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BRIEF REPORT

HSD1 and AQP7 short-term gene regulation by cortisone in 3T3-L1 adipocytes

Tania Quesada-López[a], Laura González-Dávalos[b], Enrique Piña[c], and Ofelia Mora[b]

[a]Licenciatura en Nutrición, Facultad de Ciencias Naturales, Universidad Autónoma de Querétaro (UAQ), Juriquilla, Mexico; [b]Laboratorio de Rumiología y Metabolismo Nutricional (RuMeN), Secretaría de Posgrado, Facultad de Estudios Superiores-Cuautitlán (FES-Cuautitlán), Universidad Nacional Autónoma de México (UNAM), Juriquilla, Querétaro, Mexico; [c]Departamento de Bioquímica, Facultad de Medicina, UNAM, CDMX, Mexico

ABSTRACT

Adipose Tissue (AT) is a complex organ with a crucial regulatory role in energy metabolism and in the development of obesity and the Metabolic Syndrome (MS). Modified responses and the metabolism of hormones have been observed in visceral adiposity during obesity, specifically as related with cortisone. The objective of this study was to assess, in the 3T3-L1 adipocyte cell line, the short-term effect of cortisone on the expression of 11β-Hydroxysteroid dehydrogenase 1 (Hsd1), which is responsible for activation of cortisone into cortisol, and for Aquaporin 7 (Aqp7), involved in glycerol transport through the cell membrane. Total RNA (tRNA) and complementary DNA (cDNA) were obtained from cell samples treated with cortisone (0.1, 1, and 10 μM) during different times (0, 5, 10, 15, and 20 min, and 48 h) to quantify the expression of the aforementioned genes by real time PCR employing MnSOD and Ppia as housekeeping genes. There was a time-dependent response of Aqp7, a dose-dependent response of Hsd1, and an increase observed in the expression of both genes during min 1 of treatment (5- and 6-fold, respectively), followed by a decrease during the following 5–10 min ($P < 0.05$). With the 1-μM cortisone treatment, both genes showed cubic tendencies in their expression; the Hsd1 tendency is described by the equation $y = 0.18 \times^3 - 1.65 \times^2 + 3.59x + 1.31$, while the Aqp7 tendency is described by $y = 0.33 \times^3 - 2.67 \times^2 + 4.93x + 1.84$. There are immediate and quantitatively important actions of cortisone on the expression of Aqp7 and Hsd1 in 3T3-L1 adipocytes.

Introduction

Obesity has become the major health challenge worldwide. In its most simplified version, obesity is an imbalance in Adipose Tissue (AT) with a principal rate of synthesis of triacylglycerols (TAG) compared with their degradation. TAG accumulation in visceral deposits (omental and mesenteric), identified as central obesity, confers an increased risk for triggering metabolic diseases,[1-3] regardless of total TAG accumulation in the body, and even the accumulation of subcutaneous TAG deposits, considered protective.[2-4] The similarity between the metabolic abnormalities observed in central obesity and the presence of an excess of glucocorticoids (GC) as in, for example, Cushing's syndrome, have led to the proposal that an alteration in the metabolism or in the action of GC on visceral AT plays an essential role in the pathogenesis of metabolic diseases associated with central obesity.[5] Paradoxically, under physiological conditions, the action of GC promotes lipolysis, thus the release of glycerol and fatty acids from AT.[6,7] While

excess GC does cause obesity and diabetes, circulating GC levels are normal in obese subjects.[8] This led to the knowledge that the physiological action of GC on AT depends, in part, on their circulating levels and also, and importantly, on their tissue activation prior to impacting on their receptor, which occurs through 11β-Hydroxysteroid dehydrogenase 1 (Hsd1), the latter converting inactive cortisone into active cortisol.[9-11] The Matsuzaki et al.[12] experiment solved this puzzle: it is the overexpression of Hsd1 that results in obesity and the Metabolic Syndrome (MS). In turn, knockout (KO) of Hsd1 protects against the obesity and hyperglycemia induced with a high-fat diet.[13]

