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Role of glucocorticoid receptor (GR) in white adipose tissue beiging

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ABSTRACT

Aim: Glucocorticoids (GCs) play a crucial role in energy homeostasis including white adipose tissue function; however, chronic GC excess is detrimental to mammals' health. White hypertrophic adiposity is a main factor for neuroendocrine-metabolic dysfunctions in monosodium L-glutamate (MSG)-damaged hypercorticosteronemic rat. Nevertheless, little is known about the receptor path in endogenous GC impact on white adipose tissue-resident precursor cells to bring them into beige lineage. Thus, our aim was to explore whether transient/chronic endogenous hypercorticosteronemia affects browning capacity in white adipose tissue pads from MSG rats during development.

Main methods: Control and MSG male rats aged 30 and 90 days were 7-day exposed to cold conditions in order to stimulate wet white epididymal adipose tissue (wEAT) beiging capacity. This procedure was also replicated in adrenalectomized rats.

Key findings: Data indicated that whereas epididymal white adipose tissue pads from prepubertal hypercorticosteronemic rats retained full expression of GR/MR genes resulting in a drastic reduction in wEAT beiging capacity, conversely, chronic hypercorticosteronemic adult MSG rats developed down-regulation of corticoid genes (and reduced GR cytosolic mediators) in wEAT pads and consequently partially restored local beiging capacity. Finally, wEAT pads from adrenalectomized rats revealed up-regulation of GR gene accompanied by full local beiging capacity.

Significance: This study strongly supports a GR-dependent inhibitory effect of GC excess on white adipose tissue browning, an issue strongly supporting a key role of GR in the non-shivering thermogenic process. As a consequence, normalizing the GC milieu could be a relevant factor to handle dysmetabolism in white hyperadipose phenotypes.

1. Introduction

Anti-inflammatory and immunosuppressive Glucocorticoid (GC) effects are vital for the organism's survival. GCs also modulate white adipogenesis by binding to gluco- and mineralo-corticoid receptors (GR and MR) [1,2]. We reported that rats neonatally-treated with monosodium L-glutamate (MSG) are prone to develop hypercorticosteronemia [3,4], enhanced white adiposity (WAT) [5], and dysmetabolism [6], sharing various characteristics with human Cushing's syndrome, such as: hyperlipidemia [7], hyperleptinemia [8] and hyperinsulinemia [8,9] a reduction in circulating epinephrine has also been reported [10]. Adult MSG-WAT mass [11,12] contains enlarged, insulin-resistant [8] adipocytes. However, we reported an age-dependent development of neuroendocrine-metabolic disturbances in MSG rats [13]. Specifically, whereas prepubertal MSG rat WAT mass-expansion is dominated by a hyperplastic process; conversely, WAT stromal-vascular fraction cells from adult MSG rats differentiate slowly due to reduced adipose precursor cell (APC) commitment [13] and competency [14]. Thus white adipocyte maturation slows [13] and WAT pads are replete with hypertrophic adipocytes [13]. Importantly, these changes are dependent on GC excess since adrenalectomy combined with GC replacement therapy overrides impaired adipogenesis and dysmetabolism [13].

The view of GCs as modulators of brown adipose tissue (BAT) function remains controversial [15]. BAT is an organ expressed only in mammals to ensure non-shivering thermogenesis for appropriate

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adaptation to low temperatures [16]. However, at least two different types of thermogenic AT cells are distributed in the organism: the classical brown adipocyte located in BAT pads and the beige one, a cell disseminated in WAT pads and prone to activate thermogenesis when needed [17,18]. Whereas BAT adipocytes display high levels of mitochondrial uncoupling protein 1 (UCP1), beige adipocytes have low basal UCP1 production, although UCP1 is easily inducible by low temperature conditions and physical activity [19]. However, the cell lineages from which brown and beige adipocytes derive remain unclear. In fact, despite their common adaptive thermogenic activity, brown and beige cells do not seem to derive from the same cell lineage [20–22]. Since these cells express different molecular factors [18]. Adipogenesis is a process whereby APCs differentiate into mature adipocytes, a process mediated through both GR and MR signaling on APCs [2]. Unlike the process of white and brown adipocyte differentiation in which the CCAAT/enhancer binding protein α (C/EBP-α) family and PPARγ are mainly involved [16], beige adipocytes could emerge from white adiposity due to the effects of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1α) and cell death-inducing DNA fragmentation factor-like effector A (CIDEA), a lipid-droplet-associated protein [16].

GCs have been suggested as negative modulators of UCP1 production, thereby contributing to overall GC-induced obesity. Whether GCs stimulate or inhibit UCP1 beige adipocyte production seems to depend on the interpretation of data [23]. In fact, experiments using several rodent models of hyperglucocorticoidism indicated that mitochondrial UCP1 production is dependent on the manner in which its production is expressed [23]. However, no studies were conceived to explore the mechanisms involved in the de novo browning process in WAT pads from MSG rats during development. Therefor we investigated the ability of cold-induced browning in wet epididymal WAT (wEAT) pads from GC-excess MSG, and GC-deplete non obese rats.

2. Materials and methods

2.1. Experimental animals

Adult male and female Sprague-Dawley (S—D) rats were allowed to mate in colony cages in a light (lights on between 07:00–19:00 h) and temperature (21–23 °C) controlled room. Rat chow and water were available ad libitum. Pregnant rats were transferred to individual cages. Beginning on day 2 after parturition, newborn male pups were injected i.p. with either 4 mg/g BW MSG (Sigma Chemical Co., St. Louis, MO) dissolved in sterile 0.9 % (*w/v*) NaCl or 10 % (w/v) NaCl (littermate controls; CTR) once every two days up to 10 days of age [9]. Rats were weaned at 21 days of age and immediately housed (3 rats per cage) and kept in a controlled environment (21–23 °C and lights on between 07:00–19:00 h) with free availability to rat Purina chow and tap water. Thereafter, individual daily body weight (BW) was recorded between throughout experimentation. As described below, CTR and MSG rats were euthanized on different ages for experimentations. It should be stressed that in each experiment, CTR and MSG rats were members of the same litters; however, when accumulating experiments, each different experiment was performed with CTR and MSG animals from different litters.

Additionally, sixty day-old normal (no obese) S—D rats were submitted (under light ketamine anesthesia and by the dorsal approach) to either bilateral adrenalectomy (ADX) or sham-operation (SHX). After recovered, animals were returned to their home cages (allocated as 3 rats per cage). Rats ate purina rat chow ad libitum, and while SHX rats drank tap water ADX ones drank NaCl 0.9 % solution until sacrificed.

Our Institutional Animal Care Committee approved all experiments (FCM-UNLP CICUAL's Approval Code T05012015). Animal manipulation and sacrifice followed protocols in agreement with NIH Guidelines for care and use of experimental animals.

2.2. Experimental designs (Fig. 1)

2.2.1. Experiment 1: prepubertal animals

Rats were killed by decapitation, in non-fasting condition (8–9 AM), on age 37 days (see Scheme of Experimental designs). Immediately, trunk blood was collected into plastic EDTA-coated tubes, they were rapidly centrifuged (4 °C; 2500 xg; 15 min) and plasma samples kept frozen (− 20 °C) until biomarkers measurements. Thereafter, the whole medial basal hypothalamus (MBH) [9], wEAT [24] and BAT (interscapular) [24] pads were aseptically dissected-out, weighed and placed in sterile Petri dishes containing 10 mL of sterile Dulbecco's Modified Eagle Medium-Low Glucose (1 g/L) (DMEM-LG; Gibco, purchased from Invitrogen-Argentina) [25]. wEAT pads were then used for determination of the expression levels of GR and MR genes, PGC1α and CIDEA mRNAs levels, and UCP1 (mRNA and protein levels).

2.2.2. Experiment 2: adult animals

Animals were killed by decapitation, in non-fasting condition (8–9 AM), on age 97 days. Immediately, trunk blood was collected into plastic EDTA-coated tubes, they were rapidly centrifuged (4 °C; 2500 xg; 15 min) and plasma samples kept frozen (− 20 °C) until biomarkers measurements. Thereafter, the whole medial basal hypothalamus (MBH) [9], wEAT [24] and BAT (interscapular) [24] pads were aseptically dissected-out, weighed and placed in sterile Petri dishes containing 10 mL of sterile Dulbecco's Modified Eagle Medium-Low Glucose (1 g/L) (DMEM-LG; Gibco, purchased from Invitrogen-Argentina) [25]. wEAT pads were then used for determination of the expression levels of GR and MR genes, PGC1α, CIDEA, FKBP5, GILZ and SGK1 mRNAs levels, and UCP1 (mRNA and protein levels).

