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Journal of Nutritional Biochemistry 22 (2011) 372–379
Journal of Nutritional Biochemistry

ATGL and HSL are not coordinately regulated in response to fuel partitioning in fasted rats☆

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Received 2 September 2009; received in revised form 2 March 2010; accepted 11 March 2010

Abstract

Prolonged fasting is characterized by lipid mobilization (Phase 2), followed by protein breakdown (Phase 3). Knowing that body lipids are not exhausted in Phase 3, we investigated whether changes in the metabolic status of prolonged fasted rats are associated with differences in the expression of epididymal adipose tissue proteins involved in lipid mobilization. The final body mass, body lipid content, locomotor activity and metabolite and hormone plasma levels differed between groups. Compared with fed rats, adiposity and epididymal fat mass decreased in Phase 2 (approximately two- to threefold) and Phase 3 (~4.5–14-fold). Plasma nonesterified fatty acids (NEFA) concentrations were increased in Phase 2 (approximately twofold) and decreased in Phase 3 (approximately twofold). Daily locomotor activity was markedly increased in Phase 3 (~11-fold). Compared with the fed state, expressions of adipose triglyceride lipase (ATGL; mRNA and protein), hormone-sensitive lipase (HSL; mRNA) and phosphorylated HSL at residue Ser660 (HSL Ser^{660}) were increased during Phase 2 (~1.5–2-fold). HSL (mRNA and protein) and HSL Ser^{660} levels were lowered during Phase 3 (~3–12-fold). Unlike HSL and HSL Ser^{660}, ATGL expression did not correlate with circulating NEFA, mostly due to data from animals in Phase 3. At this stage, ATGL could play an essential role for maintaining a low mobilization rate of NEFA, possibly to sustain muscle performance and hence increased locomotor activity. We conclude that ATGL and HSL are not coordinately regulated in response to changes in fuel partitioning during prolonged food deprivation, ATGL appearing as the major lipase in late fasting.

Keywords: Lipolysis; Body reserves; Prolonged fasting; Gene and protein expressions; Metabolic status

1. Introduction

During times of increased energy demand, metabolic adaptations, such as the regulation of white adipose tissue (WAT) lipolysis, are the cornerstones for a continuous fuel supply and utilization. The availability of circulating nonesterified fatty acids (NEFA), which serve as fuels for peripheral tissues, is ultimately controlled by the breakdown of fat. Lipolysis, which is governed by numerous hormonal regulatory factors that promote (β-adrenergic agonists…) or prevent (insulin…) the mobilization of lipid fuels, involves a number of lipolytic enzymes but also other proteins, like lipid droplet-associated proteins, plasma membrane transporters, and fatty acid binding proteins [1–3].

For many years, the rate-limiting step in lipolysis was believed to be controlled by hormone-sensitive lipase (HSL) [2,4], which is stimulated during fasting mainly by catecholamines, through the cAMP pathway [1]. HSL activity is regulated by site-specific phosphorylation on several serine residues. Phosphorylated hormone-sensitive lipase at residue Ser659 and Ser660 (HSL Ser^{659} and HSL Ser^{660}) have been reported as the major protein kinase A (PKA) controlling sites for lipase phosphorylation, translocation to the lipid droplet and activity (reviewed in Ref. [4]). Moreover, Ser659 has been suggested to be phosphorylated at a slower rate than Ser660 [5]. The essential role of HSL was highlighted by studies that reported firstly a strong correlation between lipolytic capacity of subcutaneous adipocytes and HSL expression in humans [6,7] and, secondly, a prevention of triacylglycerol (TAG) accumulation in adipocytes overexpressing HSL [8]. However, in vivo studies in HSL-null mice provided evidence for the existence of other lipases in WAT [9–11].

Recently, a new triglyceride lipase has been identified in WAT and simultaneously described as adipose triglyceride lipase (ATGL) [12], desnutrin [13] and calcium-independent phospholipase A2-ζ [14]. ATGL is induced early during 3T3-L1 adipocyte differentiation and is predominantly expressed in adipose tissues [12–14]. It has been established that global loss of ATGL function in mice results in the dramatic reduction of total lipase activity in WAT, increased body weight and fat gain with enlarged adipose fat depots and lipid droplets [15].

The adaptive time course response to short-term fasting in the expression of genes involved in lipid metabolism has recently been reported [16,17]. An increase in WAT ATGL and HSL gene expressions [16–18] and protein levels [18] was observed in this situation.

☆ This work was supported by grants from the Agence Nationale de la Recherche (ANR; project proteonutr, ANR-05-BLAN-0069).
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doi:10.1016/j.jnutbio.2010.03.005

All of these previous works were centred on lipolytic-related gene/protein expressions in situations of stimulated lipolysis only. Data are then still lacking regarding the relationships between changes in the expression of adipose lipases and changes of fuel partitioning during long-term food deprivation. Prolonged fasting is a good model to gain further insight into the molecular mechanisms underlying the nutritional and metabolic regulation of adipose lipid metabolism, as successive periods of stimulated and then reduced lipid mobilization occur. Indeed, prolonged fasting is characterized by the sequential mobilization of energy fuels during three distinct metabolic phases [19]. Phase 1 (P1) is a short period of adaptation, characterized by carbohydrate reserve exhaustion. It is followed by Phase 2 (P2), a period of protein sparing with concomitant and strong mobilization of lipids. Phase 3 (P3) is characterized by a metabolic shift in favor of protein breakdown over fat mobilization and utilization knowing that body lipids are not exhausted at this stage. The aim of this work was therefore to determine the changes in the expression of genes and proteins related to lipid mobilization in WAT according to the metabolic shifts elicited by prolonged fasting. We thus hypothesized that low fat mobilization in late fasting could be partly explained by down-regulation of adipose lipases and/or other lipolytic-related factors.

