Reduced fasting-induced activation of hypothalamic arcuate neurons is associated with hyperleptinemia and increased leptin sensitivity in obese mice

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Becskei C, Lutz TA, Riediger T. Reduced fasting-induced activation of hypothalamic arcuate neurons is associated with hyperleptinemia and increased leptin sensitivity in obese mice. Am J Physiol Regul Integr Comp Physiol 299: R632–R641, 2010. First published June 16, 2010; doi:10.1152/ajpregu.00674.2009.—Fasting increases c-Fos expression in neuropeptide Y (NPY) neurons of the hypothalamic arcuate nucleus (ARC) in lean, but not in hyperleptinemic mice with late-onset obesity (LOO). Although obesity is associated with leptin resistance, we hypothesized that under fasting conditions, leptin sensitivity might be restored and that hyperleptinemia may counteract the neuronal response to fasting. We investigated whether the reduced fasting response of ARC neurons in LOO is paralleled by an increase in leptin sensitivity, as measured by leptin-induced STAT-3 phosphorylation. To assess leptin’s role in the modulation of the fasting-induced ARC activation, we investigated c-Fos responses and hormone and metabolite levels in hyperleptinemic diet-induced obese (DIO) and in leptin-deficient ob/ob mice. Leptin induced a stronger STAT-3 phosphorylation in fasted LOO and lean mice than in ad libitum-fed animals. Similar to LOO, hyperleptinemic DIO mice showed no c-Fos response after fasting, while ob/ob mice showed a stronger response than lean control mice. Mimicking hyperleptinemia by repeated leptin injections in lean mice during fasting attenuated the fasting-induced c-Fos expression. Our findings indicate that high leptin levels prevent the fasting-induced activation of ARC neurons in mice. Moreover, leptin sensitivity is dynamic in obese subjects and depends on the feeding status. During short-term increases in leptin sensitivity, e.g., during fasting, leptin signaling appears to be effective, even in hyperleptinemic obesity. As reflected by the blockade of the fasting-induced ARC activation, fasting seems to interfere with the responsiveness of the ARC to signals related to the status of energy intake.

c-Fos; signal transducer and activator of transcription 3; late-onset obesity; diet-induced obesity, ob/ob mouse

THE OREXIGENIC NEUROPEPTIDE Y (NPY) neurons in the hypothalamic arcuate nucleus (ARC) are important elements of the hypothalamic circuit that controls food intake (27). They integrate peripheral feeding-related and adiposity cues, signaling the availability of ingested and stored energy (27). During food deprivation, c-Fos expression, a marker of neuronal activation, increases in NPY neurons in mice (11, 12, 14), and refeeding with chow reverses this activation (26, 31). As leptin, insulin, and glucose have an inhibitory effect and ghrelin has an excitatory effect on the NPYergic ARC neurons, these factors might be involved in attuning neuronal ARC activity to the feeding status (15, 17, 21, 24, 25, 33). Interestingly, in our previous study, hyperglycemic, hyperleptinemic, and hyperinsulinemic mice with late-onset obesity (LOO) showed no c-Fos response to fasting in the ARC and a blunted refeeding hyperphagia after food deprivation (11). It seems plausible that high fasting levels of one or several of these factors might be involved in the blockade of the fasting-induced ARC activation and the attenuation of refeeding hyperphagia in obesity.

Leptin may be particularly important among these factors. The original concept that leptin functions as an adiposity signal that prevents body weight gain has been complemented by the view that a decrease in leptin may rather represent a starvation signal that initiates adaptive neuroendocrine responses to conserve energy during food shortage (1, 3, 18). Because of the well-known phenomenon of leptin resistance, the hypothalamic feeding-related circuits have been considered unresponsive to changes in circulating leptin levels in obese individuals. However, leptin resistance can be reversed. Specifically, leptin sensitivity seems to change dynamically. For example, an acute decrease in endogenous leptin levels after fasting is associated with enhanced leptin sensitivity in lean animals (5, 6, 23). A chronic weight loss-induced decrease in leptin levels also improves leptin sensitivity in obese animals and humans (16, 27, 34). Yet, it is unknown whether short-term withdrawal of food enhances leptin sensitivity in obese subjects and whether under these conditions, hyperleptinemia prevents the neuronal activation of the ARC to fasting. Importantly, we reported previously that short-term fasting increases leptin sensitivity in LOO mice, as measured in a behavioral approach by the leptin-induced suppression of food intake (11).

To assess a possible increase in cellular leptin sensitivity as a consequence of fasting, we now compared leptin-induced phosphorylation of STAT-3 in the ARC of ad libitum-fed and fasted LOO and lean mice. STAT-3 phosphorylation is an accepted marker for leptin receptor activation, and a pharmacological inhibition of p-STAT-3 formation blocks leptin’s anorectic action (23). This supports an important role of STAT signaling for leptin’s in vivo action, although other potential signaling cascades might also be involved in the modulation of leptin sensitivity.

