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Time- and glucose-dependent differentiation of 3T3-L1 adipocytes mimics dysfunctional adiposity

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ABSTRACT

The 3T3-L1 murine adipocyte cell line remains one of the most widely used models to study the mechanisms of obesity and related pathologies. Most studies investigate such mechanisms using mature adipocytes that have been chemically induced to differentiate for 7 days in media containing 25 mM glucose. However, the dysfunctional characteristics commonly observed in obesity including adipocyte hypertrophy, increased expression of inflammatory markers, enhanced production of reactive oxygen species (ROS), increased steroidogenic enzyme expression/activity and production of steroid hormones, are not necessarily mimicked in these cells. The aim of this study was to provide an inexpensive model which represents the well-known characteristics of obesity by manipulating the time of adipocyte differentiation and increasing the concentration of glucose in the cell media. Our results showed a glucose- and time-dependent increase in adipocyte hypertrophy, ROS production and gene expression of the pro-inflammatory cytokine interleukin-6 (IL-6), as well as a time-dependent increase in lipolysis and in the gene expression of the chemokine monocyte chemoattractant protein 1 (MCP1). We also showed that gene expression of the steroidogenic enzymes 11-beta-hydroxysteroid dehydrogenase type 1 (11βHSD1), 17βHSD type 7 and 12, as well as CYP19A1 (aromatase), were significantly higher in the hypertrophic model relative to the control adipocytes differentiated using the conventional method. The increase in 11βHSD1 and 17βHSD12 expression was consistent with the enhanced conversion of cortisone and androstenedione to cortisol and testosterone, respectively. As these characteristics reflect those commonly observed in obesity, hypertrophic 3T3-L1 adipocytes are an appropriate *in vitro* model to study mechanisms of adipocyte dysfunction in an era where the rise in obesity incidence is a global health concern, and where access to adipose tissue from obese patients are limited.

1. Introduction

The 3T3-L1 clone of murine adipocytes is one of the most widely used models for studying obesity and related pathologies [1]. While the exact mechanism of obesity-associated adipose tissue expansion is still subject to dispute, it is generally accepted that cell hypertrophy (the enlargement of adipocytes) occurs to a greater extent than cell hyperplasia (the increase in adipocyte number), to meet the demand for excess energy storage [2]. Adipocyte hypertrophy is associated with several characteristics of adipocyte dysfunction such as an increase in lipid volume and reactive oxygen species (ROS) production, as well as increased secretion of inflammatory mediators and lipolysis [3,4].

Studies have documented that cell hypertrophy can be achieved in 3T3-L1 adipocytes using different methods, the most common of which is to chemically-induce adipocyte differentiation using 3-isobutyl-1-methylxanthine (IBMX), dexamethasone and insulin, in combination with various saturated fatty acids [5,6]. Several studies have shown that treating differentiated adipocytes with fatty acids such as palmitate, laurate and myristate, can increase oxidative stress and lipid content, as well as increase the expression of the inflammatory markers monocyte chemoattractant protein 1 (MCP1) and interleukin-6 (IL-6) [5,7,8]. At least one study has previously shown that in comparison to adipocytes differentiated

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Abbreviations:

11βHSD1	11-beta-hydroxysteroid dehydrogenase 1
17βHSD12	17-beta-hydroxysteroid dehydrogenase 12
CYP19A1	cytochrome P450 aromatase
MCP1	monocyte chemoattractant protein 1
IL-6	interleukin-6
NoNo	non-POU domain-containing octamer-binding protein
PAI-1	plasminogen activator inhibitor 1
ROS	reactive oxygen species

in 5 mM glucose, those differentiated in 25 mM glucose for the same amount of time displayed both an increase in lipid volume and MCP1 expression [9]. In contrast, a study differentiating adipocytes in the same concentration of glucose, but for different times, showed a significant increase in lipolysis and conversion of glucose to triglycerides after 7 days relative to adipocytes differentiated for 3 days [10]. These findings suggest that mimicking adipocyte dysfunction is a fine balance between glucose concentration and time of differentiation.