2. Methods and materials

2.1. Animals and treatment

Male Sprague Dawley rats (n=24; Centre d'élevage R. Janvier, Le Genest-Saint-Isle, France) were housed individually in a light-controlled room (light: 08:00–20:00, dark: 20:00–08:00), which was kept at a constant temperature of 25±1°C. Rats had access to chow (mass percentage: 50 % carbohydrate, 5 % fat, 24 % protein) and water ad libitum. When reaching ~260 g, the rats were randomly divided into four groups (n=6 in each): control-fed rats, rats fasted until P2 and P3 of fasting, and rats refed for one day after P3 (P3R). For all rats, water was supplied ad libitum throughout the experiment. Urine was collected daily and kept frozen at-20°C until total nitrogen determinations. Each individual cage was equipped with a running wheel (Intellibio, Nomeny, France), which allowed the animal to exercise voluntarily. A magnetic switch with LCD counter was used for recording of animal activity, expressed as the total number of wheel revolutions. Body mass was recorded daily in order to calculate the rate of body mass loss ($dm/m.dt$) which is known to be highly correlated with changes in nitrogen excretion and, hence, the rate of protein utilization [20]. Prolonged fasting is characterized by a decrease of protein utilization during P2, followed by an increase in body protein breakdown and locomotor activity during P3 [20]. We were thus able to identify the P2/P3 transition using mass loss and daily wheel-running data. The metabolic status of rats has been afterwards confirmed by plasma metabolite and hormone measurements. Interestingly, rats can be successfully refed after 3 days in P3, which demonstrates the reversibility of this late phase of fasting [21]. Depending on body mass loss, P3 rats were killed 1–2 days after the P2–P3 transition.

The research was conducted in conformity with the Public Health Service policy on Human Care and Use of Laboratory Animals. All experiments were performed in accordance with the rules of the European Committee Council Directive of November 24, 1986 (86/609/EEC) and the French Department of Agriculture (license no. 67-226 to T.R.).

2.2. Measurements on carcass, adipose tissue, plasma and urine

Rats were killed by cervical dislocation. Blood was sampled and plasma was prepared by centrifugation and kept frozen at −20°C for further analysis of metabolite and hormone levels. Epididymal WAT (EPI) was rapidly removed, weighed, frozen in liquid nitrogen and stored at-80°C for further analysis of gene/protein expression. Carcasses were frozen and kept at −20°C for body composition analysis.

Body TAG content was determined as previously reported [17]. Briefly, carcasses were ground under liquid nitrogen, lyophilized and ground again until a fine powder was obtained. Total lipids were extracted from 0.5 g of the ground carcasses by a method adapted from Folch et al. [22] and subsequently quantified. TAG were separated by thin layer chromatography (TLC), and their proportion among all the lipid fractions was determined by scanning densitometry. Body TAG content was calculated by multiplying total lipids with the proportional TAG content. Adiposity represents the percentage of TAG in the carcass. The proportional lipid content of EPI was determined gravimetrically after extraction [22]. The fat mass of EPI was then calculated as fresh mass multiplied with the proportional lipid content.

Levels of plasma insulin and corticosterone (radioimmunoassays), and NEFA and glucose (enzymatic methods) were measured using commercial kits.

Total nitrogen in urine was determined by the method of Kjeldahl, using selenium as catalyzer.

2.3. RNA isolation and Northern blot analyses

Total RNA from EPI was extracted using a guanidium isothiocyanate-phenol method [23]. The RNA (15 μg) was electrophoresed on 1.2% agarose gels, vacuum blotted onto nylon membranes (Roche Diagnostics, Mannheim, Germany) and fixed with ultraviolet light. A chemiluminescence procedure was used to detect mRNAs of interest as described previously [24]. Antisense oligonucleotide probes end-labeled (5′) with digoxigenin (Eurogentec, Seraing, Belgium) were used as previously reported for HSL, β3-adrenergic receptor (β3-AR), perilipin A (PLIN), adipocyte lipid-binding protein (ALBP/aP2), CAAT/enhancer binding protein α (C/EBPα) and 18S [17]. Other probe sequences were as follows: 5′-TGGCGTTGGCCACCAGGAAGGGCGCCGTGCT-3′ (ATGL, Genbank AY731699), 5′-GAAGATGTGCGGGCCAGGCTGCTCTGCCACTG-3′ (fatty acid synthase [FAS] Genbank M76767), and 5′-GCTCCTGGCTTTCCGCACGAGGTCCATGAGGA-3′ (adiponutrin, GenBank AY037763). After hybridization overnight at 42°C and post-hybridization washes, the membranes were incubated with an anti-digoxigenin Fab/alkaline phosphatase conjugate (Roche Diagnostics, Mannheim, Germany) and CDP-Star (Roche Diagnostics, Mannheim, Germany) as the chemiluminescence substrate. Hybridization signals were visualized by exposure of membranes to films (Amersham Biosciences Europe, Freiburg, Germany) for 5–30 min at 37°C. Signals were analyzed by densitometry. Stripping of the membranes allowed reprobing for other mRNAs or for 18S rRNA. mRNA levels were corrected for differences in gel loading or blotting by reference to the level of 18S rRNA. Densitometric data from Northern blots were normalized to fed rats, which were assigned an arbitrary value of one.

