Defective Regulation of the Ubiquitin/Proteasome System in the Hypothalamus of Obese Male Mice


In both human and experimental obesity, inflammatory damage to the hypothalamus plays an important role in the loss of the coordinated control of food intake and energy expenditure. Upon prolonged maintenance of increased body mass, the brain changes the defended set point of adiposity, and returning to normal weight becomes extremely difficult. Here we show that in prolonged but not in short-term obesity, the ubiquitin/proteasome system in the hypothalamus fails to maintain an adequate rate of protein recycling, leading to the accumulation of ubiquitinated proteins. This is accompanied by an increased colocalization of ubiquitin and p62 in the arcuate nucleus and reduced expression of autophagy markers in the hypothalamus. Genetic protection from obesity is accompanied by the normal regulation of the ubiquitin/proteasome system in the hypothalamus, whereas the inhibition of proteasome or p62 results in the acceleration of body mass gain in mice exposed for a short period to a high-fat diet. Thus, the defective regulation of the ubiquitin/proteasome system in the hypothalamus may be an important mechanism involved in the progression and autoperpetuation of obesity. (*Endocrinology* 155: 2831–2844, 2014)

**Neurons of the medial-basal hypothalamus play a central role in whole-body energy homeostasis (1, 2), and a number of interventions aimed at modulating the activity of these neurons can result in obesity (1–3). Feeding on a high-fat diet is commonly used as a method to produce experimental obesity. Work performed during the last 10 years has shown that, aside from increased caloric intake, high-fat diets can induce an inflammatory process in the hypothalamus, leading to neuronal resistance to leptin and eventually to neuronal apoptosis (4–8), providing an anatomical and functional basis for obesity.**

Notably, although most studies exploring the mechanisms behind hypothalamic dysfunction in obesity have been performed in rodents, two recent studies using neuroimaging to evaluate the human hypothalamus have provided strong evidence for dysfunction and neuronal loss associated with obesity (9, 10). Thus, defining the mechanisms that link dietary components with hypothalamic inflammation, neuronal dysfunction and eventually neuronal loss may unveil potential targets for the more efficient treatment of obesity.

An important aspect of both experimental and human obesity is that the longer obesity persists, the harder it is to reestablish correct energy homeostasis (11, 12). This is particularly evident in patients undergoing a number of dieting programs and continuously regaining body mass (12, 13). Although the reasons for the progressive increase in the defended set point for body adiposity are unknown, we suspect that diet-induced loss of hypothalamic neurons involved in the regulation of energy homeostasis may play an important role in this process (7, 14).

In this study, we hypothesized that a malfunction of the ubiquitin/proteasome system could contribute to the con-
tinuous deterioration of the hypothalamic neurons that regulate body energy homeostasis. Ubiquitination of proteins plays a broad role in cellular homeostasis. It can, through its canonical function, target old and damaged proteins for proteasomal degradation (15); in addition, it targets potentially harmful protein aggregates that cannot be degraded by the 26S proteasome for autophagic disassembly (16). Ubiquitination can also modulate and be modulated by inflammation (17, 18), which is involved in obesity-dependent insulin resistance (19). Defects in any of these functions of the ubiquitin system can potentially lead to uncontrolled inflammation and eventually to apoptosis. Here we evaluated the expression, activity, and hypothalamic distribution of proteins of the ubiquitin/proteasome system in experimental obesity. We show that prolonged but not short-term feeding on a high-fat diet results in the accumulation of ubiquitinated proteins in the hypothalamus, which is accompanied by decreased proteasome expression and formation of protein aggregates.

