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RESEARCH ARTICLES

Adipose triglyceride lipase expression and fasting regulation are differently affected by cold exposure in adipose tissues of lean and obese Zucker rats☆,☆☆

Antoni Caimari[a,b], Paula Oliver[a], Andreu Palou[a,*]

[a]Laboratory of Molecular Biology, Nutrition and Biotechnology, Universitat de les Illes Balears, and CIBER de Fisiopatología de la Obesidad y Nutrición, E-07122 Palma de Mallorca, Spain

[b]Centre Tecnològic de Nutrició i Salut (CTNS), TECNIO, CEICS, E-43204 Reus, Spain

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Abstract

Adipose triglyceride lipase (ATGL) hydrolyzes triacylglycerols to diacylglycerols in the first step of lipolysis, providing substrates for hormone-sensitive lipase (HSL). Here we studied whether ATGL messenger RNA (mRNA) and protein levels were affected by 24-h cold exposure in different white adipose tissue depots and in interscapular brown adipose tissue of lean and obese Zucker rats submitted to feeding and 14-h fasting conditions. HSL mRNA expression was also studied in selected depots. In both lean and obese rats, as a general trend, cold exposure increased ATGL mRNA and protein levels in the different adipose depots, except in the brown adipose tissue of lean animals, where a decrease was observed. In lean rats, cold exposure strongly improved fasting up-regulation of ATGL expression in all the adipose depots. Moreover, in response to fasting, in cold-exposed lean rats, there was a stronger positive correlation between circulating nonesterified fatty acids (NEFA) and ATGL mRNA levels in the adipose depots and a higher percentage increase of circulating NEFA in comparison with control animals not exposed to cold. In obese rats, fasting-induced up-regulation of ATGL was impaired and was not improved by cold. The effects of obesity and cold exposure on HSL mRNA expression were similar to those observed for ATGL, suggesting common regulatory mechanisms for both proteins. Thus, cold exposure increases ATGL expression and improves its fasting-up-regulation in adipose tissue of lean rats. In obese rats, cold exposure also increases ATGL expression but fails to improve its regulation by fasting, which could contribute to the increased difficulty for mobilizing lipids in these animals.

Keywords: ATGL; Cold exposure; Lipolysis; Adipose tissue; Fasting

1. Introduction

Fatty acids deposited as triacylglycerols in adipose tissue represent the primary energy store in animals. In periods of increased energy demand, such as fasting and cold exposure, stored fat is mobilized by lipolytic enzymes, which hydrolyze adipose triacylglycerols and release nonesterified fatty acids (NEFA) into the circulation. Adipose triglyceride lipase (ATGL) [1], also known as desnutrin [2] and calcium-independent phospholipase-A2ζ [3], is a 486-amino-acid protein that is now considered to be the main triacylglycerol lipase in adipose tissue. It hydrolyzes triacylglycerols to diacylglycerols in the first step of lipid hydrolysis, providing the substrate for hormone-sensitive lipase (HSL) in the lipolytic cascade [1,2]. The molecular mechanism by which ATGL activity is regulated is not completely understood. Differently to what has been observed for HSL, ATGL activity is not regulated directly by protein kinase A (PKA) phosphorylation. However, preliminary evidence in mammalian cells indicates that ATGL is phosphorylated by kinases (reviewed in Refs. [4,5]), and recent experiments performed by Deiuliis et al. [6] suggest that ATGL is an indirect target of PKA signaling in brown adipocytes. Also recently, Duncan et al. [7] demonstrated that although the loss of phosphorylation of two conserved C-terminal serine residues (S406 and S430) does not alter cellular localization or triacylglycerol hydrolysis by ATGL, the C-terminal region of this protein is required for triacylglycerol breakdown in live cells. Moreover, the same authors identified two critical sites in ATGL N-terminal region, S47 and D166, which are necessary for triacylglycerol hydrolysis in live cells [7]. In addition to the possible regulation of ATGL activity by phosphorylation, its lipolytic activity is strongly stimulated by an activator, comparative gene identification 58 (CGI-58) [8], and is highly dependent on the phosphorylation of perilipin A [9], which coordinates the recruitment of proteins to the lipid droplet, thus allowing the lipolytic process.

The importance of ATGL in the lipolytic process has been demonstrated in several *in vitro* experiments [1,2,10,11], and Haemmerle et al. [12] confirmed its important role *in vivo*, showing that ATGL-

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* Corresponding author. Tel.: +34 971173170; fax: +34 971173426.
E-mail address: andreu.palou@uib.es (A. Palou).

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knockout mice presented increased fat storage in the form of triacylglycerols in different tissues, weight gain and greater use of carbohydrate instead of fat as a primary fuel source during fasting. Recently, Ahmadian et al. [13] corroborated the key lipolytic role of ATGL in adipose tissue, showing that transgenic mice overexpressing ATGL specifically in white adipose tissue (WAT) displayed elevated lipolysis and increased fatty acid oxidation, resulting in higher energy expenditure and resistance to diet-induced obesity.

ATGL messenger RNA (mRNA) expression is markedly up-regulated during 3T3-L1 *in vitro* differentiation [1–3] in a process regulated by PPARγ [14], and in accordance with a protein involved in lipolysis, its expression levels are regulated by the nutritional status, being induced after fasting and decreased by refeeding [2,15]. Related with changes in nutritional status, *in vitro* experiments have demonstrated that ATGL expression is up-regulated by glucocorticoids [2] and down-regulated by insulin [10], probably through FoxO1 [16], in a dose-dependent manner, suggesting that ATGL has an important hormonal regulation. We recently demonstrated that fasting up-regulation of ATGL expression in WAT is impaired in old rats, which could contribute to explain the difficulty for mobilizing lipids when exposed to nutritional stress such as fasting and, hence, the weight gain that occurs with aging [17]. Furthermore, ATGL is down-regulated in the gonadal adipose tissue in animal models of obesity and diabetes mellitus (*ob*/*ob* and *db*/*db* mice) [2] and in obese humans with insulin resistance [18], indicating that this protein could also be related to obesity; however, there are no data on how obesity affects the regulation of ATGL by fasting.

Cold stimulus, in a process mediated by catecholamine release, produces extensive mechanisms of adaptation in adipose tissue, including an increase in lipolysis. Some *in vivo* and *in vitro* experiments in rodents indicate an important role of ATGL in lipid mobilization under cold exposure or catecholamine stimulation. Thus, Haemmerle et al. [12] showed that upon fasting, ATGL-deficient mice were extremely cold sensitive, reducing their oxygen consumption and dropping their body temperature during 4°C cold exposure. The same group demonstrated that inactivation of ATGL gene function in mice resulted in a drastic decrease (70%) of free fatty acid release from WAT in response to the β1 and β2 adrenoceptor agonist isoproterenol [12]. Moreover, Kershaw et al. [10] demonstrated that overexpression of ATGL in 3T3-L1 adipocytes increased basal and isoproterenol-stimulated glycerol and NEFA release, whereas small interfering RNA-mediated knockdown of ATGL had the opposite effect. In 2010, Deiuliis et al. [6] showed that the β3 adrenoceptor agonist CL 316,243 down-regulated ATGL gene and protein expression in brown adipocytes and that cold exposure at an early time point decreased ATGL mRNA and protein levels in brown adipose tissue of mice. However, up to now, there are no data available comparing the *in vivo* regulation of ATGL in response to cold exposure and fasting in different adipose depots, which could provide extra information concerning the role of this protein in lipolysis.

The key role of ATGL in lipid mobilization, its well-known nutritional regulation and the link between cold exposure and lipolysis, prompted us to analyze the effect of 24-h cold exposure to 4°C on mRNA and protein basal levels of ATGL and how this stimulus could affect ATGL nutritional regulation (response to fasting) in different adipose tissues. For these purposes, lean and obese Zucker rats — which display genetic obesity due to deficiencies in leptin receptor — were used.