In this study, we aimed to optimize the conditions under which adipocyte dysfunction is achieved *in vitro* by evaluating lipid droplet size, ROS production, glycerol release and the expression of markers of inflammation in 3T3-L1 cells. As enhanced steroid production due to an increase in the expression and activity of steroidogenic enzymes is also a well-known characteristic of obesity [11–13], we compared the expression and activity of selected steroidogenic enzymes in 3T3-L1 cells differentiated using the conventional method to that of the new method proposed in this study, as a validation of an *in vitro* hypertrophic model.

2. Materials and methods

2.1. Test compounds and standards

Testosterone, androstenedione, cortisone, cortisol, dexamethasone, IBMX, insulin and methyl *tert*-butyl ether (MTBE) were purchased from Sigma-Aldrich (South Africa). Deuterated steroids, d2-testosterone, d7-androstenedione, and d4-cortisol, were purchased from Cambridge Isotope Laboratories (USA). All compounds were prepared in 100% ethanol (EtOH) and used at a final concentration of 0.1% in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) (Sigma-Aldrich, South Africa).

2.2. 3T3-L1 adipocyte culture

The 3T3-L1 murine pre-adipocyte cell line (ATCC, USA) was cultured as described previously [5]. Confluent pre-adipocytes were differentiated in DMEM containing 5 mM, 25 mM or 33 mM glucose, and supplemented with 10% fetal bovine serum (FBS) and 1% PenStrep for either 7 or 14 days. Adipocyte differentiation was induced using dexamethasone, IBMX and insulin (Sigma Aldrich, South Africa) to a final concentration of 1 μM, 0.5 mM and 1 μg/mL, respectively. After 72 h, the medium was replaced with DMEM containing 1 μg/mL insulin for a further 48 h. Thereafter, the medium was replaced daily until day 7 or 14.

2.3. Oil Red O staining

To confirm the accumulation of lipid droplets, 3T3-L1 adipocytes were stained with Oil Red O following a previously described method [5]. To quantify lipid droplet size, representative images from each condition were captured using the AxioObserver Microscope (Zeiss, Germany) at 20× magnification and the areas of representative lipid droplets were measured using Zen Image Software (Zeiss).

2.4. Reactive oxygen species (ROS) assay

3T3-L1 adipocytes were seeded at a density of 4×10^3 cells/well in black-walled 96-well plates (Greiner, Germany) and differentiated for either 7 or 14 days in DMEM containing 5 mM, 25 mM or 33 mM glucose. Following differentiation, *tert*-butyl hydroperoxide (TBHP) was added to a final concentration of 50 μM for 4 h to induce ROS production as previously described [14]. Cells were then stained with 20 μM 2′,7′-Dichlorodihydrofluorescein diacetate (DCFDA) (Abcam, USA) for 45 min. DCFDA is oxidized by ROS to DCF, which emits green fluorescence at an excitation of 485 nm and an emission of 535 nm. Fluorescence was quantified using the Tecan Spark® fluorescence multi-mode microplate reader (Diagnostics Products, South Africa).

2.5. Glycerol release assay

3T3-L1 adipocytes were seeded at a density of 4×10^3 cells/well in 96-well tissue culture plates. Once confluent, the cells were differentiated for 7 or 14 days in DMEM containing 5 mM, 25 mM or 33 mM glucose. Glycerol release, a component of adipocyte lipolysis, was measured using the Lipolysis Colorimetric Assay Kit (Sigma-Aldrich, South Africa) according to the manufacturer's instructions. Briefly, lipolysis was stimulated with 100 nM isoproterenol for 3 h prior to harvesting 50 μL from each differentiation condition, and 50 μL of a reaction master mix added to allow a colorimetric reaction. Following a 30-min incubation in the dark, the absorbance was read at 570 nm, and the amount of glycerol measured using a standard curve of increasing concentrations of glycerol.