2.2.3. Experiment 3: cold-exposed prepubertal and adult CTR & MSG rats

In a separate set of experiments, rats on both 23 and 83 days of age were individually-caged and left undisturbed for one week. On the morning of ages 30 and 90 days, respectively, rats were 7 day-kept at either room temperature (Basal Conditions; BC) or low temperature (Cold Conditions, 4 °C; CC); rat BW and FI were daily recorded throughout one week. After sacrifice, in non-fasting conditions, on 37 and 97 days of age, respectively, trunk blood was collected for evaluation of the peripheral levels of several biomarkers, thereafter wEAT and BAT pads were excised, weighed and processed as described above (Experiment 2). Additionally morphometric analyses were performed in wEAT pads.

2.2.4. Experiment 4: cold-exposed GC-replete and GC-deplete adult non obese rats

On age 83 days SHX and ADX were individually caged and left undisturbed for one week. On the morning of age 90 days rats were 7 day-kept at either room temperature (Basal Conditions; BC) or low temperature (Cold Conditions, 4 °C; CC); rat BW and FI were daily recorded throughout one week. Rats were then sacrificed on 97 days of age. Trunk blood was collected for evaluation of the peripheral levels of several biomarkers and, wEAT and BAT pads were excised, weighed and processed as described above (Experiment 2).

2.3. Peripheral biomarkers measurements

Circulating levels of insulin (INS), corticosterone (CORT) and leptin (LEP) were determined by specific radioimmunoassays (RIAs) developed in our laboratory as previously validated and described [8]. LEP (standard curve 0.05–25 ng/mL) coefficients of variation (CVs) intra- and inter-assay were 4 %–7 % and 9 %–11 %, respectively; INS (standard curve 0.08–10 ng/mL) CVs intra- and inter-assay were 3 %–7 % and 8 %–11 %, respectively; CORT (standard curve 0.05–50 μg/dL) CVs intra- and inter-assay were 4 %–6 % and 8 %–10 %, respectively. Those of glucose (GLU) and triglycerides (TG) were quantified by using the respective commercial kit and following the recommendations of the

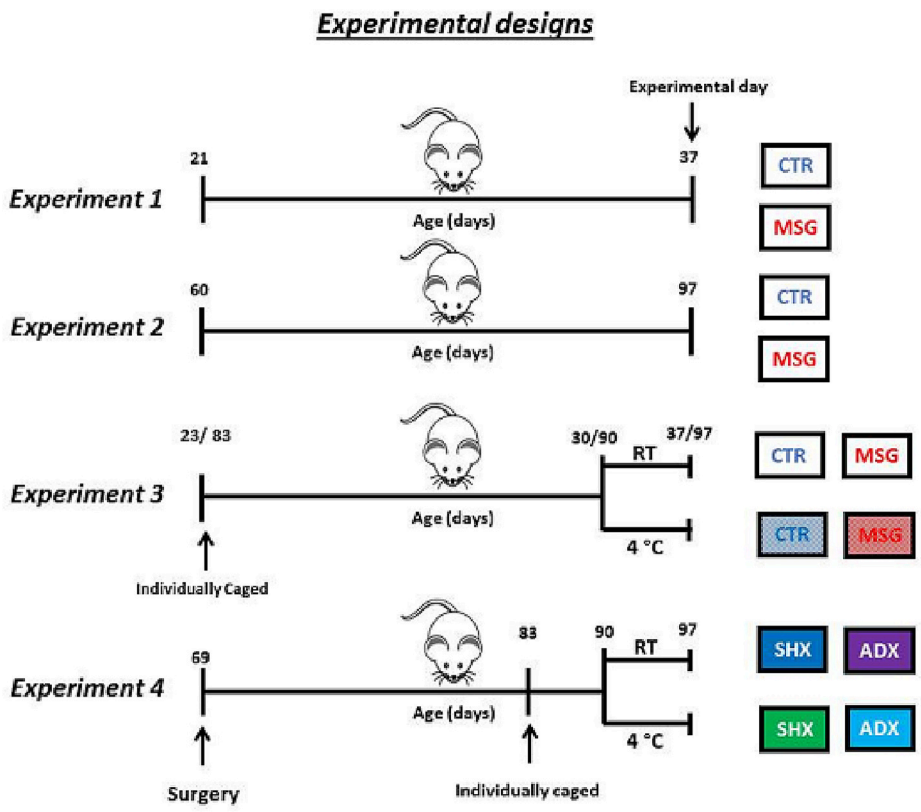


Fig. 1. Brief summary of the experimental designs: Age-matched control-litters (CTR), hypothalamic obese (MSG) rats on different ages. Sham adrenalectomized (SHX) and bilaterally adrenalectomized (ADX) non-obese adult rats (RT: Basal Conditions; 4 °C: Cold Conditions).

seller (Wiener Laboratory, Rosario-Argentina).

2.4. Adipose tissue analyses

2.4.1. Morphometric studies in wEAT pads

Freshly dissected wEAT pads were immediately fixed in 4 % paraformaldehyde (in 0.2 M phosphate buffer), at 4 °C for a maximum of 3 days. Tissues were then washed with 0.01 M PBS and immersed in 70 % (*w/v*) ethanol in distillated water for 24 h before being processed and embedded in paraffin. Sections of 4 μm were obtained at different levels of the blocks and stained with hematoxylin-eosin, then examined with a Jenamed 2 Carl Zeiss light microscope. Quantitative morphometric analysis was performed with a RGB CCD Sony camera together with the OPTIMAS software (Bioscan Incorporated, Edmons, WA, USA; 409× objective). For each pad sample ($n = 4/5$ animals per group), systematic random sampling was used to select 10 fields for each section and a minimum of 100 cells per group were examined. Each field of cells in a reference area (RA: tissue area scanned where adipocytes were scored) was measured for an average of 10 micrographs taken from two different levels. These measurements were recorded and processed; thereafter cell size (CS; expressed in μm^2) was calculated [13,14].

2.4.2. wEAT pad genes expression

wEAT pads, from CTR and MSG rats on both ages, were processed for total RNA extraction and further evaluation of mRNA expression levels of several gene-markers as mentioned above.

2.5. Quantitative real-time PCR (qrtPCR)

Total RNA was isolated [26] from wEAT pads by the Trizol extraction method (Invitrogen, Life Tech., USA). Total RNA was reverse-transcripted using random primers (250 ng) and RevertAid Reverse Transcriptase (200 U/μL, Thermo Scientific, Lithuania). Two μL cDNA were amplified with HOT FIRE Pol EvaGreen qPCR Mix Plus (Solis BioDyne, Estonia) containing 0.5 μM of each specific primer, using LightCycler Detection System (MJ Mini Opticon, Biorad). PCR efficiency was near 1. Expression levels were analyzed for the genes described above, including the reporter gene β-actin (ACTIN). PCR reactions were processed by using 3–4 samples per group, samples were run by duplicate. Data are displayed as relative expression to reporter gene. Designed primers employed are depicted in Table 1, following an alphabetical order. Relative changes in the expression level of one specific gene (ΔΔCt) were calculated by the ΔCt method. Reactions and analysis were performed as previously described [27].

Table 1
Rat-specific primers for qPCR, listed in alphabetical order.

	Primers (5′-3′)	GBAN	bp
ACTIN	se, AGCCATGTACGTAGCCATCC as, ACCCTCATAGATGGGCACAG	NM_031144	115
CIDEA	se, GGAGACCGCCAGGGACTAC as, TGCTGGCCATCACCCC	NM_001170467.1	158
FKBP5	se, AAGGCGAAGGAACACAACGA	MN_001012174.2	198
GILZ	as, CTCCTTGTCGTGGCGTCATA se, GGTGCCAAGAGATGTCCCAA as, CTGGCTTGGGGTTACTAGGC	NM_001400983.1	114
GR	se, TGCCCAGCATGCCGCTATCG as, GGGGTGAGCTGTGGTAATGCTGC	NW_047512	170
MR	se, TCGCTCCGACCAAGGAGCCA as, TTCGCTGCCAGGCGGTTGAG	NM_013131	193
PGC1α	se, AAAAGCTTGACTGGCGTCAT as, ACACCACTTCAATCCACCCAG	NM_031347.1	199
SGK1	se, GAAGATCACGCCCCCATTTA	MN_001193568.1	127
UCP1	as, TGTGACAAGGATGCTGTCAGG se, CCGAAACTGTACAGCGGTCT as, GTCATCAAGCCAGCCGAGAT	NM_012682.2	155

se, sense; as, anti-sense; GBAN: GenBank Accession Number; amplicon length: in bp.

