Milk-soluble formula increases food intake and reduces *IL6* expression in elderly rat hypothalamus

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Abstract

Malnutrition in the elderly is accompanied by several metabolic dysfunctions, especially alterations in energy homeostasis regulation and a loss of insulin responsiveness. Nutritional recommendations aim to enrich food with high protein and energy supplements, and protein composition and lipid quality have been widely studied. Despite the numerous studies that have examined attempts to overcome malnutrition in the elderly through such nutritional supplementation, it is still necessary to study the effects of a combination of protein, lipids, and vitamin D (VitD). This can be done in animal models of elderly malnutrition. In the present study, we investigated the effects of several diet formulae on insulin responsiveness, inflammation, and the hypothalamic expression of key genes that are involved in energy homeostasis control. To mimic elderly malnutrition in humans, elderly Wistar rats were food restricted (R, –50%) for 12 weeks and then refed for 4 weeks with one of four different isocaloric diets: a control diet; a diet where milk soluble protein (MSP) replaced casein; a blend of milk fat, rapeseed, and DHA (MRD); or a full formula (FF) diet that combined MSP and a blend of MRD (FF). All of the refeeding diets contained VitD. We concluded that: i) food restriction led to the upregulation of insulin receptor in liver and adipose tissue accompanied by increased *Tnfα* in the hypothalamus; ii) in all of the refed groups, refeeding led to similar body weight gain during the refeeding period; and iii) refeeding with MSP and MRD diets induced higher food intake on the fourth week of refeeding, and this increase was associated with reduced hypothalamic interleukin 6 expression.

Key Words

- aging
- inflammation
- insulin responsiveness
- milk fat
- milk protein

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Introduction

Malnutrition in older adults may be attributed to several factors, including decreased food intake (Nieuwenhuizen *et al.* 2010) and reduced energy expenditure resulting from a sedentary lifestyle. Moreover, malnutrition in elderly

subjects is often caused by an inappropriate consumption of different nutrients, including proteins and micronutrients. Indeed, it has been suggested that the impairment of energy homeostasis control is one cause of malnutrition

(Foster *et al.* 2010). Paradoxically, aging has been also associated with anorexia, which results in the diminution of body weight and in cachexia (Rolland *et al.* 2011, Biolo *et al.* 2014). Thus, aging is characterized by an alteration in energy homeostasis control and an inability to respond to physiological needs. Indeed, in elderly rats, the loss of appetite has been shown to be associated with the downregulation of the expression of hypothalamic neuropeptide Y (*Npy*), an orexigenic neuropeptide, but not with changes in the expression of α Msh or *Cart*, which are anorexigenic neuropeptides (Sohn *et al.* 2002, Wolden-Hanson *et al.* 2004). These changes in *Npy* can most probably be attributed to alterations in both leptin and insulin hypothalamic signaling. In addition to the brain, aging progressively impairs the most metabolically active tissues, such as liver, adipose tissue, and muscle, and this impairment leads to numerous defects at the molecular and cellular levels. Insulin resistance is one of the most common features of aging-associated malnutrition and metabolic disorders (Escrivá *et al.* 2007). Importantly, insulin resistance has also been shown to be a risk factor for cognitive decline and neurodegenerative diseases, such as Alzheimer's disease in the elderly (Paz-Filho *et al.* 2008, Cardoso *et al.* 2009). Thus, promoting overall insulin sensitivity in elderly subjects could contribute to a delay in the onset of some aging-related disorders, including eating disorders, inflammation, and cognitive decline. This could be achieved through nutritional intervention. Adapting protein and lipid quality and amounts to an elderly diet is one nutritional manipulation that could improve whole-body insulin sensitivity, energy homeostasis control, and inflammatory status (Akinkuoliea *et al.* 2011, Panza *et al.* 2011, Volpi *et al.* 2013). Indeed, supplementing the diet with fish oil polyunsaturated (n-3) fatty acids has been shown to improve insulin sensitivity and to reduce pro-inflammatory factors, such as interleukin 6 (IL6) (Taouis *et al.* 2002, Cancelas *et al.* 2007, Chung *et al.* 2009, Li *et al.* 2014). Aging is also characterized by a reduction in muscle protein and by slow muscle protein synthesis. Consequently, efforts have been made to improve diet protein composition in order to increase the accretion of muscle proteins and to slow sarcopenia (Guillet *et al.* 2004, Paddon-Jones *et al.* 2004, Katsanos *et al.* 2005, Hirabara *et al.* 2013). Different protein supplementations have been tested in elderly subjects. Interestingly, whey proteins resulted in higher muscle protein synthesis as compared with soy proteins (Phillips *et al.* 2009). This has been attributed to the fact that soy proteins are more preferentially directed toward splanchnic synthesis than

whey proteins are (Tang & Phillips 2009). In addition, the improvement of insulin action in muscle is crucial for maintaining a positive balance between protein synthesis and degradation as well as for slowing down sarcopenia and frailty syndrome (Fulop *et al.* 2010). Despite the tremendous number of studies that have examined attempts to overcome malnutrition in the elderly by adapting the diet through nutritional supplementations (Dangin *et al.* 2003, Nestel *et al.* 2014), it is still necessary to study the effects of a combination of protein, lipids, and micronutrients (such as vitamin D (VitD)). This can be achieved in animal models (such as elderly rodents) of undernutrition in elderly humans. Although the results obtained using such models cannot be directly extrapolated to elderly humans, they could highlight some directions for future research.