2.6. Quantitative real-time PCR (qPCR)

Total RNA was extracted from differentiated 3T3-L1 adipocytes seeded at 2×10^5 cells/well in a 12-well plate using TRIzol reagent according to the manufacturer's instructions. Briefly, 500 ng RNA was reverse transcribed using the Promega ImProm-II™ Reverse Transcription System cDNA synthesis kit (Promega, USA). The mRNA expression of MCP1 [15], IL-6 [16], 11-beta-hydroxysteroid dehydrogenase type 1 (11βHSD1) [17], CYP19A1 (aromatase) [17] and 17βHSD12 [18] (Supplementary Table 1) was determined using the CFX Opus real-time PCR System (Bio-Rad, South Africa) and KAPA SYBR FAST qPCR master mix. Non-POU domain-containing octamer-binding protein (NoNo) was used as a reference gene [19]. Relative gene expression was used to quantify gene transcripts according to the equation described by Pfaffl [20].

2.7. Enzyme-linked immunosorbent assay (ELISA)

Supernatants from control and hypertrophic 3T3-L1 adipocytes were harvested and assayed for plasminogen activator inhibitor 1 (PAI-1) using the DuoSet® ELISA Development System kits (R&D systems, USA), according to the manufacturer's instructions. The optical density and standard concentrations were plotted. Optical densities were converted to picograms/mL (pg/mL) using linear regression analysis, and the linearity range of 9.4–600 pg/mL used.

2.8. Separation and quantification of steroids by ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS)

3T3-L1 adipocytes were seeded at a density of 1×10^5 cells/well in 24-well tissue culture plates. After differentiation for 7 or 14 days in 25 mM or 33 mM glucose, respectively, the cells were treated with 100 nM cortisone or testosterone for 24 h. Culture medium containing the test compounds were added to empty wells (no cells) as a negative control for metabolism. The supernatants were harvested and 100 μL of the deuterated steroids, d2-testosterone (1.5 ng/sample), d7-androstenedione and d4-cortisol (both 10 ng/sample), were added as internal standards. Steroids were extracted using a 3:1 ratio of MTBE (v/v) to culture medium as described in Ref. [21] and Supplementary Table 2.

2.9. Statistical analysis

Graphical representation and statistical analysis of the results were performed using GraphPad Prism® version 9 (GraphPad Software, USA). To investigate the effect of differentiation time and glucose concentration on parameters of adipocyte hypertrophy, a two-way analysis of variance (ANOVA) of log-transformed data with Bonferroni's multiple comparison post-test was used. Statistical significance is indicated by the letters a, b, c, etc., where values that differ significantly from others are assigned a different letter. Unpaired *t*-tests were used for comparisons between control and hypertrophic adipocytes where *, **, *** and **** indicate $P < 0.05$, $P < 0.01$, $P < 0.001$ and $P < 0.0001$, respectively. Error bars indicate standard error of the mean (SEM) of at least 3 independent experiments performed in triplicate, unless stated otherwise.

3. Results

3.1. Enhanced glucose concentration and time of differentiation result in a dose-dependent increase in hypertrophy, ROS and expression of pro-inflammatory markers

Adipose tissue is known to expand as a means to store excess triglycerides via hyperplasia or via hypertrophy, with the latter commonly associated with oxidative stress and adipocyte dysfunction [2]. To investigate adipocyte hypertrophy, we evaluated the size of lipid droplet formation in 3T3-L1 murine adipocytes differentiated for 7 or 14 days in media containing increasing concentrations of glucose. The representative images of Oil Red O-stained lipid droplets in Fig. 1A indicate visibly larger lipids for cells differentiated for 14 days, especially those differentiated in 33 mM glucose. Quantification of lipid droplet area revealed an increase in size in both a glucose- and time-dependent manner (Fig. 1B), suggesting increased adipocyte hypertrophy under these conditions. An increase in ROS production was also observed in cells differentiated for 14 days (Fig. 1C), suggesting that in our model, adipocyte hypertrophy directly corresponds with enhanced oxidative stress, a phenomenon commonly observed in obesity [22]. On the other hand, using glycerol production as a measure of lipolysis, we showed that glycerol production was significantly enhanced in cells differentiated for 14 days relative to those differentiated for 7 days, but was not affected by the change in glucose concentration (Fig. 1D).