2.6. Western blot

Wet EAT pads, from different group-donors, were lysed with RIPA buffer (150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 50 mM Tris-HCl, 2 mM EDTA, 50 mM NaF, pH = 7.4) with 25–50 KIU aprotinin, 0.5–1 μg/mL leupeptin, 1 mM Na_2VO_4 and 0.1 mg/mL PMSF as protease inhibitors. The lysates were homogenized, incubated on ice during 30 min, and then centrifuged at 12,000 ×*g* for 20 min, at 4 °C. Supernatants were collected and total protein concentrations were determined by Lowry [28]. Thirty μg of protein were resolved by 12 % SDS-PAGE, and then transferred onto PVDF membrane. UCP1 antibody (Abcam, ab10983, at a 1:3000 dilution) was used for immunoblotting. ACTB (at a 1:4000 dilution) was used as a loading control. The horseradish peroxidase-conjugated secondary antibody was used at a 1:7500 dilution. Data detection was carried out by exposing autoradiography films (Kodak) to the membranes. Quantification Images were analyzed by Image J software. UCP1 immunoreactive band was quantified and normalized with the ACTIN protein intensity on scanned films. In most of the experiments one strip was used for CTR and MSG samples in either BC or CC; conversely in those wherein SHX and ADX samples were loaded in only one strip for both groups and conditions (see Results).

2.7. Statistical analysis

Data were expressed as mean values ± SEM, when *p* values were lower than 0.05, differences were considered statistically significant. Two-way ANOVA was performed for factorial analysis, including MSG and Cold effects and the interaction between the two factors (MSG x Cold), followed by Sidak post-test analysis, although only when interaction among factors was significant. The nonparametric Mann-Whitney *U* test was used to analyze data from mRNA expression and adipocyte size populations. For CTR and MSG comparison, Student's t-test was used, including data from western blot analyses [29]. All Statistical analyses and tests were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. The prepubertal and adult MSG rat-phenotypes

On the experimental day, MSG rats showed, degenerated optic nerves (by macroscopic observation at the time of sacrifice) and, decreased MBH NPY mRNA levels (Arbitrary Unites 55 % vs. CTR, approximately, not shown) [30] and BWs (Table 2).

Regarding AT masses (Table 2), while wEAT pads resulted significantly greater in MSG rats although at adult age only; conversely, a somewhat greater BAT mass was found in prepubertal MSG individuals only. In concordance, wEAT pad cell-size, while adipocyte hypotrophy characterized prepubertal MSG rats; conversely adipocyte hypertrophy was an indubitable characteristic from the adult MSG pad (Table 2).

Finally, while hypercorticosteronemia and hyperleptinemia were characteristics of prepubertal MSG individuals, the adult MSG rat phenotype additionally developed hyperinsulinemia and hypertriglyceridemia (Table 2).

Table 2
Body weight (BW; g), wet white epididymal adipose tissue (wEAT) weight (g/100 g BW), wEAT adipocyte area (in μm2) and brown (BAT) adipose tissue pads weights (g/100 g BW), and the peripheral levels of LEP (ng/mL), GLU (g/L), INS (ng/mL), TG (g/L) and CORT (μg/dL) in control (CTR) and MSG rats on different ages. (Mean ± SEM; $n = 8$ rats per group; a, $p < 0.000001$; b, $p < 0.01$; c, $p < 0.05$ vs. age-matched CTR rat values).

Age - group	Prepubertal		Adult	
	CTR	MSG	CTR	MSG
BW	86.1 ± 2.5	51.4 ± 2.4[a]	298.9 ± 13.3	227.6 ± 14.7[b]
wEAT	0.44 ± 0.02	0.49 ± 0.03	0.81 ± 0.04	1.52 ± 0.24[c]
Cell area	1578 ± 65	789 ± 31[b]	2061 ± 98	2888 ± 274[c]
BAT	0.11 ± 0.01	0.16 ± 0.01[c]	0.07 ± 0.01	0.09 ± 0.02
CORT	1.7 ± 0.3	5.6 ± 0.5[b]	6.7 ± 0.7	14.9 ± 0.6[c]
LEP	2.27 ± 0.32	7.97 ± 2.01[c]	8.37 ± 0.99	25.86 ± 3.19[b]
GLU	1.01 ± 0.06	0.99 ± 0.32	1.22 ± 0.03	1.24 ± 0.05
INS	0.27 ± 0.03	0.32 ± 0.04	1.78 ± 0.53	4.73 ± 0.55[b]
TG	0.82 ± 0.05	0.81 ± 0.09	0.79 ± 0.11	1.06 ± 0.19[c]

3.2. Pro-adipogenic and beige cell lineage genes expression in prepubertal and adult wEAT pads

Results from mRNA expression levels of the two main pro-adipogenic markers, GR and MR, in wEAT from prepubertal individuals indicated that no any difference was found between CTR and MSG rats (Fig. 2, panel A). Conversely, wEAT GR and MR mRNAs were both significantly lower in wEAT pads from adult MSG than CTR-litters donors (Fig. 2, panel B).

In wEAT pads from prepubertal rats, while the mRNA levels of PGC1α and CIDEA were similar in CTR and MSG rats (Fig. 3, panels A and B, respectively), interestingly, UCP1 gene expression levels was several fold lower in MSG than CTR specimens (Fig. 3, panel C).

Finally, in MSG pads from adult rats, while relative expression of PGC1α gene was significantly lower (Fig. 3 panel D), conversely, those of

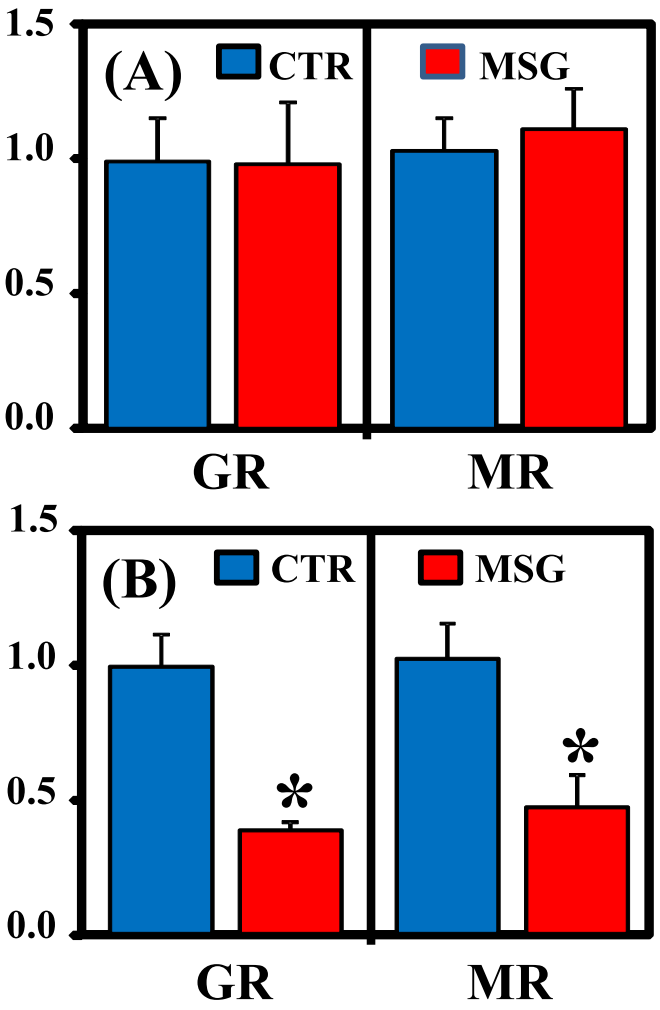


Fig. 2. mRNA expression levels (in arbitrary units; AU) of two main pro-adipogenic, GR and MR, genes in fresh wEAT pads from prepubertal (A) and adult (B) CTR and MSG rats. Values are means ± SEM. ($n = 5/6$ pads per group; * $p < 0.05$ or less vs. CTR values).