2.4. Protein extraction and Western blot analyses

After homogenization of frozen EPI samples using a laboratory ball mill, proteins were extracted in a denaturating solution [Tris–HCl 6.25 mM, sodium dodecyl sulfate (SDS) 1%, dithioerythritol 10 mM, protease inhibitor cocktail (2 μM–2 mM)]. Proteins (30 μg) were electrophoresed on 12 % SDS-acrylamide gels and blotted onto nitrocellulose membranes (Proteigene, Saint Marcel, France). Membranes were stained with Ponceau red (Sigma Diagnostics, MO, St. Louis, USA) to check that equal amounts of proteins were loaded, electrophoresed and analyzed. Membranes were saturated 1 h at 37°C in a 5% w/v dried milk/0.05% w/v Tween/phosphate-buffered saline (PBS) solution and then incubated 3 h at room temperature with rabbit polyclonal antibodies against ATGL, HSL Ser^{660} (Cell Signaling Technology, Boston, MA, USA), HSL (Affinity BioReagents, Golden, CO, USA), or PLIN (Abcam, Paris, France), or with a goat polyclonal antibody against actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). These antibodies were utilized at 1/1000 (ATGL), 1/2000 (HSL, HSL Ser^{660}, PLIN) and 1/500 (actin) dilution in a 1% w/v dried milk/0.05% w/v Tween/PBS solution (PBS-T). Washings were performed in PBS-T. Membranes were then incubated 1 h at room temperature with either rabbit (Cell Signaling Technology, Boston, MA, USA) or goat (Interchim, Montluçon, France) peroxydase conjugate anti-IgG, utilized at 1/2000 and 1/5000 dilution, respectively, in PBS-T. After washings, peroxydase activity of the second antibody was revealed using the ECL kit (Amersham Biosciences, Freiburg, Germany). Hybridization signals were visualized by exposure of membranes to films (Amersham Biosciences, Freiburg, Germany) for 5–15 min at room temperature and signals were analyzed by densitometry. Stripping of the membranes allowed reprobing for other proteins. Protein levels were corrected for differences in gel loading or blotting by reference to the level of actin. Densitometric data from Western blots were normalized to fed rats, which were assigned an arbitrary value of one.

2.5. Statistical analysis

Values are means±S.E. (n=6/group). The normal distribution of the measured variables was assessed using the Kolmogorov–Smirnov test (P>.05). Group mean differences were analyzed using one-way analysis of variance followed by the post hoc Tukey test. A P value of less than .05 was considered to be statistically significant.

3. Results

3.1. Profiles of fed, fasted and refed animals

Table 1 and Fig. 1A show variations in body mass and body lipid content between fed, fasted and refed rats. As expected, final body mass of rats was markedly affected by the nutritional treatment. Hence, body mass loss was significantly 34% greater in P3 than P2 animals. When P3 animals were refed, body mass loss values returned to levels observed during P2 within one day. The $dm/m.dt$ decreased during the first day of fasting (P1, not shown). It stabilized thereafter at low levels during P2 and later increased during P3 (Table 1). Daily changes in nitrogen excretion followed a similar pattern (Fig. 1B). The duration of each phase of fasting was similar when determined by

Table 1
Profiles of fed, fasted and refed animals, and plasma parameters

	Fed	P2	P3	P3R
Duration of fasting, days	–	4	6-7	6-7
Initial body mass, g	263.0±0.9	263.3±1.3	263.4±0.7	263.0±1.5
Final body mass, g	263.0±0.9^{a}	190.6±2.0^{b}	165.8±1.0^{c}	187.9±2.3^{b}
Body mass loss, %	–	27.6±0.8^{a}	37.0±0.4^{b}	28.5±0.8^{a}
d*m*/*m*.d*t*, g 100g^{-1} 24 h^{-1}	–	6.2±0.3^{a}	9.9±0.1^{b}	9.4±0.2^{b}
Adiposity, %	6.3±0.5^{a}	3.2±0.1^{b}	1.4±0.1^{c}	2.0±0.1^{c}
Body lipid loss, %	–	61.7±3.0^{a}	85.9±2.0^{b}	76.4±4.2^{b}
Total daily wheel-running, wheel revolutions	953±75^{a}	4712±639^{b}	10483±667^{c}	10102±423^{c}
Glucose, mM	7.06±0.02^{a}	5.45±0.21^{b}	4.18±0.39^{c}	5.72±0.16^{b}
Insulin, ng ml^{-1}	1.89±0.09^{a}	0.19±0.02^{b}	0.10±0.01^{b}	1.51±0.10^{c}
Corticosterone, ng ml^{-1}	15.3±3.1^{a}	447.2±32.8^{b}	875.4±39.4^{c}	118.0±3.7^{d}

Values are means±SE (n=6/group). Values within a same line that do not share the same superscript letter are significantly different (P<.05). R, 1 day-refed rats. d*m*/*m*.d*t* and locomotor activity values are given for the last day before sacrifice or refeeding.

using either d*m*/*m*.d*t* or daily nitrogen excretion. Locomotor activity (total daily wheel-running) was increased gradually during fasting, being greatly elevated in P2 (~5-fold) and above all in P3 (~11-fold) rats, when compared with fed animals.

In fed rats adiposity was close to 6 % and decreased significantly in P2 (approximately twofold), P3 (~4.5-fold), and P3R (approximately threefold) animals (Table 1). Interestingly, the percentage of body lipid loss was approximately twofold that of body mass loss, indicating the strong body fat mobilization during fasting (Table 1). EPI fat mass was significantly lowered in P2 (approximately threefold), P3 (~14-fold) and P3R (~6-fold) animals, when compared with fed rats (Fig. 1C). This suggests that the variations in TAG content within this fat depot were more sensitive to the nutritional treatment than changes in overall adiposity.