Materials and Methods

Experimental animals

Six-week-old male Swiss mice, male TNF receptor (TNFR)-p55−/− or TNFRp55−/− mice (knockout for the TNF-α receptor 1 and its respective control) (20) and male C3H/HeJ or C3H/HeN mice [loss of function mutation for Toll-like receptor (TLR)-4 and its respective control] (21) were fed on standard rodent chow or a high-fat diet (5.2 kcal/g; 35.0 g percentage fat, 66.0 g predominantly saturated fat) for 8 or 16 weeks. In some experiments, Swiss mice were stereotaxically instrumented using a Stoelting stereotaxic apparatus, according to a previously described method (20). Stereotaxic coordinates were as follows: anteroposterior, 0.5 mm; lateral, 0.2 mm; and depth 3.5 mm to third ventricle. The efficiency and accuracy of the cannulations were always tested by the evaluation of the drinking response elicited by the intracerebroventricular injection of angiotensin II, as previously described (22). Thereafter mice were intracerebroventricularly treated with a proteasome inhibitor, lactacystin (2.0 μM, Calbiochem) for 5 days or with small interfering RNA (siRNA) to p62, as described below. In addition, in some experiments, mice were intracerebroventricularly treated with immunoneutralizing antibodies against TNFα (40 ng) or TLR4 (40 ng) for 7 days (details of all antibodies used in the study are presented as Supplemental Material).

All experimental procedures were performed in accordance with the guidelines of the Brazilian College for Animal Experimentation and were approved by the Ethics Committee at the State University of Campinas.

siRNA treatment

An siRNA targeting p62 and a scrambled siRNA (sc29828 and sc37007; Santa Cruz Biotechnology) were complexed and diluted as previously described (23). The animals were evaluated for 6 days, and 2 μL of the mixtures containing either the siRNA to p62 or the scrambled siRNA was injected through the cannula positioned in the third ventricle on the first, third, and fifth days. In some experiments, the siRNA was delivered bilaterally directly in the arcuate nucleus according to the following stereotaxic coordinates: anteroposterior, −1.7 mm; lateral, 0.3 mm; and depth, 5.6 mm.

Hyperinsulinemic-euglycemic clamp

Glucose consumption, as previously reported (24), was assessed after 12 hours of starvation, at which time the mice were anesthetized with sodium pentobarbital (50 mg/kg body weight, ip), which has no effect on insulin action (25), and catheters were then placed in the left jugular vein (for tracer infusions) and the carotid artery (for blood sampling) (26).

Determination of oxygen consumption/carbon dioxide production and respiratory exchange ratio

Oxygen consumption/carbon dioxide production and respiratory exchange ratio were measured during a dark cycle in fasting mice using a computer-controlled, open-circuit calorimeter system (LE405 gas analyzer; Panlab-Harvard Apparatus). The airflow within each chamber was monitored with a sensor (Air Supply and Switching; Panlab-Harvard Apparatus). The O2 consumption and CO2 production were calculated with Metabolism Supplies and Switching; Panlab-Harvard Apparatus). The O2 consumption and CO2 production were calculated with Metabolism version 2.2v software based on the Withers equation, and the respiratory exchange ratio was calculated using CO2 production/O2 consumption.

Immunoblotting

The method for immunoblotting was previously described (7). Ubiquitin, proteasome, A20, p62, beclin, phospho-c-Jun N-terminal kinase (JNK), and β-actin were detected in the membranes after an overnight incubation at 4°C with primary antibodies (Supplemental Material). After incubation with a horseradish peroxidase-conjugated secondary antibody, enhanced chemiluminescence (SuperSignal West Pico; Pierce) was used for detection by autoradiography. Band intensities were quantified by optical densitometry (UN-Scan-it Gel 6.1).

RNA extraction, real-time quantitative RT-PCR (qRT-PCR), and PCR array

The TaqMan system was used in association with real-time PCR to detect TNF-α, IL-6, IL-1β, neuropeptide Y (NPY), and proopiomelanocortin (POMC) in the hypothalamus and peroxisomal proliferator-activated receptor-γ-coactivator 1 (PGC1)-α, uncoupling protein 1 (UCP1), and citrate synthase in the brown adipose tissue (Mt99999068_m1; Mm00446190_m1; Mm0434228_m1; Mm0308253_m1; Mm00435871_M1; Mm0120883_M1; Mm01244861_m1, Mm00466043_M1, respectively; Life Technologies; the mouse GAPDH gene was used as an endogenous control (number 4352339E). Gene expression was analyzed by real-time PCR using the PCR array system (RT2 Profiler PCR array mouse ubiquitin proteasome system, number PAMM099Z; QIAGEN Biotecnologia Brasil). The global analysis of 84 genes specific to the ubiquitin proteasome system was performed using a 7500 Platform and analyzed with PCR array data analysis software [Excel (Microsoft) and web based].