2. Methods and materials

2.1. Animals

The animal protocol followed in this study was reviewed and approved by the Bioethical Committee of our university, and guidelines for the use and care of laboratory animals of the university were followed.

Three-month-old male Zucker rats, both lean (−/?) and obese (*fa*/*fa*) (Charles River Laboratories España SA, Barcelona, Spain), fed with a standard chow diet (Panlab, Barcelona, Spain) were used. They were single housed at 22°C with a 12-h period of light/dark (lights on at 8:00 a.m.) and with free access to food and water. After a 7-day adaptation period, rats were distributed in two experimental groups for 24 h: control group, with animals acclimatized to 22°C, and cold group, with animals exposed to 4°C for 24 h. Animals in the control and cold groups were divided into two groups and submitted to different feeding conditions (n=5 rats for each condition): a control-fed group, animals provided with *ad libitum* access to chow diet, and a fasted group, animals deprived of food overnight (for 14 h). In the fed groups, food intake was recorded during the 24 h of the experimental period, and in the fasted groups, coprophagy was prevented by changing the cage immediately prior to food deprivation.

Different WAT depots — retroperitoneal (RWAT), mesenteric (MWAT), epididymal (EWAT) and inguinal (IWAT) — as well as the interscapular brown adipose tissue (IBAT) were rapidly removed after death, weighed, frozen in liquid nitrogen and stored at −70°C until RNA analysis. Blood was also collected, stored at room temperature for 1 h and overnight at 4°C, and then centrifuged at 1000*g* for 10 min to collect the serum.

2.2. Adiposity index

Adiposity was determined by an adiposity index computed for each rat as the sum of EWAT, IWAT, MWAT and RWAT depot weights and expressed as a percentage of total body weight.

2.3. RNA extraction

Total RNA from the different WAT depots studied (EWAT, IWAT, MWAT and RWAT) and from the IBAT depot was extracted by Tripure Reagent (Roche Diagnostic Gmbh, Mannheim, Germany) according to the manufacturer's instructions. RNA yield was quantified in a Nanodrop ND 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the integrity of the RNA was confirmed using agarose gel electrophoresis.

2.4. Real-time quantitative reverse transcriptase polymerase chain reaction (Q-PCR) analysis

Q-PCR was used to measure mRNA expression levels of ATGL in different WAT depots (EWAT, IWAT, MWAT and RWAT) and in IBAT of Zucker rats. HSL, CGI-58 and lipoprotein lipase (LPL) mRNA expression levels were also studied in selected adipose depots.

Q-PCR was performed as previously described [19]. In brief, 0.5 μg of total RNA was denatured and then reverse transcribed to complementary DNA (cDNA) using MuLV reverse transcriptase (according to Applied Biosystem's procedure) in a Perkin Elmer 9700 Thermal Cycler (Norwalk, CT, USA). For Q-PCR, the LightCycler System with SYBR Green I (Roche Diagnostic, Barcelona, Spain) was used. All Q-PCRs were made from diluted (1:40) cDNA template and performed with the following cycling conditions: after an initial Taq activation at 95°C for 10 min, Q-PCR was performed using 40–45 cycles and the following parameters: 95°C for 2 s, 60°C for 6 s and 72°C for 12 s for ATGL; 95°C for 2 s, 60°C for 6 s and 72°C for 7 s for HSL; 95°C for 2 s, 60°C for 6 s and 72°C for 8 s for CGI-58; 95°C for 2 s, 60°C for 7 s and 72°C for 8 s for LPL and 95°C for 2 s, 60°C for 6 s and 72°C for 8 s for LRP10 (used as housekeeping gene). LRP10 was chosen as housekeeping gene because it has been demonstrated as a suitable reference gene for expression studies in adipose tissue [20]. In order to verify the purity of the products, a melting curve was produced after each run by increasing the temperature of the reaction mixtures up to 95°C, by 0.1°C/s, starting at 55°C for 10 s. Values for the threshold (Ct) were determined using the LightCycler software. Primers were as follows: for ATGL, f5′-TGTGGCCTCATTCCTCCTAC-3′ and r5′-AGCCCTGTTTGCACATCTCT-3′; for HSL, f5′-TCACGCTACATAAAGGCTGCT-3′ and r5′-CCACCCGTAAAGAGGGAACT-3′; for CGI-58, f5′-CTACCTGGTGTCCCACGTCT-3′ and r5′-CAAGACCTCCTCCAAAACCA-3′; for LPL, f5′-TATGGCACAGTGGCTGAAAG-3′ and r5′-CTGACCAGCGGAAGTAGGAG-3′ and for LRP10, f5′-TCCCCTTTCTTCTCCTCCTC-3′ and r5′-TTACCGTCTGTTCCTTGCTG-3. Primers were obtained from Sigma Genosys (Sigma Aldrich Química SA, Madrid, Spain). Relative gene expression numbers were calculated as a percentage of control rats, using the $2^{-\Delta\Delta Ct}$ method [21] with the LRP10 as reference gene.

2.5. Quantification of circulating insulin, leptin, glucose and NEFA

Serum insulin and leptin levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (from DRG Instruments, Marburg, Germany, and R&D Systems, Minneapolis, MN, USA, respectively), and blood glucose was measured using an Accu-Chek Glucometer (Roche Diagnostics, Barcelona, Spain). The NEFA levels were measured in serum using an enzymatic colorimetric NEFA C kit (from WAKO, Neuss, Germany).

2.6. Homeostatic model assessment for insulin resistance (HOMA-IR) analysis

Insulin resistance was assessed by the HOMA-IR in rats submitted to overnight (14 h) fasting (n=5 for all groups). HOMA-IR score was calculated from fasting insulin and glucose concentrations using the formula of Matthews et al. [22], as follows: HOMA-IR=fasting glucose (mmol/L)×fasting insulin (mU/L)/22.5.

2.7. Total lipid content extraction and quantification

Lipid extraction was performed in the different adipose tissues of lean animals using the methods described by Hara and Radin [23] and Rodríguez-Sureda and Peinado-Onsurbe [24] with modifications. Briefly, 20–25 mg of different WAT depots (EWAT, IWAT, MWAT and RWAT) and 30–35 mg of IBAT were mixed with 1 ml of hexane/isopropanol (3:2, vol/vol). The tubes with the samples were gassed with nitrogen before being closed to minimize lipid oxidation and then left overnight under orbital agitation at room temperature protected from light. The content of each tube was transferred into a new one, and 0.3 ml of Na_2SO_4 (0.47 M) was added. Tubes were mixed for 5 min, left for 15 min in orbital agitation and centrifuged at 1000g for 10 min at 4°C. The upper phase containing lipids was dissolved in hexane and transferred to a clean, previously weighed glass tube. The hexane extract was then dried with nitrogen gas. Once the tube was dried, the percentage of lipids was determined as the weight difference between tubes with lipids extract and clean tubes, taking into account the initial amount of tissue present.

2.8. Triacylglycerol determination

To evaluate triacylglycerol content, the lipid extracts (obtained as commented above) were dissolved in LPL buffer (28.75 mM Pipes, 57.41 mM $MgCl_2.6H_20$, 0.569 mg/ml bovine serum albumin-fatty acid free (BSA-FFA free)) with sodium dodecyl sulfate 0.1%, using the method described by Rodríguez-Sureda and Peinado-Onsurbe [24]. WAT samples were resuspended in 6 ml and IBAT samples in 4 ml of LPL buffer. Then, samples were sonicated for 30 s, and tubes were left overnight in an orbital shaker at room temperature and protected from light. On the following day, the tubes were cold sonicated with three pulses of 30 s each, and their triacylglycerol levels were measured immediately using the Serum Triglyceride Determination Kit (Sigma Aldrich).