A protein that competes with HSD1 in its ability to regulate the metabolism of TAG in AT is aquaporin 7 (AQP7).[14,15] The latter is member of the AQP family, that mobilizes H_2O and metabolites through the cell membranes; AQP7 is localized in the membranes of adipocytes and mobilizes H_2O and glycerol through the membranes.[16,17] In Aqp7 KO mice, increases have

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CONTACT Ofelia Mora ✉ ofemora66@unam.mx 🖅 Laboratorio de Rumiología y Metabolismo Nutricional (RuMeN), Secretaría de Posgrado, Facultad de Estudios Superiores-Cuautitlán, Universidad Nacional Autónoma de México, Boulevard Juriquilla 3001, 76230 Juriquilla, Querétaro, México.

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been demonstrated of intracellular glycerol content and TAG, adipocyte growth, and obesity and Insulin Resistance (IR) appear.[16,18,19] Conversely, overexpression of *Aqp7* in 3T3-L1 cells stimulates the release of glycerol, glucose consumption, and the expression of phosphorylated protein kinase B (PKB-p), a well-accepted indicator for insulin sensitivity[20] and the attenuation of IR.[21] Moreover, it is known that dexamethasone (DXM), a synthetic steroid capable of inducing IR,[15] downregulates*Aqp7*[15] and that is necessary for 3T3-L1 differentiation. In particular, obesity can be caused by *Hsd1* overexpression[12] or *Aqp7* KO,[16] and viceversa, *Aqp7* overexpression protects against obesity[1,2] as well as against *Hsd1* KO.[13]

The aim of this study was to compare the action of cortisone on the expression of the two proteins analyzed previously: HSD1 and AQP7. With the intention of controlling the largest number of variables inherent to the study, it was decided to carry out the experiment *in vitro* in 3T3-L1preadipocytes after inducing their differentiation into adipocytes, and to explore the immediate responses to cortisone addition. Our working hypothesis comprised that cortisone favored *Hsd1* expression and reduced *Aqp7* expression in this manner, by means of two complementary mechanisms for strengthening the metabolism of pre-diabetic adipocytes. The results showed, contrary to our hypothesis, the ability of cortisone to induce immediate and nearly parallel mRNA (mRNA) expression for both of the genes-under-study.

Results

Preadipocyte differentiation

Cultured 3T3-L1 cells were induced as described in Methods, and Figure 1a includes Oil Red O-stained dishes to get an idea of adipose conversion efficiency from the adipocytes utilized.

Glucocorticoid response elements (GRE) for Hsd1 and Aqp7genes

The promoter sequences for the genes encoding for *Hsd1* and *Aqp7* were analyzed with the TFM-Explorer computer software to search for GREs. This software identified two potential response elements in each gene at positions −1320 and −253 for *Hsd1* (Fig. 1b) and at −748 and −29 for *Aqp7* (Fig. 1c). The presence of these GREs confirmed that cortisone cancertainly regulate the expression of the genes studied and justified the experiments performed in this work.
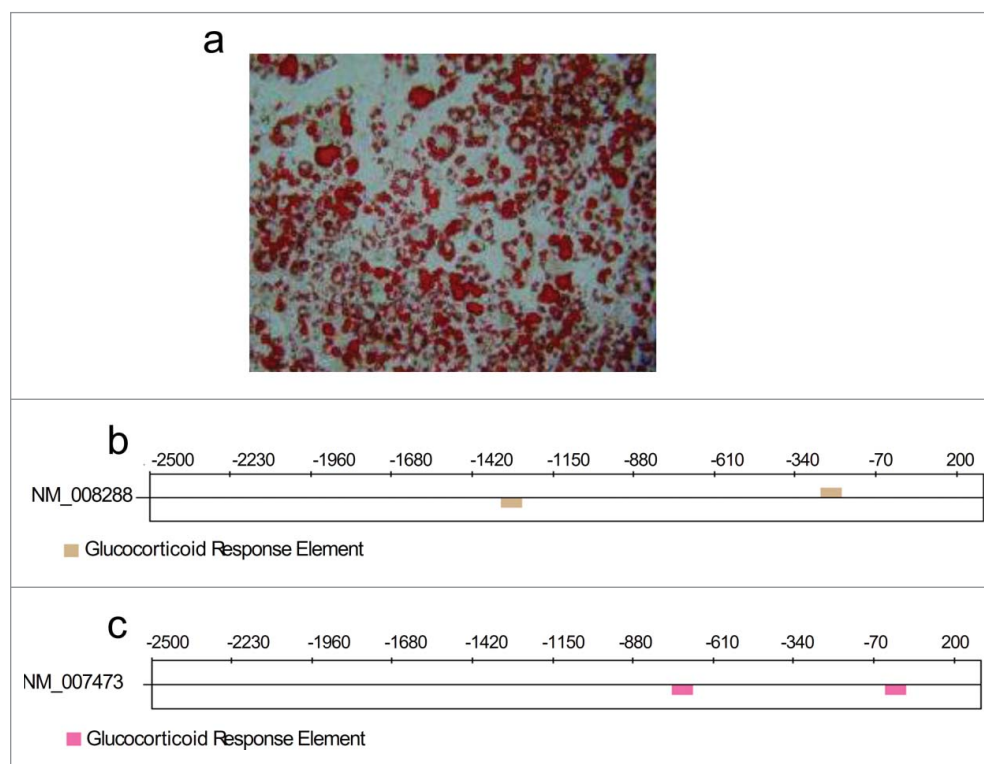

