lifespan benefits. The feasibility of such prolonged restriction may hinge on whether hunger diminishes with the time period spent restricted. We used the magnitude of hyperphagia on release from restriction as a bioassay of hunger in restricted mice. During restriction, mice develop a characteristic pattern of neuropeptide signals in the arcuate nucleus that reflect their hunger. This pattern is normalized after the postrestriction hyperphagia, validating hyperphagia as an indicator of the hunger during restriction. Mice were food restricted (80% of ad lib.) for 50 days. They initially lost weight, but then became weight stable and were maintained in CR at this lower level of energy balance for between 0 and 50 days and were then fed ad lib. for 50 days. When released onto ad lib. food, the magnitude of the hyperphagic response was independent of the prior length of CR. Hyperphagia ended when body mass was normalized. Hunger therefore did not diminish even when they were restricted for 100 days, equivalent to about 11 years in humans. The pattern of hyperphagic response suggested that signals coding body mass drive hunger during restriction, and because body mass under restriction remains depressed, this suggests that hunger would never disappear, making restriction to prolong lifespan in humans difficult to accomplish.

INTRODUCTION

Caloric restriction (CR) is at present the only experimental nongenetic paradigm known to increase both mean and maximum lifespan. It was discovered more than 70 years ago in rats,1 and since that time its effects have been shown to extend lifespan in a wide variety of species including rats, mice, yeast, Drosophila melanogaster, rotifers, bowl and doily spiders (Frontinella pyramitela), and nematodes (Caenorhabditis elegans). However, the effects of CR are not universal, because it does not appear to be effective in houseflies (Musca domestica)2 and it is also ineffective in some strains of mice.3 In some studies, the impact on lifespan has been dramatic, with increases in mean and maximum longevity of 50%.4 The impact of chronically reduced food intake has been shown to depend solely on the reduction of caloric intake, rather than intake of specific dietary nutrients.5–7, but see 8 Several studies have indicated that the impact on lifespan occurs because CR attenuates the onset of many age-related dis-

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eases, particularly cancer,\(^9\)–\(^{16}\) but see \(^{17}\) and generally reduces the expression of markers of age-related decline in function. Hence, the effect on lifespan is a consequence of a true reduction in the rate of aging. At present, CR is the only experimental manipulation of the environmental circumstances known that extends maximum lifespan through a reduction in the rate of aging.

The prospects of extending human lifespan by adopting a similar paradigm have recently generated considerable interest. This has been stimulated by the fact that studies of CR in non-human primates\(^{18}\)–\(^{20}\) have also reported an attenuation of age-related disease and markers of age-related decline.\(^{20}\)–\(^{25}\) Unsurprisingly in the light of this evidence, and doubtless encouraged by a series of popular books that have strongly advocated the CR paradigm as a route to enhanced longevity (e.g.,\(^{26}\)–\(^{28}\)), groups of individuals have already started to voluntarily restrict themselves in the hope that this will slow their aging rate and extend their lives (see http://www.cron-web.org).\(^{29}\) Moreover, appropriately randomized controlled trials of the impacts of CR have also started (e.g., the CALERIE trial in the United States; see http://calerie.dcri.duke.edu/).

We have recently used the data available across several studies of small mammals to model the likely impact of commencing on a program of CR at different times of life,\(^{30}\) assuming that the magnitude of the response in humans will mirror that observed in the rodents (which might be an optimistic assumption\(^{31}\)–\(^{35}\)). This modeling has revealed that to reap a significant longevity reward (exceeding 5 years), it would be necessary to engage in many decades of CR. The possibility that human subjects might be capable of voluntarily restricting their intake for such long periods remains open to question. One aspect that may make this more possible, however, would be if the hunger that accompanies periods of short-term calorie restriction ultimately dissipated. Anecdotally there are reports from people engaged in restriction that after time the hunger wanes. This effect might be possible because after an initial period when the body is in a dynamic state of change, energy demands appear to stabilize at a new lower level (the body weight plateau; animals\(^{36}\)–\(^{38}\) and humans\(^{39}\),\(^{40}\)) and thereafter the subject remains in energy balance. The protracted absence of energy imbalance may no longer provide a hunger signal, making continued restriction (once the dynamic phase is complete) a more likely possibility. Unfortunately, to date no controlled trials of CR protocols in nonobese subjects have been continued beyond the dynamic phase when body mass is still changing.