We and others demonstrated previously that the vast majority of fasting-activated ARC neurons are NPYergic (11, 12, 14), but only ~50% of the NPYergic ARC neurons express leptin receptors (7). We, therefore, established a functional approach to demonstrate that fasting-activated ARC neurons represent direct targets for leptin. We induced leptin-dependent STAT-3 phosphorylation in neurons that were activated by fasting (c-Fos). On the basis of the short latency of STAT-3 phosphorylation after leptin treatment, we chose an experimental paradigm, in which the leptin treatment induced p-STAT-3 formation in the ARC without altering the fasting-induced c-Fos expression. In other words, we terminated the experiments after leptin-induced STAT phosphorylation...
but before leptin-induced c-Fos expression. This allowed us to combine both functional approaches without interference between them.

In contrast to extensive literature describing leptin’s neurophysiological and behavioral effects in the context of positive energy balance (including leptin resistance), the role of leptin under fasting conditions is less well understood and less investigated. Hence, in addition to providing evidence for an increase in cellular leptin sensitivity in obese mice as a consequence of fasting, it was a further aim of our studies to substantiate the idea that leptin modulates the fasting-induced ARC activation. If this hypothesis were correct, leptin deficiency should result in an exaggerated fasting response of the ARC, while mimicking hyperleptinemia in lean animals by repeated leptin injections should attenuate the fasting response. We, therefore, investigated the fasting-induced c-Fos response in obese leptin-deficient ob/ob mice compared with their lean wild-type littermates and in leptin-treated lean mice. The latter approach eliminated most of the hormonal and metabolic alterations (e.g., hyperinsulinemia, hyperglycemia, increased free fatty acids, altered ghrelin levels) that occur in obese animal models. To dissociate the possible influence of such factors on the fasting-induced c-Fos responses, all immunohistochemical studies were complemented by the measurements of serum hormone and metabolite levels [leptin, insulin, and ghrelin; glucose, free fatty acids (FFA) and β-hydroxybutyrate (BHB)]. Finally, we validated our observation that hyperleptinemic obese mice show a blunted ARC response to fasting in diet-induced obese (DIO) mice. Similar to LOO, the DIO model is commonly used as a model for human obesity. We, therefore, considered it important to corroborate the relevance of our findings in this obesity model.

MATERIALS AND METHODS

General experimental conditions. Adult male C57BL/6n or ob/ob mice (B6.V-Lepob) and their lean littermates (Charles River, Sulzfeld, Germany) were housed individually in a temperature-controlled room (22°C) under a 12-h light-dark cycle (lights off at 0900). Mice were handled daily for at least 3 wk before experiments as recommended by Ryabinin et al. (28). Unless stated otherwise, animals had free access to chow (4.5% fat, 18.5% protein, 41.7% carbohydrates wt/wt; 12.3 MJ/kg metabolizable energy; #3430, Kliba Nafag, Switzerland), and water. Animals were anesthetized with pentobarbital (Nembutal; Abbott Laboratories, Abbott Park, IL; 80 mg/kg ip). In all experiments, mice were killed 2 h after the onset of the dark phase. Blood samples were taken before perfusion from anesthetized mice by puncturing the right ventricle. All animal procedures were approved by the Veterinary Office of the Canton of Zurich.

LOO. Chow-fed C57BL/6n mice gained weight at different rates. At the age of 12 mo, 30% of the mice reached a body weight (BW) of more than 39 g (mean BW: 42.7 g) and were defined as late-onset obese (LOO). Approximately 20% of the mice had a BW below 34 g (mean BW: 30.4 g) and were considered lean. Mice with an intermediate BW were not used in these studies.

Diet-induced obesity. From 9 wk of age, C57BL/6n mice were fed a high-fat (HF) diet (containing 23.9% protein, 35% fat, 23.2% carbohydrates wt/wt; 22.0 MJ/kg metabolizable energy; #2127; Kliba Nafag, Kaiseraugst, Switzerland) for 19 wk. Then, mice were divided into three groups based on their BW. The upper tertile was defined as DIO (BW >39 g; mean BW: 42.2 g), the middle tertile as intermediate weight gainers (INT; BW 35–39 g; mean BW: 37.7 g), and the lower tertile as diet-resistant (DR) lean (BW range: 29–35 g; mean BW: 31.9 g). The DIO and DR had a similar respective BW as the obese and the lean mice in the LOO model. Moreover, perirenal and epididymal fat pad weights were similar within the lean controls and within the obese mice of both models (not shown).