Figure 1. (a) Representative phase-contrast microscopy image for Oil Red O-stained differentiated 3T3-L1 cells. (b) and (c) Predicted sites for possible glucocorticoid response elements (GREs: AGAACANNNTGTTCT) for the genes *Hsd1*(NM_008288) and *Aqp7* (NM_007473).
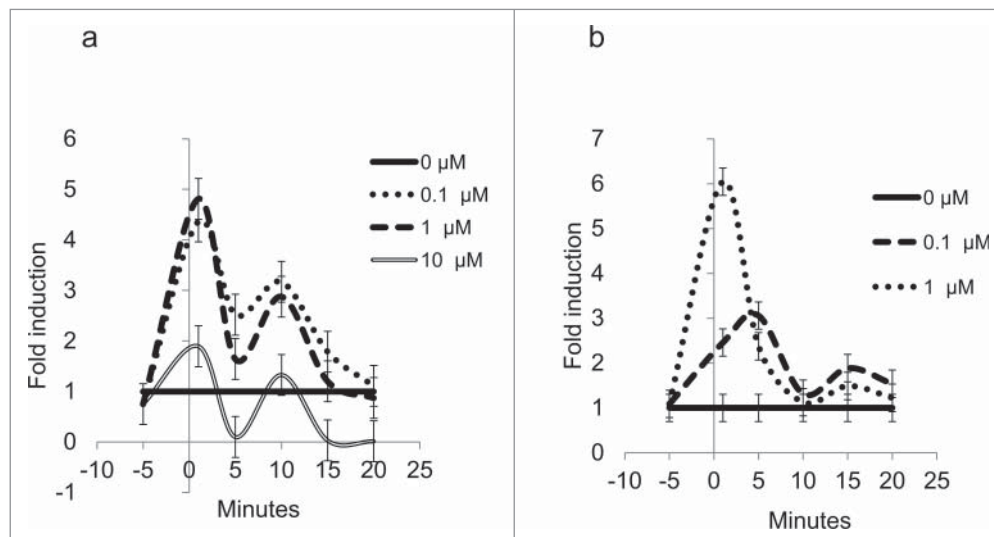
Figure 2. (a) Expression levels for *Hsd1* in 3T3-L1 adipocytes. Lines show the relative expression behavior of mRNA during the first 20 min of the experiment for treatments with 0 μM (continuous line), 0.1 μM (dotted line), 1 μM (dashed line), and 10 μM (double line) cortisone, $P < 0.05$. (b) Expression levels for *Aqp7* in 3T3-L1 adipocytes. Lines show the relative expression behavior of mRNA during the first 20 min for treatments with 0μM (continuous line), 0.1 μM (dotted line), and 1μM (double line). Data for 10 μM are not shown. All error bars represent $\pm$ SEM, $P < 0.05$ ($n = 3$).