Table 1 also shows variations of plasma glucose, insulin, and corticosterone levels between fed, fasted and refed rats. Compared to the fed group, glycemia was significantly decreased in P2 (~1.3-fold) and P3 (~1.7-fold) and partially restored upon refeeding. Insulin levels dropped significantly during fasting (up to ~19-fold in P3 vs. fed). One day of refeeding induced a significant ~15-fold increase of insulin plasma levels in P3R rats, when compared with P3 rats. Plasma corticosterone levels were increased gradually during fasting, being greatly elevated in P2 (~29-fold) and P3 (~57-fold) rats, when compared with fed animals. In refed rats (P3R), plasma corticosterone levels were significantly lowered (~7-fold) relative to P3 animals.

Fig. 1D shows variations of plasma NEFA concentrations between fed, fasted and refed rats. In comparison to the fed group, plasma NEFA concentrations were significantly increased in P2 animals (approximately twofold), and decreased in P3 animals (approximately twofold). In P3R animals, plasma NEFA concentrations were ~1.7-fold higher than in P3 rats.

3.2. Expression of ATGL, HSL, HSL Ser^{660} and PLIN in EPI

Fig. 2 shows variations in mRNA and protein levels of EPI ATGL, HSL, HSL Ser^{660} and PLIN (Fig. 2A-I). When compared with the fed state, EPI ATGL mRNA and protein levels were significantly increased in P2 (~1.5-fold). In P3R animals, control values (fed state) of ATGL mRNA levels were re-established. Relative to the fed state, HSL mRNA levels in EPI were significantly increased in P2 rats (~1.3-fold), and both HSL mRNA and protein levels were significantly decreased in P3 rats (~3.0-fold), while HSL mRNA levels were restored to control fed values in P3R animals. In comparison with fed rats, protein levels of HSL Ser^{660} were increased in P2 (~2.1-fold) and markedly decreased in P3 (~12.2-fold) animals. Relative to fed animals, PLIN mRNA and protein expressions were significantly decreased during P3 in EPI (~3.6-fold and ~2.5-fold, respectively). In P3R rats, PLIN mRNA levels were restored to control fed values. Finally, 18S rRNA and actin expressions were not affected by the treatment.

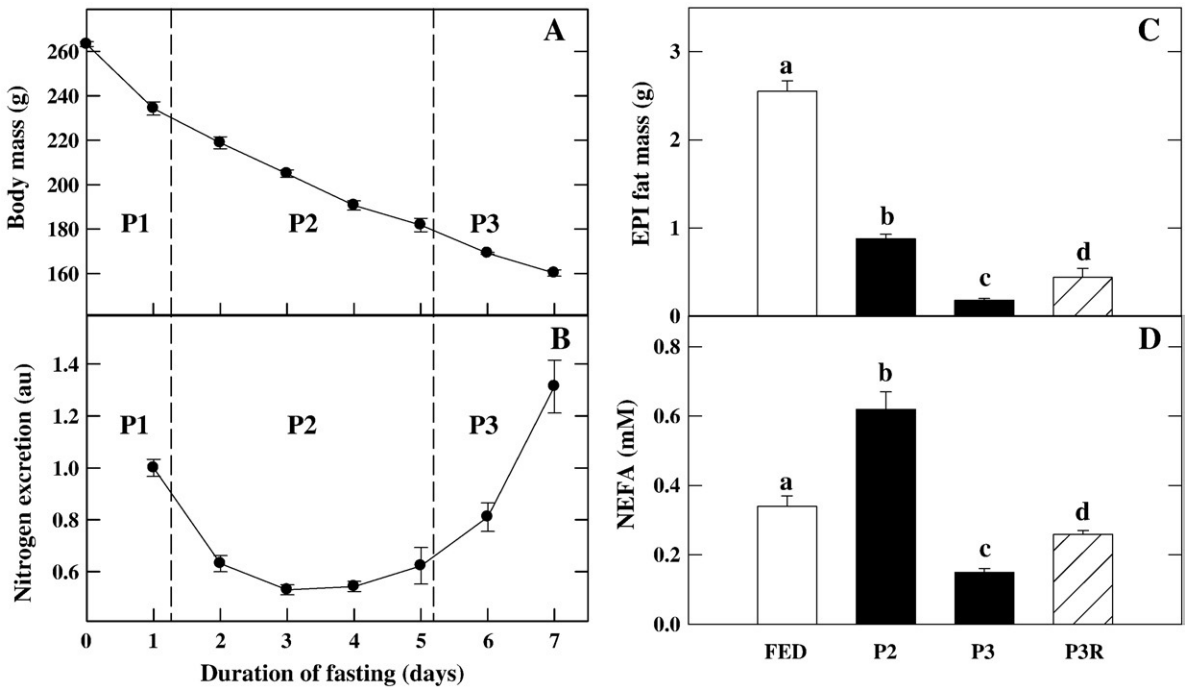

Fig. 1. Body mass (A), daily nitrogen excretion (B), EPI fat mass (C) and plasma NEFA concentrations (D) in fed, fasted and refed rats. Values are means±SE (n=6/group). Bars that do not share the same superscript letter are significantly different (P<.05). EPI, epididymal white adipose tissue; P1, P2 and P3, phase 1, Phase 2 and Phase 3 of fasting, respectively; R, one day refed rats; au, arbitrary unit. See the text (end of introduction) for detailed explanations about the three phases of fasting.