In the present study, we used new nutritional combinations that consisted of milk lipids and protein in association with polyunsaturated (n-3) fatty acid in the presence of VitD, and we investigated the effects of these combinations on elderly undernourished rats. To our knowledge, the present study is the first to combine both milk lipids and protein in elderly rats. Indeed, despite its high saturated fatty acid content, milk fat has been reported to improve insulin sensitivity (Holmberg & Thelin 2013) and to reduce central obesity (German *et al.* 2009). Furthermore, there is no consistent association between the consumption of milk fat and an increase in cardiovascular risks (Le Ruyet & Le Goer 2010). The second nutrient that we tested was milk protein that consisted of native whey protein prepared by the microfiltration of skim milk. This processing fully maintained the nutritional and functional capacities of milk protein, as previously reported (Pfeifer *et al.* 2009). Following food restriction, animals were refed with different nutritional combinations for 4 weeks. We compared a control diet (CD, a soy oil/casein semi-synthetic diet that was equivalent to chow diet) with diets where milk soluble protein (MSP) replaced casein, where a blend of milk fat, rapeseed, and DHA (MRD) replaced soy oil, or a full formula (FF) combination of the MSP and MRD diets with a specific increase in protein in the presence of high VitD concentrations.

Materials and methods

Animals and experimental design

Male Wistar rats (aged 20 months) were purchased from Janvier (Le Genest-St-Isle, France). They were housed individually in a controlled temperature

(20–22 °C) and controlled humidity (around 40%) environment with a 12 h light:12 h darkness cycle. They had free access to water.

During the adaptation period (the first 2 weeks), all of the rats were allowed to feed *ad libitum* (AL) on a control semi-synthetic diet (4% lipids from soy vegetal oil, 74% carbohydrates from sucrose and corn starch, and 14% protein from casein, supplemented with a standard vitamin and mineral mix), following classical recommendations (Table 1). All of the diets were prepared within the Institut National de la Recherche Agronomique (INRA) facilities (Jouy-En-Josas, France).

A control group was allowed to feed AL for 3 months on a diet that was similar to the adaptation-period diet (AL group, $n=18$); these rats had a daily spontaneous intake of 24.5 ± 0.8 g/day. A dietary restriction program was applied to the other animals and maintained for 3 months with an intake that was 50% of the AL diet (12 g/day, of the same composition, given every morning; Fig. 1). The duration of food restriction was chosen on the basis of results described in previous reports (Osowska *et al.* 2006, Morley 2010). At the end of the diet-restriction period, the rats were separated: one group was killed and studied as a food-restricted group (R group, $n=18$), and the others were separated into four groups ($n=18$ /group) for refeeding studies. These four groups were assessed in order to compare the effects of refeeding diets that differed in terms of their protein quality or content, lipid quality, and VitD levels had on the restoration of different physiological and biochemical parameters (Table 2). Rats continued to be

housed individually so that food intake could be measured. All of the refeeding diets were isocaloric and enriched in lipid as compared to the AL (6% versus 4%) at the expense of sucrose (26% versus 28%).

The CD group was refed with the CD, which contained 14% casein as source of protein and 6% soy oil as a source of lipids, and 1 IU/g VitD. The MSP group was refed with a similar diet to that of the CD group, except that casein was replaced by MSP (Prolacta, Lactalis Ingredients, Bourgbarre, France). The diet contained the same amounts of lipid (6% soy oil) and VitD (1 IU/g). The MRD group was refed with a similar diet as the CD group in terms of protein quantity and quality (14% casein) and VitD, but the soy oil was replaced with a 6% blend of milk fat and rapeseed (50:50) supplemented with 0.072 g of DHA (long-chain omega-3). The FF group was refed with a FF that contained increased levels of MSP (22% versus 14%) at the expense of sucrose (18% versus 26%), a 6% blend of milk fat and rapeseed (50:50) supplemented with 0.072 g of DHA, and 5 IU/g VitD (instead of 1 IU/g) (Table 1). The decision to add VitD was based on the crucial role it plays in preserving muscle mass and bone density, especially in elderly people, by affecting calcium absorption and tissue incorporation (Chernoff 2005).

To avoid digestive problems, we implemented a progressive refeeding from days 1 to 5: 20 g/day on days 1–2, 25 g/day on day 3, and 30 g/day on day 4; the animals were allowed to feed AL beginning on day 5.