Immunofluorescence staining

For histological evaluation, hypothalamic tissue samples were fixed in paraformaldehyde and processed for embedding in
paraffin blocks. Five-micrometer paraffin sections were processed for immunofluorescence staining using the ubiquitin, p62, Cd11b, and HuR antibodies (Supplemental Material) and secondary antibodies conjugated to fluorescein isothiocyanate or rhodamine (sc2777 and sc2092, respectively; Santa Cruz Biotechnology). The images were obtained using a confocal laser microscope (LSM510; Carl Zeiss).

Transmission electron microscopy (EM)

Hypothalamic sections were examined in a Tecnai G2 Spirit Twin (FEI) transmission EM operated at 80 kV. Neurons were photographed, and the digital images were used for ultrastructural analysis. For each condition, eight randomly selected distinct neurons were initially photographed in medium-magnification, 10-μm² fields. Distinct areas of the selected neurons were then photographed in high-magnification, 5-μm² fields and autophagosomes, and protein aggregates were counted in four to six high-magnification fields of each of the eight randomly selected neurons. Autophagosomes, the most reliable markers of autophagy (27), were identified by the presence of a double membrane partly visible as two parallel membrane bilayers separated by an electron-lucent cleft surrounding cytosol and organelles. The details of the transmission EM features of autophagosomes are described elsewhere (27). Protein aggregates were identified as heterogeneous electron-dense amorphous structures with mean diameters of at least 50 nm, as previously described (28, 29).

Statistical analysis

The results are presented as the means ± SE. Levene’s test for the homogeneity of variances was initially used to check the fit of data to the assumptions for parametric ANOVA. All results were analyzed by a t test or a one-way ANOVA complemented by the Tukey test to determine the significance of individual differences. The level of significance was set at P < .05.

Results

Phenotypic characterization of Swiss mice fed on a high-fat diet

Swiss mice are an outbred strain genetically related to the diabetes-prone AKR mice (30). When fed on a high-fat diet (obese), Swiss mice present 1.2- and 1.5-fold increases in body mass, compared with mice fed on chow (lean), at 8 and 16 weeks, respectively (Figure 1A). This is not accompanied by any significant change in caloric intake (Figure 1B) at the time of evaluation, ie, at 8 or 16 weeks on the high-fat diet. Increased caloric intake occurs only during the first 2–3 weeks after introduction of high-fat diet, normalizing thereafter (data not shown). At 16 weeks, the serum levels of leptin (Figure 1C) and insulin (Figure 1D) are increased, whereas glucose consumption during a hyperinsulinemic-euglycemic clamp is reduced (Figure 1E) (additional results regarding the hyperinsulinemic-euglycemic clamp studies are presented as Supplemental Figure 1, A and B). The serum levels of TNF-α (Figure 1F), IL-1β (Figure 1G), and IL-6 (Figure 1H), as well as the hypothalamic expression of the mRNAs of TNF-α (Figure 1I), IL-6 (Figure 1J), and IL-1β (Figure 1K), are increased in the obese mice. Finally, at 16 weeks, the obese mice are resistant to the anorexigenic effect of leptin in the hypothalamus (Figure 1L).