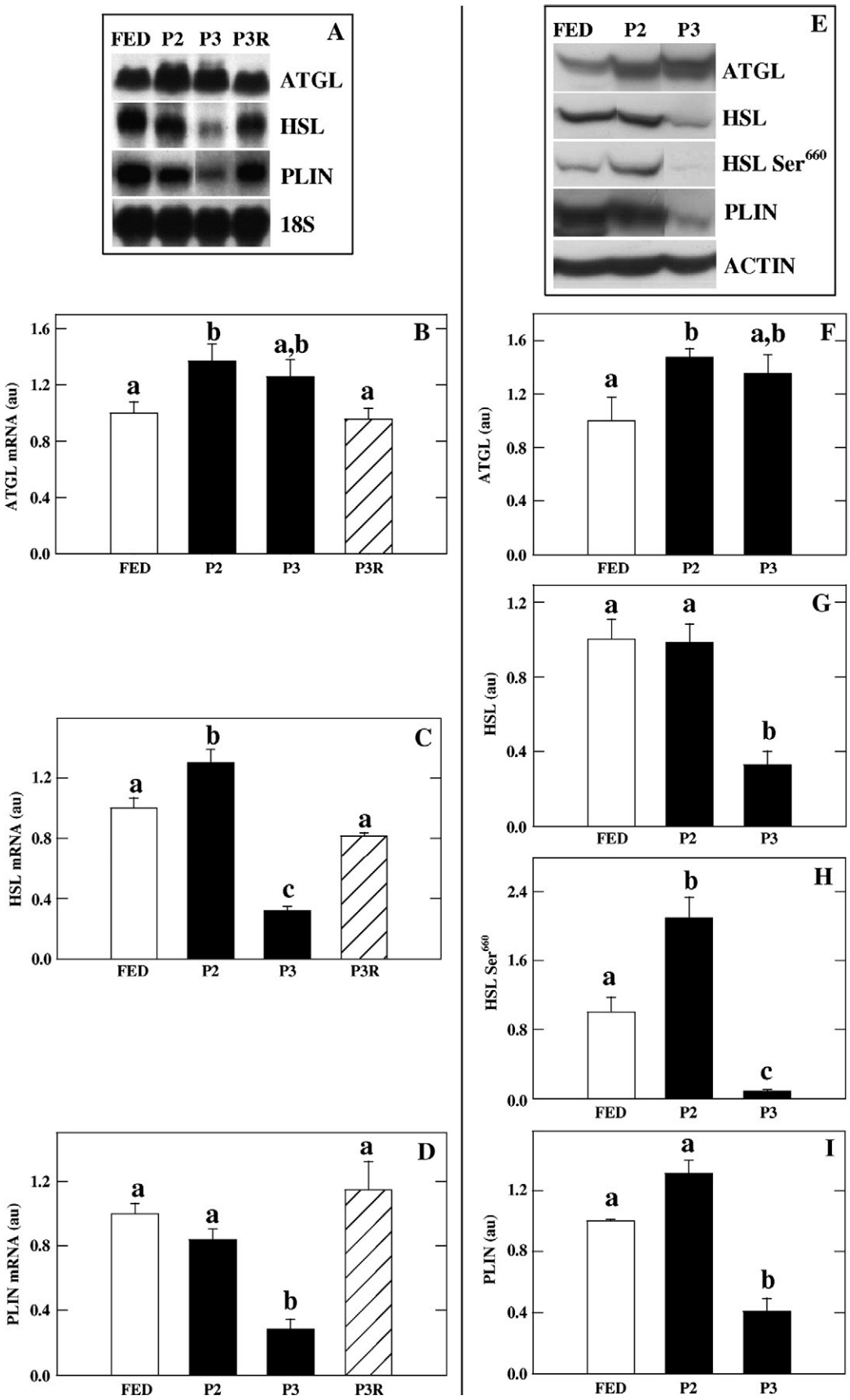

Fig. 2. Representative Northern (A) and Western (E) blots, mRNA and protein levels of ATGL (B and F, respectively), HSL (C and G, respectively), HSL Ser^{660} (H) and PLIN (D and I, respectively) in EPI of fed, fasted and refed rats. Values are means±S.E. (n=6/group). Bars that do not share the same superscript letter are significantly different (P<.05). R, 1 day-refed rats; au, arbitrary unit.

3.3. Relationships between plasma NEFA concentrations and EPI lipase levels

We found a significant correlation between plasma NEFA concentrations and EPI mRNA and protein levels of HSL (Fig. 3A). A significant correlation was also found between plasma NEFA concentrations and EPI protein levels of HSL Ser^{660} (Fig. 3B). However, there was no significant relationship between plasma NEFA concentrations and either ATGL mRNA or protein levels (Fig. 3C). To investigate whether this lack of relationship might be attributable to data originating from a specific group during fasting, we calculated the ratio between ATGL levels (mRNA and/or protein) and plasma NEFA concentrations of fed, P2 and P3 animals. The calculated ATGL/NEFA ratio reached a value of 2–3 in fed and P2, but was closer to 8–9 in P3 rats, indicating that the lack of relationship between ATGL mRNA levels and circulating NEFA levels is mainly explained by data from this latter group. In the same way, we did not find a significant correlation between EPI ATGL and HSL mRNA or protein levels (not shown).