All of the procedures were conducted according to the guidelines for laboratory animal care and were approved

Table 1 Composition of diets

Components	<i>Ad libitum</i> (AL)	Restricted (R) (12 weeks)	Refeeding (4 weeks)			
		R	Control diet (CD)	Milk soluble protein (MSP)	Milk fat, rapeseed, and DHA (MRD)	Full formula (FF)
Protein (g)						
Casein	14	14	14	0	14	0
MSPs	0	0	0	13.5	0	21.2
Fat (g)						
Soy	4	4	6	6	0	0
Milk fat	0	0	0	0	3	3
Rapeseed	0	0	0	0	3	3
DHA	0	0	0	0	0.072	0.072
Carbohydrates (g)						
Sucrose	28	28	26	26	26	18
Cornstarch	46	46	46	46	46	46
Cellulose	4	4	4	4	4	4
Mineral mix + standard vitamins (g)	4	4	4	4	4	4
Vitamins						
Vitamin D (IU/g)	1	1	1	1	1	5
Total (g)	100	100	100	100	100	100

Male wistar rats
age 20 months

Figure 1

Food restriction and refeeding protocol for elderly Wistar rats. Elderly (20-month-old) Wistar rats were fed chow diet for 2 weeks and were then divided into six groups, as described in the 'Materials and methods' section. AL, *ad libitum*; R, restricted; CD, control diet; MSP, milk soluble protein; MRD, milk fat, rapeseed, and DHA; FF, full formula.

by the Local Ethics Committee for animal experimentation, Paris Center and South no. 59 (France), with authorization no. 91-519.

Insulin responsiveness assay

To evaluate insulin sensitivity following refeeding, each group was divided into two subgroups. Rats that were fasted overnight and then received either saline solution ($n=6$) or insulin (1 U/kg of body weight, $n=6$) bolus by i.p. injection 30 min before being killed.

Metabolic and endocrine measures

Body weight and food intake were measured once a week during the food restriction period and daily in the refeed groups ($n=18$ /group).

Blood samples were collected from starved rats from all of the groups ($n=6$ /group) in heparinized tubes and centrifuged (3500 *t*/min for 20 min), and plasma was stored at -80°C until analysis. Plasma glucose levels were measured using an Accu-Chek Performa kit (Roche). Plasma levels of insulin and leptin were measured using ELISA Kits (Millipore, Molsheim, France). Plasma *Tnf α* and *Il6* levels were assayed by ELISA rat *Tnf α* and *Il6* ELISA Kits (Invitrogen, Life Technologies), respectively, according to the manufacturer's instructions.

Western blot analyses

Following hormonal treatment, protein lysates from the hypothalamus, liver, and muscle were quickly removed, frozen in liquid nitrogen, and stored at -80°C . Samples were prepared for western blot analyses as previously described (Berthou *et al.* 2011). Briefly, samples were homogenized in 1 ml lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 0.5% Nonidet-P40, and 1% Triton X-100), protease inhibitor cocktail (0.35 mg/ml phenylmethylsulphonyl fluoride, 2 mg/ml leupeptin, and 2 mg/ml aprotinin), and phosphatase inhibitor cocktail (10 mM sodium fluoride, 1 mM sodium orthovanadate, 20 mM sodium β -glycerophosphate, and 10 mM benzamidine) with Precellys 24/Cryolys (hypothalamus: 20 s; liver and adipose tissue: 2×20 s). Homogenates were incubated for 2 h at 4°C and then centrifuged (at 14 000 *g* for 1 h at 4°C), and the supernatants were stored at -80°C . Protein concentrations of the supernatants were determined using a protein assay kit (BCA Protein Assay Kit, Thermo

Table 2 Endocrine parameters and pro-inflammatory factors. Results are expressed as means $\pm$ S.E.M.

Parameters	<i>Ad libitum</i> (AL)	Restricted (12 weeks)	Refeeding (4 weeks)			
		Restricted (R)	Control diet (CD)	Milk soluble protein (MSP)	Rapeseed, milk fat, and DHA (MRD)	Full formula (FF)
Endocrine parameters						
<i>n</i>	6	6	6	6	6	6
Glycemia (mg/ml)	1.1 ± 0.05^a	0.71 ± 0.02^b	0.94 ± 0.04^c	$0.87 \pm 0.08^{b,c}$	0.93 ± 0.08^c	0.92 ± 0.05^c
Insulinemia (ng/ml)	1.07 ± 0.33^a	0.3 ± 0.06^c	$1.16 \pm 0.17^{a,d}$	$1.69 \pm 0.22^{b,d}$	1.97 ± 0.23^b	$0.88 \pm 0.16^{a,c}$
Leptinemia (ng/ml)	10.79 ± 1.15^b	2.64 ± 2.03^a	10.64 ± 1.83^b	12.18 ± 1.03^b	13.03 ± 0.8^b	12.83 ± 1.83^b
HOMA index	$0.36 \pm 0.92^{a,b}$	0.05 ± 0.01^c	0.2 ± 0.03^b	$0.33 \pm 0.04^{a,b}$	0.46 ± 0.08^a	$0.2 \pm 0.05^{b,c}$
Pro-inflammatory factors						
<i>n</i>	6	6	6	6	6	6
TNF α (pg/ml)	17.44 ± 2.42	13.5 ± 1.35	19.82 ± 2.93	16.95 ± 3.76	14.62 ± 1.25	13.47 ± 0.86
IL6 (ng/ml)	145.87 ± 8.24	158.73 ± 21.19	143.49 ± 4.48	142.54 ± 4.92	154.13 ± 5.43	173.97 ± 10.73

Different letters indicate significant differences with at least $P < 0.05$.