To explore the impact of protracted CR on perceptions of hunger, we studied the responses of MF1 mice. We have previously shown in these mice that the dynamic phase during which animals on restriction reach a new energy balance takes approximately 30–50 days to develop.\(^{41}\) After release from restriction, the mice show a profound hyperphagic response, increasing their food intake to well above the levels of age-matched control animals maintained without restriction. We used the magnitude of this hyperphagic response on release from restriction as a bioassay of the levels of hunger in the mice, although this is not the only method of hunger assessment available (other alternatives include behavioral operant tasks that evaluate how much the animal is willing to work for food). We placed mice on a 20% restriction for 50 days and then fed them in energy balance for further periods between 0 and 50 days, recording the magnitude of their hyperphagic responses on release from restriction.

**METHODS**

**Animals and housing**

MF1 mice aged 6–8 weeks were purchased from Harlan (UK) and housed individually in M3 cages (NKP Kent, UK) under a 12 light:12 dark photoperiod at 20 ± 2°C. All mice had *ad lib.* access to water throughout the study and were provided with wood shavings and shredded paper bedding for enrichment. The diet used in this study was pelleted rat and mouse, breeder and grower diet, (Special Diets Services, BP Nutrition, UK), which has a gross energy content of 17.4 MJ/kg (9.2% fat by energy).
Effect of duration of restriction on postrestriction hyperphagia

To determine the hunger of mice under prolonged restriction, 40 mice were placed on 20% CR for 50 days. Ten control mice continued feeding ad lib. for the remainder of the study. Restriction was calculated on an individual basis relative to the measured food intake at the start of the study (prerestriction). After 50 days, the restricted mice were randomly assigned to one of 4 groups (n = 10 in each). These continued to be fed in restriction but had body mass “clamped” to maintain energy balance for either 0, 10, 30, or 50 days. To achieve this, we adjusted the amounts of food they ate every second day to keep body mass constant. Actual achieved levels of restriction differed slightly, therefore, between the treatment groups and are reported in the results. After the period of body mass clamp, each group was released from the imposed restriction and allowed free access to food for a further 50 days. Body mass and food intake were carefully monitored throughout, while body composition was determined using dual-energy x-ray absorptiometry (Lunar Piximus methodology as described previously and calibrated as described previously) on three separate occasions: at the start of the study when all mice were feeding ad lib, at the end of the restriction phase, and at the end of the final ad lib. phase.

Validation of the “hunger bioassay”

We have previously observed that when mice under CR are permitted access to ad lib. food, they exhibit a period of hyperphagia. We used the magnitude of this hyperphagic response as a measure of the hunger of the mice under restriction. To evaluate whether hyperphagia reflects “hunger,” we performed a validation study to compare the feeding behavior of the mice, under restriction and after release from restriction, with patterns of neuropeptide gene expression in their brains. When mice are food deprived, they develop a characteristic neuropeptide gene expression profile in the arcuate nucleus (ARC) of the hypothalamus. This profile involves upregulation of so-called orexigenic neuropeptides (notably neuropeptide Y [NPY] and Agouti-regulated peptide [AgRP]) that stimulate food intake, and downregulation of anorexigenic neuropeptides that inhibit food intake (notably pro-opiomelanocortin [POMC] and cocaine and amphetamine regulated transcript [CART]). These neuropeptide patterns are believed to underpin the drive to eat and are a molecular marker of the phenomenon of “hunger.” If the mice under restriction are “hungry,” we would expect to observe upregulation of NPY and AgRP and downregulation of POMC and CART in their arcuate nuclei, relative to control mice. If the hyperphagia serves to abolish this “hunger,” then we would anticipate that the gene expression would be normalized relative to ad lib.-fed controls after the hyperphagic period.

To test this idea, we placed 30 mice on a 50% CR for a period of 25 days, and kept 10 control mice on ad lib. food. We split the 30 mice on restriction into 3 groups. Group 1 was culled after 25 days of restriction before being given the daily food ration on day 25. Group 2 was culled after 25 days of restriction 2 hours after being given the daily food ration on day 25. Group 3 was released from restriction on day 25 and fed ad lib. for 4 days and then culled.

Mice were dissected immediately postmortem and their brains were removed and frozen on dry ice. Hypothalamic gene expression was quantified using in situ hybridization techniques. We measured POMC, AgRP, CART, and NPY in the ARC. Brain sections were collected onto sets of 8 slides spanning the ARC from approximately –2.7 to –1.22 mm relative to bregma, according to the atlas of the mouse brain. After undergoing in situ hybridization, the slides were exposed to film (Kodak, Biomax MR film) to determine the intensity of the hybridization signal, which was then quantified using Image Pro Plus (Media Cybernetics) after calibration using a standard curve.

