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Multipotent Stromal Cells from Subcutaneous Adipose Tissue of Normal Weight and Obese Subjects: Modulation of Their Adipogenic Differentiation by Adenosine A_1 Receptor Ligands

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Abstract: Adenosine A_1 receptor (A_1R) activation, stimulating lipogenesis and decreasing insulin resistance, could be useful for metabolic syndrome management in obese subjects. Since full A_1R agonists induce harmful side-effects, while partial agonists show a better pharmacological profile, we investigated the influence of two derivatives of the full A_1R agonist 2-chloro-*N*^{6}-cyclopentyladenosine (CCPA), C1 and C2 behaving as A_1R partial agonists in animal models, on the adipogenic differentiation of stromal/stem cells (ASCs) from human subcutaneous adipose tissue, which mainly contribute to increase fat mass in obesity. The ASCs from normal-weight subjects showed increased proliferation and A_1R expression but reduced adipogenic differentiation compared to obese individual-derived ASCs. Cell exposure to CCPA, C1, C2 or DPCPX, an A_1R antagonist, did not affect ASC proliferation, while mainly C2 and DPCPX significantly decreased adipogenic differentiation of both ASC types, reducing the activity of glycerol-3-phosphate dehydrogenase and the expression of PPARγ and FABP-4, all adipogenic markers, and phosphorylation of Akt in the phosphatidylinositol-3-kinase pathway, which plays a key-role in adipogenesis. While requiring confirmation in in vivo models, our results suggest that A_1R partial agonists or antagonists, by limiting ASC differentiation into adipocytes and, thereby, fat mass expansion, could favor development/worsening of metabolic syndrome in obese subjects without a dietary control.

Keywords: adipose stromal cells (ASCs); subcutaneous adipose tissue; adenosine A_1 receptors; adipogenic differentiation; adipogenic markers

1. Introduction

Severe pathologies such as diabetes and/or cardiovascular diseases are caused by or associated with obesity [1], whose prevalence has tripled worldwide in the last four decades. Thus, the World Health Organization (WHO) has coined the term "globesity", defining this disorder as a global epidemic [2].

Obesity is linked to a prevailing dysfunction of the white adipose tissue (WAT), which, in addition to the primary function of energy storage in healthy condition, produces and secretes various signaling molecules playing a crucial role in regulating feeding and metabolism [3]. WAT is constituted by two fat depots named subcutaneous (SAT) and visceral (VAT) adipose tissues, due to their different locations below the skin and in the trunk cavity, respectively [4]. Although these tissues are morphologically similar, it has been recently demonstrated that they exhibit different metabolic characteristics. Thus, in obesity, while the increase in SAT is regarded as protective, by improving insulin sensitivity and decreasing the risk of developing type 2 diabetes, the accumulation of visceral fat is associated with metabolic disease and its harmful sequelae (insulin resistance, type 2 diabetes, dyslipidemia, hypertension, atherosclerosis, hepatic steatosis, and cancer) [5]. These differences can be correlated to a distinct behavior of the cells constituting these two tissues. Indeed, in obese individuals, hypertrophic adipocytes are more frequently present in VAT and contribute to systemic insulin resistance. In contrast, a greater number of small adipocytes in SAT exhibit increased glucose uptake upon insulin exposure, as reviewed in [4,6]. Moreover, after a long debate on the origin/identity of the adipocyte progenitors in WAT, there is now a general consensus that inside SAT and VAT several cell types are present, including a population of stem-like cells which can contribute to the weight gain in obese individuals. These cells are mainly found in the perivascular stroma and show many features similar to the classic adult mesenchymal stem cells, namely high self-renewal and multipotency, enabling them to differentiate towards different phenotypes [7]. However, the comparison between adipose stromal/stem cells from SAT (S-ASCs) and VAT (V-ASCs) has pointed out some functional and metabolic differences, with S-ASCs displaying a proliferation rate and adipogenic potential significantly higher than V-ASCs and giving rise to more functional and better organized adipocytes [8,9]. Therefore, S-ASCs are more frequently investigated as a more manageable experimental model, even though V-ASCs could also be a valuable cell model for investigating the molecular mechanisms implicated in the pathophysiology of metabolic disorders such as obesity.

Despite the spread of obesity and the severity of the metabolic syndrome frequently associated with it, the current pharmacological therapy for this disorder is mostly ineffective. Hence, the need to discover novel efficient drugs. Among the molecules explored for their potential as anti-obesity drugs, there are adenine-based purines (ABPs). They are ubiquitous substances produced and released from virtually all cells/tissues. At the extracellular level, ABPs, in particular ATP or its nucleoside, adenosine, modulate a wide variety of physiological/pathological processes by interacting with specific receptors [10,11]. These latter are classified as P1 receptors, which are primarily stimulated by adenosine, and P2 receptors, which respond to nucleotides. The P1 receptors comprise four subtypes (A_1, A_{2A}, A_{2B}, A_3), whereas P2 receptors are further subdivided into seven P2X ligand-gated ion channels ($P2X_{1–7}$) and eight P2Y G-protein-coupled receptors ($P2Y_{1,2,4,6,11–14}$) [12–14]. Each of these receptors has been cloned and characterized for distinct tissue expression and function. As for adipose tissue, research has emphasized a possible modulatory role of adenosine and A_1 receptors (A_1R) on fat turnover. Indeed, it has been demonstrated that:

1. Adenosine is present in the interstitial fluid of the adipose issue at concentrations able to modulate lipolysis;
2. This effect is prevailingly mediated by the A_1R, which are dominant in mature adipocytes;
3. The stimulation of A_1R in mature adipocytes leads to a decreased release of non-esterified fatty acids (NEFA) into plasma due to the inhibition of cAMP formation, which in turn leads to increased sensitivity to insulin. Thus, A_1R agonists may be useful for treating hyperlipidemia and also diabetes [15].

However, although effective as antilipolytic agents, full A_1R agonists may cause unpleasant cardiovascular effects (bradycardia, systemic vasodilation, etc.) and their use leads to rapid A_1R desensitization thus shortening the duration of the therapeutic effects [16]. Therefore, research is currently aimed at investigating partial A_1R agonists that can exert a greater effect on adipose tissue provided with a larger reserve of A_1R than cardiac tissue, at the same time inducing fewer side-effects and a later tachyphylaxis. Some of them have also been tested up to phase 2 of a clinical trial [17].

Based on the evidence that S-ASCs derived from SAT, hereafter referred to simply as ASCs, appear to play an important modulatory role in fat turnover and can, therefore, represent a good target for the pharmacological treatment of obesity, the aims of this study have been to:

1. Compare some properties, such as the proliferation and ability to differentiate towards an adipogenic phenotype of these ASCs, derived from the SAT of normal or obese individuals, which have not been systematically characterized;
2. To evaluate the expression and activity of A_1R in the above-mentioned ASCs in relation to their adipogenic differentiation potential. In particular, we thought to compare the effects of one of the best known full A_1R agonists, 2-chloro-N6-cyclopentyladenosine (CCPA), with those evoked by cell exposure to partial A_1R agonists, using two compounds, synthesized and kindly provided by colleagues from the University of Camerino (Italy), who are included in the authorship of this paper.