2.9. Western blot analysis of ATGL

Immunoblot analyses of ATGL (antibody anti-ATGL from Cayman Chemical, Ann Arbor, MI, USA) in IBAT and in IWAT and MWAT depots of fed and fasted control and cold-exposed Zucker rats were performed as previously described [17]. The signal for ATGL protein was normalized to the signal of the black amide B10 staining, and the results were expressed as the ATGL/black amide B10 ratio.

2.10. Statistical analysis

All data are expressed as the mean±S.E.M. Differences between groups were analyzed using two- or one-way analysis of variance (ANOVA) or Student's t test. The test used for each comparison is specified in the footnotes of the tables and figures. Linear relationships between key variables were tested using Pearson correlation coefficients. The analyses were performed with SPSS for windows (SPSS, Chicago, IL). Threshold of significance was defined at P<.05 and is indicated when different.

3. Results

3.1. Body weight, adiposity and circulating parameters: effect of obesity, cold exposure and fasting

Obese Zucker rats presented 22% greater body weight and 260% higher adiposity index both in the control and the cold groups; food intake was also higher in obese Zucker (28%) at the two temperatures studied (22°C and 4°C; Student's t test, P<.05; Table 1). Cold exposure for 24 h did not significantly affect body weight or adiposity of the studied animals, either in lean or in obese rats; however, in lean animals, cold produced a significant decrease in the MWAT (one-way ANOVA, P<.05), a depot that has been previously shown to be especially responsive to stimuli such as food intake [25] (Table 1). As a result of cold exposure, there was a reduction in food intake, approximately 38%, in both lean and obese rats (Student's t test, P<.05; Table 1).

As shown in Table 1, according to their higher body weight and adiposity, obese Zucker rats of both the control and the cold groups were hyperleptinemic and hyperinsulinemic and presented a higher HOMA-IR index (one-way ANOVA, P<.05). It has been described that animals exposed to cold present low levels of circulating leptin [26–28] and insulin [28,29]. In our animals, as a result of cold exposure, circulating leptin levels, but particularly insulin levels, decreased in obese rats (1.3- and 3.3-fold, respectively; Student's t test, P<.05), while the decrease in lean animals was not significant (P=.145, Student's t test, for leptin).

Cold exposure induced a decrease in NEFA levels; however, as a consequence of 14-h fasting, there was an increase in circulating NEFA in lean and obese rats, both in animals housed at 22°C and in those housed at 4°C (Table 2). In lean animals, the percentage increase in circulating NEFA levels in response to fasting was higher in cold-exposed than in control animals (119% vs. 50.4%; Table 3).

3.2. Effect of obesity on ATGL and HSL mRNA expression levels and on ATGL protein levels

ATGL mRNA expression in lean Zucker rats was higher in IBAT than in the different WAT depots studied (one-way ANOVA, P<.05; Fig. 1A). In obese Zucker rats, ATGL mRNA levels were decreased in comparison to lean rats in IBAT and in all the WAT depots, with the exception of IWAT (Student's t test, P<.05; Fig. 1A), although this was not translated into a decrease in protein levels (data not shown). The mRNA expression pattern of HSL in the different adipose tissue depots

Table 1
Biometric parameters, food intake, serum parameters and HOMA-IR in control and cold-exposed lean and obese Zucker rats

	Control group		Cold group		
	Zucker lean	Zucker obese	Zucker lean	Zucker obese	
Body weight (g)	328±9	404±17[a]	321±10	391±16[a]	O
Adiposity index (%)	2.52±0.22	8.69±0.13[a]	2.31±0.13	8.68±0.22[a]	O
EWAT (g)	1.75±0.21	6.61±0.74[a]	1.55±0.19	5.24±0.37	O
IWAT (g)	3.77±0.42	21.4±0.7[a]	3.29±0.19	19.8±1.2	O
MWAT (g)	1.20±0.12	2.95±0.27[a]	0.85±0.07[b]	3.83±0.26[b]	OxT
RWAT (g)	1.64±0.14	4.04±0.26[a]	1.74±0.14	5.27±0.55	O
IBAT (g)	0.39±0.02	0.60±0.06	0.49±0.05	0.57±0.08	O
Food intake (g)	22.2±1.0	30.5±0.8[a]	13.6±0.9[b]	19.1±0.8[a,b]	O, T
Insulin (μg/L)	0.79±0.11	19.2±4.9[a]	0.68±0.21	5.81±1.58[a,b]	OxT
Leptin (μg/L)	3.47±0.24	41.1±2.9[a]	2.63±0.46[a]	31.7±2.0[b]	OxT
HOMA-IR (%)	1.83±0.21	21.2±3.3[a]	1.21±0.30	16.4±2.8[a]	O

The adiposity index was computed as the sum of EWAT, IWAT, MWAT and RWAT depot weights and expressed as a percentage of total body weight. Food intake was recorded in fed groups during the 24 h of the experimental period. HOMA-IR was computed using the formula of Matthews et al. [22]. Insulin and leptin levels were measured by ELISA. Results represent mean±S.E.M. (n=5). O, effect of body weight; T, effect of temperature; OxT, interaction of body weight and temperature (two-way ANOVA, P<.05) considering lean and obese animals of the control and the cold group.

[a] Obese vs. lean animals of the same group (Student's t test, P<.05).

[b] Lean/obese rats of the cold group vs. lean/obese rats of the control group (Student's t test, P<.05).

Table 2
Circulating NEFA levels in *ad-libitum*-fed and 14-h-fasted control and cold-exposed lean and obese Zucker rats

	Zucker lean		Zucker obese	
	Fed	Fasted	Fed	Fasted
Control group				
Serum NEFA (mM)	0.82±0.06	1.23±0.09[a]	1.36±0.02[b]	2.21±0.13[a,b]
Cold group				
Serum NEFA (mM)	0.40±0.08[c]	0.88±0.07[a]	0.97±0.10[b,c]	2.01±0.34[a,b]

Circulating NEFA levels in *ad-libitum*-fed and 14-h-fasted lean and obese Zucker rats housed at 22°C (control group) or at 4°C for 24 h (cold group) were measured in serum using an enzymatic colorimetric kit. Results represent means±S.E.M. (n=5).

[a] Effect of fasting: lean/obese fasted rats vs. lean/obese fed rats of the same group (Student's t test, P<.05).

[b] Effect of obesity: obese fed/fasted rats vs. lean fed/fasted rats of the same group (Student's t test, P<.05).

[c] Effect of cold exposure: lean/obese fed rats of the cold group vs. lean/obese fed rats of the control group (Student's t test, P<.05).

studied and the effect of obesity on HSL expression were similar to those observed for ATGL (Fig. 1B).

3.3. Effect of cold exposure and fasting on ATGL mRNA expression and protein levels in lean and obese Zucker rats

3.3.1. Lean rats

In lean rats, cold stimulus affected ATGL mRNA and protein levels, but the effect was dependent on the adipose depot. In both fed and fasted animals, ATGL mRNA expression was inhibited as a result of cold exposure in IBAT but was increased in EWAT and in RWAT depots (two-way ANOVA, P<.05; Fig. 2A). The inhibitory effect of cold exposure on ATGL mRNA levels in the IBAT was confirmed in the fed group by studying the protein levels, although they did not reach statistical significance (Student's t test, P=.066; Fig. 2B). In the IWAT depot, cold exposure only increased ATGL mRNA expression in fasted animals (Student's t test, P<.05), and the same pattern was found when ATGL protein levels were assessed (Fig. 2A and B). No effect on ATGL mRNA expression in response to cold exposure was found on the MWAT (Fig. 2A); however, an important increase was observed in the MWAT depot when considering protein levels, both in fed and in fasted animals (two-way ANOVA, P<.05; Fig. 2B).