Return to chow results in more pronounced body mass loss in mice fed for 8 weeks on the high-fat diet, compared with 16-week-fed mice

Both humans and experimental animals with long-lasting, severe obesity are expected to be resistant to body mass loss when undergoing caloric restriction (12, 31). To evaluate the impact of diet change in body mass reduction in our experimental model, Swiss mice fed ad libitum for 8 or 16 weeks on a high-fat diet were transferred to chow ad libitum and followed up for 7 days. At the beginning of the experimental period, mice fed on the high-fat diet for 16 weeks had a significantly higher body mass compared with mice fed on the high-fat diet for 8 weeks (Figure 1M). Although total caloric intake was similar between the groups (Figure 1N), mice fed for 8 weeks on a high-fat diet presented a significantly greater reduction of body mass compared with 16-week-fed mice (Figure 1O). To exclude the possible effect of age on the regulation of body mass loss after a return to chow, a group of mice was introduced to the 8-week high-fat diet at the age of 14 weeks such that, at the end of the experimental period, they were age matched with the mice fed for 16 weeks on the high-fat diet. As depicted in Figure 1, M–O, theagematched mice behaved exactly as the original 8-week group.

Modulation of hypothalamic ubiquitin-related proteins in diet-induced obesity

The expression levels of 84 ubiquitin-related genes were evaluated, using a qRT-PCR array, in the hypothalami of diet-induced obese Swiss mice fed for 16 weeks on a high-fat diet. In general, there was a 15.4% modulation of gene expression, in which 7.1% of the genes were up-regulated and 8.3% were down-regulated (Figure 2A). Of all the regulated genes, those coding for proteins with E3 ligase activity were the most affected, followed by those coding for E2 conjugating activity (Figure 2B). Genes coding for proteins with E3 ligase activity were predominantly down-regulated (Figure 2, C and D). All the transcripts evaluated in the array can be seen at the manufacturer’s site (RT² Profiler PCR array mouse ubiquitin proteasome system, number PAMM099Z; QIAGEN), and the modulated genes are presented in Table 1.

Accumulation of ubiquitinated proteins in the hypothalamus in mice fed on high-fat diet for 16 but not 8 weeks

To determine the impact of duration of obesity on proteins of the ubiquitin/proteasome system in the hypothalamus, we evaluated the expressions of four proteins in-
involved in the regulation/function of this system: ubiquitin, the subunit 26S of proteasome, the deubiquitinase A20, and the adaptor protein p62, involved in the connection between proteasome and autophagy. After 8 weeks, the amounts of ubiquitinated proteins in the hypothalamic extracts were lower in obese mice than in lean mice (Figure 2E, upper left-hand panel). This was accompanied by the increased expression of the A20 (Figure 2E, upper right-hand panel) and proteasome (Figure 2E, lower right-hand panel), whereas the levels of p62 were similar between lean and obese mice (Figure 2E, lower left-hand panel). Conversely, at 16 weeks, there was an increase in the amount of ubiquitinated proteins in the hypothalami of obese mice (Figure 2F, upper left-hand panel). This was accompanied by reductions in A20 (Figure 2F, upper right-hand panel) and proteasome (Figure 2F, lower right-hand panel) expression and by increased expression of p62 (Figure 2F, lower left-hand panel).

**Increased colocalization of ubiquitin and p62 in the hypothalamus in mice fed on a high-fat diet for 16 weeks**

The formation of intracellular aggregates containing ubiquitin and the adaptor protein p62 occurs in neurode-
generative conditions such as Parkinson’s and Alzheimer’s diseases (32, 33) and also in the hypothalami of obese mice overexpressing the E4 enzyme E4B (34). Here we found ubiquitin and p62 distributed throughout the hypothalamic areas involved in the control of energy homeostasis: the arcuate nucleus (Figure 3A), the paraventricular nucleus (Figure 3B), and the lateral hypothalamus (Figure 3C), with a clear predominance in cells in the arcuate nucleus. However, the colocalization of ubiquitin and p62 was much more evident in the hypothalami of mice fed on the high-fat diet for 16 weeks, compared with 8 weeks, particularly in the arcuate nucleus and the lateral hypothalamus (Figure 3, A and C, bar graphs).