2. Materials and Methods

2.1. Chemicals

Disposables for tissue culture were from Falcon (Steroglass, Perugia, Italy). Alpha modified Eagle's minimum essential medium (α-MEM) was purchased from EuroClone S.p.A. (Milan, Italy). L-Glutamine for culture medium, penicillin/streptomycin, amphotericin B, ascorbic acid, dexamethasone were from Sigma-Aldrich (Milan, Italy) as well as CCPA, 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and all the other chemicals, unless differently indicated. As for the two partial A_1R agonists, namely 2′-dCCPA, compound C1, and 3′-dCCPA, compound C2, were synthesized at the University of Camerino (Camerino, Italy). They are derivatives of the A_1AR full agonist CCPA (Figure 1) and the lack of the hydroxyl groups of the ribose moiety leading the compounds to behave as partial agonists of A_1R should be noted, as previously demonstrated [18–20].

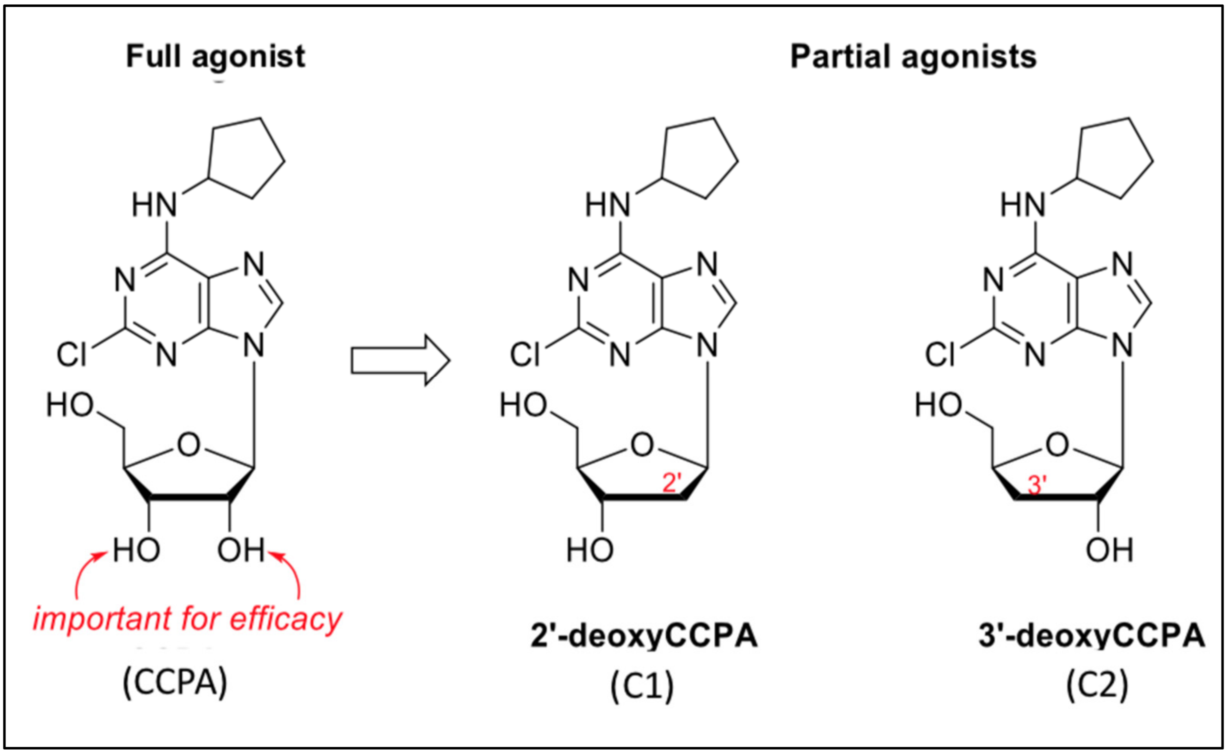


Figure 1. Chemical structure of the compounds under study. The presence of OH groups in the formula of CCPA is highlighted. These are fundamental for the affinity of this agent to the A_1R and the consequent pharmacological activity, and the absence of one of the two OH groups in the formula of the compounds C1 and C2, synthesized ex novo starting from CCPA.

2.2. Cell Cultures

We used stromal ASCs isolated from explants of subcutaneous adipose tissue of normal weight (BMI = 21 ± 2) and obese (BMI = 36 ± 2) subjects (*n* of subjects for each type: 5; mean age of human subjects, all females, was 35 ± 3 years), which are commercially available from the Zen-Bio Inc. Company (Research Triangle Park, NC, USA). This allowed us to overcome ethical issues. Cells were cultured using a growth medium consisting of α-MEM medium, 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Thermo Fisher Scientific, Milan, Italy), 1% penicillin/streptomycin, 1% amphotericin B. Cultures were incubated at 37 °C and 5% CO_2, and the medium was changed twice a week. Experiments were performed only in the first five-eight cell passages. The adipogenic differentiation was induced by culturing the human ASCs with Adipocyte Differentiation medium for the first 7 days in vitro (DIV) and Adipocyte Maintenance medium (Zen-Bio Inc., Research Triangle Park, NC, USA) up to 28 DIV, with medium change every 7 days.

Cultures were stained at different time points with Oil Red O (ORO, Sigma-Aldrich, Milan, Italy) dye to identify lipid vacuoles that were subsequently quantified by spectrophotometric analysis. Briefly, cell monolayers were rinsed with cold phosphate buffered saline (PBS, pH 7.4) and fixed with cold 4% paraformaldehyde. After rinsing in PBS, cells were stained with 60% working ORO solution for 25 min, washed and subsequently imaged by using a Cool-SNAPcf digital CCD camera (PhotoMetrics, Huntington Beach, CA, USA), before incubation with cold 100% isopropanol to extract the incorporated stain. Absorbance of extracted dye was measured in triplicate at 500 nm by a spectrophotometer (SpectraMax SM190, Molecular Devices, Sunnyvale, CA, USA).

As for the pharmacological treatments of the cells, the experimental protocol was the following: we administered each drug at each medium change (that is, once every seven days, starting drug administration at time 0). When the antagonist was present together with the other drugs, it was added to the culture medium 1 h prior to the second compound. The doses of CCPA and DPCPX were chosen on the basis of previous experience [21,22], while for the doses of compounds C1 and C2, pilot experiments were conducted to determine their effect on the adipogenic differentiation of ASCs. Those that gave a sub-maximal and maximal effects were chosen and are those indicated in the Results section.

2.3. Cell Proliferation Analysis

Cell proliferation was assayed by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay, using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega Italia, Milan, Italy), according to the manufacturer's instructions. The absorbance was measured at 490 nm, using a microtiter plate reader (Spectracount, PerkinElmer, Waltham, MS, USA). Moreover, we also measured the number of viable cells at different days of culture, using the trypan blue exclusion method. Briefly, cells were harvested after different time periods, incubated with trypan blue, and counted with a hemocytometer (three different fields for each sample evaluated in triplicate). Results are expressed as number of live cells/well.