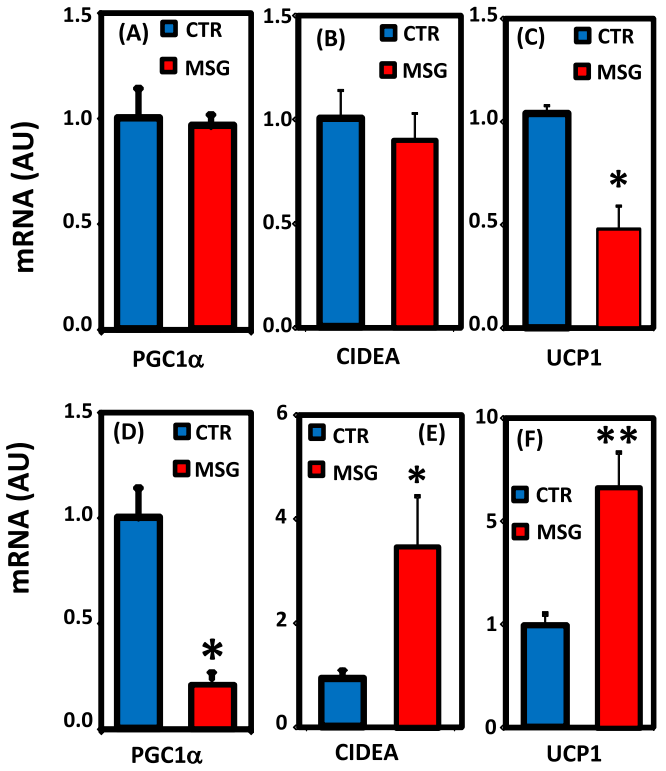


Fig. 3. mRNA expression levels of beige cell lineage (PGC1α, CIDEA and UCP1) markers in fresh wEAT pads from CTR and MSG rats, on prepubertal (panels A-C, respectively) and adult (panels D-F, respectively) ages. Values are means ± SEM. (n = 5/6 pads per group; * $p < 0.05$ and ** $p < 0.01$ vs. CTR values).

CIDEA and UCP1 genes were significantly higher than those found in CTR pads (Fig. 3, panels E and F, respectively).

3.3. Cold-induced browning activation in CTR and MSG rats on different ages

In this set of experiments, we explored the in vivo browning capacity in hypercorticosteronemic and control-litter rats on both ages. To achieve this aim, individually-caged rats on both ages were 7 day-maintained at either room (basal conditions; BC) or cold (4 °C; cold conditions; CC) temperature conditions. Data indicate that in BC, MSG rats on both ages displayed significantly lower BW, and exposition to CC reduced BW in both rat-groups, regardless of age (Table 3).

Regarding FI, NPY-deficient MSG rats were hypophagic in BC and CC, and cold exposure significantly increased (vs. BC values) FI in CTR (on both ages) and in 37 day-old MSG animals only (Table 3). On age 37 days, wEAT pad pass were similar in CTR and MSG rats in BC, and both mass values decreased similarly after cold exposition (Table 3).

With respect to BAT pad mass, data indicate that 37 day-old MSG rats in BC displayed enlarged pad than CTR-litters ones, and group-differences remained in CC, although both rat groups decreased their BAT pad mass after cold exposure (Table 3). Conversely, in 97 day-old individuals, BAT pad mass in BC was similar in CTR and MSG rats, and CC was able to increase BAT pad mass in both groups examined (Table 3).

Glycemias resulted similar in CTR and MSG rats on either age, and it was not influenced by CC, regardless of the group. While the peripheral levels of TG were similar among 37 day-old CTR and MSG rats, hypertriglyceridemia characterized MSG rats on adult age (Table 3). Finally, hypercorticosteronemia was a characteristic of MSG rats in BC, regardless of age, and CC significantly increased those values in both rat-groups (Table 3).

Cold exposition significantly diminished (CC x MSG interaction; $p <$ 0.05) cell size in both 37 day-old CTR and MSG wEAT pads examined in BC and CC (Fig. 4, panels A-D, and right-side table). Exposure to low temperature of 97 day-old rats, as expected, also resulted effective to significantly decrease (CC x MSG interaction; $p < 0.05$) cell size in pads from CTR rats (Fig. 4, panels E & F, respectively, and right-side table) and also in MSG rat-pads (Fig. 4 G & H, respectively, and right-side table).

Table 3
Body weight (BW; in g), 7 day-average of food daily intake (7dFI; in g), wet white epididymal adipose tissue (wEAT) and brown adipose tissue (BAT) pads weights (in g/100 g BW), and the peripheral levels of GLU (g/L), TG (g/L) and CORT (μg/dL) in control (CTR) and MSG rats on different ages after being 7 day-exposed to either room temperature (basal conditions, BC) or low temperature (cold conditions, CC). (Mean ± SEM; $n = 8$ rats per group; *, $p < 0.05$ vs. CTR-BC values; +, p < 0.05 vs. MSG-BC values; a, $p < 0.000001$, b, $p < 0.01$, c, p < 0.05 vs. age-matched CTR values in similar conditions).

	37 days		97 days	
	CTR	MSG	CTR	MSG
BW-BC	89.6 ± 4.1	59.8 ± 1.6^{a}	312.8 ± 6.3	236.1 ± 6.7^{b}
BW-CC	69.7 ± 2.1*	45.4 ± 2.3^{+}	286.1 ± 7.8*	214.2 ± 5.8 $^{+b}$
7dFI-BC	13.3 ± 0.7	10.7 ± 0.5^{a}	22.4 ± 0.7	18.5 ± 0.7^{c}
7dFI-CC	15.2 ± 0.6*	13.8 ± 0.5^{+c}	25.7 ± 1.2*	19.1 ± 1.3^{c}
wEAT-BC	0.42 ± 0.03	0.46 ± 0.04	0.77 ± 0.05	0.66 ± 0.03
wEAT-CC	0.28 ± 0.03*	0.27 ± 0.02^{+}	1.25 ± 0.06*	1.19 ± 0.13^{+}
BAT-BC	0.11 ± 0.01	0.15 ± 0.01^{c}	0.07 ± 0.01	0.09 ± 0.02
BAT-CC	0.19 ± 0.02*	0.27 ± 0.04^{+c}	0.14 ± 0.02*	0.17 ± 0.03^{+}
GLU-BC	1.03 ± 0.08	0.99 ± 0.33	1.17 ± 0.03	1.05 ± 0.05
GLU-CC	1.02 ± 0.25	0.83 ± 0.11	1.08 ± 0.03	1.07 ± 0.04
TG-BC	0.82 ± 0.05	0.81 ± 0.09	0.79 ± 0.11	0.91 ± 0.19^{c}
TG-CC	0.22 ± 0.06*	0.21 ± 0.02^{+}	0.41 ± 0.16*	0.53 ± 0.09^{+c}
CORT-BC	1.71 ± 0.31	5.64 ± 0.56^{b}	6.74 ± 0.73	14.99 ± 1.58^{c}
CORT-CC	8.3 ± 0.6*	8.8 ± 1.8^{+b}	17.1 ± 2.7*	26.6 ± 6.9^{+b}

No group- nor temperature-dependent changes for PGC1α mRNA levels were noticed (Fig. 5, panel A). Conversely, a significant reduction in CIDEA mRNA values had taken place in MSG rats in CC (Fig. 5, panel B). Regarding UCP1 (both, gene expression and protein level), this marker was enhanced 7 days after cold exposition in CTR rats only; importantly, UCP1 (gene expression and protein) was several fold lower in MSG than in CTR pads analyzed in BC, and cold exposure did not induce any significant UCP1 change in MSG tissues (Fig. 5, panels C and D).

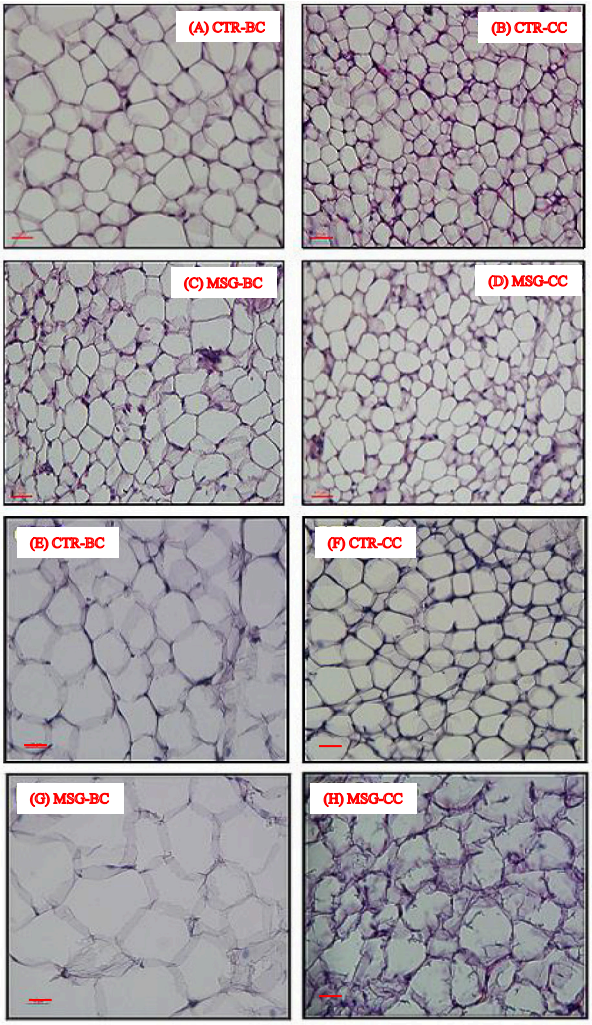
Finally, data analyses from genes expression (PGC1α, CIDEA and UCP1) in wEAT pads from 37 day-old rats indicated that a significant CC × MSG interaction occurred for both, CIDEA and UCP1 genes (see Fig. 5, panels B and C, respectively).