Chronic low-grade inflammation is a distinctive feature of obesity, and markers of inflammation are commonly used to identify adipocyte dysfunction [23]. Thus, we next investigated the effect of glucose concentration and time of adipocyte differentiation on the mRNA expression of the well-known inflammatory markers, MCP1 and IL-6. Relative to adipocytes differentiated for 7 days, both MCP1 (Fig. 2A) and IL-6 (Fig. 2B) expression were consistently enhanced after 14 days. Collectively, these results suggest that increasing the time of differentiation consistently enhances markers of adipocyte dysfunction, while increasing the concentration of glucose dose-dependently increases hypertrophy, ROS production and IL-6 expression. Compared to the conventional 7-day method of adipocyte differentiation in media containing 25 mM glucose [24], we showed that all markers of dysfunctional adiposity were elevated in adipocytes differentiated for 14 days in media containing 33 mM glucose. Thus, for the remainder of this study, we refer to adipocytes differentiated using the conventional method as 'control' and those differentiated for 14 days in 33 mM glucose as 'hypertrophic'.

3.2. The secretion of PAI-1 as well as the expression and activity of selected steroidogenic enzymes is enhanced in hypertrophic 3T3-L1 adipocytes

Since adipokines such as PAI-1 are strongly linked to obesity-associated adipocyte dysfunction, we used ELISA to compare the secretion of PAI-1 in control and hypertrophic adipocytes, and observed a 1.9-fold increase in hypertrophic adipocytes (Fig. 3A). Moreover, the expression and activity of several steroidogenic enzymes are also known to be increased in obesity [11–13]. For instance, the expression of 11βHSD1, the enzyme that converts cortisone to cortisol is upregulated in the adipose tissue of both obese women and mice [13,25], while the expression of CYP19A1 which is responsible for the aromatization of androgens to estrogens is commonly overexpressed in obese women [12]. Indeed, real-time qPCR results showed significant upregulation of both 11βHSD1 and CYP19A1 mRNA expression in hypertrophic relative to control 3T3-L1 adipocytes (Fig. 3B and C). Similarly, we showed a significant increase in the expression of 17βHSD12 which is known to catalyze the conversion of androstenedione to testosterone in murine adipocytes (Fig. 3C) [18]. We next sought to evaluate whether a concomitant increase in steroid production would be observed. As CYP19A1 is not active in 3T3-L1 adipocytes [26], the activities of only 11βHSD1 and 17βHSD12 were subsequently assessed using UHPLC-MS/MS. The results showed that the production of cortisol from cortisone (Fig. 4A) and testosterone from androstenedione (Fig. 4B) was significantly greater in hypertrophic adipocytes, suggesting that both the expression and activity of 11βHSD1 and 17βHSD12 are increased under hypertrophic conditions.

4. Discussion

The global incidence of obesity and related pathologies such as type 2 diabetes, hypertension and several cancers has risen dramatically, including in countries within sub-Saharan Africa [27]. For this reason, there is a critical need to understand the mechanisms underlying adipogenesis and obesity-related pathologies, in a model representing the characteristics of obesity. However, access to adipose tissue from overweight/obese patients is challenging and typically involves a rigorous ethical undertaking. While animal studies have been used to investigate obesity-associated mechanisms [28], these models are costly and do not necessarily allow for high-throughput experiments. The *in vitro* murine 3T3-L1 adipocyte cell line remains the most widely used to study mechanisms of adipogenesis, in which exogenous fatty acids are commonly used to induce adipocyte dysfunction [5,7,8]. However, the use of these cellular models is limited by several negative effects, the most common being lipotoxicity [6]. Here, we developed a novel model of adipocyte hypertrophy by modifying the glucose concentration and time of differentiation used in the conventional method of 3T3-