2.4. Lactate Dehydrogenase Assay

Lactate dehydrogenase (LDH) levels are widely used to evaluate necrotic cell death, LDH being a cytoplasm enzyme released upon cell membrane damage. Cells, seeded (2×10^3 cells/well) in 96-well plates, were incubated with drugs according to the usual protocol. At different time points (14 and 28 days), 50 µL of supernatant from each well, transferred to a new 96-well plate, were added to 50 µL of substrate buffer consisting of 0.7 mM p-iodonitrotetrazolium Violet, 50 mM L-lactic acid, 0.3 mM phenazine methoxysulfate, 0.4 mM NAD and 0.2 M Tris-HCl pH 8.0. The plate, suitably blanketed, was incubated in the dark at room temperature for 30 min, and finally the reaction was stopped by addition of 50 µL/well of stop solution. The absorbance was measured spectrophotometrically at 490 nm and the results were expressed as a percentage of total LDH released from positive controls, which were cells previously incubated at 37 °C and 5% CO2 for 45 min with specific lysis buffer (25 µL of 10% Nonidet P-40) and then centrifuged at 250× g for 4 min. Thus, the results were calculated, adopting the following formula:

$$\text{percent cytotoxicity} = 100 \times (\text{experimental LDH release}/\text{maximum LDH release}) \quad (1)$$

All reagents were purchased from Promega Italia (Milan, Italy).

2.5. Caspase 3/7 Activity Assay

Apoptosis of cells, exposed or not to A_1R agonist/antagonist, was evaluated by the Caspase-Glo Assay (Promega Italia, Milan, Italy), which contains a peptide (DEVD-aminoluciferin) that can be cleaved by caspases 3/7 liberating aminoluciferin from which a thermostable luciferase, included in the kit, is able to generate a luminescent signal, which is proportional to caspase-3/7 activity. Briefly, caspase-3/7 detection reagent was added at 1:1 ratio to the culture medium and cells were incubated at 25 °C for 1 h. The luminescent signal was revealed by a luminometer (VeritasTM Microplate Luminometer Turner Biosystems, Sunnyvale, CA, USA). The background value, measured in a sample containing only growth medium and caspase-3/7 detection reagent but no cells, was subtracted from each measurement.

2.6. Quantitative Real Time Polymerase Chain Reaction (qRT-PCR)

This technique was used for the evaluation of the mRNA expression of adenosine receptors. Total RNA was extracted from S-ASCs with Trizol reagent (Invitrogen, Thermo Fisher Scientific, Milan, Italy). RNA content was determined by Nanodrop 2000 spectrophotometer (Thermo Scientific, Milan, Italy) and RNA integrity was checked by electrophoresis on 1.5% agarose gel in Tris Borate EDTA (TBE) (89 mM Tris, 89 mM boric acid, 20 mM EDTA, pH 8.0). Gels were analyzed by a RED analyzer (Cell Biosciences, Santa Clara, CA, USA). All samples were amplified by Turbo DNA-free kit (Invitrogen, Thermo Fisher Scientific, Milan, Italy). Reverse transcription was performed using 1 µg of total RNA/sample and High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. The reaction mixture was loaded to the Gene Amp PCR system 9700 (Applied Biosystem) undergoing the cycle at 37 °C for 120 min.

Real-time PCR was carried out with the ABI Prism 7900 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression of adenosine receptors (A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R) was evaluated in undifferentiated ASCs and in ASCs submitted to adipogenic differentiation at different time points. Gene expression analysis was carried out using TaqMan assays (assay ID: Hs00181231_m1 for A_1R, assay ID: Hs00169123_m1 for $A_{2A}R$, assay ID: Hs00386497_m1 for $A_{2B}R$ and assay ID: Hs04194761_s1 for A_3R; Applied Biosystems, Foster City, CA, USA). Moreover, the TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used according to standard protocols. Gene expression levels were normalized (ΔCt) by using the housekeeping glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control (assay ID: Hs00187842_m1, Applied Biosystems, Foster City, CA, USA). The results were analyzed for relative quantitation among groups using the comparative $2^{-\Delta\Delta Ct}$ method.

2.7. Western Blot Analysis

Cells, harvested at 4 °C in lysis buffer containing a protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy), were centrifuged at 14,000 rpm (10 min, 4 °C). Protein amount was measured by BioRad protein assay (Bio-Rad Laboratories, Milan, Italy). Samples (usually 50–60 µg), diluted in sodium dodecyl sulphate (SDS)-bromophenol blue buffer, were boiled (5 min) and separated on 10% SDS polyacrylamide gels. Proteins, once transferred on polyvinylidene fluoride membrane, were blocked with PBS/0.1% Tween20/5% nonfat milk (Bio-Rad Laboratories) for 2h at 4 °C and then incubated overnight at 4 °C with primary antibodies (polyclonal rabbit: anti-Adenosine A_1 Receptor, dilution 1:200, Alomone Labs, Jerusalem, Israel; anti-peroxisome proliferator-activated receptor gamma, PPARγ, dilution 1:1000, from Abcam, Cambridge, UK; anti-fatty acid-binding protein 4, FABP4, dilution 1:1000 and anti-phosphorylated Akt, pAkt, dilution 1:1000, both from Cell Signaling, Danvers, MS, USA). Subsequently, the membranes were exposed to goat anti-rabbit HPR-conjugated secondary antibody (final dilution 1:5000, incubation for 1h at room temperature; Bethyl Laboratories Inc., Montgomery, TX, USA). Sample equal loading was determined by stripping and re-probing the blots with an anti-ß-actin antibody (dilution 1:1000, incubation overnight at 4 °C; Santa Cruz Biotechnologies, Heidelberg, Germany) or, as for pAkt detection, with an anti-Akt antibody (dilution 1:1000, Cell Signaling, Danvers, MS, USA). Immunocomplexes were visualized by chemiluminescence (ECL) detection system (GE Healthcare Life Sciences, Milan, Italy) and quantified by densitometric analysis (ImageJ V 1.53 software; U.S. National Institutes of Health, Bethesda, MD, USA).

2.8. Glycerol 3-Phosphate Dehydrogenase Activity

The activity of the enzyme glycerol 3-phosphate dehydrogenase (GPDH), which is important for both carbohydrate and lipid metabolism, was evaluated by an assay kit suitable for its detection in tissue and cell culture samples (Sigma-Aldrich, Milan, Italy). Following manufacturer's instructions, GPDH activity was determined by measuring a colorimetric product with absorbance at 450 nm proportional to the enzymatic activity present in each sample. One unit of GPDH corresponds to the amount of enzyme required to generate 1.0 µmol of NADH per minute at pH 8 at 37 °C.