Scientific, Courtaboeuf, France). Protein extracts (70 µg) were subjected to SDS-PAGE and transferred on to Immobilon-FL membranes (Millipore). Blots were blocked with 5% BSA (Euromedex, Strasbourg, France) and then immunoblotted with primary antibodies raised against phospho (p)-AKT(Ser473), AKT, insulin receptor (IR), p38-MAPK, β and tubulin (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. For protein detection, we used HRP-conjugated secondary antibodies and chemiluminescence (Amersham Biosciences, Life Technologies). The blots were finally scanned and quantified using the Carestream Molecular Imaging System 4000MM PRO (Carestream Health, Inc., Bagnolet, France). Relative protein quantities were normalized using β -tubulin antibodies.

RNA extraction and quantitative RT-PCR

Hypothalamus, liver, and adipose tissue (visceral fat) of elderly rats from all of the groups ($n=18/\text{group}$) were quickly removed under RNase-free conditions, immediately frozen in liquid nitrogen, and stored at -80°C . Total RNA was prepared for quantitative RT-PCR (qRT-PCR) as previously described (Benoit *et al.* 2013). Briefly, frozen samples of hypothalamus, liver, and adipose tissue were homogenized using a tissue homogenizer (Precellys 24), and RNA was extracted with TRIzol LS reagent (Invitrogen) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed (F-572L M-MuLV, Finnzymes, Fontenay-sous-bois, France) and subjected to qRT-PCR (Step-One, Applied Biosystems) using adequate primers and Fast SyberGreen Master Mix (Applied Biosystems). Relative cDNA quantities were calculated from cycle thresholds (C_t) and normalized with the housekeeping gene *S18* (Rps18). All of the qRT-PCR primers were purchased from Sigma, including those for *Ucp2* (forward: 5'-TGGCGGTGGTCGGAGATAC-3', reverse: 5'-GGCAAGGGAGGTCGTCTGTC-3'), *Ucp3* (forward: 5'-CCCAAAGGAACGGACCAC-3', reverse: 5'-GGTTCTGTAGGCATCCATAGTC-3'), *Adipor1* (forward: 5'-GCTGGCCTTTATGCTGCTCG-3', reverse: 5'-TCTAGGCCGTAACGGAATTC-3'), *Adipor2* (forward: 5'-CCACAA CCTTGCTTCATCTA-3', reverse: 5'-GATACTGAGGG GTGGCAAAC-3'), *ObRb* (forward: 5'-ACCACATACCTC-CTCACACTA-3', reverse: 5'-AGCAGTCCAGCCTACACT-CTT-3'), *IR* (forward: 5'-TGCCACCAATCCTTCCGTTCC-3', reverse: 5'-TCCTCCGCCTGCCTCTCC-3'), *Npy* (forward: 5'-ATGCTAGGTAACAAACG-3', reverse: 5'-ATGTAGTG-TCGCAGAG-3'), *Il6* (forward: 5'-GTTGCCTTCTTG GGAC-TGATGTT-3', reverse: 5'-ACTGGTCTGTTGTGGGT GG

TATC-3'), *Tnfa* (forward: 5'-CTCATTCCTGCTCGT GGC GG-3', reverse: 5'-CCGCTTGGTGGTTTGCTACGA-3'), *Ampk* (forward: 5'-GAATGGAAGGTAGTGAATGC-3', reverse: 5'-TAAAGTCTAGAAGATAGCTCCG-3'), and *18S* (forward: 5'-TCCCCGAGAAGTTTCAGCACAT-3', reverse: 5'-CTTCCCATCCTTCACGTCCTTC-3').

Statistical analysis

Statistical analyses were performed using the Mann-Whitney *U* test for the endocrine parameter, signaling, and gene expression experiments. Two-way repeated-measures ANOVA was used to test changes in body weight and energy intake over time followed by the Bonferroni's *post hoc* test. The results are expressed as means $\pm$ S.E.M., and $P < 0.05$ was considered statistically significant.

Results

The effects of food restriction and diet composition during refeeding on body weight gain in elderly Wistar rats

We investigated the effect of food restriction and refeeding using different diets (AL, R, CD, MSP, MRD, and FF) on the body weight gain of elderly (20-month-old) Wistar rats. Thus, there were six groups of rats. Rats were killed 30 days after the commencement of the refeeding period, except for the AL group members, which were killed before the food restriction period began. Rats were feed-restricted (R; -50% of their regular

Figure 2

The effects of food restriction and refeeding in elderly Wistar rats. Elderly (20-month-old) Wistar rats were divided into two groups: *ad libitum* (AL) and restricted (R, -50% of energy intake). These groups were maintained for 90 days under these regimens. The AL group members were killed at day 90, and the R group was then divided into five groups; one group was killed under the R state, and the other groups were refed with a control diet (CD), a milk soluble protein diet (MSP), a milk fat, rapeseed, and DHA (MRD) diet, or a full formula (FF) diet for an additional 30 days. Body weight was measured daily, and results are expressed as means $\pm$ S.E.M., $n=18$. $**P < 0.001$.