Time-dependent effects of cortisone on the expression of mRNA for Hsd1 and Aqp7 in 3T3-L1 differentiated adipocytes

The differentiated 3T3-L1 cells used herein to test the effects of the cortisone are depicted in Figure 1a. To test the metabolic response on the expression of *Hsd1* and *Aqp7*, an experiment employing three different concentrations of cortisone (0.1, 1, and 10 μM) during six different time intervals (1, 5, 10, 15, 20 min, and 48 h) was performed. The cortisone treatments led to an immediate and significant increase ($P<0.05$) in a waved manner in mRNA expression for both genes during the experiment (Fig. 2a and 2b). The expression of the *Hsd1* mRNA that increased during min 1 was observed with 0.1, 1, and 10 μM cortisone, but the response was greater with the 0.1- and 1-μM treatments, with 4.5- and 4.8-fold induction, respectively. It is noteworthy that the three concentrations exhibited a similar waved manner (as depicted in Fig. 2a), and it should be noted that the response with 0.1 and 1 μM after 20 min returned to control values, while mRNA expression with 10 μM was diminished ($P < 0.05$).

In the case of *Aqp7* (Fig. 2b), mRNA expression was increased 6-fold during min 1 with 1-μM cortisone and 3-fold after min 5 with 0.1-μM cortisone, but similar expressions of mRNA after min 5 until the end of the experiment were observed with both treatments ($P < 0.05$). Quantification of mRNA relative expression by quantitative Polymerase Chain Reaction (qPCR) was not possible with the 10-μM cortisone treatment, and

an increase in amplification cycles was considered unnecessary, because the results would not have been veracious.

There are three aspects of the results included in Figure 2a and 2b that attract attention: a) the rapid response of 1-μM cortisone, a minute lag for the 5-fold increase in *Hsd1* mRNA, and the 6-fold increase for *Aqp7* mRNA (Fig. 2a and 2b); b) the strong tendency to return to mRNA baseline values within the next 5 min for *Hsd1* (Fig. 2a) and within 10 min for *Aqp7* (Fig. 2b); and c) the rough parallelism of both mRNA in response to the hormone treatment. Responses to 0.1 μM cortisone, as compared with 1 μM cortisone, were similar for *Hsd1* (Fig. 2a) and smaller for Aqp7 (Fig. 2b).

After 48 h, mRNA expression for both genes was repressed by all treatments (0.2- and 0.4-fold induction, respectively) (Fig. 3).