Experiments 1–3: effects of 26-h food deprivation on ARC activity and leptin sensitivity in the late-onset obesity model. The aim of these studies was threefold. In experiment 1, we tested whether LOO mice also show an attenuated ARC activation after an extended fasting period of 26 h [vs. 14 h in our previous studies (11)]. The lack of fasting-induced ARC activation after this stronger fasting stimulus most likely would exclude the possibility that the obese mice (including the DIO mice; see experiment 6) are unresponsive because of an insufficient duration of fasting. In experiment 2, we used leptin-induced STAT-3 phosphorylation as a marker to investigate whether fasting enhances leptin sensitivity in the ARC of obese mice, similar to what has been reported for lean animals (5, 6, 23). In experiment 3, we assessed the colocalization of p-STAT-3 and c-Fos in fasted lean control mice following leptin injection, to investigate whether leptin has a direct effect on the fasting-activated neurons.

LOO and age-matched lean mice were used. Mice of each BW group were fed ad libitum or were fasted (26 h). The fasted and ad libium-fed groups were further divided into two groups each, one group that received a subcutaneous injection of leptin (2.6 mg/kg) and one group that received saline (0.1 ml/10 g) 45 min before death. This resulted in four groups in each BW group: fed+leptin, fasted+leptin, fasted+saline (for all n = 5 for LOO and n = 4 for lean) and fed+saline (n = 5 for LOO and lean). The 45-min time interval between injection and death was chosen because leptin-induced STAT-3 phosphorylation is high at this time point, while leptin-induced c-Fos expression does not yet occur (20, 32); therefore, leptin-induced c-Fos does not interfere with the fasting-induced c-Fos expression. Furthermore, this dose of leptin does not affect c-Fos expression in fasted or ad libitum-fed mice 2 h after treatment (12), and there was no differential effect of leptin on c-Fos expression in NPY and POMC neurons (inhibition vs. stimulation) in fasted mice. Therefore, such an effect was excluded as a confounding factor in our approach.

In each group, three sets of hypothalamic slices were collected. One set was stained for c-Fos (experiment 1) or for p-STAT-3 immunoreactivity (experiment 2) only. The third set was double-stained for both c-Fos and p-STAT-3 (experiment 3, lean mice only).

Experiment 4: effect of food deprivation on ARC activity in ob/ob mice. Here, we tested whether the fasting-induced c-Fos expression in the ARC is enhanced in leptin-deficient mice compared with normal lean controls. Ob/ob mice (mean BW: 58.1 g) and their lean littermates (mean BW: 29.9 g) were fed ad libitum with chow (n = 6 for lean and n = 5 for ob/ob) or were fasted (n = 6/group) for 14 h before death.

Experiment 5: effect of repeated leptin injections during fasting on ARC activation in lean mice. Lean mice (mean BW: 27.8 g) were food deprived for 14 h starting at the onset of the light phase. At this time point, animals received a leptin (0.1 mg/kg sc) or saline injection (n = 6/group). Injections were given every 3 h (4 in total), and mice were killed 2 h after the last injection i.e., 2 h after dark onset. The brains were processed for c-Fos immunohistochemistry as described below.

Experiment 6: effect of food deprivation on ARC activity in diet-induced obesity. Here, we investigated whether similar to LOO mice (11) hyperleptinemic DIO mice also show a blunted c-Fos expression after 14 h of fasting. Mice were fed ad libitum (n = 5 for DR; n = 4 for DIO and for INT) with HF diet or were fasted (n = 6/group) for 14 h (i.e., during the light phase until 2 h into the dark phase) before death. Similar to experiment 1, one group of DIO mice (n = 6) was subjected to 26 h of food deprivation to achieve a stronger fasting stimulus.

To exclude that HF vs. chow feeding biases the basal or the fasting-induced c-Fos expression, two groups of chow-fed lean mice (BW range: 28–34 g; mean BW: 30.7 g) were also used. They were either food deprived for 14 h (n = 5) or fed ad libitum (n = 5) and...
were killed at the same time as the HF-fed mice. At the age of 9 wk, these mice had been randomly selected from the same batch as the HF-fed mice. They were kept under the same conditions, in neighboring cages to the HF-fed mice but received chow at all times. In these mice, only c-Fos expression was analyzed, and it was compared only to that in lean HF-fed mice.

**Immunohistochemistry.** c-Fos immunohistochemistry was performed as described previously (9, 13). For the detection of p-STAT-3 immunoreactivity (ir), mice were perfused with saline followed by ice-cold 0.1 M PBS containing 2% paraformaldehyde. After removal, the brains were postfixed (1 h), cryoprotected [24 h in 20% sucrose solution in 0.02 M potassium-PBS (KPBS)], and snap frozen in CO2. Coronal sections (20 μm) were cut in a cryostat. The sections were air-dried at room temperature, rehydrated in KPBS, pretreated with 0.3% NaOH/0.3% H2O2 (20 min), followed by 0.3% glycine (10 min) and 0.03% SDS (10 min; all in 0.02M KPBS). Unspecific binding was blocked by 20-min incubation in blocking solution (KPBS containing 4% normal donkey serum, 0.4% Triton X-100, 1% BSA) and avidin (Vector Laboratories, Burlingame, CA). The primary antibody (rabbit anti-p-STAT-3 1:1,000, in blocking solution/biotin; Cell Signaling, Beverly, MA) was applied for 48 h at 4°C, followed by the secondary antibody (biotinylated donkey anti-rabbit 1:200; Jackson Immunoresearch, Bar Harbor, ME) for 2 h at room temperature. After incubation in ABC solution (Vector Laboratories), diaminobenzidine was used as a chromogen (0.04% in PBS with 0.02% H2O2, 0.08% NiCl2·6H2O, 0.01% CoCl2·6H2O). Finally, the sections were dehydrated in graded alcohols, cleared in xylenes, and coverslipped with Entellan (Merck, Darmstadt, Germany).