Respecting data from gene expression levels in wEAT pads from 97 day-old individuals, they indicate that in BC, wEAT pad PGC1α mRNA levels were lower in those from MSG rats examined in BC, and CC enhanced it in both groups examined (Fig. 6, panel A). Regarding CIDEA mRNA levels, they were higher in MSG than CTR pads; and cold exposure similarly enhanced, vs. respective BC values, this marker levels in both groups studied (Fig. 6, panel B). No CC x MSG interaction was evinced for both above mentioned genes.

Finally, in BC both mRNA and protein UCP1 levels were significantly higher in MSG than in CTR pads (Fig. 6, panels C and D, respectively), wherein UCP1 gene levels displayed a significant CC × MSG interaction (Fig. 6, panel C). In CC a significantly increase in UCP1 (mRNA and protein) values was noticed in CTR and MSG rats (Fig. 6, panels C and D, respectively); although UCP1 protein resulted greater in MSG than in CTR tissues (Fig. 6, panel D).

3.4. Cold-induced changes in GR mediators in wEAT pads from adult CTR and MSG rats

Due to data found in browning-inducer factors in wEAT pads submitted to different temperature conditions (data depicted in Section



Prepubertal		Adult	
CTR-BC	MSG-BC	CTR-BC	MSG-BC
1,284±102	1,135±101	1,997±116	2,988±286
CTR-CC	MSG-CC	CTR-CC	MSG-CC
837±57*	487±24*	1,185±99*	2,027±123*

Fig. 4. Representative fields of wEAT pads from 37 day-old (panels A-D) and 97 day-old (panels *E*-H) CTR and MSG rats 7-day exposed to room temperature (Basal Conditions; BC) or cold temperature (Cold Conditions, CC) conditions. Cells were stained with hematoxylin-eosin, cells show cytoplasmic rim of mature adipocytes. Cell area (in μm^2) variations, accordingly to temperature conditions (in both groups), are depicted in the right-side table. Values are Means ± SEM; n = 8 pads per group; *p < 0.05 CC vs. respective BC values. (Objective 40×; scale bar in red represents 10 μm).

3.3), and in down-regulation of GR/MR noticed in adult MSG rats in BC, we determined whether different mediators involved in GR function varied in fat pads when adult MSG rats were exposed to either ambience (BC) or low (CC) temperature conditions. For this purpose, gene expression levels of FK506 binding protein 5 (Fkbp5, a protein able to interact functionally with GR), glucocorticoid-induced leucine zipper (GILZ; a cytosolic protein wherein its gene expression is activated by GCs) and serum and glucocorticoid-regulated kinase 1 (SGK1, a kinase that its transcription is acutely induced by GCs) were measured in wEAT pads from 97-day old CTR and MSG rats in both BC and CC.

Data indicated that in fat pads from adult CTR rats, the expression levels of all three mediators were significantly enhanced, and significant CC x MSG interaction was evinced for the above mentioned genes after animal exposition to low temperature conditions (Fig. 7, panels A-C, respectively). Conversely, these mediators did not vary in fat pads from adult MSG rats exposed to low temperature conditions (CC) (Fig. 7, panels A-C).

3.5. GR and MR genes expression in wEAT pads from GC-deplete (ADX) and GC-replete (SHX) adult non obese rats

In a preliminary set of experiments, we evaluated the mRNA levels of wEAT GR and MR from SHX and ADX adult (97 day-old) rats studied at room temperature. Data indicated (Fig. 8) that GR mRNA levels in ADX rat pads (individuals displaying non detectable plasma CORT concentrations) were significantly ($p < 0.05$) greater than those found in SHX-litter pads (rats displaying 5.89 ± 1.71 μg/dL CORT). Conversely, no group-differences in MR gene expression levels were noticed (Fig. 7).

3.6. Cold-induced browning activation in GC-deplete (ADX) and GC-replete (SHX) adult non obese rats

Additionally, in a separate set of experiments (see Materials & Methods, Experiment 4), 7 days exposition of SHX and ADX rats to either room temperature (BC) or 4 °C environment (CC), we noticed that plasma CORT significantly ($p < 0.05$) increased in SHX rats (Table 4); whereas in ADX ones, the circulating CORT levels remained undetectable, regardless of temperature condition (Table 4). In both groups of animals exposed to CC, a significant ($p < 0.0001$) reduction in BWs (vs. respective values recorded in BC) was found (Table 4), although it occurred in a different manner: –20 % for SHX rats and –12 % for ADX ones (approximately). CC also significantly ($p < 0.005$ vs. respective values obtained in BC) augmented rat FI (Table 4). Both groups of rats significantly ($p < 0.05$) loose wEAT pad mass in CC (Table 4). It should be stressed that for those parameters mentioned above, statistical analyses revealed no any significant CC x ADX interaction. However, both groups of rats significantly ($p < 0.003$) gained BAT pad mass in CC (Table 4); interestingly, a significant ($p < 0.002$) CC x ADX interaction had taken place for such a BAT pad mass variation.

With respect to wEAT pad PGC1α mRNA levels, they were significantly lower in specimens from ADX rats examined in BC, and CC significantly enhanced it in both groups examined, although in a lesser

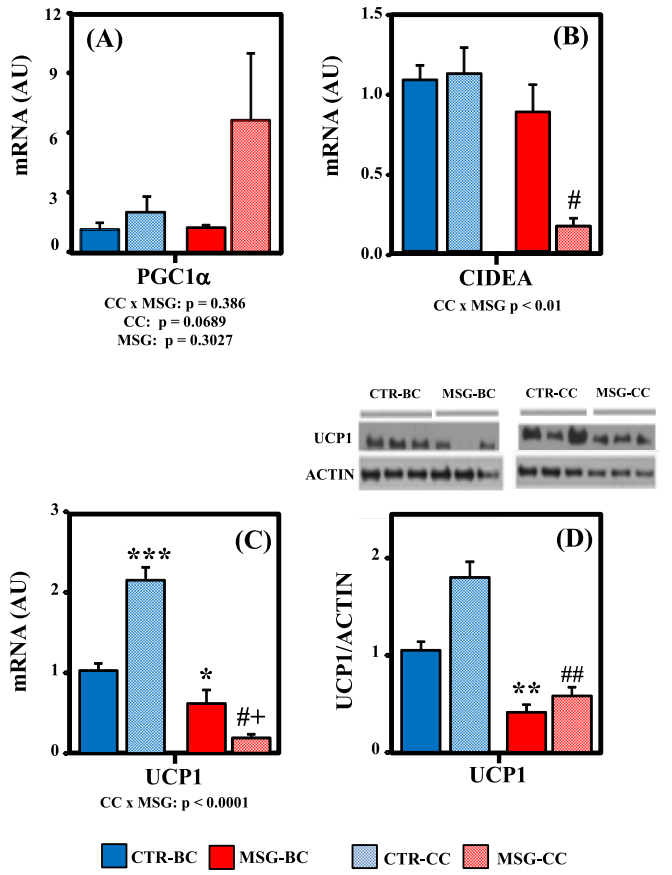


Fig. 5. PGC1α, CIDEA, and UCP1 genes expression (panels A, B and C, respectively) and UCP1 protein levels (panel D) in wEAT pads from 37 day-old CTR and MSG rats 7 day-exposed to room (Basal Conditions: BC) or low (Cold Conditions: CC) temperature conditions. Values are means ± SEM (n = 4/5 pads per group-conditions). A representative plot of UCP1 protein is shown. Two-way ANOVA was performed for factors (MSG and Cold) and interaction (MSG x Cold) analysis followed by Sidak post-test for data from panels A, B and C. Student's t-test was used for statistical analysis for data from panel D. *, $p <$ 0.05 vs. CTR-BC; **, p < 0.05 vs CTR-BC; ***, p < 0.0001 vs CTR-BC; +, p < 0.05 vs. MSG-BC; ++, p < 0.002 vs. MSG-BC; #, p < 0.001 vs. CTR-CC; ##, p < 0.0003 vs. CTR-CC.