3.4. mRNA levels of genes associated with lipid metabolism in EPI

Table 2 shows variations in mRNA levels of EPI FAS, adiponutrin, β3-AR, ALBP/aP2, peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα in fed, fasted and refed rats. FAS mRNA levels were drastically decreased by fasting (~25–50-fold), while being partially restored after refeeding. Due to a very strong repression, adiponutrin mRNA expression was not detectable during fasting. Refeeding allowed restoration of adiponutrin mRNA levels, which were nonetheless significantly lower (~3.4-fold) in P3R rats, when compared with fed animals. Alterations in β3-AR mRNA levels were also significant during fasting, being ~2.2 times greater in P2 rats and reduced to half in P3 rats, in comparison to fed animals. β3-AR mRNA levels were then augmented by refeeding, being ~3.8 times greater in P3R vs. P3 rats. In comparison with the fed state, ALBP/aP2 mRNA levels were significantly decreased in P3 and P3R rats (~1.2–1.5-fold). During fasting, mRNA concentrations of PPARγ were significantly decreased (~1.5–2.3-fold) and a reverse trend was observed after refeeding. Finally, a significantly lower C/EBPα mRNA expression, in comparison to fed animals, was only observed in P3 rats (~2.5-fold).

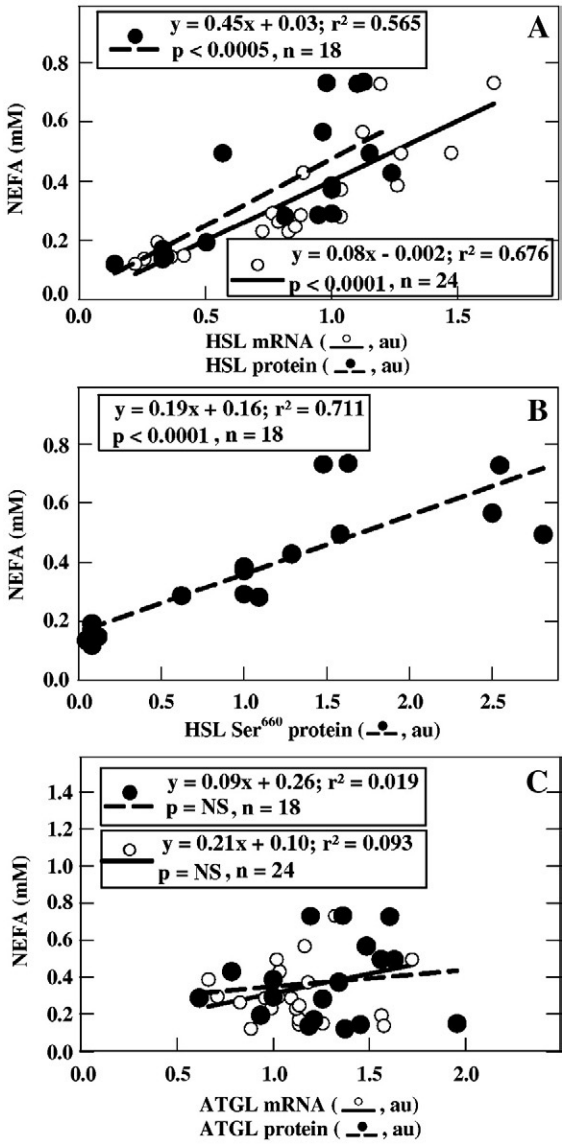

Fig. 3. Relationship between plasma NEFA and mRNA and/or protein levels of HSL (A), HSL Ser^{660} (B) and ATGL (C) in EPI of fed, fasted and refed rats. Values are means±S.E. (n=6/group).

4. Discussion

Several adaptations occur during fasting to maintain energy homeostasis. Mobilization of lipid fuels from adipose tissues is essential during P2 of fasting, while it tends to diminish during P3 with concomitant increase in the use of body proteins as energy fuels [19]. HSL and ATGL are the main lipases involved in TAG catabolism [1]. In addition, the crucial role of lipid droplet-coating proteins, such as PLIN and of other cytosolic adipocyte proteins, for the hydrolysis of TAG stored in fat cells has been well documented [4,25]. In particular, PLIN and CGI-58 (Comparative Gene Identification 58) are known to interact in non-stimulated adipocytes. Phosphorylation of PLIN in adipocytes stimulated by lipolytic hormones releases CGI-58, which binds ATGL to initiate lipolysis. At the same time, phosphorylated HSL translocates to the lipid droplet, associates with phosphorylated PLIN and exerts its lipolytic action.

Although the increase in NEFA flux during short-term fasting appears to involve either post-translational control of HSL or the regulation of other enzymes, HSL activity is also known to be regulated by pre-translational mechanisms during prolonged fasting [26]. Fasting in mice has moreover been reported to induce a transient increase of WAT ATGL mRNA expression, which reaches a maximal level after 12 h before gradually decreasing to basal levels after 48 h [13]. A very short-term (14 h) fast has also been shown to increase ATGL gene expression and protein levels in different WAT locations of rats [18]. This is in line with previous results in HSL-deficient mice, where HSL-independent lipolysis was shown to be markedly increased during fasting and to mediate a normal flux of fatty acids in such situations [27]. It is of note that ATGL mRNA and protein expression have been demonstrated to be highly correlated under several different experimental conditions [28]. In line with these previous results, we found here in rat adipose tissue that HSL, PLIN and ATGL protein levels essentially followed the same pattern as mRNA during prolonged fasting (Fig. 2). We observed selective regulation of adipose lipase expression according to the metabolic status of fasted rats. In particular, EPI ATGL and HSL mRNA as well as ATGL and HSL Ser^{660} expressions were significantly increased during P2, but only HSL mRNA and HSL Ser^{660} and PLIN expressions were lowered during P3. These patterns of expression suggest that pre-translational and post-translational regulation of lipases and lipid droplet-coating proteins may constitute complementary mechanisms that control long term lipid availability. Accordingly, EPI HSL and PLIN regulation paralleled changes in plasma NEFA, especially HSL and HSL