Ubiquitin and p62 are expressed in neurons and microglia of the hypothalamus

Labeling cells with the neuron-and microglia-specific markers, HuR and CD11b, respectively, provided evidence that both ubiquitin and p62 are expressed in both cell types in the arcuate nucleus of obese mice (Figure 3, D–G). However, coexpression was more evident in the neurons than in the microglia (Figure 3, D–G). In addition, using transmission EM, we demonstrate that neurons of the medium-basal region of the hypothalamus of obese mice have increased levels of protein aggregates (Figure 3H).

Reduced expression of markers of autophagy in the hypothalamus in obese mice

Reduced autophagy in hypothalamic neurons results in increased adiposity (35, 36). Because p62 is involved in connecting the ubiquitin and the autophagy pathways, we evaluated the impact of prolonged feeding with a high-fat diet on markers of autophagy in the hypothalamus in Swiss mice. As depicted in Figure 4, 8 weeks of a high-fat diet is insufficient to change the expression of beclin and LC3 (Figure 4A). Conversely,
after 16 weeks on a high-fat diet, the expression levels of both beclin and LC3 were significantly reduced (Figure 4B). This was accompanied by a significant reduction in the number of autophagosomes detected by transmission EM (Figure 4C).

Stability of the ubiquitin/proteasome system in the hypothalamus of obesity-resistant mutants

Knockouts for the TNFR1 and TLR4 genes are protected from diet-induced obesity, at least in part, because they do not present hypothalamic inflammation upon high-fat feeding (5, 20, 37). In fact, after 16 weeks on high-fat feeding, TNFR1 (Figure 5A) and TLR4 (Figure 5B) knockout mice present similar body masses to their respective chow-fed controls. In the hypothalamus of TNFR1 knockout mice fed a high-fat diet, the amount of ubiquitin (Figure 5C, upper left-hand panel) was reduced compared with that in mice fed on chow, whereas the expression levels of A20 (Figure 5C, upper right-hand panel), p62 (Figure 5C, lower left-hand panel), and proteasome (Figure 5D, lower right-hand panel) were all similar to those of chow-fed mice. The expression of ubiquitin was lower in either TNFR1 or TLR4 mutants fed for 16 weeks on a high-fat diet as compared with their genetic background control, C57BL/6J (Supplemental Figure 1C). To further evaluate the role of hypothalamic inflammation on the regulation of the ubiquitin/proteasome system, Swiss mice fed on high-fat diet for 16 weeks were treated with intracerebroventricular injections of immunoneutralizing antibodies against TNF-α/H9251 or TLR4. There was no significant change in body mass (Figure 5E); however, the amount of ubiquitin in the hypothalamus was reduced (Figure 5, F and G).

Chemical inhibition of hypothalamic proteasome results in body mass gain

To test the hypothesis that the stability of the ubiquitin/proteasome system in the hypothalamus of mice fed for 8 weeks on a high-fat diet plays an important role in the maintenance of energy homeostasis, mice were treated via intracerebroventricular injections with lactacystin, a chemical inhibitor of the proteasome, for 5 days (Figure 6, A and B), leading to the increased accumulation of ubiquitin in the hypothalamus (Figure 6, C and D). The lac-