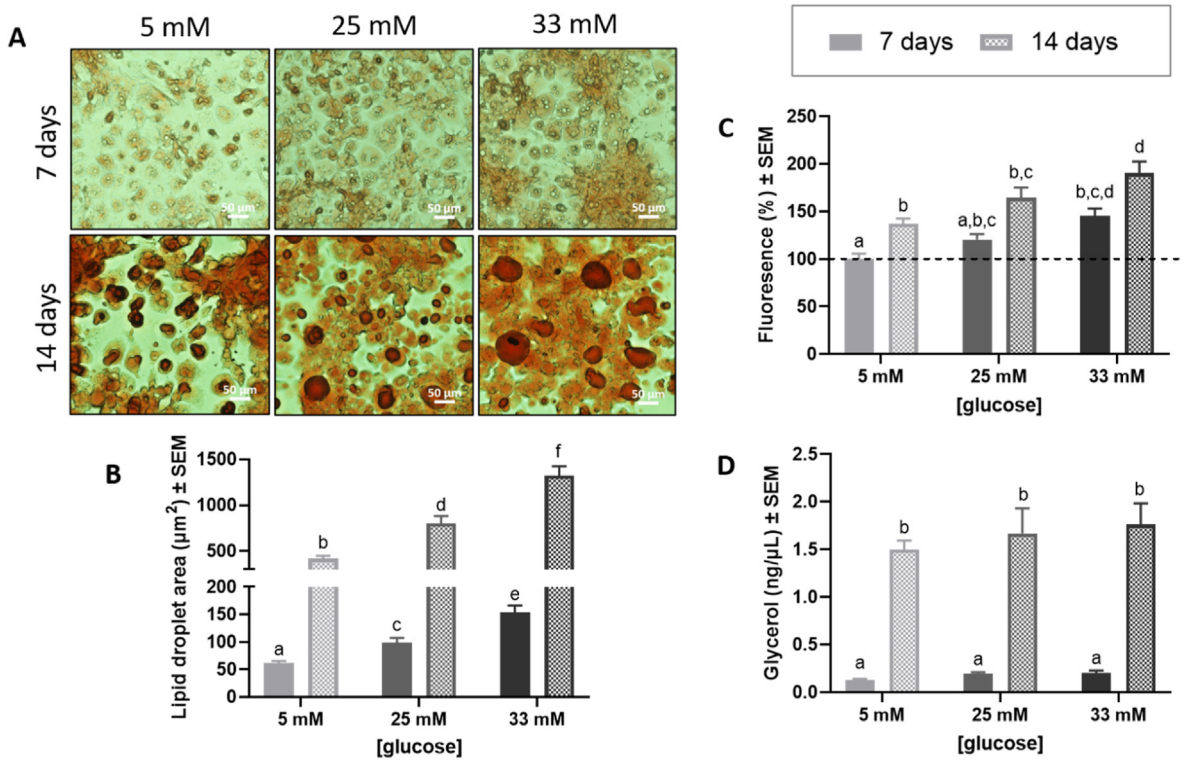

Fig. 1. Increased time and glucose concentration for differentiation dose-dependently increases 3T3-L1 adipocyte hypertrophy and ROS production. 3T3-L1 pre-adipocytes were differentiated for 7 or 14 days in 5 mM, 25 mM or 33 mM glucose and stained with Oil Red O dye. **(A)** Representative images are shown (scale bars: 50 μm) and **(B)** the areas (in μm^2) of representative Oil Red O-stained lipid droplets were quantified. Changes in **(C)** ROS production or **(D)** glycerol production (in ng/mL) were quantified with adipocytes cultured in 5 mM for 7 days in **(C)** set as 100%, and the values of all other conditions set relative to this. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

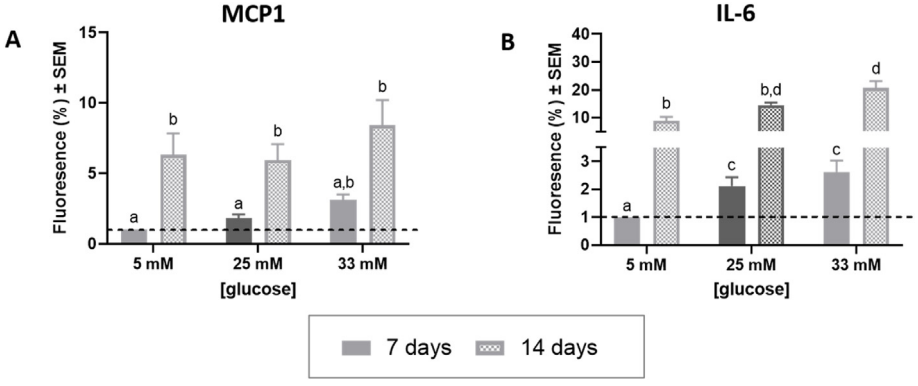

Fig. 2. The mRNA expression of MCP1 and IL-6 are significantly increased after 14 days of adipocyte differentiation. Total RNA was isolated from 3T3-L1 adipocytes differentiated for 7 or 14 days in 5 mM, 25 mM or 33 mM glucose, reverse transcribed and the mRNA expression of **(A)** MCP1 and **(B)** IL-6 was determined relative to that of NoNo (reference gene) using real-time qPCR. The relative mRNA expression of each gene was expressed as fold change with the data for adipocytes cultured in 5 mM glucose for 7 days set as 1 and the values of all other conditions set relative to this. Results represent the averages (±SEM) of three independent experiments performed in duplicate.