Table 2
mRNA levels of various genes associated with lipid metabolism in EPI of fed, fasted and refed rats

	Fed	P2	P3	P3R
FAS (au)	1.00±0.23[a]	0.04±0.02[b]	0.02±0.02[b]	0.59±0.15[a,b]
Adiponutrin (au)	1.00±0.18[a]	–	–	0.29±0.06[b]
β3-AR (au)	1.00±0.13[a]	2.16±0.27[b]	0.49±0.09[c]	1.91±0.13[b]
ALBP/aP2 (au)	1.00±0.16[a]	1.14±0.10[a]	0.66±0.05[b]	0.77±0.07[b]
PPARγ (au)	1.00±0.06[a]	0.66±0.08[b]	0.43±0.05[b]	0.96±0.09[a]
C/EBPα (au)	1.00±0.11[a]	1.02±0.10[a]	0.39±0.10[b]	0.92±0.22[a]

Values are means±SE (n=6/group). Values are normalized to those of fed animals, which were assigned the arbitrary value of one. Values within a same line that do not share the same superscript letter are significantly different (P<.05). au, arbitrary unit.

Ser^{660} expressions (Fig. 3A and B), which therefore appear to change in accordance with lipid fuel availability. However, this was not the case for ATGL expression (Fig. 3C). This lack of relation was mostly explained by data from animals in P3. The global loss of ATGL function in mice results in a shift in favor of carbohydrate over fat as the primary fuel source during fasting [15]. As carbohydrate reserve exhaustion occurs early during P1 of fasting, it can be suggested that the stimulated expression of ATGL during P2 is consistent with the strong lipid fuel mobilization and utilization at this stage. On the other hand, the phase-specific regulation of HSL mRNA levels and HSL Ser^{660} expression would coincide with activated/repressed lipolysis and increased/decreased plasma NEFA levels (see above) in P2/P3, respectively. During prolonged fasting, adipocytes essentially shrink and elongate with numerous irregular cytoplasmic projections, and they still exhibit unilocular but slimmed lipid droplets [29,30]. The resulting decrease in the overall droplet surface would thus be in line with a reduced lipolytic potential in P3, associated with the down-regulation of HSL expression reported here at this stage of fasting. In addition, it has already been reported that after protracted fasting, visceral fat pads of mice are completely delipidised [29]. In the current study, about 15% of the initial lipid content is still present in P3 animals (see Table 1). We thus hypothesize that the reduced lipid mobilization at this stage is not due to lipid reserves exhaustion but is a combination of the reduced access (diminished exchange surface) and hydrolysis (diminished expression and activity of HSL) of the remaining TAG stores. Our results thus provide a molecular basis that is consistent with the higher mobilization of lipid fuels during P2 vs. P3. An essential role for both muscle ATGL and HSL in maintaining adequate NEFA supply to sustain normal substrate metabolism at rest and during exercise has recently been reported [31]. Here we show that the rise in locomotor activity during prolonged food deprivation, especially in P3, occurs at the same time EPI HSL expression is decreased while ATGL expression is maintained. This would argue for the essential role of ATGL, but not HSL, possibly to maintain a low mobilization rate of NEFA for muscle performance in late fasting.

The relative contribution of the two adipose lipases to the overall lipolytic process remains to be clarified. Whether ATGL is involved in basal lipolysis only or also in stimulated lipolysis is still debated in humans, where ATGL appears to be of lesser importance than HSL for controlling lipolysis [32]. In ATGL-deficient mice, a reduced isoproterenol-stimulated lipolysis has been reported, despite unchanged basal release of fatty acids and glycerol [15]. Moreover, both basal and catecholamine-induced lipolysis in 3T3-L1 adipocytes are stimulated by adenoviral and retroviral-mediated ATGL overexpression [33], while being consistently reduced by inhibition via antisense technologies [12,33]. In rodents, the current view is that adipose lipases may work hierarchically, with ATGL initiating lipolysis by hydrolysis of TAG, thus producing diacylglycerols, which would then be hydrolyzed by HSL [4]. In that sense, the down-regulation of HSL and HSL Ser^{660}, but not of ATGL expression reported here in late fasting would contribute to induce diacylglycerols accumulation within fat cells. Knowing that some lipases (such as HSL) can be inhibited by intermediary lipid metabolites [34], this could lead to a progressive inactivation of lipolysis, and thus insufficient production of NEFA to satisfy whole energy expenditure. This would consequently favor protein breakdown to sustain energy requirements in P3.

In accordance with previous studies [35,36], adiponutrin, which is a gene closely related to ATGL, with a high degree of sequence homology but differences in function, exhibited the inverse pattern of expression. Indeed, adiponutrin was dramatically down-regulated during fasting and restored by refeeding (Table 2). Thus, although they are both members of the adiponutrin family, ATGL and adiponutrin do not share the same functional nor nutritional regulation. HSL and PLIN mRNA levels are known to be more or less regulated in a coordinated manner in WAT during short-term fasting [17]. This coordinated regulation is in agreement with previous data showing that PLIN is a lipid droplet-associated protein that can modulate HSL activity and ATGL-dependent lipolysis [37,38]. We report here that EPI HSL and HSL Ser^{660} mRNA and/or protein expressions are also regulated in a coordinated fashion with PLIN for longer durations of fasting characterized by changes in body fuel availability, while this is not the case for ATGL. ALBP/aP2 is an intracellular lipid-binding protein that could play a critical role during lipolysis by interacting with HSL [39]. However, compared to HSL, its gene expression was less affected here by the fasting/refeeding manipulation.