2.9. Statistical Analysis

All experiments were performed from three to four times. Some results such as those related to ORO staining or Western blots are presented as representative images of more similar experiments in which cells from sister cultures were used. The quantitative data are expressed as mean ± SD (standard deviation). Time-response curves were calculated by using nonlinear regression (GraphPad Prism 6.0 software, San Diego, CA, USA). Statistical analyses were performed by Prism 6.0 software, using Student's t test using the Holm–Sidak method or one-way analysis of variance (ANOVA) followed by a post hoc comparison test (Dunnett's or Tukey's test). Group differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Evaluation of Some Biological Characteristics Shown by ASCs Derived from Normal Weight and Obese Subjects

We first characterized some properties of the ASCs derived from the subcutaneous adipose tissue of normal weight and obese subjects. Thus, we observed that the number of viable cells counted by the trypan blue exclusion method was lower for both cell types when they were induced towards adipogenic differentiation (Figure 2A); moreover, the number of live ASCs from normal-weight subjects was mostly greater than those of obese subjects both when undifferentiated and grown in adipogenic medium for 10 DIV. These findings were substantially confirmed by the MTS assay, generally used to measure the viability of the cells and thereby, their proliferative capacity (Figure 2B). Finally, both cells exhibited a significant adipogenic differentiation, as revealed by the ORO staining and spectrophotometric evaluation performed 28 days after the adipogenesis induction; however, in the ASCs of obese subjects the differentiation ability was greater than in the ASCs of normal-weight subjects, this also being evident from the increase in the number of lipidic vacuoles inside cells from obese subjects (Figure 2C).

3.2. Expression of Adenosine A_1 Receptors in ASCs from Normal Weight and Obese Subjects

We then examined the expression of adenosine receptors A_1, A_{2A}, A_{2B} and A_3 in the ASCs under study by real-time PCR (Figure 3A and Supplementary Figure S1). They were all present, although there were some fluctuations in the respective mRNAs, since their levels, except those of A_3 receptors, were higher in undifferentiated cells after a prolonged period (21 DIV). The A_{2B} receptors showed the greatest expression in differentiated ASCs, while the A_{2A} and A_3 receptors were the least expressed, the latter showing mRNA levels higher in ASCs of normal weight and obese subjects at 7 DIV after the adipogenesis induction. Focusing on A_1R, their expression was fairly stable in cells over the adipogenic differentiation period up to 21 DIV (Figure 3A), while resulting higher in undifferentiated ASCs at 21 DIV. Western blot analysis of A_1R protein content substantially confirmed the data obtained by real-time PCR in ASCs from normal weight individuals either when undifferentiated or during their commitment towards the adipogenic differentiation, while showing an increase or decrease of the A_1R protein, respectively, in undifferentiated or differentiated ASCs of obese individuals at 21 DIV (Figure 3B).

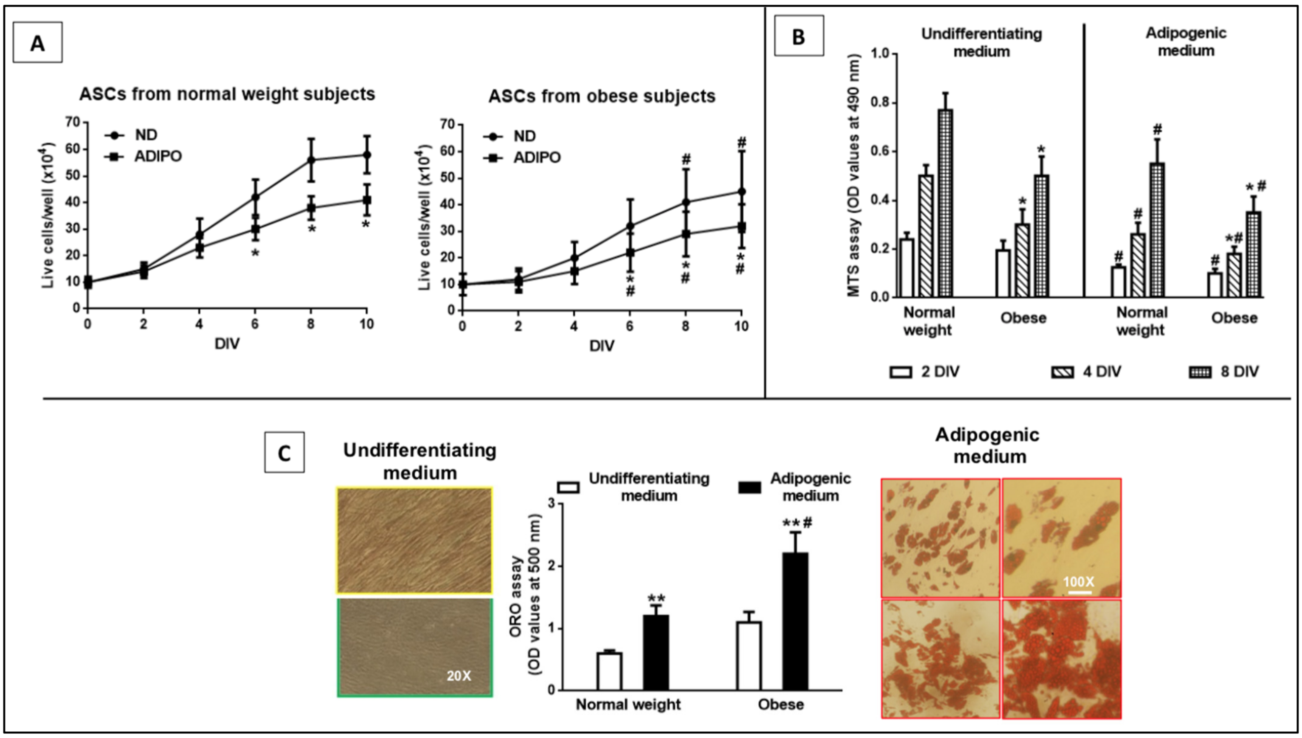


Figure 2. Characterization of the proliferation rate and the adipogenic differentiation ability of ASCs from the subcutaneous tissue of normal weight and obese individuals. (**A**) ASCs were initially seeded at 10^4 cells/well in 35 mm dishes. The count of the cell number was performed using the trypan blue exclusion method (see in the Methods section) every two days from 0 up to 10 days in vitro (DIV) in ASCs of both types grown in undifferentiating or adipogenic medium. (**B**) Proliferation of ASCs evaluated by MTS assay in cells undifferentiated or induced to adipogenic differentiation, seeded at a concentration of 1000 cells/well in 96 well plates, for a period of 2 to 8 DIV. The proliferation rate was measured as absorbance detected at 490 nm in the cells mentioned above and the results are expressed as units of optical density (OD). In the panels A and B, the values are the mean ± SD of 4 independent experiments using for each one different cell donors. Panel A statistical significance: * $p < 0.05$ normal-weight or obese ASCs induced to differentiation vs. the corresponding cells maintained in undifferentiating medium (one-way ANOVA plus Dunnett's test); # $p < 0.05$ ASCs of obese patients grown in undifferentiating or adipogenic medium vs. cells of normal-weight subjects maintained in the corresponding experimental condition (Student's t test using the Holm–Sidak method). Panel B statistical significance: * $p < 0.05$ obese vs. normal-weight ASC; # $p < 0.05$ ASCs grown in adipogenic medium vs. cells maintained in undifferentiating medium (one-way ANOVA plus Dunnett's test). (**C**) The adipogenic differentiation was evaluated in ASCs from the subcutaneous adipose tissue of normal (top) and obese individuals (bottom) subjected to differentiation for 28 days by staining with Oil Red O (ORO) stain. The stained vacuoles were visualized under an optical microscope (20× and 100× magnification; scale bar: 100 µm) while the quantification of the staining was performed by reading the isopropanol extracts on the spectrophotometer at 500 nm. The images are representative of cells from three normal weight and three obese subjects. The graphs show the units of optical density (OD) on the ordinate scale. The results are the mean ± SD of three independent experiments. ** $p < 0.01$: statistical significance measured against undifferentiated cells; # $p < 0.05$: obese vs. normal weight ASCs induced to differentiation (Student's t test using the Holm–Sidak method).