L1 adipocyte differentiation.

Both glucose concentration and time of differentiation resulted in a dose-dependent increase in lipid droplet size in 3T3-L1 adipocytes (Fig. 1A and B), which corresponded with a similar increase in ROS production (Fig. 1C). Indeed, it has previously been suggested that increased accumulation of hypertrophic adipocytes corresponds with an enhanced oxidative environment [22]. Similarly, ROS production was previously shown to be significantly greater in mature bone marrow adipocytes cultured in high glucose media for 21 days compared to cells cultured for 14 days in both high and low glucose media [14]. While cell hypertrophy and ROS production were enhanced in response to both increased glucose and time, an increase in lipolysis only occurred after 14 days of differentiation and was unaffected by the changes in glucose concentration (Fig. 1D). In agreement with this finding, a previous study showed that isoproterenol-induced lipolysis was unaffected by the addition of extracellular glucose [29]. Together, these findings suggest that lipolysis may be more sensitive to changes in time than changes in glucose concentration.

Low-grade chronic inflammation is one of the most distinguishing features of obesity [23]. Although it is a complex phenomenon involving multiple pathways, it is commonly

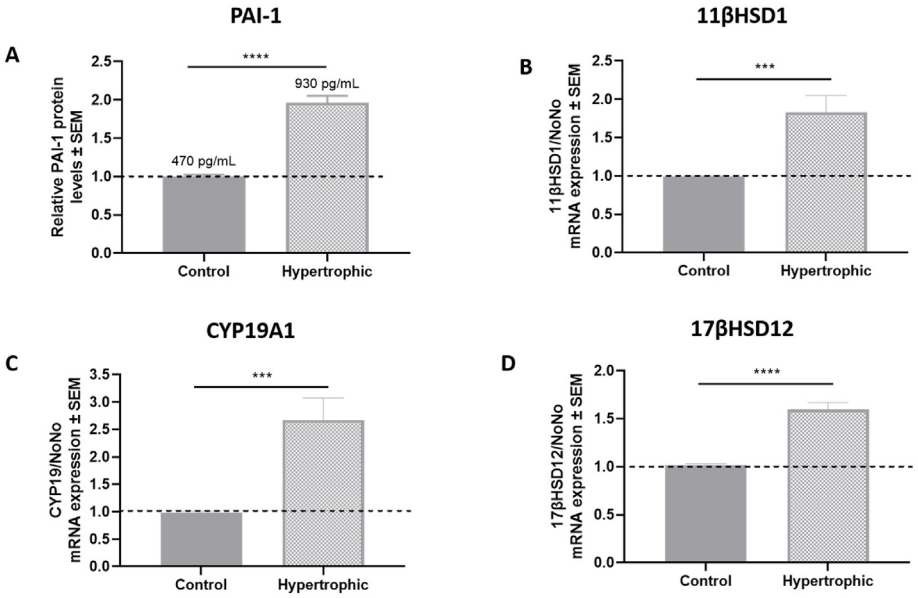

Fig. 3. PAI-1 protein secretion and the mRNA expression of selected steroidogenic enzymes is elevated in hypertrophic 3T3-L1 adipocytes. (A) Cell supernatants from control or hypertrophic adipocytes were harvested, and PAI-1 protein secretion was measured using ELISA. Total RNA was isolated from 3T3-L1 adipocytes differentiated for 7 days in 25 mM glucose (control) and 14 days in 33 mM glucose (hypertrophic), reverse transcribed and the mRNA expression of **(B)** 11βHSD1 **(C)** CYP19A1 and **(D)** 17βHSD12 was determined relative to that of NoNo (reference gene) using real-time qPCR. The relative mRNA expression of each enzyme in the hypertrophic adipocytes was determined relative to the expression determined in the control adipocytes, which was set as 1. Results represent the averages (±SEM) of three independent experiments performed in duplicate.