Lipolysis is under tight regulation by hormones, especially the lipolytic catecholamines and anti-lipolytic insulin (reviewed in [40]). Insulin has notably been reported to negatively regulate HSL activity and ATGL mRNA expression [33,41]. The drop in insulinemia upon food deprivation could thus favour the increased levels of ATGL during fasting (Fig. 2). The phosphorylation state of HSL is of major importance in regulating its activity [4]. The negative regulation of HSL activity by insulin was presumably imputed to the hormone's actions on the phosphorylation state of the enzyme [41]. Accordingly, the drop in insulinemia during P2 could favour the phosphorylation-induced activation of the enzyme, which is corroborated by the increased levels of HSL Ser^{660} at this stage of fasting (Fig. 2H). However, the drop in HSL Ser^{660} levels during P3 remains unexplained and may involve other regulating factors. In addition, possible insulin-induced pretranslational alterations in HSL (i.e., decreased mRNA levels) have been putatively suggested to require more than 2 days [41]. The drop in insulinemia could thus also be involved in the increased HSL mRNA levels during P2, but fails to explain the drop in HSL mRNA expression during P3 (Fig. 2C). Altogether, these results suggest that lower insulin levels would play a role in the nutritional regulation of ATGL during both P2 and P3 and of HSL only in P2. In rodents, catecholamines action essentially depends on their effects on the β3-AR. We found that β3-AR mRNA expression was in line with lipid reserve mobilization (Tables 1 and 2). Following the example of HSL (see above), β3-AR mRNA expression was indeed well correlated to plasma NEFA levels (r^2=0.501; P<.0006; y=2.79x+0.41). This would be in line with the control of lipolysis through the cAMP pathway via catecholamine's action on β-adrenergic receptors. Although visceral WAT β3-AR mRNA levels are commonly regarded as being modulated by insulin in such circumstances [17,42], the fact that insulinemia did not parallel EPI β3-AR mRNA level variations in P3 do not support this view in late fasting. ATGL expression is also positively regulated by glucocorticoids [13]. As circulating corticosterone gradually increased to high levels during prolonged fasting and decreased during refeeding (Table 1), the glucocorticoid inducing effects on ATGL expression might partially explain its elevated levels during P2 and P3.

Knowing that FAS gene expression and activity have been shown to be closely related [43], the drastic reduction (near extinction) of FAS mRNA levels during P2 and P3 of fasting supports the view that fatty acid synthesis is greatly inhibited in rat adipose tissue in such situations. This could be driven by the decreased plasma glucose and

insulin concentrations during food deprivation. FAS gene expression is indeed known to be primarily regulated by these factors [44]. Rat perirenal and EPI adipocytes have been reported to actively oxidize endogenous fatty acids [45,46]. However, although the net amount of fatty acids disposed through the oxidative pathway has been expected to be significant and increased with fasting, the percentage of endogenous fatty acids oxidized (0.2%) might be small compared with those partitioned in other pathways (49.7% of endogenous fatty acids re-esterified and 50.1% released) [46]. Even augmented, the contribution of the oxidation pathway to the metabolic fate of fatty acids within adipose tissue is then expected to remain at a low level during P2 and P3 of fasting. PPARγ and C/EBPα are transcription factors predominantly involved in adipogenesis [47]. PPARγ has also been reported to increase in vivo WAT lipolysis, regardless of tissue location, notably by directly and positively regulating ATGL mRNA and protein expression in mature adipocytes in vitro and in murine WAT in vivo, suggesting a role for ATGL in mediating PPARγ's effects on lipid metabolism [28,48,49]. Here we show that mRNA levels of PPARγ and C/EBPα are not closely related to ATGL gene expression in WAT. Indeed, variations in mRNA concentrations of these transcription factors did not parallel those of ATGL in our study. PPARγ is a ligand-activated nuclear transcription factor, which binds to specific DNA response elements as heterodimers with RXR (retinoid X receptor). Interaction of ligand with PPARγ is indeed required for the induction of ATGL [50]. In order to determine whether the increase of ATGL (and HSL) gene expression observed here in P2 is mediated by PPARγ activation, future studies should investigate PPARγ heterodimerization with RXR during prolonged fasting.

In conclusion, our results showed that ATGL and HSL are not coordinately regulated during long-term food deprivation. ATGL appears as the major lipase in adipose tissue of rats in P3 of fasting, whilst HSL, HSL Ser^{660} and ATGL expressions seem essential in P2. This may represent a new mechanism that contributes to the control of lipolysis in relation to changes in fuel partitioning during prolonged fasting. Powerful tools from the field of analytical chemistry have already successfully allowed a number of proteins associated to lipid metabolism to be described [51]. Indeed, proteomic strategies can help gaining insights into metabolic disorders according to body reserves availability [52]. In future studies, such approaches would be useful to bring key functional information on the adipose proteome and, in particular, regarding residue-specific post-translational modifications (e.g., phosphorylation) of lipolytic enzymes and/or regulatory factors according to changes in animal metabolic status after nutritional manipulations.

Acknowledgments

The authors are grateful to S. Zahn and C. Husser for skilled technical assistance.

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