RESULTS

Effect of duration of restriction on postrestriction hyperphagia

Throughout the study, control animals maintained a constant food intake averaging 5.17 ± 0.24 g on day 1 and 5.20 ± 0.22 g on the last
day of study. Their body mass, however, increased from 35.6 ± 1.10 to 44.7 ± 2.18 g, involving increases in both lean and adipose tissue. Lean tissue increased from 32.9 to 36.5 g, while fat increased from 3.4 to 6.9 g. Restricted mice did not grow at the same rate as controls (GLM [General Linear Model] using day of restriction and group as factors $F_{19,998} = 80.94$, $p < 0.0012$), and consequently on day 50 of restriction, they had a significantly lower body mass than the controls by an average of 2.6 g (analysis of variance [ANOVA] $F_{1,50} = 4.35$, $p = 0.04$). Fat mass was not significantly different from that of the controls. Lean mass, however, was significantly lower in the restricted mice (ANOVA $F_{1,49} = 6.72$, $p = 0.013$). By the end of the 50-day restriction period, the restricted mice were in a new state of energy balance achieved by reducing both activity and resting metabolic rate.41

Control animals continued to increase in body mass beyond day 50. In contrast, the body mass of the mice on restriction remained depressed. Body mass was lower than that of the controls by an average of 5.6 g for the mice restricted for 50 days, 2.5 g for those restricted for 60 days, 2.8 g for the 80-day restriction, and 3.8 g for those restricted for 100 days. Actual achieved levels of restriction relative to the initial food intakes were 18% for those exposed for 60 days, 21% for those restricted for 80 days, and 23% for those restricted for 100 days. When mice were released from restriction, they all showed a marked hyperphagia; however, this only lasted for 1 day before they reduced their food intake to levels that were not significantly different from the control animals measured at the same time point (Fig. 1a). For the mice restricted for 50 and 100 days, this food intake level was not significant different from the intake rate in the same animals before they were placed on restriction. However, in the mice restricted for 60 and 80 days, the food intake after the period of hyperphagia was slightly but significantly lower (Max of 14%) than their initial rate of food intake (paired t-tests $p < 0.05$).

The extent of the hyperphagia did not vary significantly with the length of time that the mice had been on restriction (ANOVA $F_{3,37} = 0.37$, $p = 0.78$; Fig. 1b). When food intake was summed over the first week of release from restriction, there was also no effect of the time on restriction on intake (ANOVA $F_{3,37} = 0.93$, $p = 0.14$), although a trend suggested that, if anything, hyperphagia increased with prolonged restriction (Fig. 1c). The response in body mass to re-feeding was extremely rapid (Fig. 2a), showing a marked increase by day 2 of re-feeding, to a level that in all cases was not significantly different from the controls. By the final measurement period, none of the restricted groups differed significantly from the controls or each other (Fig. 2b) (ANOVA Body mass: $F_{4,49} = 1.18$, $p = 0.33$, Fat mass: $F_{4,49} = 0.68$, $p = 0.61$, Fat free mass: $F_{4,49} = 0.70$, $p = 0.60$).

![FIG. 1. The extent of hyperphagic response after release from caloric restriction. a: Daily variations after release from clamp. b: Extent of hyperphagia on day 1 after release from clamp. c: Combined food intake over the first week after release from clamp. In the latter two cases there were no significant differences with time held on caloric restriction (n = 10 per group).](image-url)
Validation of the hunger bioassay by comparison to neuropeptide gene expression levels

When the mice were killed after 25 days of 50% food restriction but before receiving their daily food ration, they exhibited the expected pattern of hypothalamic gene expression, with expression levels of the orexigenic NPY (ANOVA with Tukey test $F_{3,35} = 6.00, p = 0.002$) and AgRP (ANOVA with Tukey test $F_{3,35} = 10.30, p < 0.001$) elevated in the ARC relative to control ad lib.-fed mice, and expression levels of the anorexigenic neuropeptides POMC (ANOVA with Tukey test $F_{3,35} = 10.23, p < 0.001$) and CART (ANOVA with Tukey test $F_{3,35} = 7.26, p = 0.001$) reduced (Fig. 3). When mice were placed under the same level of restriction for 25 days but were given their daily ration of food 2 h before they were sacrificed, the pattern of restriction gene expression did not normalize relative to the controls. However, if mice were removed from restriction and fed ad lib. for 4 days, until they had completed their postrestriction hyperphagia, their gene expression patterns completely normalized relative to the controls (Fig. 3).