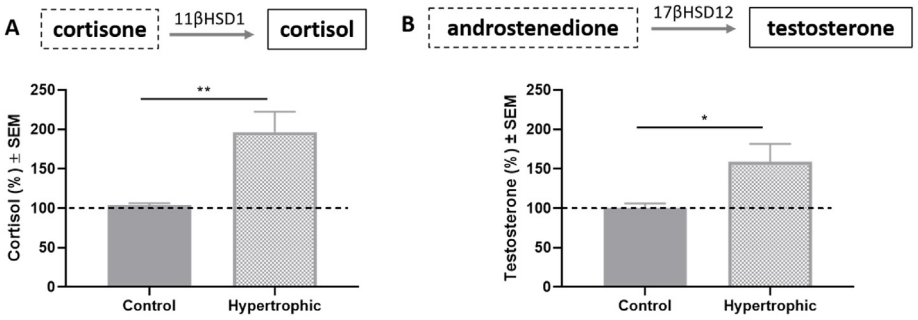

Fig. 4. Cortisol and testosterone production are elevated in hypertrophic 3T3-L1 adipocytes. 3T3-L1 adipocytes differentiated for 7 days in 25 mM glucose (control) and 14 days in 33 mM glucose (hypertrophic) were treated with 100 nM of the steroid precursors **(A)** cortisone and **(B)** androstenedione (indicated in the dashed-line blocks), and the subsequent conversion to their respective steroid products cortisol and testosterone (indicated in the solid-line blocks) was measured after 24 h by UHPLC-MS/MS. The relative production of steroid in the hypertrophic adipocytes was determined relative to the total amount of steroid produced in the control adipocytes, which was set as 100%.

characterized by an increase in inflammatory markers including cytokines, adipokines and chemokines. We showed a significant increase in MCP1 and IL-6 mRNA expression after 14 days of differentiation (Fig. 2A and B). While the results for MCP1 mRNA expression in 7-day differentiated adipocytes correlated with that of a previous study showing a glucose-dependent increase in MCP1 [9], we observed a trend of both a glucose- and time-dependent increase in IL-6 mRNA expression (Fig. 2B), which has also been shown in a 3T3-L1 adipocyte model using fatty acids to mimic adipocyte dysfunction [16]. Although increases in these markers are observed in obese patients [23], these findings alone do not necessarily confirm that 3T3-L1 adipocytes differentiated for 14 days in high glucose media mimic the dysfunctional conditions typically seen in obese patients. For this reason, we also evaluated whether levels of the adipokine, PAI-1 and that of selected steroidogenic enzymes, both known to be elevated in obese patients [4,25], would also be increased in our model of hypertrophic adipocytes. Indeed, we showed a significant increase in the secretion of PAI-1, as well as the mRNA expression of 11bHSD1, CYP19A1 and 17bHSD12 (Fig. 3A–D) in the hypertrophic adipocytes. The increase in 11βHSD1 expression correlated with the increased conversion of cortisone to cortisol (Fig. 4A), while the increase in 17βHSD12 expression correlated with increased conversion of androstenedione to testosterone (Fig. 4B). The latter is in line with at least one study showing a significant increase in the expression of 17βHSD12 in the adipose tissue of mice fed a high fat diet, which corresponded with a marginal increase in testosterone levels [30].

Taken together, we have successfully shown an increase in adipocyte hypertrophy, oxidative stress, expression of inflammatory markers, as well as an increase in the expression and activity of selected steroidogenic enzymes in the widely used 3T3-L1 adipocyte cell line cultured in 33 mM glucose for 14 days. We thus provide an *in vitro* 3T3-L1 hypertrophic adipocyte model mimicking obesity *in vivo*, without the unwanted negative effects observed in models using fatty acids. This model is an inexpensive and physiologically representative alternative with which to study the mechanisms of adipocyte dysfunction, particularly when access to adipose tissue from obese patients is limited.

Declaration 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2023.06.026.

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