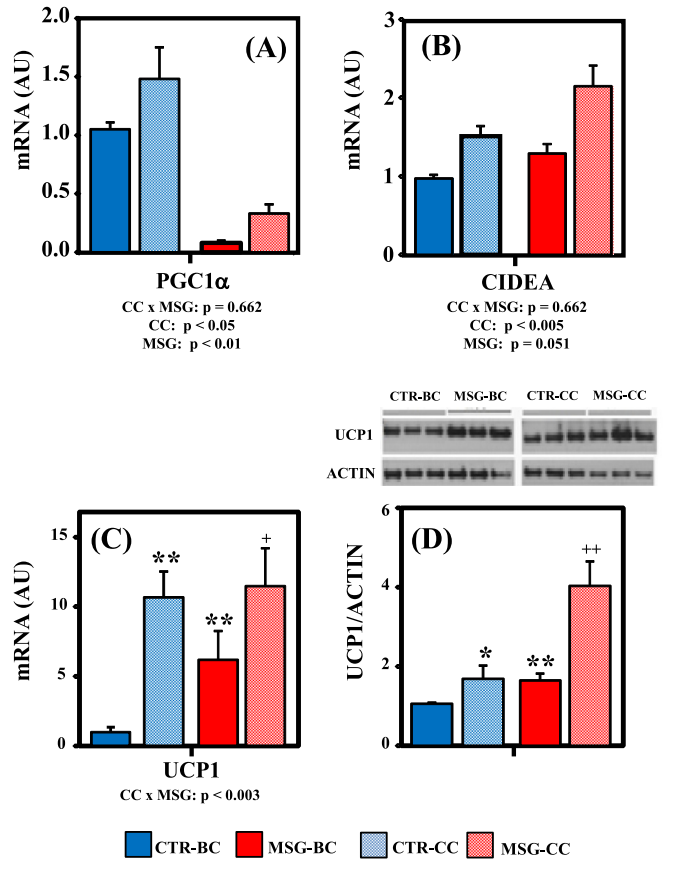


Fig. 6. PGC1α, CIDEA and UCP1 genes expression (panels A-C, respectively) and UCP1 protein levels (panel D) in wEAT pads from 97 day-old CTR and MSG rats 7 day-exposed to room (Basal Conditions: BC) or low (Cold Conditions: CC) temperature conditions. Values are means ± SEM (n = 4/5 pads per group-conditions). A representative plot of UCP1 protein is shown. Two-way ANOVA was performed for factors (MSG and Cold) and interaction (MSG x Cold) analysis followed by Sidak post-test for data from panels A, B and C. Student's t-test was used for statistical analysis for data from panel D.*, $p < 0.05$ vs. CTR-BC; **, $p < 0.02$ CTR-BC; +, $p < 0.05$ vs. MSG-BC; ++, $p < 0.03$ vs. MSG-BC.

extent in ADX pads (Fig. 9, panel A). CIDEA mRNA level was higher in ADX than in SHX pads in BC; and cold exposure significantly enhanced, vs. respective BC values, CIDEA mRNA level in SHX pads only (Fig. 9, panel B).

In BC, mRNA and protein UCP1 levels were significantly greater in ADX than in SHX pads (Fig. 9, panels C and D, respectively). Cold exposition (CC) significantly increased (vs. respective BC values) UCP1 mRNA levels in both SHX and ADX rat-pads (Fig. 9, panel C). A significant CC x ADX interaction applied for UCP1 mRNA levels only (Fig. 9, panel C). As for wEAT pad UCP1 protein content, while it was significantly enhanced in SHX pads exposed to CC (Fig. 9, panel D); conversely, ADX pads already displayed significantly elevated UCP1 protein pad content, regardless of temperature condition (Fig. 9, panel D).

4. Discussion

Our studies demonstrated that white adipose tissue pads (namely, the epididymal one) from prepubertal MSG rats, characterized by a relatively short period of hypercorticosteronemia with no local GR/MR down-regulation, displayed absent local browning capacity or at least expressed it at its minimal activity. wEAT pad UCP1 production (mRNA and protein) was several times lower than that displayed by CTR-litter rats. Conversely, when hypercorticosteronemia was chronically established at high levels, as occurred in adult MSG rats, their wEAT pads displayed down-regulation in GR/MR expression and almost abrogated GR/MR function cytosolic mediators [34–36] after exposed to cold, consequently pad beiging capacity reappeared. These observations indicate that substantial GC excess inhibition on wEAT pad browning capacity took place when local corticoid receptors were fully expressed (MSG rats at prepubertal age) but not when they were at least partly functional (adult MSG rats). This GC dependency was later corroborated by examining beiging capacity in wEAT pads obtained from both non-obese SHX and ADX adult donors. wEAT pads from ADX rats were characterized by the presence of high UCP1 (gene and protein) levels compared to those found in SHX-litter. wEAT pads from ADX rats displayed an UCP1 increase over the respective baseline (BC) values. Important to stress is the observation that ADX wEAT pads showed a clear GR gene up-regulation, an effect that took place within a non-detectable corticosteronemia context. All together, these data could be indicative of established glucocorticoid-resistance at the wEAT level from the adult MSG rat that enabled development of a non-shivering thermogenic process that could mitigate, at least partially, the unhealthy adult MSG rat phenotype.

Despite the controversy whether GCs inhibit the thermogenic process [15], our study fully supports data from previous studies [15,31–33], indicating that GCs act by lowering mammals' thermogenic activity,