Figure 3

The effects of refeeding on food intake and the hypothalamic expression of *Npy*, *Pomc*, *Ir*, and leptin receptor. (A) Food intake (kcal/BW) was measured from days 4 to 26 of the refeeding period. Until day 8, all of the refeed groups received the same amount of food, and then all of the groups were allowed to refeed *ad libitum* with an adequate diet. Results are expressed as means $\pm$ s.e.m., $n = 18$. *** $P < 0.001$. The hypothalamic expression of *Npy*

(B), *Pomc* (C), insulin receptor (*Ir*) (D), and leptin receptor (*ObRb*) (E) was measured by qRT-PCR analysis of total RNA extracted from all of the groups at the end of the experiment. Results were normalized to 18S rRNA. Results are expressed as means $\pm$ s.e.m., $n = 18$. * $P < 0.05$ when the FF group was compared with all of the other groups (B) and when the MSP group was compared with all of the other groups (D and E).

Figure 4

The effects of food restriction and refeeding on the hypothalamic expression of genes involved in energy homeostasis. After the rats were killed and the hypothalami were extracted, total RNA from each of the groups was subjected to qRT-PCR using adequate primers to quantify (A) *Ampk*, (B) *Adipor1*, (C) *Ucp2*, and (D) *Ucp3* expression. Results were

normalized to 18S rRNA. Results are expressed as means $\pm$ s.e.m., $n = 18$. For *Ampk* expression, * $P < 0.05$ when the FF group was compared with all of the other groups. For *Adipor1* and *Adipor2*, * $P < 0.05$ when the MSP group was compared with all of the other groups. For *Ucp2*, different superscript letters denote significant differences at $P < 0.05$.

Figure 5

The effects of food restriction on insulin signaling in the liver and on hypothalamic inflammation in elderly Wistar rats. (A) Liver mRNA samples from the AL and R groups were subjected to qRT-PCR using primers to amplify IR, and results were normalized using 18S rRNA. Results are expressed as means $\pm$ S.E.M., $n = 18$. * $P < 0.05$ when comparing the AL group with the R group. (B) Proteins were prepared from the livers of the AL and R groups and were subjected to western blot analysis using antibodies directed toward IR, and results were normalized to β -tubulin. Results are expressed as means $\pm$ S.E.M., $n = 18$. * $P < 0.05$ when comparing the AL group with the R group. (C) Adipose tissue samples from the AL and R groups were subjected to qRT-PCR using primers to amplify IR, and results were normalized using 18S rRNA. Results are expressed as means $\pm$ S.E.M., $n = 18$. * $P < 0.05$ when comparing the AL group with the R group. (D) To evaluate insulin responsiveness, the AL and R groups received a bolus insulin or

placebo treatment, and then liver proteins were solubilized and subjected to western blot analysis using antibodies directed toward phosphorylated AKT (pAKT). Results were normalized using antibodies directed toward total AKT (tAKT). Results are expressed as means $\pm$ S.E.M., $n = 6$. *** $P < 0.001$ when comparing insulin-treated animals with animals treated with saline. (E) To determine the effects of restriction on the phosphorylation of p38-MAPK, solubilized proteins from the livers of AL and R groups were subjected to western blot analysis using antibodies directed toward phosphorylated p38 (pp38), and results were normalized to total p38 (p38). Results are expressed as means $\pm$ S.E.M., $n = 6$. * $P < 0.05$. (F) Hypothalamic RNA samples from the AL and R groups were subjected to qRT-PCR using primers to amplify TNF α , and results were normalized using 18S rRNA. Results are expressed as means $\pm$ S.E.M., $n = 18$. * $P < 0.05$ when comparing the AL group with the R group.

intake) for 90 days and were then allowed to refeed AL for 30 days with a CD, MSP, MRD, or FF diet, except for the R group, which was killed at the end of the food restriction period (Fig. 1). Before the food restriction, rats exhibited a body weight of 581.8 ± 12.9 g, and following restriction, they had lost 104.63 ± 4.3 g. All of the refeed groups gained a similar amount of weight despite their different diets (Fig. 2), with a mean body weight of 623.6 g.

The effects of diet composition during refeeding on food intake and the hypothalamic expression of *Npy*, *Pomc*, *IR*, and leptin receptor

During the early stage of refeeding (until day 18) all of the groups ingested similar amounts of food, but at days 22 and 26, the MRD and FF groups exhibited a higher food intake as compared with the CD and MSP groups (Fig. 3A). To investigate whether these changes were due to changes

in the expression levels of hypothalamic neuropeptides and receptors that are involved in the control of food intake, we measured the expression levels of *Npy*, *Pomc*, *IR*, and *ObRb*. *Npy* expression was significantly increased in the FF group as compared with the other groups (Fig. 3B), whereas *Pomc* expression was not modified (Fig. 3C). In addition, we observed downregulation of both *IR* and *ObRb* in the MSP group as compared with all of the other groups (Fig. 3D and E). No changes were observed between the CD and R groups.