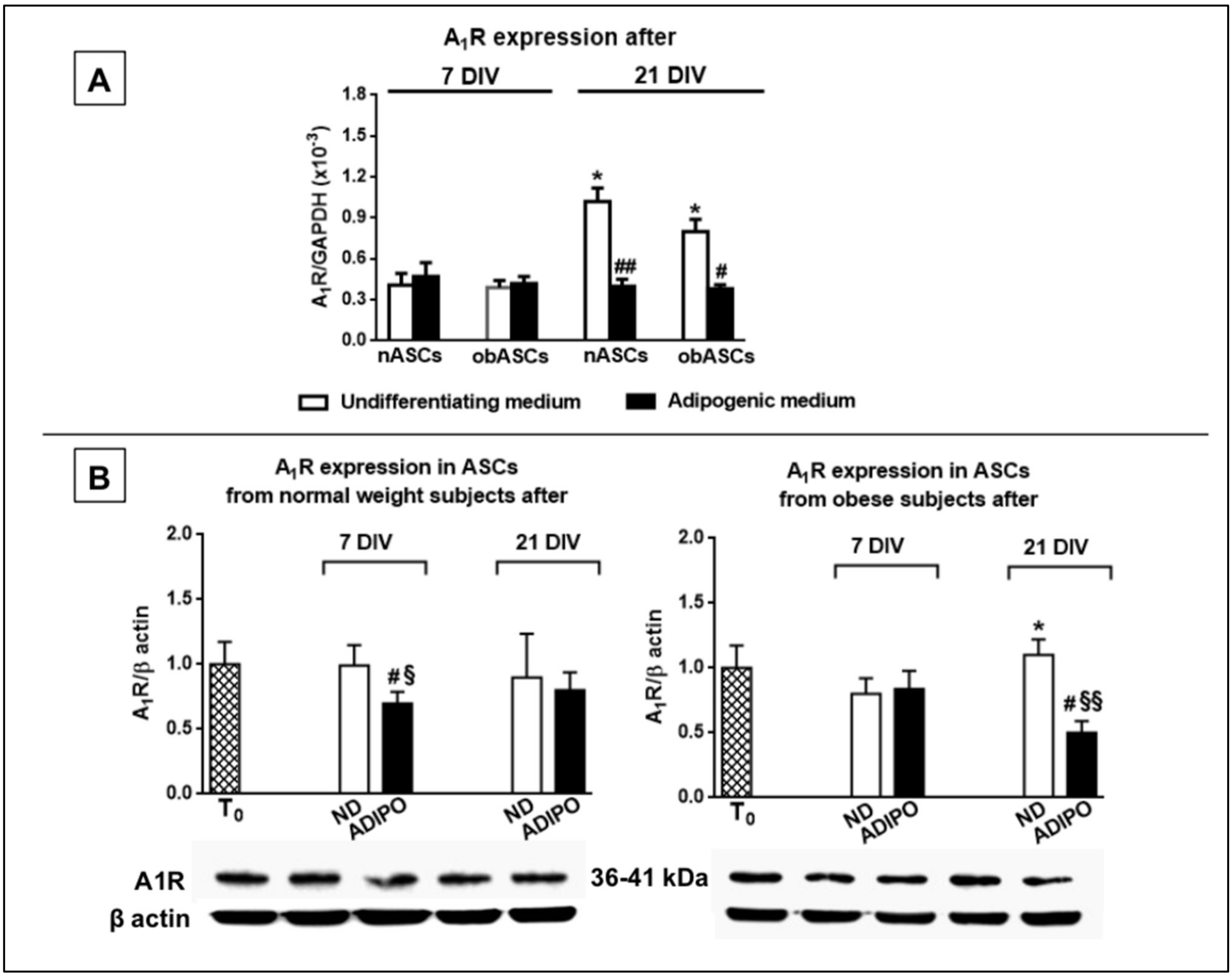


Figure 3. Expression of adenosine A_1 receptors (A_1R) in ASCs of normal-weight and obese subjects (nASCs and obASCs, respectively) grown in undifferentiating or adipogenic medium for different time periods. The presence of the A_1R has been evaluated by RT-PCR (panel A) and Western blot (panel B) analyses. In the latter, the immune-detected bands have been obtained from ASCs grown as undifferentiated cells (lane 2 and 4 in each blot) or in adipogenic medium (lane 3 and 5 in each blot) for 7 or 21 DIV. T_0 corresponds to cells at the time of the adipogenesis induction. Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or β-actin was used as internal control in (**A**) and (**B**), respectively. The molecular weight (expressed as kilodaltons, kDa) reported in the figure relating to Western blot analysis is that currently indicated in literature for the A_1R subtype and also in the datasheet of the antibody used for detecting it. Results are the mean ± SD of three independent experiments for each type of ASCs deriving from 3 different donors. The images are representative of blots obtained from these independent experiments, which gave very similar results. Statistical significance measured for: * $p < 0.05$, undifferentiated ASCs at 21 DIV against the same cells at 7 DIV; # $p < 0.05$ and ## $p < 0.01$, ASCs induced to differentiation vs. undifferentiated ASC at the same time period (7 or 21 DIV); § $p < 0.05$ and §§ $p < 0.01$, ASCs induced to differentiation vs. ASCs at T_0 (one-way ANOVA plus Tukey's test).

3.3. Effect of Cell Exposure to A_1R Ligands on ASC Proliferation

We investigated the effect of the pharmacological treatment of ASCs with A_1R ligands on their growth. We used for this and further experiments herein reported the well-known full A_1R agonist, CCPA, and antagonist, DPCPX, plus two home-made partial agonists A_1R agonists, C1 and C2, whose binding data and functional activity were previously reported in detail, demonstrating less affinity and potency of the compounds C1 and C2 at the A_1R level with respect to CCPA (Martire et al., 2019). The exposure of ASCs derived from normal weight or obese subjects to the full or partial A_1R agonists, alone or in the presence of the antagonist DPCPX, did not modify the proliferation of these cells when cultured in adipogenic medium (at 7 DIV), as assessed by MTS assay (Figure 4A).

Since MTS is not an efficient assay when the cells become confluent in the culture plate, that is after 8–10 DIV, we used two other assays to evaluate eventual cell death by necrosis (LDH assay) or apoptosis (caspase 3/7 assay), which may occur during the in vitro permanence of tested ASCs and/or upon their pharmacological treatments. Both assays demonstrated that longer cell exposure to the same drugs above mentioned up to 28 DIV did not cause necrosis or apoptosis since we determined similar values under basal conditions (i.e., without any drug treatment) (Figure 4B,C).