p-STAT-3 and c-Fos colocalization was assessed by immunofluorescence. After pretreatment as described above for p-STAT-3 single staining, the sections were incubated with the primary antibodies simultaneously (anti-p-STAT-3: 1:500/goat anti-c-Fos; 1:1,000; Jackson Immunoresearch, Bar Harbor, ME) for 2 h at room temperature. After incubation in ABC solution (Vector Laboratories), diaminobenzidine was used as a chromogen (0.04% in PBS with 0.02% H2O2, 0.08% NiCl2·6H2O, 0.01% CoCl2·6H2O). Finally, the sections were dehydrated in graded alcohols, cleared in xylenes, and coverslipped with Entellan (Merck, Darmstadt, Germany).

**Blood chemistry.** Blood glucose concentrations were measured immediately by a portable glucometer (Glucometer Elite, Bayer, Leverkusen, Germany). Plasma concentrations of insulin and leptin were analyzed by the LINCoplex technology (Bio-Rad, Hercules, CA, USA) using LINCO test kits (mouse endocrine lincoplex kit; LINCO Research, St. Charles, MO). Total ghrelin was determined by radioimmunoassay (total ghrelin RIA kit; Linco Research). FFA, BHB, and triglycerides (TG) were measured by an automated serum chemistry analyzer (Cobas Mira; Roche, Mannheim, Germany).

**Statistics.** The mean value of the cell count/section of an individual animal was used for statistical analyses. All data were compared by Student’s t-test or by one-way ANOVA, followed by Student-Newman-Keuls test. The mean values ± SE are presented in the figures. Differences were considered significant at *P* < 0.05.

**Fig. 1.** The effect of 26 h of food deprivation on c-Fos expression in the arcuate nucleus (ARC) and on metabolic and hormonal parameters of 1-yr-old mice with late-onset obesity (LOO) and of age-matched lean controls injected with saline or leptin. Fasting induced very low c-Fos expression in the LOO mice compared with lean controls. FFA, free fatty acids; BHB, β-hydroxy-butyrate; TG, triglycerides. a,b,c,dDifferent letters indicate significant differences between groups (*P* < 0.05).

**Fig. 2.** Quantification of p-STAT-3-positive neurons in the ARC of fed and 26-h fasted 1-yr-old lean and obese (LOO) mice injected with leptin. Saline-injected LOO mice had higher p-STAT-3 levels than lean mice under both feeding conditions. Leptin induced stronger STAT-3 phosphorylation in lean compared with LOO mice. Leptin induced higher STAT-3 phosphorylation in fasted mice than in fed animals. a,b,c,dDifferent letters indicate significant differences between groups (*P* < 0.05).
RESULTS

Experiment 1: effects of 26-h food deprivation on ARC activity in LOO model. The effect of 26 h of food deprivation on c-Fos expression was evaluated in saline-treated LOO mice and lean controls. Only a few c-Fos-positive neurons were detected in the ARC in both groups of ad libitum-fed mice (Fig. 1). Fasting induced a large increase in c-Fos expression in the lean animals ($P < 0.001$) but only a small increase in LOO mice ($P < 0.05$). The number of c-Fos-immunoreactive (ir) neurons was almost 3 times lower in the fasted LOO than lean mice ($P < 0.001$).

Plasma hormone and metabolite concentrations are summarized in Fig. 1. Compared with the respective lean controls, leptin levels were 9 times higher in the ad libitum-fed and the fasted LOO mice ($P < 0.001$ both). After 26 h of food deprivation, leptin levels decreased significantly in both groups. Insulin levels were significantly higher in the ad libitum-fed LOO than lean mice. Fasting induced a significant reduction of insulin levels only in the LOO mice. The fasting insulin levels did not differ between obese and lean mice. LOO mice had lower ghrelin levels under both feeding conditions than lean animals ($P < 0.05$), but ghrelin increased to a similar degree in both groups during fasting.