Expression of mRNA for Hsd1 and Aqp7

After analyzing the results for *Hsd1* and *Aqp7*, their behaviors were compared and adjusted to a quadratic response curve. mRNA expression for *Hsd1* and *Aqp7* under treatment with 1 μM cortisone was adjusted with correlations of 0.5 and 0.39, respectively. Although these are not strong correlations, it is noteworthy that mRNA expression for both genes demonstrated an imminent increase at the beginning of, and a repression late in, the experiment. For the 1-μM cortisone treatment, both protein expressions exhibited cubic tendencies: the
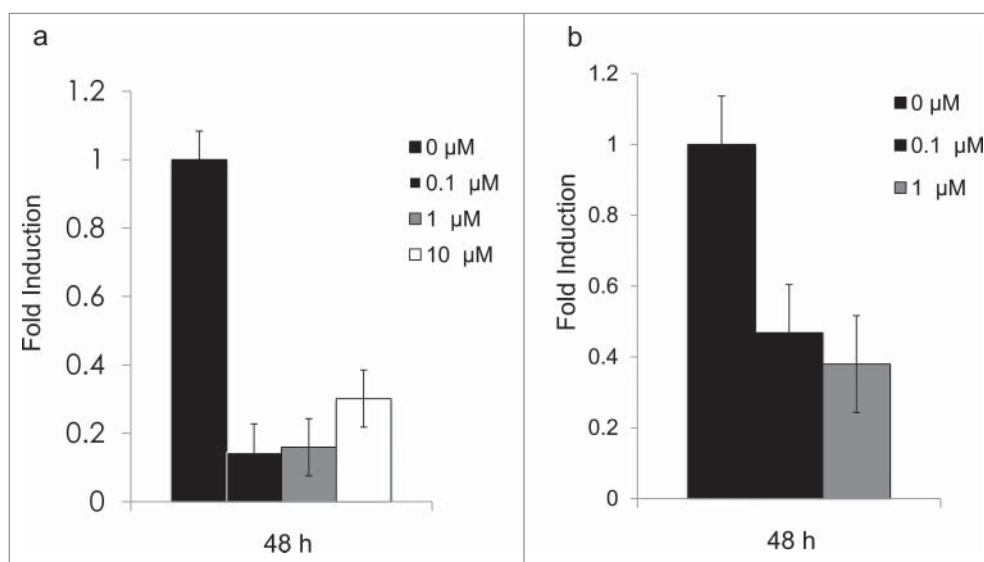
Figure 3. (a) Expression levels for *Hsd1* in 3T3-L1 adipocytes. Bars show the relative expression of mRNA after 48 h for treatments with 0 μM, 0.1 μM, 1 μM, and 10 μM cortisone, $P < 0.05$. (b) Expression levels for *Aqp7* in 3T3-L1 adipocytes. Bars show the relative expression of mRNA after 48 h for treatments with 0μM, 0.1 μM, and 1μM cortisone. The 10 μM treatment revealed undetectable levels for the*AQP7* gene, but no signs of cell-increased mortality nor detachment. All error bars represent mean $\pm$ SEM, $P < 0.05$ ($n = 3$).

Hsd1 tendency is described by the equation $y = 1.31+3.59x-1.65 \times^2+0.18 \times^3$, while the *Aqp7* tendency is characterized by $y = 1.84+4.93x-2.67 \times^2+0.33 \times^3$ (Fig. 4). This mathematical adjustment underlies the fact that the kinetic effect of cortisone during the time employed is not linear and that this is both time- and dose-dependent.
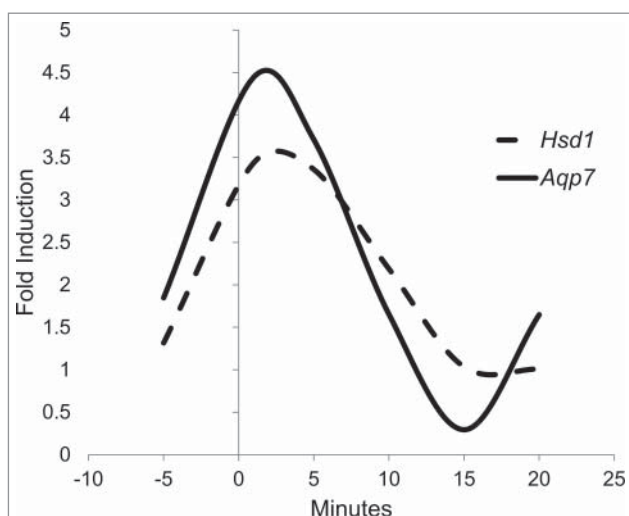
Glucose and glycerol quantification during the treatment time

The treatment with the highest expression of mRNA (1μM) was considered for quantification of glucose and free glycerol in the culture medium. The glucose concentration in the culture media was found to have a minor and transitory decrease after 10 and 20 min of cortisone treatment with respect to control samples (Fig. 5).