DISCUSSION

Validation of the hunger bioassay by comparison to neuropeptide gene expression levels

The pattern of gene expression in the hypothalamic ARC, with the orexigenic neuropeptides upregulated and the anorexigenic neuropeptides downregulated, clearly indicated that prior to receiving their daily food ration, mice on restriction were hungry relative to ad lib.-fed controls. Under this level of restriction, the data also clearly suggested that even after they had been given their daily food ration, they were still hungry relative to the control animals, because the neuropeptide gene expression levels were not normalized. After release onto ad lib. food, however, the mice underwent a postrestriction hyperphagia, after which their hypothalamic gene expression levels were normalized relative to the ad lib.-fed controls. These patterns provide us with confidence that the postrestriction hyperphagia is driven by these neuropeptide patterns and is a reflection of the extent of the animals’ “hunger.”

Effect of duration of restriction on postrestriction hyperphagia

When the mice were placed on restriction, they rapidly modified their behavior and reduced their energy demands to compensate for the reduced energy intake. We have previously shown that they achieved this by a combination of both reduced activity and reduced resting metabolism, consistent with the responses observed in other small rodents when placed under restriction. Despite the fact that the mice under restriction were feeding in energy balance for a protracted period, the magnitude of the hyperphagic response they demonstrated showed no abatement with the time on restriction.

That the hyperphagia observed on the day after release from restriction was not related to
the duration of prior restriction may have been because there is a limit in the amount of energy that these mice can process over a 24-h period. Possibly there was no point in the animals eating more than the 7.3 g that they ate (Fig. 1) because they could not process any additional intake. Considerable work has been performed to elucidate the limits on sustained energy intake. These studies suggest that a processing limit may occur at around 7 × basal metabolic rate. In our mice, the food intake on the day after restriction was equivalent to 126.3 kJ/day. We have previously measured RMRt (the resting metabolic rate [RMR] at thermoneutral but not postabsorptive: slightly higher than basal metabolic rate [BMR];55) in these mice at 17.5 kJ/day. Hence, the mice were feeding at around the supposed limit of 7 × BMR.

Despite the fact the mice appeared to be feeding at around their physiological capacity on the day after restriction ended, the hyperphagia, in all but the 50-day clamp group, was not extended beyond the first day of derestricted feeding. Hence, while there may have been a ceiling on the capacity of the animals to ingest and process food on the first day after restriction, the signals driving food intake (presumed to be the physiological basis of hunger) were insufficient to sustain the hyperphagia beyond that first day, independent of the period the animals had been on restriction. This evidence suggests that for these mice under these durations and magnitude of restriction, hunger does not dissipate over the duration of restriction. The maximum period of 100-day restriction used in our study was about one seventh the lifespan of an average MF1 mouse. In human terms, therefore, this would be similar to being on CR for a period of about 11 years (using the mean human life expectancy of around 78 years). If human responses are similar, this suggests that even after more than a decade of restriction, there would still be a feeling of hunger in subjects under restriction. Whether humans would respond in the same manner,
however, is questionable and data from rhesus monkeys (*Macaca mulatta*) suggested that the drive to perform an operant task for food did not differ between monkeys under prolonged CR compared to controls.\(^5^7\) The authors suggested that this was an indication of diminished hunger during prolonged CR.

Our understanding of the physiological factors that drive food intake has expanded enormously in the last decade. In particular, the roles of hormones released from the alimentary tract, in combination with other peripheral signals (such as leptin and insulin) and their targets in the brain, have been elucidated.\(^5^8\)–\(^6^5\) In the current animals, the hyperphagic response abated and neuropeptide levels in the ARC were normalized as soon as the mice had returned to the same body mass as the control animals. This suggests that signals from the periphery reflecting the body mass may have been a key component driving the neuropeptide gene expression profiles and the postrestric- tion hyperphagia. Once the body mass and these signals were “normalized,” the animals stopped overeating.

If this interpretation that the drive to eat was signaled primarily by the low body mass is correct, it has some important implications. First, because body mass of subjects under restriction remains permanently reduced relative to control subjects not under restriction,\(^3^8\),\(^6^6\) then hunger on restriction would never dissipate. Second, if these peripheral signaling compounds were supplied exogenously to subjects under restriction, this might remove the sensation of hunger and make it more likely that subjects would remain compliant with the restriction protocol. However, we have also shown here that during restriction there are profound changes in the gene expression levels of neu- ropeptides that are involved in the regulation of food intake. It is possible that these neuroendocrine responses are also an integral part of the mechanism that triggers the beneficial physiological responses to restriction. Consequently, by normalizing these patterns to remove the “hunger” pain of restriction, one might also inadvertently remove the longevity gains. How tightly these pathways are linked, if at all, would merit additional study.

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### REFERENCES


35. de Grey AD. The unfortunate influence of the weather on the rate of ageing: why human calor restriction or its emulation may only extend life expectancy by 2–3 years. Gerontology 2005;51:73–82.


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