As a result of 14-h fasting, ATGL mRNA expression was significantly increased in IBAT, EWAT, MWAT and RWAT depots when both control and cold-exposed animals were considered (two-way ANOVA, P<.05; Fig. 2A). However, this increase in response to fasting was more evident in cold-exposed than in control animals in most of these adipose depots [IBAT: control (percentage increase: 20.4%), cold (percentage increase: 67.5%); MWAT: control (51.5%), cold (89.4%); RWAT: control (39.9%), cold (46.9%)], with the exception of EWAT, in which a higher percentage increase in ATGL mRNA levels in response to fasting was found in control (61%) compared to cold-exposed animals (47.4%). In the IWAT depot, ATGL mRNA expression increased in response to fasting only in cold-exposed animals (Student's t test, P<.05), in which a percentage increase of 139.4% was found, compared to the cold-fed animals (Fig. 2A). Consequently, when the effect of fasting was analyzed considering the mean percentage increase of ATGL mRNA expression obtained from each adipose tissue depot studied, a greater increase was observed in cold-exposed lean animals in comparison with the controls (Table 3). Coincident with mRNA levels, ATGL protein levels significantly increased in response to fasting in MWAT depot in both control and cold-exposed animals, although the effect was more evident in cold-exposed than in control animals (two-way ANOVA, P<.05; Fig. 2B). In control animals, ATGL protein levels did not increase either in the IBAT depot or in the IWAT depot as a result of fasting. However, as also happened with mRNA levels, in cold-exposed rats, ATGL response to fasting was improved at the protein level in both tissues, although the increase observed as a result of 14-h fasting did not reach statistical significance either in IBAT or in IWAT (P=.1 and P=.076, respectively; Student's t test).

3.3.2. Obese rats

In obese Zucker rats, as a general trend, cold exposure increased ATGL mRNA and/or protein levels in the different adipose tissue depots studied, even in IBAT (Fig. 3A and B). The different regulatory pattern observed in response to cold exposure in IBAT between lean and obese Zucker rats was confirmed by studying the protein levels (Figs. 2B and 3B).

Fasting response was partially impaired in obese animals, as ATGL mRNA levels only increased in EWAT and MWAT of both control and cold-exposed animals (two-way ANOVA, P<.05) and in IBAT of control animals (Student's t test, P<.05; Fig. 3A). Moreover, contrary to what was observed in lean animals, regulation by fasting did not improve as a result of cold exposure in any of the adipose depots, with the exception of EWAT [control (percentage increase: 44.8%), cold (percentage increase: 70%)] (Fig. 3A). ATGL protein levels were affected by fasting in IBAT and MWAT depots of both control and cold-exposed animals (two-way ANOVA, P<.05). It is worth noting that, differently to what was observed for mRNA levels, ATGL response to fasting improved in response to cold exposure in MWAT of obese Zucker rats [control (percentage increase: 62.6.%), cold (percentage increase: 115.2%)]. No changes were found in ATGL protein levels of IWAT in response to fasting either in control or in cold-exposed obese animals (Fig. 3B).

3.4. Effect of cold exposure and fasting on HSL mRNA expression in lean and obese Zucker rats

The effect of cold exposure on HSL expression was very similar to that observed for ATGL in both lean and obese rats (Fig. 4 and B). It is worth noting that, in lean rats, the effect of cold exposure on HSL mRNA levels was also dependent on the adipose tissue depot studied and, as observed for ATGL, decreased in IBAT, while it increased in EWAT but was not affected in MWAT (two-way ANOVA, P<.05; Fig. 4A).

When considering HSL mRNA expression, as happened for ATGL, 24-h cold exposure also improved the response to fasting in lean animals (Fig. 4A). Thus, the fasting response increase in HSL mRNA

Table 3
Percentage changes (increase or decrease) in ATGL and HSL mRNA expression, in circulating NEFA and in adipose tissue lipid and triacylglycerol content in response to 14-h fasting in lean Zucker rats housed at 22°C (control group) or at 4°C for 24 h (cold group)

Fasted/fed conditions	Control group	Cold group	P value
▲ ATGL mRNA expression (%)	36.4±9.6	78.1±17.2	.067
▲ HSL mRNA expression (%)	11.0±8.4	64.1±14.3*	.033
▲ Circulating NEFA (%)	50.4	119	
▼ Lipid content (%)	5.45±0.57	9.30±1.64	.078
▼ TG content (%)	6.90±3.09	11.7±6.4	>.1

▲, percentage increase; ▼, percentage decrease. ATGL and HSL mRNA expression (%) represents the mean±S.E.M. of the percentage change of ATGL and HSL expression in response to fasting taking into account all the adipose depots studied in control and cold-exposed lean Zucker rats (n=5 depots for ATGL; n=3 depots for HSL, in each group of animals). Circulating NEFA (%) represents the percentage change of circulating NEFA levels in response to fasting obtained in control and cold-exposed lean Zucker rats. Lipid content (%) and TG content (%) represent the mean±S.E.M. of the percentage change of lipid and triacylglycerol content in response to fasting in control and cold-exposed lean Zucker rats obtained when considering all the adipose depots studied (n=5 depots in each group of animals, control and cold). The statistical significance of each percentage between control and cold-exposed animals was assessed by Student's t test, defined at P<.05, and is indicated with *. All the P values are given.

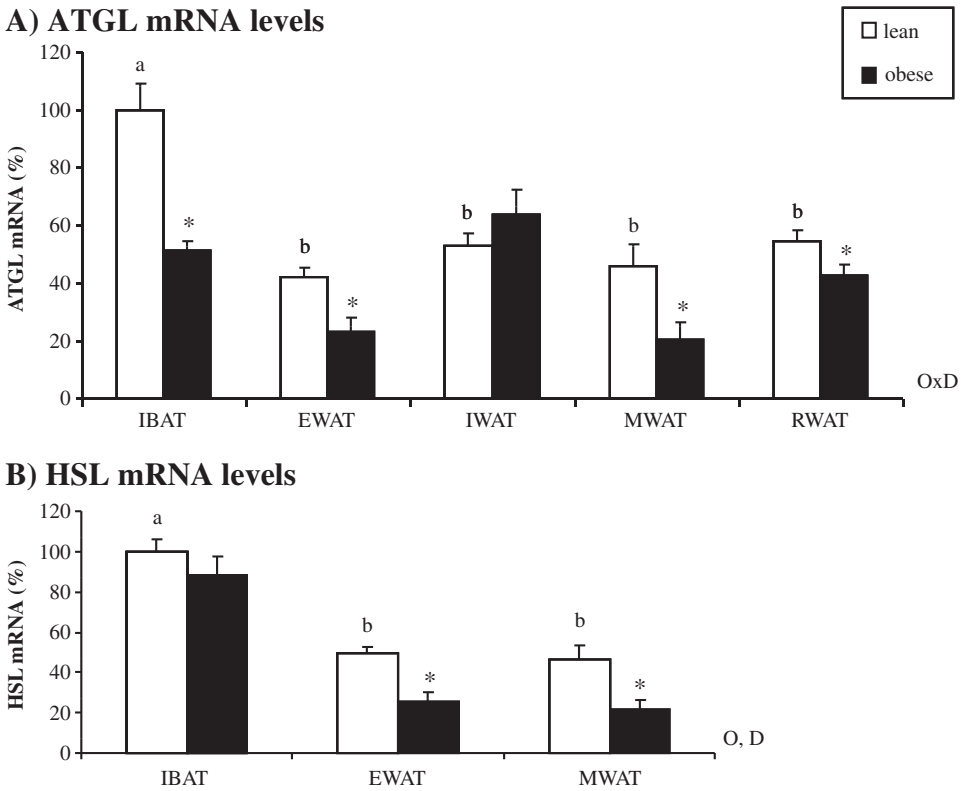

Fig. 1. ATGL (A) and HSL (B) mRNA expression levels in IBAT and in different WAT depots — EWAT, IWAT, MWAT and RWAT — in *ad libitum* lean and obese Zucker rats housed at 22°C, measured by real-time Q-PCR analysis. Results represent mean±S.E.M. (n=5) of ratios of specific mRNA levels to LRP10, expressed as a percentage vs. lean IBAT of the control group that was set to 100%. O, effect of body weight; D, effect of adipose tissue depot; OxD, interaction between body weight and adipose tissue depot (two-way ANOVA, P<.05). Within the different adipose depots of lean animals, bars not sharing a common letter (a, b) are significantly different (one-way ANOVA, P<.05). *Obese vs. lean animals (Student's t test, P<.05).