The effects of diet composition during refeeding on the hypothalamic expression of genes involved in cellular energy sensing

To analyze the effects of diet composition during refeeding on hypothalamic markers of energy sensing, the expression levels of *Ampk*, *Adipor1*, *Ucp2*, and *Ucp3* were

Figure 6

The effects of refeeding on insulin responsiveness in the livers of elderly Wistar rats. (A) Liver protein from the AL, R, CD, MSP, MRD, and FF groups was solubilized and subjected to western blot analysis using antibodies directed toward phosphorylated AKT (pAKT). Results were normalized to total AKT (tAKT) using specific antibodies. Bands were quantified using a Carestream apparatus and quantified using Carestream Software

(Carestream, Bagnolet, France). Results are expressed as means $\pm$ S.E.M. of pAkt/tAkt (%insulin/NaCl), $n=6$. *** $P<0.0005$ and **** $P<0.0001$.

(B) Comparison between the refed groups regarding the insulin-dependent AKT phosphorylation response amplitude (insulin/saline). Results are expressed as means $\pm$ S.E.M., $n=6$ and different superscript letters denote significant differences at $P<0.05$.

determined. We observed that *Ampk* expression was significantly increased in the FF group as compared with the other refed groups (Fig. 4A). However, we showed that the mRNA levels of *Adipor1* (Fig. 4B) and *Ucp3* (Fig. 4D) were significantly reduced in the MSP group as compared with the other groups. Figure 4C shows that *Ucp2* expression was downregulated in the CD, MSP, and MRD groups as compared with the FF, AL, and R groups.

The effects of food restriction and refeeding on endocrine parameters and insulin responsiveness in elderly rats

We showed that food restriction was associated with a significant decrease in the plasma levels of glucose and insulin and a reduced HOMA index as compared with the AL group (Table 2). Refeeding for 30 days increased the plasma levels of insulin and leptin in all of the refed groups so that they reached the levels of the AL group (Table 2). In addition, the HOMA index was also restored. It is noteworthy that plasma insulin levels were lower in the FF group as compared with the other refed groups.

However, pro-inflammatory factors (*Il6* and *Tnf α*) were not affected by restriction or by the quality of diet during the refeeding (Table 2).

The effects of food restriction and refeeding on liver insulin sensitivity

We investigated the effects of food restriction on insulin sensitivity in elderly rats by measuring IR expression in liver and adipose tissue. We showed that restriction induced the upregulation of IR in both liver and adipose tissue (Fig. 5A, B and C). However, insulin-dependent Akt phosphorylation in the liver was not significantly modified by food restriction, and insulin was able to phosphorylate AKT in both the AL and R groups (Fig. 5D). However, the amplitude of response in the R group was less pronounced than that in the AL group. In addition, food restriction led to an augmentation of inflammation markers in the liver and the hypothalamus. Indeed, food restriction increased p38-MAPK phosphorylation in the liver and *Tnf α* expression in the hypothalamus (Fig. 5E and F respectively).

Figure 7

The effects of refeeding on insulin responsiveness in the muscle of elderly Wistar rats. Muscle protein from the AL, R, CD, MSP, MRD, and FF groups was solubilized and subjected to western blot analysis using antibodies directed toward phosphorylated AKT. Results were normalized to total AKT

using specific antibodies. Bands were quantified using a Carestream apparatus and quantified using Carestream Software. Results are expressed as means $\pm$ S.E.M., $n=6$. * $P<0.05$.

To investigate the effects of diet composition during refeeding on insulin-responsiveness, AKT phosphorylation was measured in response to insulin challenge. We showed that insulin significantly increased AKT phosphorylation in the liver in all of the groups (Fig. 6A). However, the amplitude of response was significantly lower in the MSP group as compared with the other refeed groups (Fig. 6B).

The effects of food restriction and refeeding on muscle insulin-dependent AKT phosphorylation

To investigate the effects of food restriction and diet composition during refeeding on insulin-responsiveness, muscle AKT phosphorylation was measured in response to insulin challenge. We showed that insulin significantly increased muscle AKT phosphorylation in the AL group and that this augmentation was abolished in the R group. Concerning the refeed groups, insulin responsiveness was restored in the MRD group, which exhibited a significant increase in insulin-dependent AKT phosphorylation. The FF group exhibited a slight increase in insulin-dependent AKT phosphorylation ($P=0.06$), whereas the CD and MSP groups showed lower insulin responsiveness (Fig. 7).

The effects of food restriction and refeeding on the expression of adiponectin receptor, IR, and AMPK in the liver

To investigate the effects of diet composition on cellular energy sensing in liver, we studied the changes in adiponectin receptor and AMPK expression. Following food restriction and refeeding with different diets, the expression levels of ADIPOR1, ADIPOR2, and AMPK in the liver were assessed by qRT-PCR. No differences were observed between the different groups before or during food restriction nor during refeeding, except that the expression of ADIPOR1, ADIPOR2, and AMPK was significantly increased in the CD group as compared with the AL, R, MSP, MRD, and FF groups (Fig. 8A, B and C). We also showed that IR was upregulated in the R group as compared with the AL and FF groups, whereas IR in the CD, MSP, and MRD groups was significantly increased as compared with the AL group but not as compared with the FF and R groups (Fig. 8D).