3.4. Effect of Cell Exposure to A_1R Ligands on ASC Adipogenic Differentiation

We then evaluated the activity of A_1R ligands administered for different periods on the differentiation of ASCs towards an adipogenic phenotype. Both cells derived from normal-weight and obese subjects accumulated lipids within the cytosolic vacuoles without any significant influence from their exposure to the full A_1R agonist for 7 or 14 days. In contrast, the adipogenic differentiation was reduced by the compound C2, as shown by cell staining with ORO and its spectrophotometric analysis at 7 and 14 days, whereas the compound C1 was active only at the highest concentration used, starting from the 14th DIV onwards (Figure 5A,B).

Even at the end of the adipogenic differentiation period (28 days), the lack of effect by CCPA, as well as the inhibitory activity on the differentiation process by the partial agonists in ASCs from normal-weight and obese subjects was still observed (Figure 6A,B).

Cell pretreatment with the antagonist DPCPX alone significantly inhibited cell differentiation and, when given together with each of the other drugs, this effect was prevalent and caused a further significant decrease of the adipogenic differentiation induced by both partial A_1R agonists, mainly at 28 DIV, in ASCs of normal-weight subjects (Figures 5A and 6A). Again, a similar behavior was observed in the cells from obese patients (Figures 5B and 6B).

3.5. Effect of Cell Exposure to A_1R Ligands on the Expression/Activity of Selected Markers of Adipogenic Differentiation

The exposure of both ASC types to the partial agonist C2, at the highest dose (500 nM), but not to the compound C1, was able to limit the expression of early markers involved in adipogenic differentiation such as PPARγ and FABP4, as shown by Western blotting at 10 DIV after the induction of the differentiation, while CCPA caused a decrease of only FABP4 content in ASCs from obese individuals (Figure 7A).

DPCPX showed effects similar to those exerted by the compound C2, except for PPARγ, whose content was reduced by the A_1R antagonist in ASCs of obese subjects, however, without reaching statistical significance.

We also evaluated the activity of another marker of adipogenic differentiation, namely GPDH, which catalyzes the reversible conversion of dihydroxyacetone phosphate and NADH to glycerol-3-phosphate and NAD^+, thus, being important for lipid metabolism. In pilot experiments, GPDH has been shown to reach its maximum activity around 12–14 days of differentiation into ASCs derived from normal weight and obese individuals, respectively (data not shown). Thus, the activity of this enzyme, assessed at 14 DIV, was increased by induction towards adipogenesis to a greater extent in ASCs from obese subjects than in those from normal weight individuals. In both cell types, such an increase was not modified by cell exposure to CCPA, whereas the compound C1 and even more the compound C2 or the A_1R antagonist significantly reduced the enzyme activity (Figure 7B).

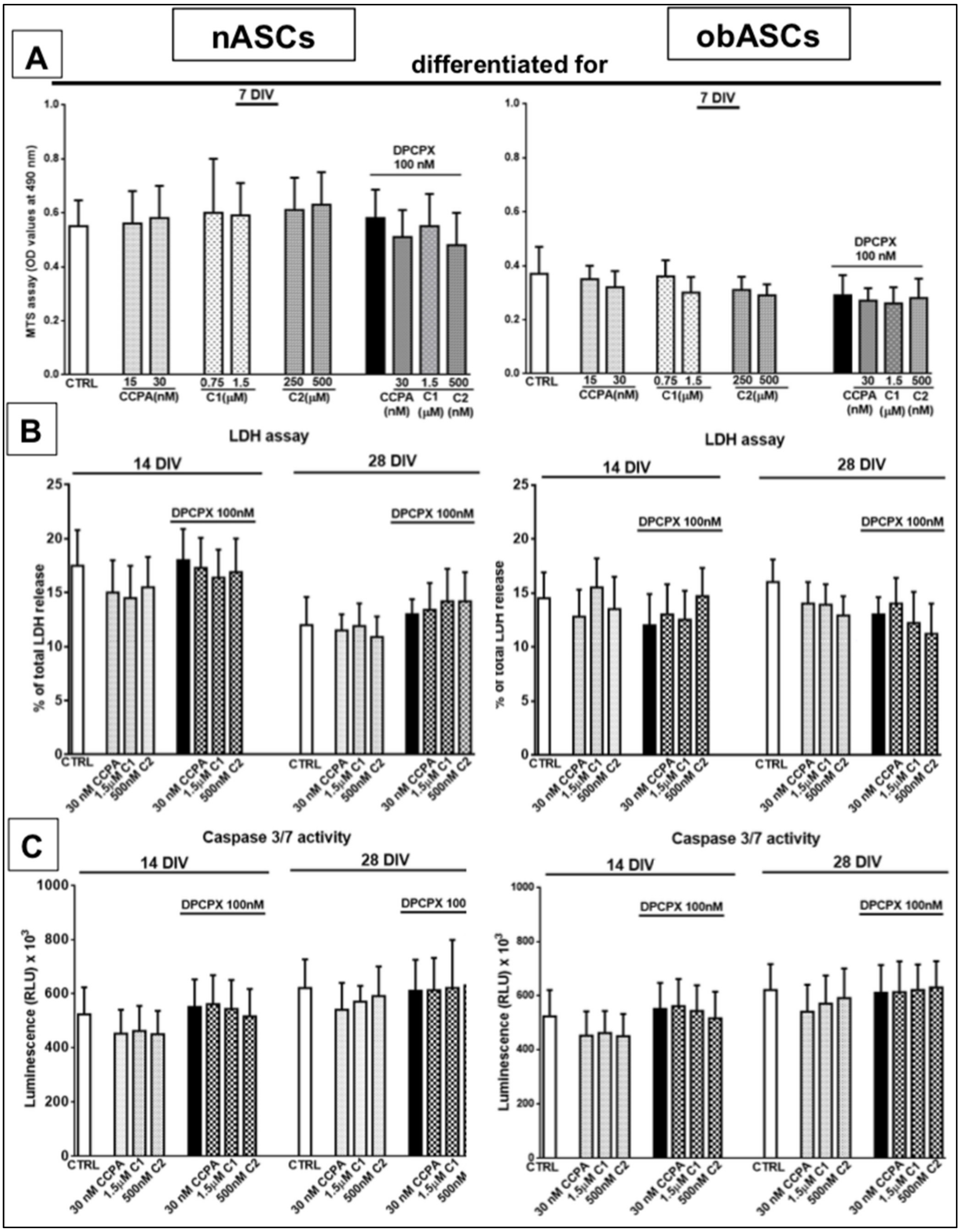


Figure 4. Effect of A_1R ligands on growth and necrotic or apoptotic death of ASCs from the subcutaneous tissue of normal weigh or obese subjects. ASCs induced towards an adipogenic differentiation were treated with CCPA or C1 or C2 compounds, in the absence or presence of the A_1R antagonist DPCPX, administered 1 h prior to the full or partial agonists. After different time periods from the beginning of the pharmacological treatments, the proliferation rate of the cells was evaluated by MTS assay, activity of LDH or caspases 3/7. (**A**) MTS assay performed at 7 DIV, following the protocol reported in the Methods section and in the legend of the panel B of Figure 2. (**B**) LDH release from cells, assumed as an index of necrotic death, was measured by a commercially available kit. Values are expressed as the percentage of the total amount of the enzyme released in the medium from the cells after their lysis. (**C**) Apoptotic death was assessed by the evaluation of the release of caspases 3 and 7, the most involved in this process, by luminescence using a commercial kit and following the manufacturer's instructions. All values in the graphs are the mean ± SD of four independent experiments in which each sample was tested in triplicate. Statistical significance was performed showing that all pharmacological treatments did not significantly affect values measured in basal condition (control: CTRL) (one-way ANOVA plus Dunnett's test).