Blood glucose levels did not differ between lean and obese ad libitum-fed mice, and the difference just failed to reach significance between the two fasted groups ($P = 0.052$). The fasting-induced decrease in glucose levels was only significant in the LOO group. The LOO mice had higher ad libitum FFA, BHB, and TG levels than lean mice. FFA and BHB levels increased, while TG levels decreased to similar levels in LOO and lean mice after fasting.

Experiment 2: effect of 26-h food deprivation on leptin sensitivity in LOO model. Saline-injected lean mice exhibited similarly low numbers of p-STAT-3-positive ARC neurons whether fed or fasted (Fig. 2). Leptin increased the number of
p-STAT-3-ir neurons in both groups ($P < 0.001$ vs. saline), but the number of p-STAT-3-positive cells after leptin was significantly higher in fasted than in fed lean mice. The saline-injected LOO mice had significantly more p-STAT-3-positive ARC neurons than lean mice under both feeding conditions. Leptin significantly increased the number of p-STAT-3-ir neurons in fasted but not ad libitum-fed LOO mice, so that the number of p-STAT-3-positive ARC neurons after leptin was significantly higher in 26-h food-deprived than ad libitum-fed mice. Representative photomicrographs of animals from each treatment group are shown in Fig. 3.

Experiment 3: leptin-induced STAT-3 phosphorylation in c-Fos-expressing ARC neurons in fasted lean mice. As expected, leptin did not affect the number of fasting-activated c-Fos-expressing neurons (66.9 ± 7.8 vs. 75.6 ± 18.2 cells/section; Fig. 4) under our experimental conditions. Leptin also had no effect on c-Fos expression in ad libitum-fed mice assessed 45 min after injection (data not shown). Hence, leptin did not seem to interfere with the fasting-induced ARC activation, at least as far as the number of c-Fos-positive neurons is concerned; this was an important prerequisite for this experiment.

Functional phenotyping revealed that leptin significantly increased p-STAT-3 formation in fasting-activated neurons (21.6 ± 7.9 vs. 68.4 ± 17.8 double-labeled cells/section; $P = 0.042$) in lean mice. Hence, 90% (vs. 32% in saline-treated controls) of all c-Fos-positive neurons also showed immunostaining for p-STAT-3 in leptin-treated fasted lean mice. Therefore, the proportion of the leptin-sensitive neurons among the c-Fos-expressing cells can be estimated to be at least 58%. In other words, because c-Fos-positive neurons that already show p-STAT-3 immunoreactivity under unstimulated basal conditions are not necessarily leptin insensitive, the number of fasting-activated and leptin-sensitive ARC neurons ranged between 58% and 90%. Representative photomicrographs demonstrating the colocalization of fasting-induced c-Fos and leptin-induced p-STAT-3 immunoreactivity are shown in Fig. 4.

Experiment 4: effect of 14-h food deprivation in obese ob/ob mice. c-Fos expression was similarly low in the ARC of ad libitum-fed ob/ob mice and their lean littermates (Fig. 5). Compared with the fed groups, fasting induced a large increase in c-Fos expression in both lean and ob/ob animals (both $P < 0.001$), but the fasted ob/ob mice had significantly more (27%) c-Fos-positive neurons than lean littermates.

Insulin levels were higher in ob/ob mice under both feeding conditions compared with lean littermates ($P < 0.001$; Fig. 5). Food deprivation resulted in a decrease in insulin concentration in both genotypes, but insulin was significantly higher in fasted ob/ob than lean mice. Ad libitum ghrelin levels were similar in

Fig. 4. Leptin-induced STAT-3 phosphorylation in c-Fos-expressing ARC neurons of fasted lean mice. Representative ARC sections immunostained simultaneously for fasting-induced c-Fos (green nuclear staining) and leptin-induced p-STAT-3 (red nuclear staining) of 26-h fasted 1-yr-old lean mice injected. The merged image shows that most of the c-Fos-positive neurons that were activated by fasting show p-STAT-3 in response to leptin treatment (yellow nuclear staining). 3V: 3rd ventricle. Right: bar chart shows that leptin treatment did not affect fasting-induced c-Fos expression but significantly increased p-STAT-3 formation in c-Fos-positive neurons ($^{*}P = 0.042$).
both groups of mice; ghrelin increased in lean but not in ob/ob mice \((P < 0.001)\) during fasting.

Glucose levels were significantly higher in ad libitum-fed ob/ob mice than in lean controls. In two of five ad libitum-fed ob/ob mice, glucose was above the upper detection level (>33 mmol/l); these values were not included in the statistical analysis. After 14 h of fasting, glucose decreased significantly in both groups, but it was still significantly higher in the ob/ob mice. Ad libitum-fed ob/ob mice had significantly higher plasma BHB levels than lean littermates. After 14 h of fasting, BHB increased to similar levels in both groups. Plasma FFA levels were similar in ad libitum-fed mice; fasting values were significantly higher in the ob/ob mice than in the lean controls.