levels was more evident in cold-exposed than in control animals in all the adipose depots [IBAT: control (percentage increase: 17.1%), cold (percentage increase: 77.8%); EWAT: control (−5.7%), cold (35.5%); MWAT: control (21.4%); cold (79.1%)]. Therefore, the mean percentage increase of HSL mRNA expression obtained from each adipose tissue depot studied was higher in cold-exposed lean animals in comparison with control lean animals (Table 3). In the same way as that occurred with ATGL, HSL mRNA levels increased in response to fasting in EWAT and MWAT of both control and cold-exposed obese Zucker rats (two-way ANOVA, P<.05) and in IBAT of control animals (Student's t test, P<.05), but the exposure to cold did not improve the response to fasting in any of the adipose depots.

3.5. Effect of cold exposure on LPL and CGI-58 mRNA expression

We also analyzed the mRNA expression of LPL and CGI-58 (which has been found to act as a co-lipase for ATGL, increasing ATGL activity in the lipolytic process) in IBAT and EWAT of lean and obese fed-Zucker rats.

In EWAT, LPL mRNA expression was not affected by cold exposure either in lean or in obese Zuker rats (lean rats: control group: 100%±12%, cold group: 81.4%±4.2%; obese rats: control group: 81.2%±23%, cold group: 103%±5.5%). Differently to that observed in EWAT, LPL mRNA expression was increased in IBAT of lean Zucker rats in response to 24-h cold exposure (control group: 100%±6.3%, cold group: 234%±22%; Student's t test; P<.05), while no changes were found in obese rats (control group: 98.3%±9.5%, cold group: 86.9%±12%).

Comparative gene identification 58 mRNA expression was not affected by cold exposure in any of the selected adipose depots studied either in lean or in obese rats (data not shown), in spite of the clear effect that cold produced on ATGL expression in these depots.

3.6. Correlation of ATGL mRNA and protein levels with serum parameters

ATGL mRNA expression correlated positively with circulating NEFA (r=0.263, P<.05) only when considering lean animals, both control and cold exposed (fed and fasted). When control and cold-exposed lean animals were considered separately, a positive correlation between both parameters was also observed in both groups, but the correlation was stronger in the case of cold-exposed in comparison to control animals (r=0.491, P<.01 vs. r=0.310, P<.05). This positive correlation between ATGL mRNA expression and circulating NEFA was also stronger in cold-exposed animals in comparison with control ones when each adipose tissue depot was considered separately, and was statistically significant for the EWAT, IWAT and MWAT depots (r=0.802, P<.01; r=0.730, P<.05 and r=0.741, P<.05, respectively), while statistical significance was not reached in any of the adipose depots of animals of the control group. When considering only the obese animals, a positive correlation between ATGL mRNA expression and circulating NEFA was observed in the MWAT depot of the cold group (r=0.750, P<.05). ATGL protein levels and circulating NEFA only correlated positively (r=0.824, P<.01) when MWAT of cold-exposed lean animals was considered.

In accordance with their opposite roles in lipid metabolism (lipolytic/lipogenic), a negative correlation between ATGL mRNA expression and serum insulin levels was found when all the animals and all the adipose tissue depots were considered together (r=−0.319, P<.01) and also when lean and obese animals were considered