The effects of refeeding on liver pro-inflammatory factors

To determine the effect of refeeding on liver inflammation, we compared the expression levels of TNF α and IL6 in the livers of refeed groups with the AL and R groups. The expression of TNF α was significantly higher in the AL

Figure 8

The effects of refeeding on liver genes that are involved in energy homeostasis and inflammation. After the rats were killed and the livers were extracted, total RNA from all of the groups was subjected to qRT-PCR using appropriate primers for *Adipor1* (A), *Adipor2* (B), *Ampk* (C), *Tnfα* (D),

and *Il6* (E). Results were normalized to *18S* rRNA and are expressed as means $\pm$ S.E.M., $n=18$. * $P<0.05$ when samples were compared with all of the groups (A, B, C and E), and different superscript letters denote significant differences at $P<0.05$ (D and F).

group as compared to the R group, and this diminution was maintained independently of the composition of the refeeding diet (Fig. 8E). IL6 expression was significantly higher in the AL and R groups as compared with all of the refed groups, and this increase was maintained independently of diet composition (Fig. 8F).

The effects of food restriction and refeeding on the hypothalamic expression of TNFα and IL6

To determine the potential effects of the different diets that were used during the refeeding period on hypothalamic inflammation, we measured the expression of two markers: TNFα and IL6. We showed that food restriction significantly increased the expression of TNFα, and refeeding, independently of diet composition, reduced the expression of TNFα so that it reached the same level as that for the AL group (Fig. 9A). We also showed that IL6 expression was significantly reduced in the FF group as compared with all of the other groups, including the R and AL groups (Fig. 9B).

Discussion

Nutritional manipulation is now considered one of the most promising solutions for overcoming, or at least attenuating, many age-related disorders and diseases, such as atherosclerosis, cancer, diabetes, sarcopenia, metabolic syndrome, inflammation, and obesity. Indeed, many reports have revealed increased malnutrition in the elderly that is associated with frailty, a decline in muscle mass, and an impairment of energy homeostasis control. Among the nutrients that have been extensively studied as potential candidates for nutritional manipulation during aging, protein takes an important place. Indeed, the focus on protein has been driven by the important loss of skeletal muscle that could be, at least partially, attributed to amino acid availability (Fukagawa 2013). Besides protein, carbohydrates, and fat have been also given much attention because of the metabolic disorders that are associated with these macronutrients in the elderly (such as insulin resistance, type 2 diabetes, and obesity). However, nutritional manipulations are complex because

Figure 9

The effects of food restriction and refeeding on hypothalamic genes that are involved in inflammation. After the rats were killed and the hypothalami were extracted, total RNA from all of the groups was subjected to qRT-PCR using appropriate primers for *Tnfα* (A) and *Il6* (B) expression. Results were normalized to 18S rRNA and are expressed as means $\pm$ S.E.M., $n = 18$. * $P < 0.05$ when samples were compared with the AL group.

macronutrients such as protein, fat, and carbohydrates are involved in the regulation of food intake through amino acids, fatty acids, and glucose respectively, and this could interfere with the theoretical efficacy of each nutrient. To add to this complexity, the combination of nutrients with different qualities, compositions, and energy values could also play a role in the diet of the elderly and its efficiency and availability.

In the present study, we aimed to analyze the effects of diets that differed in terms of their protein and fat quality on insulin responsiveness, inflammation, and the expression of genes that are involved in the control of energy homeostasis in elderly undernourished Wistar rats. Special attention was paid to the combination of MSP and milk lipids.

To mimic malnutrition in elderly humans, we subjected elderly (20-month-old) rats to food restriction (-50%) for 12 weeks, and this led, as expected, to a significant reduction in body weight accompanied by diminished glucose, insulin, and leptin plasma levels. Food restriction induced the upregulation of IR in liver and adipose tissue, and it maintained liver insulin-dependent AKT phosphorylation as compared with the AL group. However, it is noteworthy that the insulin responsiveness of the R group was weaker as compared with the AL group. This was mostly a result of the striking reduction in plasma insulin levels. Food restriction in elderly rats induced the augmentation of p38-MAPK

phosphorylation in the liver, which is generally considered to be a marker of cellular inflammation and is believed to promote insulin sensitivity (Qi *et al.* 2013). Food restriction also increased pro-inflammatory factor TNF α in the hypothalamus. This impairment is in accordance with the alteration in insulin signaling that we observed, but it contradicts our hypothesis that food restriction would not alter insulin-dependent AKT phosphorylation in the liver; it may be explained by a degree of insulin alteration. Indeed, food restriction reduced insulin plasma levels but most probably increased animal stress, which may have contributed to the onset of hypothalamic inflammation and the activation of liver p38-MAPK (García-San Frutos *et al.* 2012). It is noteworthy that food restriction has the opposite effect on liver TNF α to that which it has on the hypothalamus. Indeed, food restriction downregulated liver TNF α as compared with the AL group. It has been suggested that aging increases liver TNF α and apoptosis and that food restriction reduces hepatic apoptosis, and this may explain the hepatic downregulation of TNF α (Ando *et al.* 2002). Importantly, we also showed that muscle insulin responsiveness exhibited different features from that of the liver. Indeed, food restriction abolished muscle insulin-dependent AKT phosphorylation as compared with the AL group. Interestingly both the MRD and FF groups exhibited total or partial restoration of muscle insulin responsiveness respectively. This effect could contribute to better protein deposition in these two groups, which indicates a beneficial effect of milk fat combined with DHA and MSP. Further experiments are needed to directly measure muscle protein synthesis.