Experiment 5: effect of repeated leptin injections during fasting on ARC activation in lean mice. Leptin-treated 14-h food-deprived mice had significantly less c-Fos-positive cells in the ARC than saline-treated controls \((35.7 \pm 6.7\) vs. \(96.8 \pm 12.5\) cells/section; \(P < 0.01)\) (Fig. 6). Leptin levels in the leptin-treated mice were 6 times higher than in saline-injected controls \((6.02 \pm 0.82\) vs. \(1.06 \pm 0.26\) ng/ml; \(P < 0.01)\) at the time of death, i.e., 2 h after the last injection; the concentration was similar to 26-h fasted LOO mice or DIO, or to ad libitum-fed DR mice (compare Fig. 6 with Figs. 1 and 7). There was no significant difference between the leptin- and saline-treated groups in any other parameter measured (leptin vs. saline; insulin: \(0.6 \pm 0.1\) vs. \(0.9 \pm 0.3\) ng/ml; ghrelin: \(3.82 \pm 0.36\) vs. \(4.64 \pm 0.43\) ng/ml; glucose: \(7.68 \pm 0.63\) vs. \(6.77 \pm 0.35\) mmol/l; FFA: \(0.63 \pm 0.03\) vs. \(0.48 \pm 0.08\) mmol/l; BHB: \(1.16 \pm 0.14\) vs. \(1.04 \pm 0.09\) mmol/l; and TG: \(0.36 \pm 0.02\) vs. \(0.40 \pm 0.01\) mmol/l).

Experiment 6: effect of food deprivation on ARC activity in diet-induced obesity. Only few c-Fos-ir cells were present in the ARC of ad libitum-fed DR, INT, and DIO mice (Fig. 7). Fourteen-hour food deprivation increased the number of c-Fos-positive cells only in DR mice \((P < 0.001)\). In DIO mice, a significant increase in c-Fos expression was detected after 26-h fasting, but the number of activated neurons was still significantly lower than in 14-h fasted DR mice. There was no significant difference in the number of c-Fos-positive cells between fasted DR and fasted age-matched lean mice that were adapted to chow (63.6 ± 16.7 cells/section). This indicates that the type of maintenance diet did not have an influence on the fasting-induced ARC activation.

Fig. 5. The effect of food deprivation on c-Fos expression in the arcuate nucleus (ARC) and on metabolic and hormonal parameters of young leptin-deficient ob/ob mice and lean wild-type littermates. Representative ARC sections immunostained for c-Fos of fed and 14-h fasted animals show that fasting induced stronger c-Fos expression in the ob/ob mice than in the lean controls. Bar charts show the quantification of the c-Fos expression in the ARC, and the fasting-induced changes in plasma insulin, ghrelin, glucose, BHB, FFA, and TG levels. a,b,c,d Different letters indicate significant differences between groups \((P < 0.05)\). 3V, 3rd ventricle. Scale bar: 100 μm.
Leptin levels were higher in ad libitum fed INT and DIO than in DR mice (Fig. 7; \( P < 0.01 \)). After 14 h of fasting, leptin concentrations significantly decreased in DR and INT, but not DIO mice. Of note, the leptin concentration in fasted INT mice was similar to ad libitum-fed DR mice. Fasted DIO mice had markedly higher leptin levels than fasted DR mice (\( P < 0.001 \)). Ad libitum insulin levels did not differ significantly among groups. The decrease in insulin during 14 h of food deprivation just failed to reach significance in the INT (by 77%; \( P = 0.058 \)) and DR mice (by 60%; \( P = 0.10 \)). Insulin levels of DIO mice did not decrease after 14 h of fasting. After 26 h of food deprivation, insulin levels of the DIO mice were lower than after 14 h of fasting (\( P < 0.05 \)). Ghrelin levels were similarly low in ad libitum-fed DR, INT, and DIO mice. After 14 h of food deprivation, ghrelin increased significantly in all three groups but remained lower in DIO mice than in the other two groups (\( P < 0.05 \)). After 26 h of fasting, ghrelin levels increased further in DIO mice (\( P < 0.001 \) vs. ad libitum and 14-h fasted DIO) and were not significantly different from that of 14-h fasted DR and INT mice.

There was no statistically significant difference in the blood glucose levels among the three groups under ad libitum feeding condition; there was no significant decrease after 14 h fasting in any group. Compared with DR mice, the fasting blood glucose values were higher in the INT mice (\( P < 0.05 \) ) and just failed to reach significance in the DIO group (\( P = 0.055 \)). Following 26 h of food deprivation, glucose was significantly lower in DIO mice compared with the levels in ad libitum-fed animals and did not differ from that of 14-h fasted DR mice. BHB levels were similar in the three groups when fed ad libitum, and 14 h of food deprivation induced a similar increase in all groups. Following 26 h of food deprivation, BHB levels increased further in the DIO mice (\( P < 0.01 \) vs. DIO ad libitum and DIO 14 h fasted). Plasma FFA and TG levels were similar in all groups under all feeding conditions; fasting had no significant effect in any group.