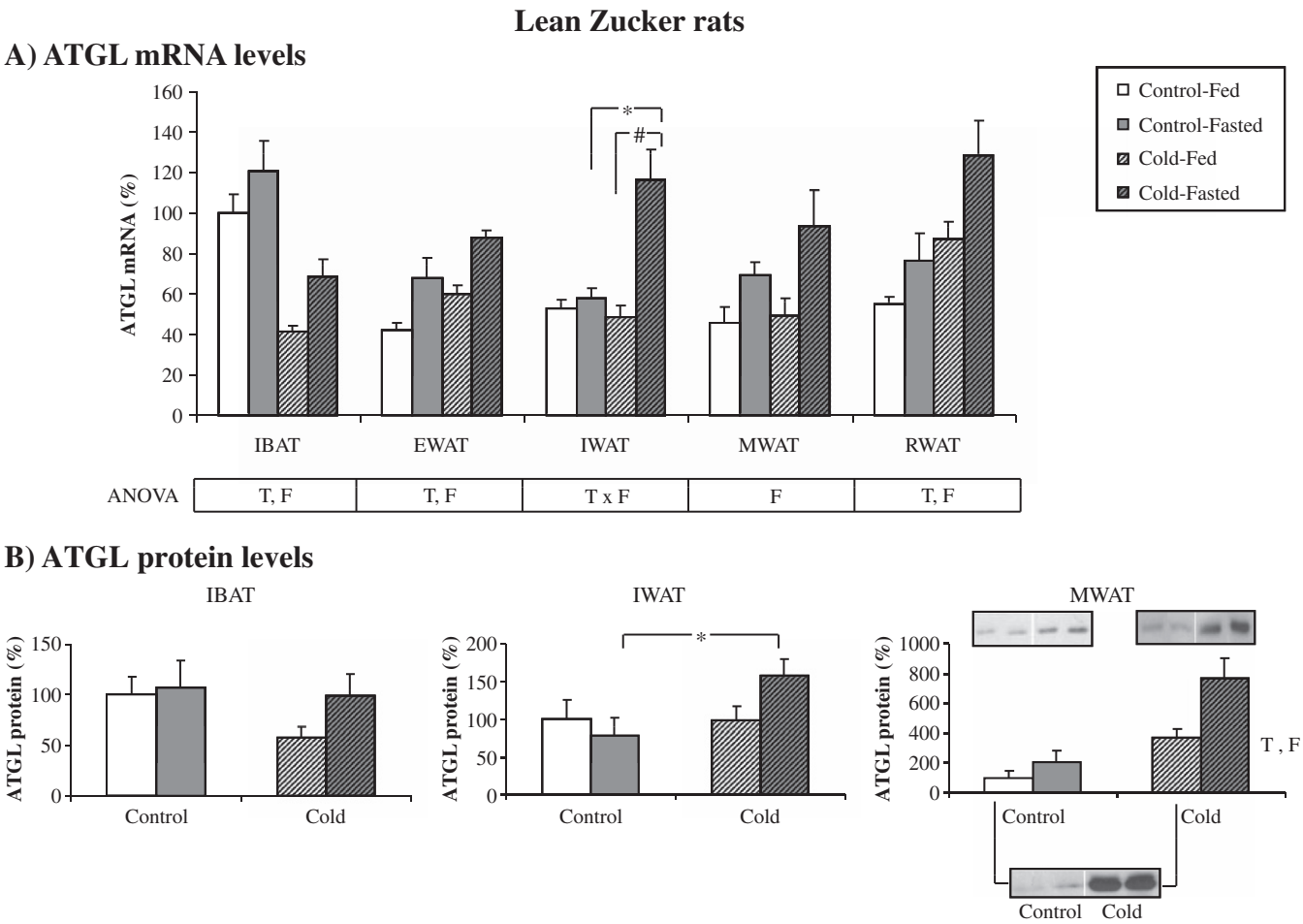

Fig. 2. (A) ATGL mRNA expression levels in IBAT and in different WAT depots — EWAT, IWAT, MWAT and RWAT — in *ad-libitum*-fed and 14-h-fasted lean Zucker rats housed at 22°C (control) or at 4°C for 24 h (cold), measured by real-time Q-PCR. Results represent mean±S.E.M. (n=5) of ratios of specific mRNA levels to LRP10, expressed as a percentage vs. IBAT of the control-fed group that was set to 100%. The values of ATGL mRNA expression of control-fed animals in the different adipose depots are common in Figs 1A and 2A. F, effect of fasting; T, effect of temperature; T×F, interaction between temperature and fasting (two-way ANOVA, P<.05). *Control vs. cold animals; #fed vs. fasted animals (Student's t test, P<.05). (B) ATGL protein levels in IBAT, IWAT and MWAT in *ad-libitum*-fed and 14-h-fasted lean Zucker rats housed at 22°C (control) or at 4°C for 24 h (cold), measured by Western blot. Results represent mean±S.E.M. (n=5) of ratios of protein levels normalized to the signal of black amide B10 staining. Values of the control-fed animals are set to 100%, and the other values are expressed relative to them. Representative bands obtained in the Western blot are shown for MWAT, which was chosen as a representative adipose depot. Ten micrograms of protein was loaded per lane. F, effect of fasting; T, effect of temperature (two-way ANOVA, P<.05).

separately (r=−0.245, P<.05 and r=−0.312, P<.01, respectively). When all the animals were taken into account and each adipose tissue depot was considered separately, this negative correlation was found in EWAT, MWAT and RWAT depots (r=−0.395, P<.05; r=−0.482, P<.01 and r=−0.363, P<.05, respectively), while statistical significance was not reached in IWAT and IBAT depots. A negative correlation between ATGL protein levels and serum insulin levels was only observed in MWAT (r=−0,325, P<.05) when both lean and obese animals were considered.

3.7. Lipid and triacylglycerol content in adipose tissues: effect of fasting and cold exposure

Lipid and triacylglycerol content was measured in lean animals (control and cold exposed) because these were the ones with a clearer response to fasting conditions in terms of ATGL mRNA and protein levels.

Lipid content was only affected by fasting in the MWAT and EWAT depots of cold-exposed animals, where a decrease was observed (feeding: 648±18 and fasting: 562±24 mg/g tissue, P=.061, Student's t test, for the MWAT; feeding: 744±12 and fasting: 684±26 mg/g tissue, P=.084, Student's t test, for the EWAT). When the effect of fasting was analyzed considering the mean percentage decrease in lipid content obtained from each of the adipose tissue depots studied, a higher percentage decrease was observed in cold-exposed animals in comparison with control ones (Table 3).

A decrease in triacylglycerol content in fasted animals was observed only in the IWAT depot of cold-exposed rats (442±33 in fed vs. 307±41 mg/g tissue in fasted rats, P<.05, Student's t test). As what happened with lipid content, when the effect of fasting was analyzed considering the mean percentage decrease in triacylglycerol content obtained from each adipose tissue depot studied, a higher percentage decrease was observed in cold-exposed animals than in control ones, although the decrease did not reach statistical significance (Table 3). The difference in this percentage decrease was higher when not considering the triacylglycerol values from the IBAT (15.9%±6.2% vs. 5.42%±3.50% of decrease in cold-exposed and control animals, respectively).

An effect of temperature was only observed in the IBAT, in which lipid and triacylglycerol levels decreased as a consequence of cold exposure (control group: 413±19 and 121±15 mg/g tissue, respectively; cold group: 198±21 and 87.2±3.22 mg/g tissue, respectively; P<.05; Student's t test).

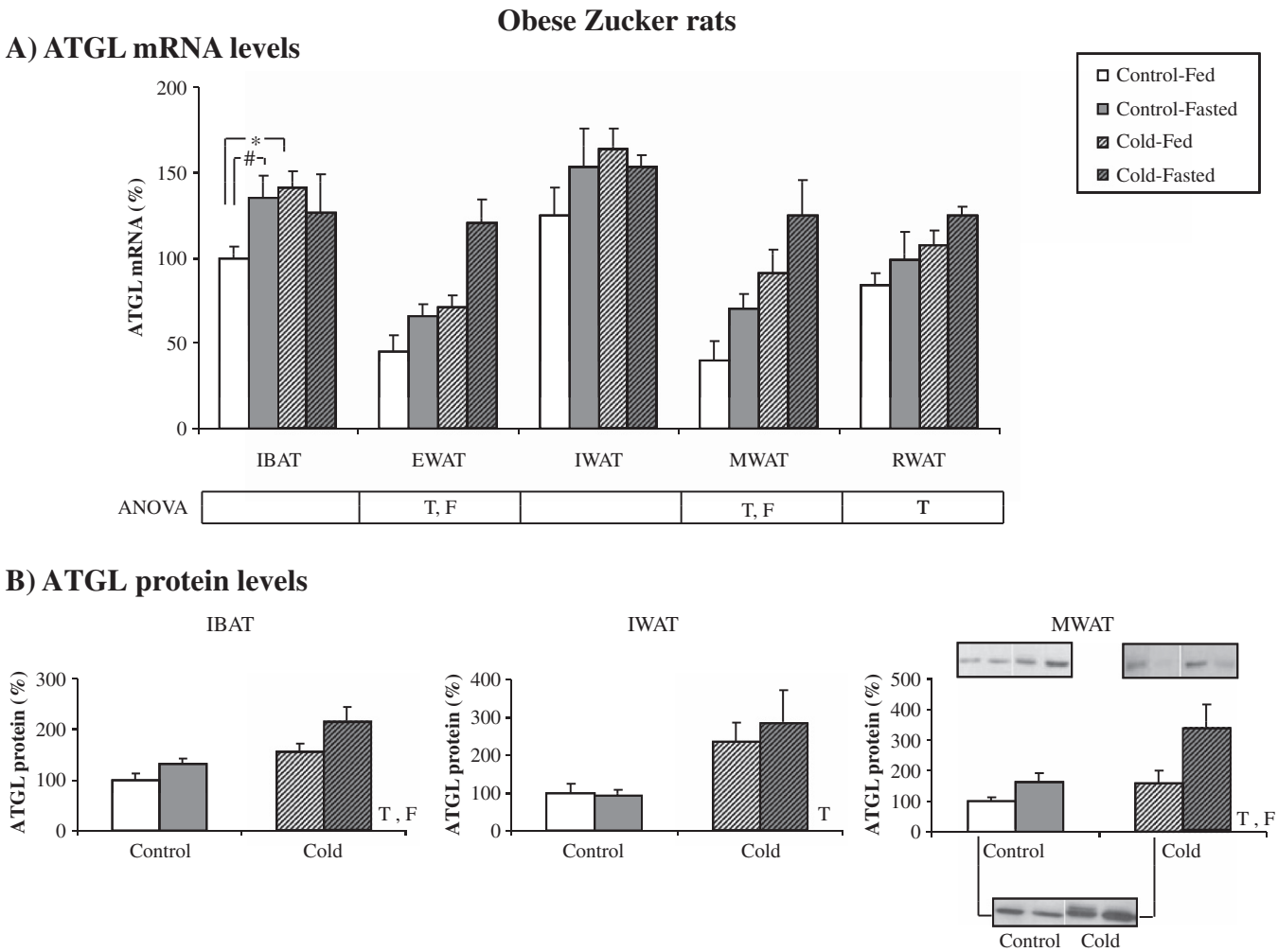

Fig. 3. ATGL mRNA expression levels (A) and protein levels (B) in *obese* Zucker rats in the same conditions and tissues described in Fig. 2 for lean animals.

