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

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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.

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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