DISCUSSION

The NPYergic ARC neurons are key components of the central network controlling food intake and energy balance, and their activity correlates with the energy status in lean mice (11, 14). Here, we show that these neurons were not activated after 14 h of fasting in two models of hyperleptinemic obese mice, and even an extended period of food deprivation (26 h) had little effect. In contrast, obese, but genetically leptin-deficient \( ob/ob \) mice showed a strong response under comparable experimental conditions. Further, the fasting-induced c-Fos response in the ARC was reduced in lean mice that were made hyperleptinemic by repeated leptin injections during fasting. Cellular leptin sensitivity as measured by STAT-3 phosphorylation increased during fasting in obese mice. These results strongly suggest that high leptin levels interfere with the neuronal response of the ARC to fasting signals as it is the case in animal models of hyperleptinemic obesity. Because leptin induced STAT-3 phosphorylation in the majority of the c-Fos-positive ARC neurons in fasted lean mice, these cells seem to be direct targets for peripheral leptin.

The experiments with the DIO mice are consistent with our previous studies using LOO mice (11). Hence, the lack of neuronal activation to 14 h of fasting seems to be a common feature of obesity models characterized by hyperleptinemia. Even after an extended food deprivation period of 26 h, both obesity models showed only a weak fasting-induced c-Fos response. It appears likely that these few activated cells might also be NPYergic, although it cannot be excluded that these cells belong to a phenotypically or functionally different subpopulation of ARC neurons. The fasting-induced changes in metabolic parameters, e.g., the increase in lipid oxidation, were similar in DIO, LOO, and lean mice. We, therefore, believe that the fasting-induced changes in lipid signals do not seem to account for the lack of neuronal activation in hyperleptinemic obese mice.

One intriguing finding of this study was the different neuronal response to fasting in obese hyperleptinemic vs. genetically leptin-deficient obese mice under the same experimental condition. This suggests that the responsiveness of the ARC to fasting-induced perturbations in energy homeostasis critically depends on leptin. While high-leptin levels seem to attenuate the neuronal response to fasting, the lack of leptin has the opposite effect, i.e., the fasting-induced ARC activation is enhanced. Consistent with this idea, we demonstrated here that similar to the hyperleptinemic obese animals, repeated leptin injections in lean mice effectively blocked the fasting-induced c-Fos expression in the ARC. Notably, a 26 times higher dose of a single leptin injection to 12-h fasted lean mice had no effect on the fasting-induced c-Fos expression in NPY neurons in the ARC 2 h after application (12). Therefore, it is unlikely that the effect of leptin on c-Fos expression in the current experiment was only due to the last bolus leptin injection 2 h before death.

It may sound paradoxical to argue that high leptin levels prevent the fasting-induced neuronal activation of the ARC in common models of obese mice (including our LOO and DIO...
models) because these mice are typically considered leptin resistant. In fact, exogenous leptin did not increase STAT-3 phosphorylation in the ARC of ad libitum-fed LOO mice in our studies, which was in contrast to lean controls. Of note, however, the leptin-induced STAT-3 phosphorylation was increased after food deprivation not only in lean, but also in obese mice. This indicates that the increase in leptin sensitivity during fasting may allow the high leptin levels to exert some action in the ARC, even in obese mice and thus to prevent the fasting-induced activation of ARC neurons in these animals. In lean animals, fasting increases leptin receptor expression, enhances leptin binding to the receptor, and reduces the expression of inhibitors of the leptin-signaling pathways (5, 6, 22, 23). Whether the same mechanisms underlie the enhanced leptin sensitivity of the obese mice requires further studies.

Transport of leptin across the blood-brain barrier seems to be dynamic and sensitive to changes in the feeding status. While longer periods of fasting (starvation for more than 2 days) appear to reduce leptin transport into the brain, short-term fasting has the opposite effect. In particular, it has been demonstrated in age-related obese and in DIO mice that fasting for 24 h and 16 h, respectively, increases the transport of leptin into the brain (2, 4). Interestingly, in the former studies, the rate of leptin transport in fasted obese mice was similar to lean control animals. These studies not only clearly show that leptin has access to the brain in fasted obese mice, but they also support our general idea that hyperleptinemia may interfere with the responsiveness of the ARC to fasting signals.