Following food restriction, we refeed four groups with diets that differed in terms of their protein content, fat quality, and VitD. Independently of diet quality, we showed that all of the refeed groups displayed similar gains in body weight and almost reached the body weight of the AL group. This body weight catch-up reached almost 80% within 4 weeks of refeeding and was associated with a significant increase in plasma leptin levels in all of the groups. However, plasma insulin levels were significantly increased in all of the refeed groups as compared with the R groups, but the FF group exhibited significantly lower plasma insulin levels as compared with the CD, MSP, and MRD groups. Thus, in the FF group, the plasma glucose level was similar to that of all of the other refeed groups, and this could be attributed to better insulin responsiveness. Indeed, the FF contained both milk fat and MSP with high concentrations of VitD. Furthermore, the FF group exhibited a higher food intake, which was

correlated with the upregulation of orexigenic neuropeptide *Npy* in the hypothalamus. Similar results concerning food intake were obtained in the MRD group but this group did not exhibit significant changes in *Npy* and *Pomc* expression. The MRD group's increased food intake could probably be attributed to other mechanisms that have not yet been elucidated. The FF and MRD diets share in common a similar content of MRD. Thus, we suggest that fat quality increased the appetite for these two diet combinations. However, the combination of milk fat with MSP and high VitD concentrations seems to have had a beneficial effect as compared with the MRD group, as was evidenced by the reduced expression of pro-inflammatory cytokine *Il6* in the hypothalamus of the FF group. Indeed, it has been reported that hypothalamic inflammation is implicated in the alteration of neural circuitry and in the subsequent impairment of energy homeostasis regulation (Meng & Cai 2011). Thus, these beneficial effects of FF formulation are probably a result of the combination of MSP, milk fat-associated LC-omega-3 fatty acids, and high doses of VitD. Indeed, whey protein has been shown to improve insulin responsiveness (Arciero *et al.* 2014), and VitD supplementation has been shown to reduce neuroinflammation (Adzemovic *et al.* 2013). Furthermore, VitD deficiency promotes cognitive decline (Keeney *et al.* 2013). In addition, the protective effects of omega-3 fatty acids have been previously described in terms of their ability to prevent insulin resistance, inflammation, and neuroinflammation (Figueras *et al.* 2011, Kalupahana *et al.* 2011, Oliver *et al.* 2012). The FF group also exhibited the upregulation of hypothalamic *Ampk* as compared with all of the other groups, and this finding provides evidence supporting the beneficial effect of this formula. Indeed, hypothalamic *Ampk* has been described as a crucial energy sensor that is essential in the hypothalamic integration of hormonal and nutritional signals (Schneeberger & Claret 2012). It is noteworthy that *Ucp2*, *Ucp3*, and *Adipor1* were downregulated in the MSP group, which indicates a potential defect in glucose sensing at the hypothalamic level. Indeed, *Ucp2* and *Adipor1* (the receptor that activates the *Ampk* cascade) are important regulators of neuronal glucose and energy sensing (Guillod-Maximin *et al.* 2009, Toda & Diano 2014). This accords with the reduced expression levels of hypothalamic leptin and IRs.

At the hepatic level, we showed that in all of the groups *Adipor1*, *Adipor2*, and *Ampk* were not significantly modified, except in the CD group, where these cellular energy sensors were upregulated. This was most probably caused by the higher sensitivity of the CD group to the fasted state, seeing as the entire experiment was performed

in a fasted state. It has been previously shown that the expression of *Adipor1* is increased under fasting conditions and is decreased after refeeding (Dridi & Taouis 2009).

Taken together, the present results indicated that: i) food restriction led to the upregulation of IR in the liver, hypothalamus, and adipose tissue and was accompanied by increased pro-inflammatory TNF α in the hypothalamus of elderly Wistar rats; ii) in all of the refeed groups, refeeding led to similar body weight gain by the end of a 4-week refeeding period; iii) refeeding with a blend of MRD (or FF) induced higher food intake; and iv) the combination of MSP and milk fat in the presence of VitD and omega-3 PUFA (the FF group) reduced hypothalamic *Il6* expression.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

H O H performed most of the experiments and participated in writing the manuscript; B D participated in designing the experiment, interpreting the nutritional data, and revising the manuscript; Y B performed some of the experiments; D C performed most of the qRT-PCR experiments; L R performed most of the ELISA analyses; P L and C B provided the specific diets and contributed to the experimental design and to the manuscript revision; M T designed and supervised the study and wrote the paper. All of the authors read and approved the final manuscript.

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