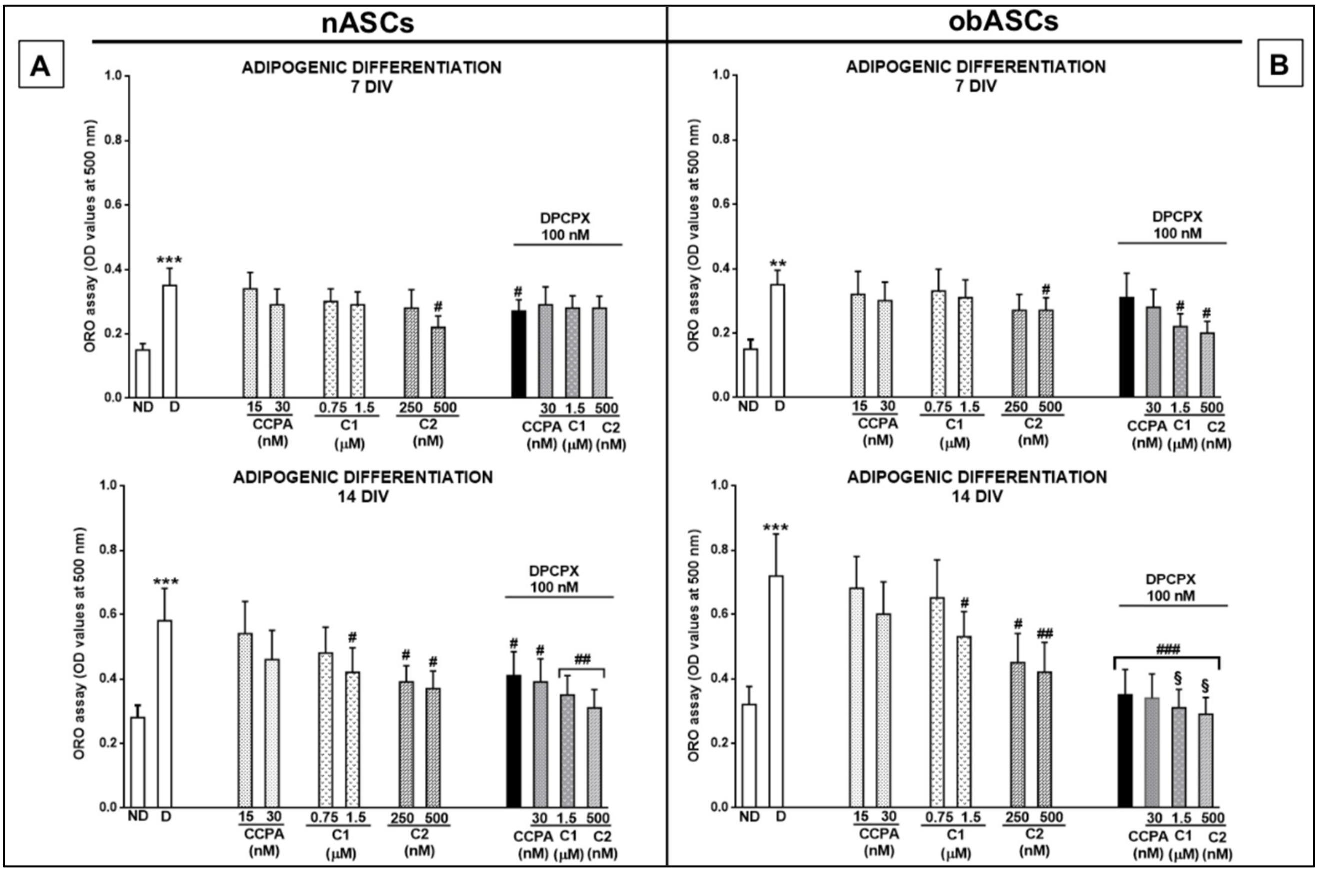
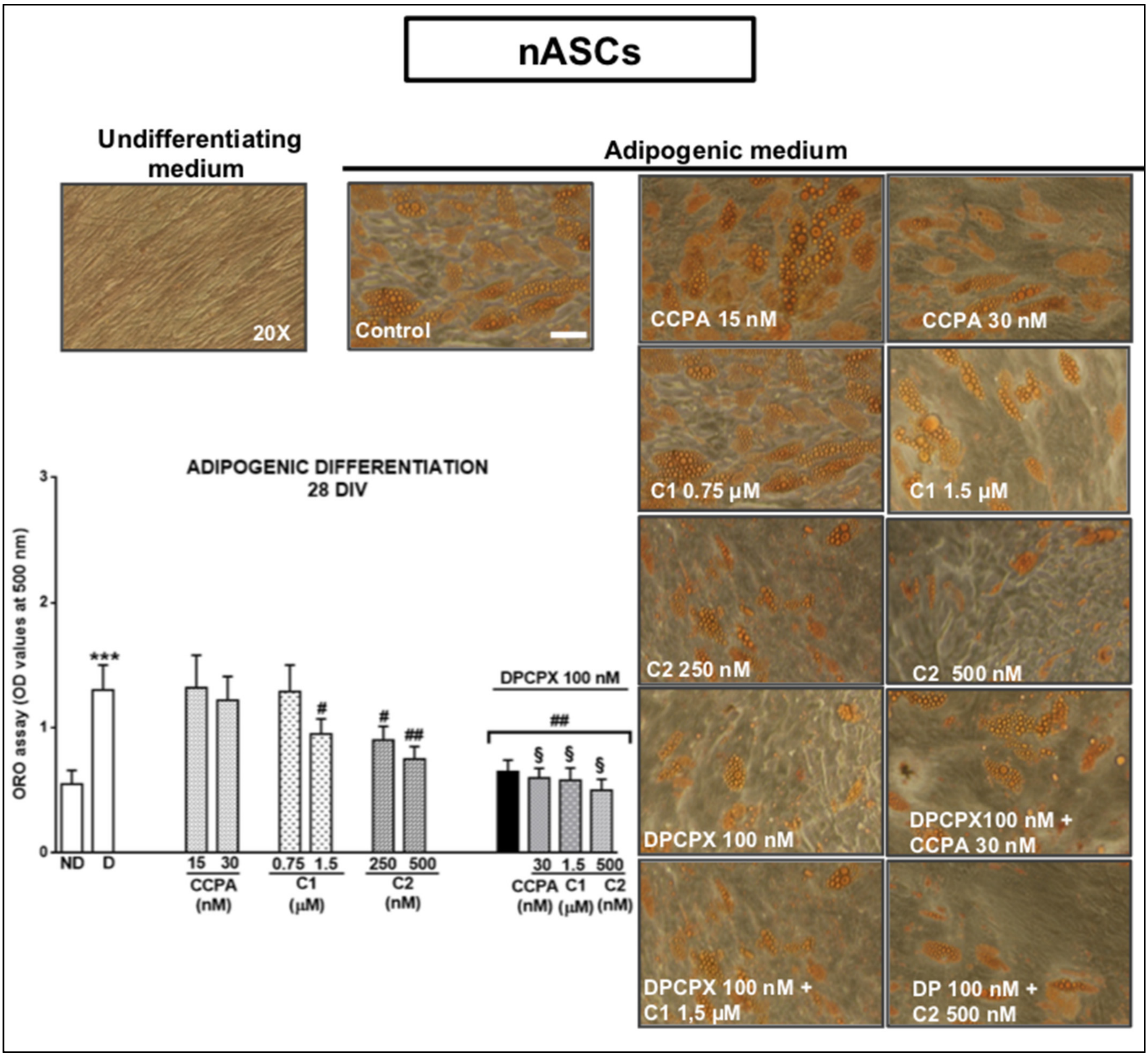