There were no significant differences between glycerol concentrations in the culture medium throughout the

Figure 4. 3T3-L1 cell line precursors were differentiated and subsequently treated during 0, 5, 10, 15 and 20 min with different cortisone concentrations (0, 0.1, 1, and 10 μM). Because 1 μM exhibited the greatest impact on the relative expression of both genes, we provide a quadratic response curve for HSD1 expression (dashed line) and AQP7 (continuous line). Data represent mean $P < 0.05$ (n = 3).
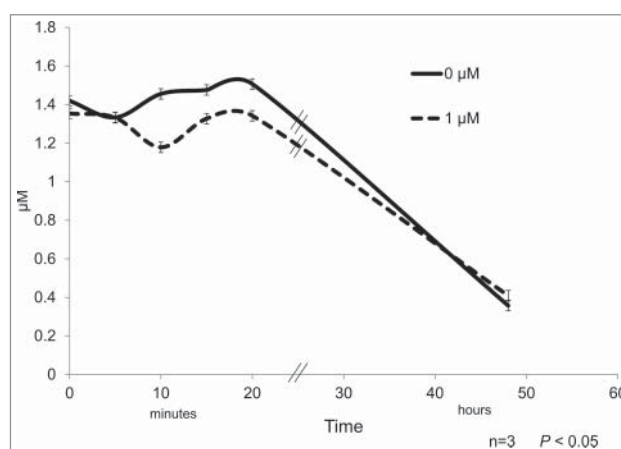

Figure 5. 3T3-L1 cell line precursors were differentiated and subsequently treated during 0, 5, 10, 15, and 20 min with 1 μM cortisone. Glucose was quantified and the different time points of the treatment for controls (continuous line) and differentiated adipocytes (dashed line). Data represent mean $\pm$ SEM, $P < 0.05$ ($n = 3$).

experiment as compared with control samples (data not shown).

Discussion

The anti-inflammatory action of glucocorticoids is well known; however, under certain pathological circumstances, such as MS, they can favor inflammation. For this reason, only after knowing the non-pathologic genomic response of Hsd1 and Aqp7 to glucocorticoids/cortisone, it would be possible to determine whether their modified expressions comprise a cause or a consequence of the disease.[7,22,23] Therefore, this study was performed on differentiated adipocytes derived from a non-obese source after confirming the possible role of cortisone in regulating the expression of these genes through the two GREs predicted in each of their promoters. Even when some authors have reported changes in the expression of either Hsd1[24] or some aquaporins[25-28] in response to glucocorticoid stimuli, no reports have been found that link both of the genes of this research to the MS.

An immediate increase in DNA transcription after appropriate prokaryote cell stimulation is frequently reported. Scarce information is available for eukaryotic cells; yeast subjected to chemical stress demonstrated two upregulated genes that appeared extremely rapidly: between 30 s and 2 min after their stimulation,[29] and about 7% of genes from the yeast genome were induced >5-fold after 10 min in a medium saline shock.[30] Our results demonstrated an immediate cortisone-mediated mRNA increase in both enzymes studied: HSD1 and AQP7 (Fig. 2a and 2b), which are two leading proteins of lipid metabolism and which have been related with obesity. Such an increase was followed, at once, by fast mRNA degradation of both genes, subsequently demonstrating a second wave of mRNA synthesis and degradation, and also for both genes. Interestingly, mathematical response-curve analysis showed a cubic tendency for the 0.1- and 1-μM treatments (Fig. 4). Such changes in genes expression are unrelated with glucose and free glycerol quantification in the cultured medium (Fig. 5). To date, to the best of our knowledge, no studies have reported minute-dependent modifications in the expression of these proteins in response to cortisone. All of these responses render our experimental model an excellent one for considering emerging principles or mRNA production and degradation dynamics in eukaryotic cells: first, upon proper stimulation, an increase in mRNA synthesis might occur at <1 min (Fig. 2a and 2b); second, coupling between transcription and mRNA decay are connected in some fashion in a so-called "circular process;"[31] third, changes in degradation rates are important for shaping sharp, "peaked" responses;[32] and fourth, coupling between transcription and mRNA decay most frequently accompanies a cellular process that involves, among others, the response to cellular stress,[32] as the present case might represent.

An alternative to understanding the actions of cortisol at higher doses might be an eventual "cortisone-resistance" phenomenon. This is a well-characterized phenomenon in lymphocytes[33] and it is observed when concentrations of corticoid hormones exceed the physiological concentrations and when the bulk of cells expires.[34] Therefore, a cortisone-induced toxicity at 10 μM cannot be discarded.