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Abbreviation: RefSeq, reference sequence.
Figure 3. Increased colocalization of ubiquitin and p62 in the hypothalamus in mice fed on a high-fat diet for 16 weeks. Six-week-old, male Swiss mice were randomly assigned to standard rodent chow (lean) or high-fat diet (obese), ad libitum, for 8 or 16 weeks. At the end of the respective experimental periods, hypothalami were obtained for the immunofluorescence study of the expression, distribution, and colocalization of ubiquitin (Ubi) and p62 (A–C); Ubi and the neuron marker HuR (D); Ubi and the microglia marker CD11b (E); p62 and HuR (F); and p62 and CD11b (G). In addition, hypothalamic samples were obtained for transmission EM studies (H). Protein aggregates (arrows, shown in the caption) were identified and counted in four to six high-magnification fields of eight distinct neurons selected randomly. The microphotographs (A–G) are representative of four independent experiments. Arc, arcuate nucleus; LH, lateral hypothalamus; PVN, paraventricular nucleus. In panel H, the bar graph depicts mean protein aggregates per square micrometer. The micrographs are representative of 32–48 distinct acquisitions. *, P < .05 vs lean.
tacystin-treated mice presented a greater increase in body mass (Figure 6, E and F), a higher caloric intake (Figure 6G), an increased expression of hypothalamic NPY (Figure 6H), and no change in POMC (Figure 6I). Lactacystin treatment produced no changes in energy expenditure (Figure 6, J–L) or in the expression of markers of thermogenesis in the brown adipose tissue, such as citrate synthase (Figure 6M), PGC1α (Figure 6N), and UCP1 (Figure 6O). In addition, lactacystin treatment was not sufficient to modulate the hypothalamic expression of TNF-α (Figure 6P) or phospho-JNK (Figure 6Q), but it did increase the expression of IL-1β (Figure 6R). The intracerebroventricular treatment with lactacystin had no impact on the expression of an inflammatory marker in peripheral tissue (Supplementary Figure 1D).

**Inhibition of hypothalamic p62 results in body mass gain**

The anomalous expression of the adaptor protein p62 is known to be involved in the formation of protein aggregates in neurodegenerative diseases, and changes in its expression can lead to obesity (32–35). The intracerebroventricular treatment of Swiss mice, fed for 8 weeks on high-fat diet, with an siRNA targeting p62 (Figure 7, A–C) resulted in a 70% reduction in hypothalamic p62 expression (Figure 7D, lower, panel, left-hand panel), which was accompanied by increased accumulation of ubiquitin (Figure 7D, upper panel, left-hand panel), increased proteasome expression (Figure 7D, lower panel, right-hand panel), and no change in the expression of A20 (Figure 7D, upper panel, right-hand panel) in the hypothalamus. The inhibition of hypothalamic expression of p62 resulted in increased body mass and increased caloric intake (Figure 7, E–G) with no change in the hypothalamic expressions of NPY (Figure 7H) and POMC (Figure 7I).

To evaluate the anatomic specificity of this process, some mice were submitted to bilateral cannulation of the arcuate nucleus (Figure 7B) and treated...
with the p62 siRNA according to the same protocol as in Figure 7C. As shown in Figure 7J, the inhibition of p62 specifically in the region of the arcuate nucleus resulted in an even more pronounced increase of body mass, with no significant modification of caloric intake (Figure 7K).