3.8. Correlation of ATGL mRNA and protein levels with lipid and triacylglycerol content

ATGL mRNA expression correlated negatively with lipid content only in the MWAT depot, both in control and in cold-exposed lean animals, although this correlation was stronger in animals of the cold group ($r=-0.680$, $P<.05$ and $r=-0.785$, $P<.01$, in control and cold-exposed animals, respectively). No correlation was observed between ATGL mRNA expression and triacylglycerol content in any adipose tissue depot. ATGL protein levels did not correlate with lipid or triacylglycerol content in any of the different depots studied.

4. Discussion

An important role for ATGL in fasting-induced lipolysis has been suggested [2,15], thus prompting the idea that an alteration in its expression and/or nutritional regulation could contribute to increased fat deposition. Previously, we have described that impairment in ATGL fasting response with age in different adipose tissue depots can be associated with the increase in body weight characteristic of aging [17]. A relation of ATGL mRNA expression with obesity has also been suggested, as ATGL is down-regulated in subcutaneous adipose tissue of insulin-resistant overweight/obese humans [18] and in the gonadal adipose depot of obese *ob*/*ob* and *db*/*db* mice [2]. Our data show that ATGL mRNA levels in obese Zucker rats are lower in the different adipose depots studied, with the only exception of the subcutaneous IWAT, suggesting a relation of the obese state with regulatory mechanisms affecting ATGL expression. Genetically obese Zucker rats lack the functional leptin receptor, which determines the development of obesity accompanied by hyperleptinemia, hyperinsulinemia and insulin resistance [30–32]. Different *in vitro* studies have demonstrated that ATGL mRNA expression is down-regulated by insulin [10] and up-regulated by leptin [33], while Kershaw et al. [10] showed that ATGL regulation by insulin is impaired in adipose tissue of streptozotocin-induced diabetic mice. Thus, taking into account the regulatory effect of insulin and leptin on ATGL mRNA levels, the deregulation on ATGL mRNA expression observed in the different adipose depots of obese rats could be related to the evident alteration in leptin and insulin signaling pathways that these rats presented. However, as this clear effect in ATGL mRNA expression levels was not translated into a decrease in protein levels, which could be due to a fast translational rate, further investigation is necessary to clarify the role of both hormones in ATGL regulation in the obese state.

Exposure to cold is a situation that requires physiological adaptations to maintain body temperature, such as increases of heat production (thermogenesis) and food intake, which are frequently accompanied by hypoinsulinemia [28,29] and hypoleptinemia [26–28]. In the present study, insulin and leptin circulating levels were lower in cold-exposed obese rats; however, a reduction in food intake was observed in both lean and obese rats in response to cold stimulus. Although we do not have a clear explanation for this, we hypothesize that such an effect could be due to an initial stress

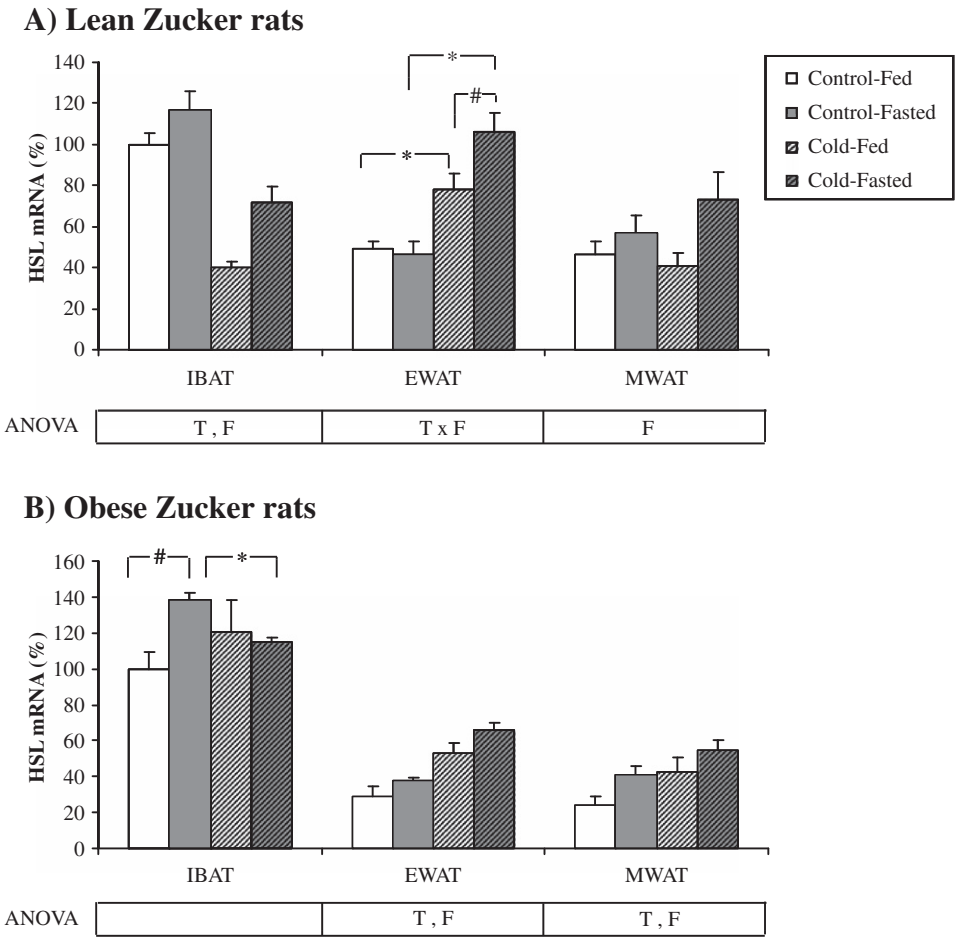

Fig. 4. HSL mRNA expression levels in IBAT, EWAT and MWAT in *ad-libitum*-fed and 14-h-fasted lean (A) and obese (B) Zucker rats housed at 22°C (control) or at 4°C for 24 h (cold), measured by real-time Q-PCR. Results represent mean±S.E.M. (n=5) of ratios of specific mRNA levels to LRP10, expressed as a percentage vs. IBAT of the control-fed group that was set to 100%. The values of HSL mRNA expression of control-fed lean animals in the different adipose depots are common in Figs 1B and 4A. F, effect of fasting; T, effect of temperature; T×F, interaction between temperature and fasting (two-way ANOVA, P<.05). *Control vs. cold animals; #fed vs. fasted animals (Student's t test, P<.05).

response to cold exposure, as 24 h could be not enough time to achieve an optimal adaptation to a cold environment. Related with this, Leung and Horwitz [34] demonstrated that rats exposed to cold for a long time showed an immediate reduction in nocturnal meal frequency and did not increase the nocturnal food intake until 8 days of cold exposure. Therefore, it would be expected that after a longer period of cold exposure, optimal adaptation to this environment would be achieved, and thus, an increase in food intake would be observed.