Balachandran et al.[35] performed, with the HSD1 enzyme, a similar experiment in vivo and in vitro, finding a diminished and an increased response in expression, respectively; however, it is important to consider that the treatment time was different (6 and 48 h). Also, the in vitro experiment was performed in an isolated environment, while the experiment performed in vivo preserved the adipose niche. This suggests that it may be necessary to evaluate the acute response in the isolated adipocytes of healthy and obese individuals, and also the in vivo response, in order to be able to determine whether the increase is a consequence of the hypertrophy and could exert influence the development and progression of the different, but related, diseases due to the genomic effect.

Because the presence of AQP(s) and their functions in the cell are of recent discovery, available information on these is limited. In the case of Aqp7, studies are focused on its expression and translation in MS (tissue-specific) and how these are modified during the differentiation process. As in the case of Hsd1, preadipocytes lack the expression of mRNA and protein for Aqp7. Its messenger is first detected after 6 days of differentiation, and the protein appears to be increased and maintained in the mature adipocytes.[15,36]

This study suggests an important hormone response, which would be worthwhile to explore in greater depth, and the study also questions the real, fast regulation mediated by corticosteroids that favors a dramatic increase in expression during short time periods and the purpose of this. We have now identified an important effect on genomic responses mediated by GC, and its impact on metabolic complications is our new assignment. Response during short intervals should be explored in metabolically compromised cells in order to determine the role of cortisone in metabolism and the pathogenesis of obesity in adipose tissue.

Methods

Identification of GRE for Hsd1 and Aqp7 genes

The promoters for the genes encoding for Hsd1 (ID 15483, Ref sequence NM_008288) and Aqp7 (ID 11832,

Ref sequence NM_007473)were analyzed using the Transcription Factor Matrix-Explorer (TFM-E) from LIFL (Laboratoired'InformatiqueFondamentale de Lille, Université Lille, FR; http://bioinfo.lifl.fr/TFM).[37] The sequences corresponding to the region between +200 and −2500 bpwere examined with the TRANSFAC-JASPAR matrices database and then searched for the GRE.

3T3-L1 cell culture

The 3T3-L1 preadipocyte cell line was cultured in DMEM (Gibco, Life Technologies, 11995-065) containing 10% Fetal Bovine Serum (FBS; Gibco, 16000-044) and 1% Penicillin/Streptomycin (Gibco, 15140-122). Cells were seeded at a density of 1×10^4 per ml. On day 2 post-confluence, the differentiation process was induced with a 2-day treatment, changing the medium to DMEM supplemented with 5 μg/ml insulin (Gibco, A11382IJ), 0.25 μM dexamethasone (DXM; Sigma-Aldrich, D4902), and 0.5 mM isobutylmethylxanthine (IBMX; Gibco, PHZ1124). Differentiation was followed by a single-day treatment without DXM and IBMX and 4 additional days without insulin. FBS and antibiotics were included during the entire differentiation process. To ensure that differentiation was properly complied with, oil-red O staining was performed as previously reported by Nicholson et al.[22]

Cell treatment

Differentiated cells in 6 well-plates (procedure described previously) were incubated in a restricted medium for 2 h (DMEM and Penicillin/Streptomycin). After restriction, the cells were treated with three different concentrations (0.1, 1, and 10 μM) of cortisone (Sigma-Aldrich, C2755) during 5 different time periods (5, 10, 15, and 20 min, and 48 h). The treatment medium was collected and utilized to identify time-dependent changes through quantification of glucose and free glycerol using the glucose oxidase-peroxidase method and the free glycerol determination kit (Sigma-Aldrich, FG0100-1KT), respectively. To maintain control of the starting point, cells were harvested with no treatment (illustrated as −5-min timing in Fig. 2a and 2b), and these harvested cells were employed as the starting point for all treatments, because increased expressions were detected immediately after treatment.