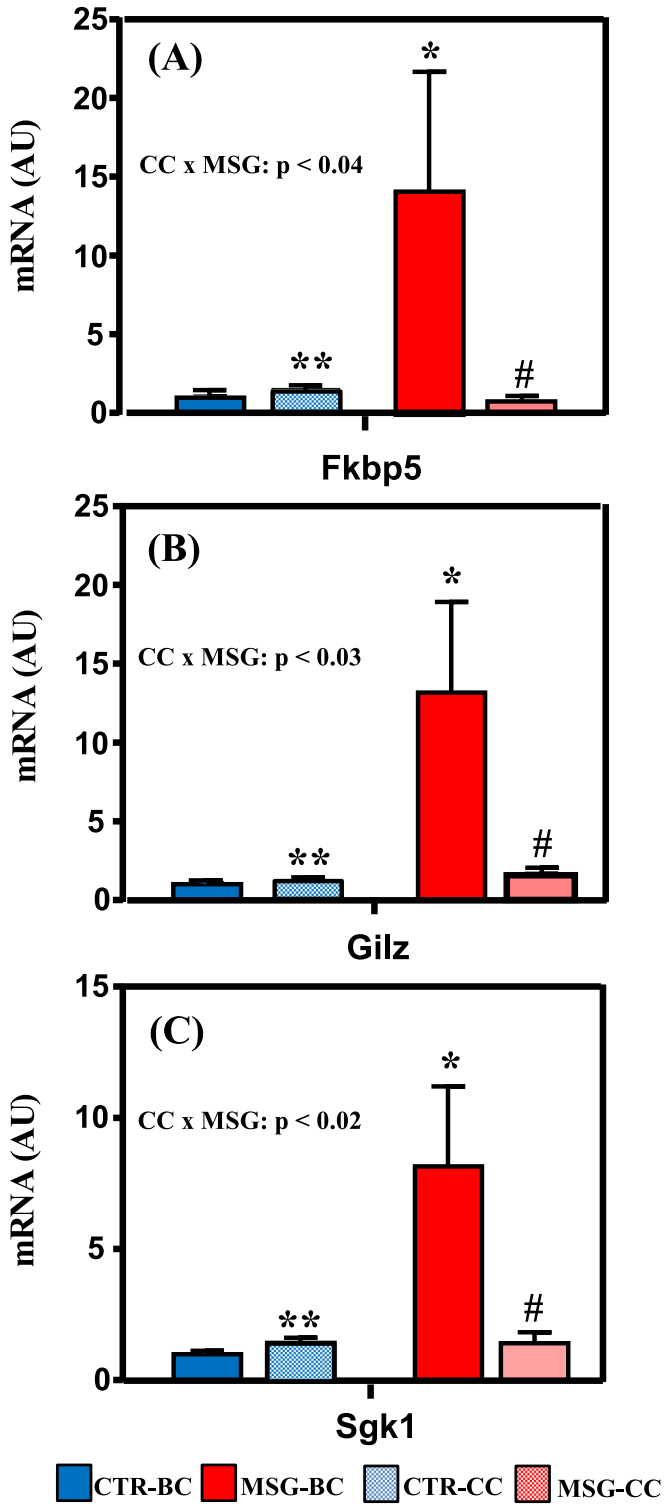


Fig. 7. FKBP5, GILZ and SGK1 genes expression (panels A, B and C, respectively) in wEAT pads from 97 day-old CTR and MSG rats 7 day-exposed to room (Basal Conditions: BC) or low (Cold Conditions: CC) temperature conditions. Two-way ANOVA was performed for factors (MSG and Cold) and interaction (MSG x Cold) analysis followed by Sidak post-test for data from panels A, B and C. Values are means ± SEM (n = 4/5 pads per group-condition). *, $p < 0.05$ vs. CTR-BC; **, $p < 0.05$ vs. CTR-CC; #, $p < 0.05$ vs CTR-CC.

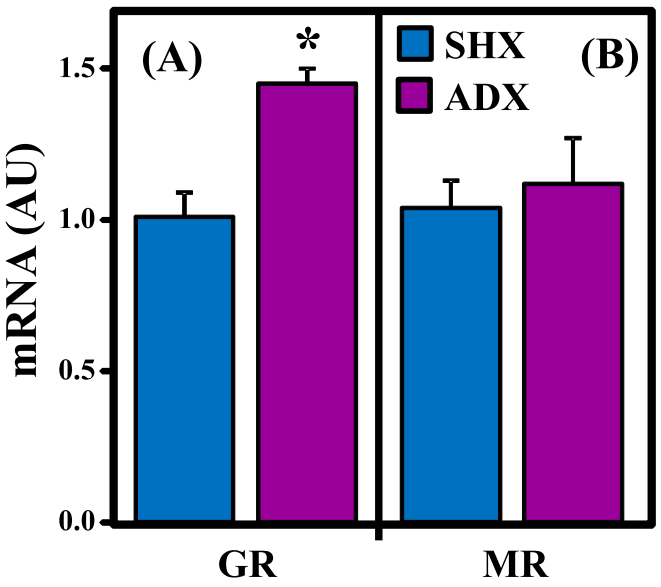


Fig. 8. mRNA expression levels (in arbitrary units; AU) of GR (A) and MR (B) genes in fresh wEAT pads from GC-replete (SHX) and GC-deplete (ADX) rats. Values are means ± SEM. ($n = 5/6$ pads per group; * $p < 0.05$ vs. SHX values).

Table 4
Body weight (BW; in g), 7 day-average of food daily intake (FI; in g), wet white epididymal adipose tissue (wEAT) and brown adipose tissue (BAT) pads weights (in g/100 g BW), and the peripheral levels of CORT (μg/dL) in sham (SHX) and adrenalectomized (ADX) adult non-obese rats after 7 day-exposed to either room temperature (basal conditions, BC) or low temperature (cold conditions, CC). (Mean ± SEM; $n = 6$ rats per group; a, $p < 0.0001$ vs. age-matched SHX-BC and ADX-BC, respectively; b, $p < 0.005$ vs. age-matched SHX values in similar conditions; c, $p < 0.05$ vs. age-matched SHX-BC and ADX-BC, respectively, d, $p < 0.003$ vs. age-matched SHX-BC and ADX-BC, respectively; *, $p < 0.05$ vs. SHX-BC values).

	BC		CC	
	SHX	ADX	SHX	ADX
BW	324.2 ± 11.2	298.5 ± 6.1	261.4 ± 7.2[a]	262.4 ± 5.3[a]
FI	23.9 ± 0.7	17.1 ± 0.5[b]	27.7 ± 0.4	20.5 ± 0.6[b]
wEAT	0.99 ± 0.12	0.81 ± 0.09	0.76 ± 0.11[c]	0.62 ± 0.09[c]
BAT	0.611 ± 0.002	0.069 ± 0.003	0.176 ± 0.028[d]	0.116 ± 0.001[d]
CORT	6.07 ± 1.59	ND	17.11 ± 3.21*	ND

regardless of the manipulation of GC excess applied. We previously demonstrated that the WAT pad studied in prepubertal (37 day-old) MSG rats, individuals in which GC excess was moderate, showed increased adipogenic capacity due to a hyperplastic expansion of their WAT pads [14], a mechanism considered a metabolic protective process [14]; conversely, when hypercorticosteronemia is chronically established, such as in the adult MSG rat, its WAT pad was characterized by reduced precursor cell differentiation capacity (impaired APC competency) into the white adipocyte lineage [14]. Resulting in an unhealthy hypertrophic WAT mass expansion [13,14]. Moreover, our previous studies support that age-dependent changes in WAT function are GC excess time-dependent given that by normalizing GC peripheral levels (ADX combined with GC replacement therapy) at adult age, retarded adipogenesis is counteracted and consequently, MSG rats become able to correct dysmetabolism [13].

We now demonstrated that in prepubertal MSG rats, individuals fully expressing GR/MR genes in wEAT pads, short-term GC excess could be exerting a clear inhibitory effect on local fat-pad beiging capacity. Conversely, wEAT pads in which GR/MR genes are not fully expressed (down-regulated, and consequently only partly functional) could indicate an incomplete inhibitory GC effect, thereby enabling recovery of a

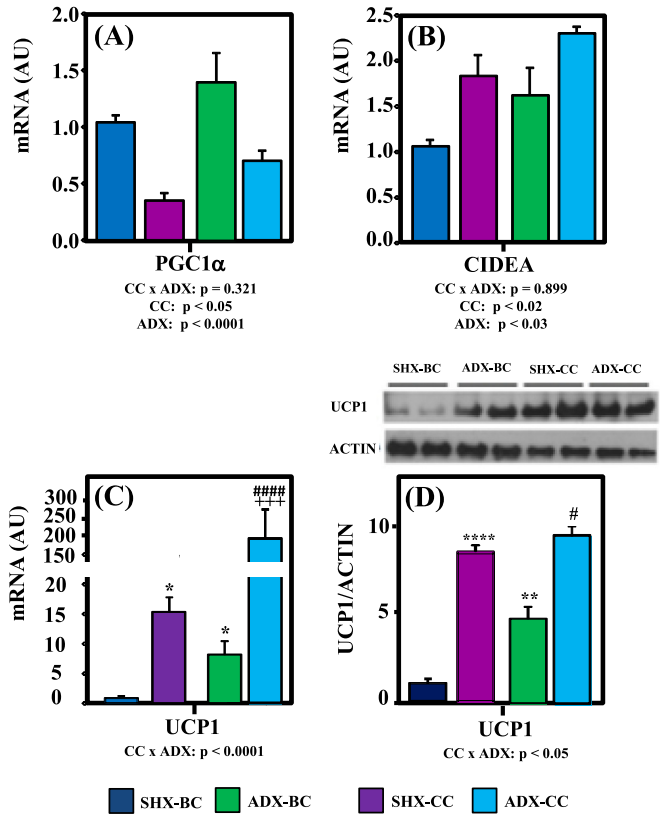


Fig. 9. PGC1α, CIDEA and UCP1 genes expression (panels A, B and C, respectively) and UCP1 protein (panel D) levels in wEAT pads from 97 day-old SHX and ADX rats 7 day-exposed to either room (Basal Conditions: BC) or low (Cold Conditions: CC) temperature conditions. Values are means ± SEM ($n = 4$/5 pads per group-conditions). A representative plot of UCP1 protein is shown. Two-way ANOVA was performed for factors (MSG and Cold) and interaction (MSG x Cold) analysis followed by Sidak post-test for data from panels A, B, C and D. *, $p < 0.05$ or less vs. SHX-BC; **, $p < 0.001$ vs. SHX-BC; ****, $p < 0.0005$ vs. SHX-BC; +++, $p < 0.0002$ vs. SHX-BC; #, $p < 0.03$ vs. ADX-BC; ####, $p < 0.0001$ vs. SHX-CC).

low local pad beiging capacity, as observed in the 97-day-old hypothalamic obese (MSG) rat.