**Discussion**

One of the most relevant questions in obesity is why prolonged and severe increases in body mass result in changes in the defended set point of adiposity, which contributes to the autoperpetuation of the disease (12, 31). A few recent studies have provided some functional and anatomical bases for this phenomenon. In diet-induced obesity, there is selective activation of apoptosis in the neurons of the hypothalamus (7, 10). Genetic predisposition to obesity favors increased apoptosis of neurons, exerting catabolic functions (7), whereas diet-induced activation of inhibitory-κB kinase-β/nuclear factor-κB signaling in the hypothalamus leads to neuronal apoptosis and the impairment of the neural stem cell-dependent replacement of neuronal loss (14). On the whole, these processes can lead to an
Figure 6. Inhibition of proteasome in the hypothalamus accelerates obesity. Male, 14-week-old Swiss mice fed on a high-fat diet for 8 weeks were stereotaxically cannulated (anteroposterior, 0.5 mm; lateral, 0.2 mm; and depth 3.5 mm) (A), tested for cannula patency with angiotensin II, and then treated with lactacystin (2.0 μM, 100 μM; intracerebroventricular) for 5 days (B). At the end of the experimental period, hypothalamic protein extracts were obtained and separated by SDS-PAGE, transferred to nitrocellulose membranes, and submitted to immunoblotting determination of the expression of ubiquitin (Ubi) (C). All membranes were stripped and rebotted with anti-β-actin antibody (C). The bar graph (D) present the means ± SEM of the arbitrary scanning units obtained from the densitometric determination of the respective bands in the blot depicted in panel C. Body mass during the experimental period is presented as a relative variation as compared with the vehicle-treated mice (E) and as the cumulative absolute change during the experimental period (F). Daily food intake is presented as the ratio of the food intake on the first day of experiment (G). The expression of NPY (H) and POMC (I) were determined by real-time PCR in the hypothalamus at the end of the experimental period. Determination of O2 consumption (J), CO2 production (K), and respiratory quotient (RQ) (L) was used with permission of Richard L. Sidman. Richard L. Sidman performed at the end of the experimental period. The expressions of citrate synthase (CitS) (M), PGC1α (N), and UCP1 (O) were determined by real-time PCR, in the brown adipose tissue at the end of the experimental period. The expressions of genes encoding TNF-α (P) and IL-1β (R) were determined by real-time PCR, and the expression of phospho-JNK (Q) was determined by immunoblot, in the hypothalamus, at the end of the experimental period. In all experiments, n = 6. *, P < 0.05 vs vehicle. Brain image in panel A was modified from Figure 255 from Sidman et al, High Resolution Mouse Brain Atlas; 1999 (www.hms.harvard.edu/research/brain) (used with permission of Richard L. Sidman).
imbalance in the subpopulations of the hypothalamic neurons, promoting orexigenic/antithermogenic vs anorexigenic/prothermogenic effects (7, 10, 14). Thus, diet-induced apoptosis of selected neuronal groups in the hypothalamus may be at least one of the factors contributing to the refractoriness of obesity to conventional therapy.

A further advance in the characterization of the roles played by distinct hypothalamic neuronal subpopulations and prolonged obesity in the autoperpetuation of the disease was obtained by the generation of a reversible mouse model of obesity in which the expression of the POMC gene was selectively blocked in the neurons of the hypothalamus. POMC reactivation during early obesity resulted in a complete reversal of the phenotype, whereas a late reactivation of POMC was insufficient to promote a complete normalization of body weight (31).

In the present study, we evaluated the regulation of the ubiquitin/proteasome system in the hypothalamus of mice with short- and long-term diet-induced obesity. The regulation of protein turnover is essential for cellular homeostasis (38), and the ubiquitin/proteasome system is the most important mechanism regulating protein degradation in eukaryotic cells, promoting proteolysis of up to 80% of short-lived proteins (39, 40). Most of the remaining proteolysis is performed in lysosomes or by autophagy, in which a molecular connection between the ubiquitin/proteasome system and autophagy has recently been identified and shown to be dependent on the activity of p62 (32). In neurons, the correct regulation of protein turnover is crucial for cell viability and particularly for synaptic plasticity (41). The impairment of this process leads to the accumulation of protein aggregates in the cells, which is a common feature of neurodegenerative conditions such as Parkinson’s and Alzheimer’s diseases (42, 43).

In the first part of the study, we show that long-term diet-induced obesity has a substantial impact on the hypothalamic expression of ubiquitin-related genes, as
shown by the PCR array. Most of the genes undergoing changes belong to the E3 family, which suggests that the regulation imposed by the diet and prolonged obesity is somewhat specific because E3 ligases and some E3 enzymes with deubiquitinase activity are more specific for their targets than proteins with E1 and E2 activities (44). Next, we explored the hypothesis that the activity of the ubiquitin/proteasome system in the hypothalamus differs between mice with short and prolonged periods of obesity. As in humans with obesity for long periods of life, who are extremely resistant to body mass reduction (12), prolonged experimental obesity resulted in a less pronounced loss of body mass after the transfer from high-fat diet to chow. This was accompanied by increased accumulation of ubiquitin in the hypothalamus and by changes in the expression of proteins exerting distinct functions in the ubiquitin/proteasome system.