Similar to altered rates of leptin transport into the brain, changes in leptin receptor expression might influence leptin sensitivity. Food deprivation increases leptin receptor expression in lean mice and rats but not in ob/ob mice (8, 22). Interestingly, ob/ob mice have a more than 2 times higher baseline leptin receptor gene expression compared with lean control, which is reversed by leptin treatment over a period of 5 days (8). Hence, leptin regulates the expression of its own

Fig. 7. The effect of food deprivation on c-Fos expression in the ARC and on metabolic and hormonal parameters of young mice fed a high-fat diet for 19 wk. Representative ARC sections immunostained for c-Fos of fed and 14-h fasted animals show that fasting induced a strong c-Fos expression in the diet-resistant (DR) lean, but not in the intermediate weight gainers (INT) and obese (DIO) mice. Bar charts show the quantification of the c-Fos expression in the ARC, and the fasting-induced changes in plasma leptin, insulin, ghrelin, glucose, BHB, FFA, and TG levels. Different letters indicate significant differences between groups (P < 0.05). 3V, 3rd ventricle. Scale bar: 100 μm.
receptor leading to increased expression under leptin deficiency. Whether changes in leptin receptor gene expression contribute to the changes in leptin sensitivity under our experimental conditions remains to be investigated.

In addition to the high leptin-induced inhibitory tone, other factors may also contribute to the lack of ARC activation in hyperleptinemic obesity. The NPYergic neurons are well-established targets for several feeding-regulatory factors. Increasing ghrelin and decreasing glucose levels have excitatory effects, and insulin has a direct inhibitory action on these neurons (15, 17, 21, 24, 25, 33). After 14 h of food deprivation, plasma insulin and glucose levels did not decrease in the DIO mice, and ghrelin levels increased less than in the lean controls. Therefore, the smaller fasting-induced changes in these parameters might have contributed to the lower c-Fos expression. However, taking together the results of the other experiments of the current study, we believe that neither relative changes nor the absolute levels of these signals are of primordial significance for the differences of ARC activity observed between fasted lean and obese mice.

Fasting glucose levels were highest in the ob/ob mice, while these mice showed the strongest c-Fos response after fasting. Further, the relative drop in blood glucose was larger in the 26-h fasted DIO mice than in 14-h fasted lean animals, but it was only associated with a mild ARC activation. Finally, glucose levels did not change during fasting in the lean DR mice, but these mice showed a strong c-Fos response. Hence, despite the well-established role of glucose in modulating ARC activity (10, 17, 24), neither relative changes nor absolute values of blood glucose seem to predict the response in the ARC to fasting when comparisons are made across conditions characterized by different leptinemia.

The situation may be similar in respect to a potential role of ghrelin and insulin. For example, the moderately obese and hyperleptinemic INT mice showed no c-Fos response to fasting in spite of similar fasting-induced changes in insulin and ghrelin levels as DR mice. Similarly, 26 h of fasting in the DIO and LOO mice induced comparably large relative changes in insulin and ghrelin, but these mice showed a low c-Fos response in the ARC. In contrast, high c-Fos expression was present in fasted ob/ob mice, although they had the highest fasting insulin levels and their ghrelin levels did not increase during fasting. Therefore, across the different models, the fasting-induced ARC activation is not reflected by relative changes or absolute values of insulin and ghrelin. In relation to fasting-induced changes in ghrelin levels, our results are consistent with previous studies showing that ghrelin levels in ob/ob do not increase in response to fasting (30). Hence, ghrelin does not seem to be involved in the strong fasting-induced ARC activation in these mice. Further, we demonstrated previously that neutralization of circulating ghrelin has no effect on the fasting-induced c-Fos expression in the ARC of lean mice (9). Therefore, there is currently no evidence that endogenous ghrelin is necessary for the fasting-induced activation of ARC neurons. Probably multiple fasting signals act in concert to bring about this response. According to our current findings, the leptin level seems to reliably predict the responsiveness of the ARC to these fasting signals. In line with this concept, leptin deficiency alone does not cause an activation of the ARC when other fasting signals are absent, because similar to wild-type mice, c-Fos expression was low in ad libitum-fed ob/ob mice.

In summary, our results show that a blunted response in orexigenic ARC neurons to acute negative energy balance is associated with obesity-related hyperleptinemia and increased leptin sensitivity, but not with hyperinsulinemia or hyperglycemia. We consistently demonstrated across leptin-deficient, normoleptinemic, and hyperleptinemic conditions that leptin seems to set a tone, which determines the responsiveness of the ARC to fasting signals. Leptin sensitivity seems to be dynamic both in lean and in hyperleptinemic obese mice, and it increases during fasting. This substantiates a modulatory role for leptin in obese subjects who are generally considered leptin resistant.

Perspectives and Significance

It is tempting to speculate about the pathophysiological implications of our findings in the context of obesity. As we recently demonstrated, the lack of fasting-induced ARC activation in obese mice is associated with a lack of refeeding hyperphagia after 14 h of fasting in LOO mice (11). Hence, our immunohistological findings are reflected in behavioral changes. Extending the concept of leptin resistance, our current observations suggest that hyperleptinemia in combination with dynamic increases in leptin sensitivity has an impact on the responses of the ARC to physiologically occurring short-term perturbations of energy intake. The potential long-term consequences of these findings for energy homeostasis need to be investigated.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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