RNA isolation

After the culture treatment, total RNA was isolated employing the TRIzol reagent (Life Technologies, 15596018) according to the manufacturer's instructions and stored at −70°C. The RNA obtained was quantified with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific) at 260 nm, and purity was assessed utilizing 260:280 ratios. RNA integrity was confirmed by electrophoresis in a 1% agarose gel.

cDNA synthesis

After evaluating the concentration and purity of the RNA extractions, 1 μg RNA was taken from each sample to perform the transcription of a cDNA (cDNA) brand, with oligo dT_{15} and Superscript II Reverse Transcriptase (Life Technologies, 18064071). To avoid genomic DNA contamination, RNA samples were pre-treated with DNAse (Roche, 776785).

Real-time PCR (qPCR)

Specific primers for the genes *Hsd1, Aqp7, MnSOD*, and *Ppia* were designed using Oligo7 software (Table 1). PCR products were sequenced to verify primer-specific identity. Quantitative RNA expression for *Aqp7* and *Hsd1* was determined utilizing a StepOne Real-Time PCR System (Applied Biosystems) and the LightCycler-FastStart DNA Master Sybr Green I kit (Roche Applied Science, 12 239 264 001). The program was performed as follows: denaturation at 96°C for 10 min, followed by 40 cycles of 1 s at 95°C for denaturation, 10 s at 65°C for primer alignment, and 10 s at 72°C for elongation; the melting curve started at 60°C for 1 min and ended with 15 s at 95°C. Subsequently, the relative abundance of mRNA was calculated by normalization to *MnSOD* and *Ppia* [best housekeeping gene candidates suggested by NormFinder (MDL, Aarhus, Denmark)] according to the $2^{-\Delta\Delta CT}$ method.

Special care was taken to validate the data presented in Fig. 2a: a) each experiment was performed three times with the coordinated participation of three experienced technicians; b) systematic experimental control was included 5 min prior to the addition of cortisone; c) in all cases, two genes were included to normalize the relative abundance of mRNA; d) mRNA controls values

Table 1. Primers for qPCR.

Gene	Primers	Size in bp
Aqp7	Fwd – TATGGTGCGAGAGTTTCTGG	174
	Rev – GCCTAGTGCACAATTGGTGA	
Hsd1	Fwd – GACCCATTGCAAGGAACAA	69
	Rev – GTAGTAAGCGTGCTCCCACAC	
MnSOD	Fwd – TCAGACCAGAAATGCTCCAG	200
	Rev – GCCAGCAATGTAGTGAGCAG	
Ppia	Fwd – AGCACTGGGGGAGAAAGGATT	148
	Rev – AGCCACTCAGTCTTGGAGT	

Note. bp: base pairs.

remained virtually unchanged; and e) the dispersion of individual experimental values was small and this was reflected in small standard errors.

Glucose quantification in the culture medium

For glucose quantification in the medium, the glucose oxidase-peroxidase method was employed.[38] This assay consists of the synthesis of H_2O_2 from glucose by the glucose-oxidase enzyme. Thishydrogen peroxide reacts with ortho-Dianisidine and peroxidase to yield a colored product, which is subsequently quantified in a spectrophotometer (Agilent 8453) at a 540-nm wavelength.

Free glycerol quantification in the culture medium

Glycerol quantification was performed following the protocol suggested by the free glycerol determination kit (Sigma-Aldrich). For this assay, the free glycerol present in the sample was phosphorylated and subsequently oxidized to produce H_2O_2. As in the glucose quantification assay, the reagents produced a quinoneimine dye that can be detected and measured at a 540-nm wavelength in a spectrophotometer (Agilent).

Statistical analysis

The experiment comprised a completely randomized design with a 4×6 factorial arrangement (treatment per time). The results were analyzed with the SAS general linear model (SAS Institute), and least-square-means comparison testing was performed.

Abbreviations

Aqp7	Aquaporin 7
AT	Adipose Tissue
GC	Glucocorticoids
GRE	Glucocorticoid Response Elements
Hsd1	11β-Hydroxysteroid dehydrogenase type 1
IR	Insulin Resistance
MS	Metabolic Syndrome
TAG	Triacylglycerols

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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