These statements are strongly supported by drastic local pad reduction in the expression levels of the key mitochondrial marker UCP1, in wEAT from 37-day-old MSG rats examined after cold temperature exposition, a process accompanied by remarkably reduced CIDEA mRNA levels as well. Conversely, pads from the chronic hypercorticosteronemic (adult) MSG rats exposed to low temperature display an increase in the mRNA levels of CIDEA [37] and UCP1, as well as UCP1 protein [38,39]. Interestingly, cold-induced UCP1 protein increase was more pronounced than that of the expression of its gene. It must be stressed that only UCP1 protein, but not mRNA, are able to induce mitochondrial thermogenesis [23].

Studies trying to clarify the relevance of GR in AT's biology used models of GR knockout; namely, regarding adaptive thermogenesis, most of them were focused on BAT. Indeed, in one study authors found that BAT^{GRKO} mice displayed no changes in BW, FI, AT histology and BAT thermogenic genes expression, thus suggesting that GR is not essential for energy control [40]. However, in Adipocyte-specific GR KO (AGRKO) mice, the GC-induced BAT whitening in WT mice was reversed in GR-deficient mice, without any change in UCP-1 and respiratory chain protein levels [41,42]. The latter do not concord with that found by Hayashi et al. who showed that expression of UCP-1, DIO-2 and PRDM16 is increased in BAT from AGRKO mice [41]. These authors suggested that this effect could be due to a not-impaired BAT lipolysis, contrary to what was observed in other tissues. It is known that the main source of energy for adaptive thermogenesis comes from B-oxidation of fatty acids. In models of Cushing's syndrome, due to glucocorticoid administration, GR deletion decreased NEFA and glycerol peripheral levels. Indeed, this deletion of GR reversed GC-induced adipose triglyceride lipase (ATGL) in inguinal WAT (iWAT) [41,43]. Interestingly, Hayashi et al. reported that the decreased ATGL was accompanied by inhibition of lipogenic enzymes, such as LPIN1, FASN and ACACA [42]. Even that, iWAT explants from AGRKO mice developed a low enough response to isoproterenol to stimulate cold-induced thermogenesis [40]. In the present study we have no evaluated lipid metabolism, thus it could be of relevance to further develop approaches to better understand the relationship between the GCs-GR system, lipid metabolism and WAT browning.

The decreased key thermogenic marker (UCP1) gene expression in prepubertal MSG wEAT pads could be related to a local pad inhibited post-corticoid receptor signaling mechanism. Indeed, reduced PGC1α mRNA in epicardial white adipose pad but not in other white pads has been described in DMT2 patients with coronary artery disease [44], thus indicating reduced development of brite-like adipocytes. However, in a GC excess context, our data agree with those showing that white adipose tissue CIDEA gene increase after synthetic corticoid treatment in humans [45]; in turn, high CIDEA signaling could be able to enhance the expression levels of the main thermogenesis-reflecting brown/beige adipocyte factor, UCP1 [46]. These results could point, at least in part, to a compensatory effect to turn on browning capacity of white adiposity from adult MSG rats. It is accepted that individuals' cold exposition (a sympathetic stimulus), among others increasers of UCP1 production (such as leptin and glucagon release during exercise, thyroid hormones, fibroblast growth factor 2, circadian rhythm variations, as well as several factors dependent on the quality of food intake [47]), is highly effective for browning activation [48], although it is also claimed that this stimulus does not seem to be fully dependent on β3-AR stimulation [49]. In this regard, it should be mentioned that in MSG rodents, reduced catecholamine production has been reported [10], although we did not find any change in wEAT pad β3-AR gene expression (data not shown). Moreover, it is known that there is functional communication between AT and different tissues. Among them, muscle is one of the main target of GCs biological action, where it acts by promoting protein catabolism. In turn, several myokines secreted from muscle could modulate, positively or negatively, non-shivering thermogenesis; indeed, it has been claimed that irisin, IL-6, follistatin, and meteorin-like stimulate this process [50]. Information on these myokines in patients with Cushing's Syndrome is scarce. IL-6, the most studied of these myokynes, is elevated in patients with Cushing's Syndrome; however, it is important to highlight that a relevant source of IL-6 secretion is WAT [51]. Regarding irisin, a decrease in its plasma levels has been found in CS patients [52]; conversely, in spite of follistatin and meteorin-like, to our knowledge, there are no studies on these myokines in patients with Cushing's Syndrome . Although in the present study we did not measure these myokines, it cannot be ruled out that at least partially they could contribute to increase thermogenesis in adult MSG animals.

In addition to changes in wEAT pad biomarkers, the effectiveness of cold temperature exposition of rats was evaluated by modifications in the phenotypes from both 37 day- and 97 day-old rats. We found not only changes in BW and AT (wEAT and BAT pads) masses but also in local pad white adipocyte size reduction, regardless of the rat group (CTR and MSG) analyzed. When we examined the adult MSG phenotype from rats 7-day exposed to low temperature we found that they in general responded to cold stimulus in a somewhat similar fashion to the CTR litter. Undeniably, the animals' cold exposition resulted in a significant and quite similar reduction in BWs; however, the 7 day-average of FI was lower in the already hypophagic [3–6] MSG rats than in the CTRs. No significant loss in wEAT pad mass was observed between the two experimental groups at different ages. However, as expected [53], BAT masses were significantly augmented in a similar fashion in CTR

and MSG rats exposed to cold temperature. Moreover, adrenal CORT hyper-response to cold is an invariable characteristic in rodents [54]. Also, GLU and TG peripheral levels were unvaried and diminished [55], respectively, in a similar fashion in both rat groups at both ages studied.

Thus, despite in vivo chronic exposition to endogenous GC excess, while GR/MR partial down-regulation was established at local fresh fat pad level, the wEAT from adult MSG rats remained able to retain a modest health-alleviatory browning capacity [40]. Moreover, a deleterious GC excess in rodents (3–4 weeks submitted to corticosterone treatment) on BAT function was reported [56,57]. In fact, those rats developed deep dysmetabolism and decreased rat BAT mitochondrial content, switching its BAT phenotype to a white-like one [56], thereby aggravating the metabolic consequences induced by GC excess on WAT functionality. Both MR and GR are widely accepted key pro-adipogenic markers. However, whereas Caprio et al. [2] claimed a key role for MR activation in the new adipogenesis process, Lee & Fried claimed this role for GR [58]. Nevertheless, whether GCs inhibit mitochondrial UCP1 production and if so, through which signaling element they do remains largely unclear. We have now assessed, by combining experiments using both transient/chronic hypercorticosteronemic (MSG) rats and those lacking endogenous corticosterone (ADX) that GR, as well as its cytosolic mediators [34–36], could be representing a good candidate for mediating an inhibitory GC effect on mitochondrial UCP1 production by inducing poor non-shivering thermogenesis when corticoid receptors are fully functional. However, it remains to be determined whether the specific intracellular receptor GR alpha or GR beta [59] (or both) is responsible for this effect.

5. Conclusions

We conclude that poor browning capacity in white adipose tissues from hypercorticosteronemic MSG rats could represent an important disability of this phenotype to dissipate excess energy, since increased WAT mass in the MSG rat involves the development of relevant mitochondrial-dependent dysmetabolism. Chronic MSG hypercorticoidism induces a deep disruption due to ceaseless hypothalamo-pituitary-adrenal axis hyperactivity combined with hyperinsulinemia which together ensure constant unhealthy WAT mass expansion. We hypothesize that MSG WAT-resident cells when needed, might be minimally activated to counteract chronic lipid-dependent energy accumulation, consequently resulting in substrate-dependent ATP over-accumulation [60]. Our study strongly suggests that reduced WAT beiging capacity in the transient/chronic hypercorticosteronemic rat could perpetuate impaired non-shivering thermogenesis, and consequently its deep unhealthy phenotype. This information could be relevant for a future approach to treatments in human obesity-related patients seeking to ameliorate the dysmetabolism occurring in hypothalamic-obese and other obese phenotypes.

CRediT authorship contribution statement

F.F.M. performed the research. A.A. gave her continued support on different techniques employed in the study, mainly in qPCR assays and analyses and histological data analysis. A.E.H. collaborated with F.F.M., mainly in performing experiments; D.C. performed adrenal surgeries; E. S. and A.G. conceived and designed the experimental designs; M.G.Z., E. S. and A.G. analyzed all data; E.S. wrote the manuscript. All authors participated in the revision and correction of the manuscript. All authors read and approved the final version of the manuscript.

Declarations of competing interest.

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Declarations of competing interest

The authors declare no conflict of interest.

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