On the whole, the differences between 8 and 16 weeks of high-fat feeding suggest that the efficiency of the machinery for protein degradation is lost over time, a phenomenon that is not related to aging. The reason for this is because, at the same age as the obese mice, our lean controls did not display similar alterations. It is particularly interesting that in mice fed a high-fat diet for 8 weeks, the amount of ubiquitinated protein is reduced. This finding is in accordance with other studies showing that acute inflammatory stimuli can accelerate proteolysis by increasing proteasome activity (45, 46). In fact, there are a number of studies showing differential regulation of the ubiquitin/proteasome system in inflammatory conditions (reviewed in reference 47). Accordingly, depending on the magnitude and duration of the inflammatory process, components of the ubiquitin/proteasome system can be affected in different ways. Thus, what we propose here is that, during the initial steps of hypothalamic inflammation in obesity, there is a mild increase of the activity of the system, which compensates for the increased rate of protein damage imposed by the inflammatory process.

The impact of the anomalous regulation of ubiquitin expression on body adiposity was demonstrated recently in two studies that either removed or increased the expression of ubiquitin in mice (34, 48). Surprisingly, in both conditions, the resulting phenotype was increased adiposity, suggesting that the fine-tuning of protein degradation is crucial for whole-body energy homeostasis. Here we show that the system can be regulated by a nutritional factor, which is the most important determinant of obesity in human populations (49, 50). Moreover, we show that changes in the regulation of the ubiquitin/proteasome system in the hypothalamus are connected with inflammation because inflammation-protected, obesity-resistant mutants such as TLR4- and TNFR1-knockout mice (5, 20) were also protected from diet-induced modulation of the ubiquitin/proteasome system in the hypothalamus. In addition, the immunoneutralization of either TNF-α or TLR4 in the hypothalamus of obese mice also reduced the amount of ubiquitin.

Interestingly, the overexpression of E4B ligase, which increases protein ubiquitination, was shown to result in the formation of protein aggregates in the hypothalamus (34), leading to obesity. The presence of protein aggregates is supported by the physical association between ubiquitin and p62 (32). Here we detected an increased association between p62 and ubiquitin and also an increase in the presence of protein aggregates, particularly in the arcuate nucleus of obese mice fed for 16 weeks on the high-fat diet. This is important additional evidence of anatomical and molecular features of neurodegeneration in the hypothalamus of an animal model of diet-induced obesity.

In the final part of the study, we used two distinct approaches to accelerate the impairment of the ubiquitin/proteasome system in the hypothalamus in mice fed on the high-fat diet for 8 weeks. For this experiment, mice were treated with lactacystin, a chemical inhibitor of proteasome, or with an siRNA targeting p62. In both cases, there was accumulation of ubiquitin in the hypothalamus and increased body mass gain, accompanied by increased caloric intake. We have no current explanation for the fact that impairment of the ubiquitin/proteasome system in the hypothalamus results in a preferential change in caloric intake and not in whole-body energy expenditure. However, because a similar result was reported in the ubiquitin knockout mouse (48), we suspect that neurons primarily involved in the control of food intake are more sensitive to altered regulation of the system.

Combining data from our present study with those of former studies evaluating diet-induced hypothalamic inflammation, we conclude that high-fat dietary content initially induces an inflammatory process in the hypothalamus, which leads to leptin resistance, increased caloric intake, and reduced energy expenditure, resulting in the progressive increase of adiposity (4, 10, 37). Until a certain point of progression of the disease, the return to a low-fat diet will result in the complete rescue of the obese phenotype (the present data and reference 31). However, as high-fat feeding persists, hypothalamic neuronal damage will progressively contribute to the irreversibility of the disease. We propose that the anomalous activity of the ubiquitin/proteasome system in the hypothalamus is one of the mechanisms contributing to the deterioration of the system that regulates whole-body energy homeostasis. Thus, long-term obesity is accompanied by neuronal changes commonly found in classical neurodegenerative condi-
tions, which can explain not only the refractoriness of obesity but also its frequent association with Alzheimer’s and Parkinson’s diseases (51–53).

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