Cold stimulus increases sympathetic activity, and the catecholamines released from sympathetic innervations, acting mainly through β3 adrenergic receptors, stimulate lipolysis (and fatty acid release) in WAT and BAT and thermogenesis in BAT [35,36]. Detailed *in vivo* and *in vitro* studies have suggested an important role of ATGL in lipid mobilization in response to cold stimuli and isoproterenol treatment [10,12]. In this sense, Haemmerle et al. [12] demonstrated that ATGL null mice display defective cold adaptation, suggesting that the enzyme provides NEFA for fuel thermogenesis in IBAT, and Deiuliis et al. [6] showed that ATGL mRNA and protein levels drop after short- and/or medium-term cold exposure in IBAT of mice, probably via a PKA-dependent mechanism. However, as far as we know, there are no data available concerning how cold exposure affects ATGL mRNA and protein basal levels in different WAT depots. Our results in cold-exposed animals show that, as a general trend, ATGL mRNA expression is increased in different WAT depots, both in lean and in obese Zucker rats. When studying protein levels in selected depots, we observe an important increase as a result of cold exposure in MWAT of lean rats. In obese rats, the increase in ATGL mRNA levels was also observed at the protein level in MWAT and IWAT depots and could be tentatively related to the important decrease observed with cold exposure in circulating insulin basal levels in combination with increased sympathetic stimulation that occurs under cold exposure. All in all, these data demonstrate for the first time that ATGL mRNA and protein basal levels are affected by cold exposure in WAT, which is in agreement with the previously suggested role of ATGL in cold-stimulated lipolysis.

In IBAT, however, ATGL mRNA expression increases in obese cold-exposed rats but decreases in lean rats, this pattern being confirmed when studying ATGL protein levels. When we study the effect of cold exposure on HSL mRNA expression, the results are very similar to those observed for ATGL, even in the different response to cold in IBAT of lean and obese animals, suggesting similar regulatory mechanisms for both proteins in the different adipose tissues studied. The current results obtained in lean rats are mainly in agreement with those obtained recently by Deiuliis et al. [6], who demonstrated that cold exposure at different times produced significant decreases in ATGL and HSL mRNA levels (at 3, 6 and 24 h) and also in ATGL protein levels (at 3 h) in IBAT of mice. A differential cold response regulatory pattern, like that observed here for ATGL and HSL mRNA expression in WAT and IBAT of lean rats, has also been observed for other lipases, such as LPL (reviewed in Ref. [37]), and could be tentatively

associated with the different size and form of fat stores in the two types of tissues. Thus, in a situation of energy demand as is cold exposure, while WAT could use (or release) its own fatty acids derived from the hydrolysis of triacylglycerols through the up-regulation of ATGL and HSL in white adipocytes, brown adipose fat is more directed to be quickly burned, and therefore, ATGL and HSL mRNA expression is down-regulated. In this situation, the mobilization of fatty acids from WAT and/or the activation in IBAT of LPL, which is essential for the hydrolysis and distribution of triglyceride-rich lipoprotein-associated fatty acids among extrahepatic tissues [38], would be necessary in order to replenish the tissue supply of fatty acids. In fact, several studies have demonstrated that LPL activity is stimulated rapidly in response to cold exposure in IBAT [38–42] but not in EWAT [39,42] or IWAT [38] depots of rodents. In agreement with these reports, in the present study, we have found an increase in LPL mRNA levels in IBAT of lean rats in response to cold exposure, but not in EWAT. Due to the greater amount of lipids present in IBAT, this mechanism would not be evident in obese rats, in which ATGL mRNA and protein levels are increased in response to fasting in the same way as happens in the WAT depots.

Regarding ATGL regulation by fasting, our data show that in lean animals, ATGL mRNA expression is up-regulated in all the depots studied in both control and cold-exposed rats, with the exception of the IWAT of control animals. A very similar pattern is found for HSL. However, it is worth noting that in lean rats, cold exposure highly improves fasting up-regulation of ATGL and HSL: under a cold stimulus, both ATGL and HSL mRNA levels rise sharply in fasted animals in most of the WAT depots studied and also in IBAT, with a much more evident response to fasting than that observed in rats acclimated to 22°C. In fact, in lean rats, the percentage increase of ATGL and HSL mRNA expression in response to fasting taking into account all the adipose depots studied is higher in cold-exposed than in control animals for both proteins (78.1% vs. 36.4% for ATGL and 64.1% vs. 11% for HSL). This improvement in the response to a fasting situation under cold exposure is also observed at the protein level in all the adipose depots studied and could contribute to a better capacity for mobilizing lipids in a situation that deeply affects energy homeostasis. All these data suggest a synergic effect between catecholamines, released as a result of exposure to low temperatures, and other regulating factors involved in the fasting response in the regulation of ATGL. Moreover, regarding the putative role of ATGL in cold-induced lipolysis, a stronger positive correlation was found between circulating NEFA and ATGL mRNA expression in the different adipose tissue depots in cold-exposed in comparison with control lean Zucker rats. Furthermore, a positive correlation between circulating NEFAs and ATGL protein levels was only found in cold-exposed animals, specifically in MWAT. All in all, these data in lean animals, together with the fact that the percentage decrease in lipid content considering the different adipose tissue depots and the percentage increase of circulating NEFA in response to fasting are higher in cold-exposed animals, reinforce the hypothesis of an important role of ATGL in cold-stimulated lipolysis.

On the other hand, in obese rats, in spite of the increase in circulating NEFA levels in response to fasting, there is a partial impairment of ATGL regulation by fasting in WAT, as ATGL mRNA levels only increased in EWAT and MWAT of both control and cold-exposed animals and ATGL protein levels increased only in the MWAT depot. However, it is worth noting that in obese animals, an up-regulation of ATGL in response to fasting is observed in IBAT at mRNA level in control rats and at protein level in both control and cold-exposed rats. Contrary to that observed in lean rats, cold exposure has no evident effect improving the response to fasting in the obese state, as this improvement is only observed in the EWAT and MWAT depots (at the mRNA and protein level, respectively), which are probably more sensitive to the ATGL regulatory mechanisms. Moreover, in obese rats, the fasting up-regulation of ATGL mRNA expression observed in IBAT of rats acclimated to 22°C disappears in cold-exposed animals. Obesity has been shown to be associated with a resistance to sympathetic action on adipose tissue [43]; thus, this insensitivity could be related to the impaired response to cold, which would explain the lack of increase in ATGL expression by fasting that we observe in obese animals and the lack of improvement in nutritional regulation in obese cold-exposed rats. Moreover, and taking into account the effect of leptin increasing sympathetic stimulation [43,44], the lack of a functional leptin receptor in obese Zucker rats could contribute to the impairment in ATGL fasting regulation by cold exposure observed in these animals.

MWAT seems to be the most sensitive to ATGL regulation by fasting and cold stimulus in both lean and obese rats. In both groups, ATGL mRNA and protein levels increase in response to fasting, and this regulation is improved after cold exposure, which is translated into a decrease in the lipid content in the depot of lean animals. Moreover, it is the only tissue where a significant positive correlation between circulating NEFA levels and ATGL mRNA levels was observed in both lean and obese rats. ATGL protein levels in MWAT depot of lean and obese rats are also importantly increased as a result of cold exposure, and a significant positive correlation between circulating NEFA levels and ATGL protein levels was observed in lean rats. The fact that in lean animals, the size of the MWAT depot is reduced can be taken as a proof of the physiological relevance of the effects of cold exposure increasing ATGL expression. We have previously described a close relation of the MWAT depot, a tissue especially sensitive to regulation by feeding, and regulation by nutrients of other adipose-specific products, such as leptin [25,45].

In conclusion, here we describe a remarkable effect of cold exposure on ATGL regulation, up-regulating both mRNA and protein levels in different WAT depots of lean and obese rats and improving its increase with fasting mainly in lean cold-exposed animals. A more important than previously considered role in cold-induced lipolysis of ATGL in rat adipose tissue is suggested. In addition, the widespread decrease of ATGL mRNA expression in adipose tissue of obese rats and the impairment of its regulation by fasting compared with lean rats can contribute to a worse capacity to mobilize lipids and, thus, to fat accumulation and obesity.

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