Figure 5. Effect of A_1R ligands on the adipogenic differentiation of ASCs of normal-weight and obese subjects. ASCs were grown in adipogenic medium for 7 and 14 DIV and exposed to the indicated pharmacological treatments along these time periods. Each drug was added to the cultures at each medium change, that is every 7 days. (**A**,**B**) Quantitation of the Oil Red O (ORO) staining by spectrophotometer reading of the isopropanol extracts at 500 nm. The bar graph shows units of optical density (OD). Results are the mean ± SD of four independent experiments, with cells from different donors. ** $p < 0.01$; *** $p < 0.001$: statistical significance of differentiated (D) vs. undifferentiated ASCs (ND) (one-way ANOVA plus Dunnett's test); # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$: statistical significance of values obtained in differentiated ASCs exposed to pharmacological treatments vs. the same cells not exposed to drugs; § $p < 0.05$: statistical significance of values obtained in differentiated ASCs exposed to A_1R full or partial agonists plus DPCPX vs. the differentiated ASCs exposed to the same pharmacological treatments without the presence of DPCPX (one-way ANOVA plus Tukey's test).

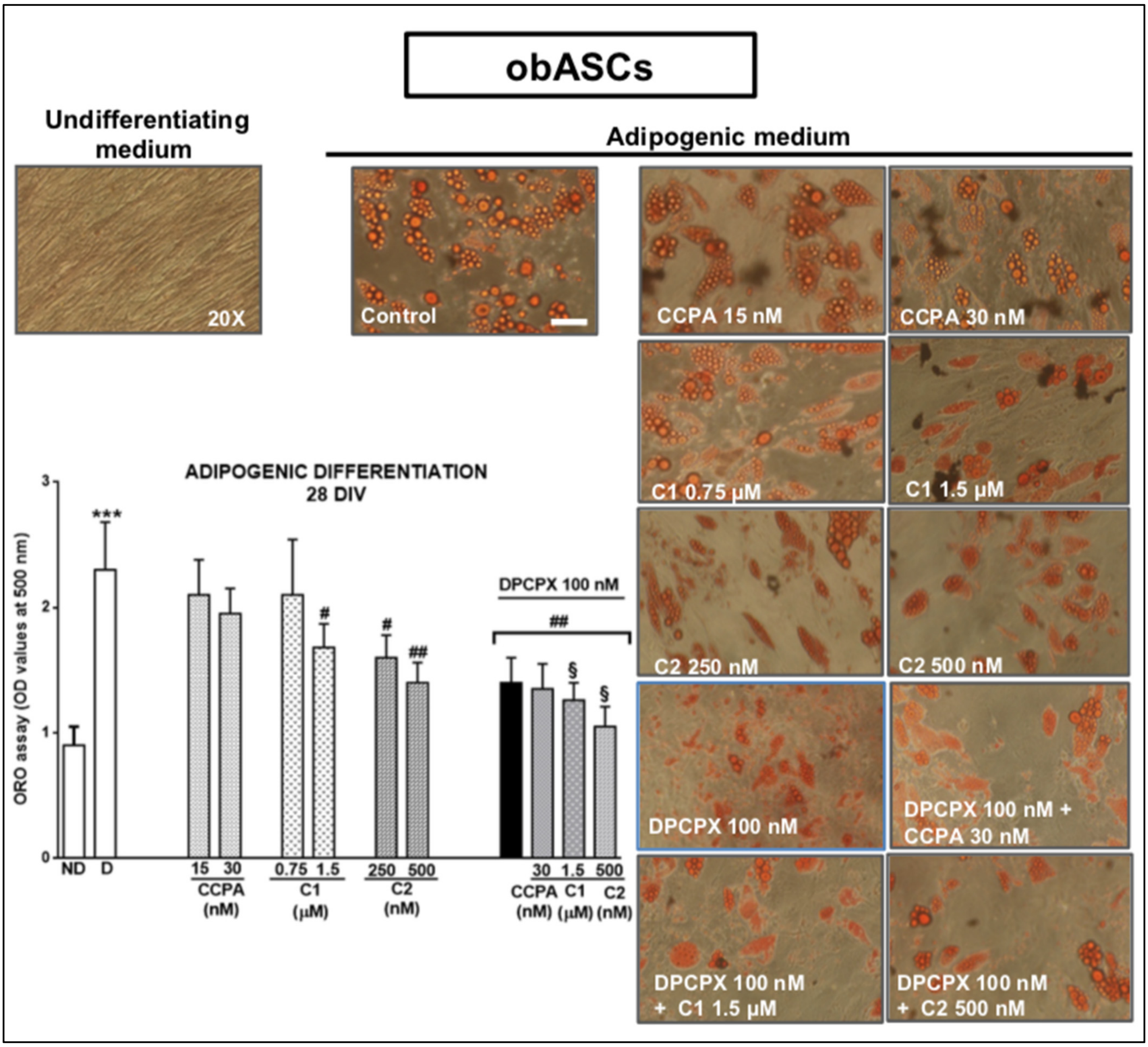
3.6. Effect of Cell Exposure to Full or Partial A1R Agonists on the Expression of Akt/Protein Kinase B, a Downstream Effector in the Phosphoinositol-3 Kinase (PI3K) Pathway, Which Is Involved in the Adipogenic Differentiation Process

Finally, we investigated the involvement of the PI3K pathway, which is normally activated by insulin, one of the agents present in the mixture added to the culture medium to favor ASC adipogenic differentiation [23]. Indeed, we found that during this process there was an increase in the phosphorylation of Akt (pAkt), also known as protein kinase B (PKB), a downstream effector of PI3K, which was evident in the ASCs of normal-weight subjects starting from the 3rd DIV onwards, while it reached a maximum expression at 14 DIV in the ASCs of obese individuals, thereafter, decreasing mainly in the latter. The Akt phosphorylation, evaluated at 14 DIV, was not significantly affected by CCPA or by the compound C1 in either cell types, whereas it was remarkably decreased by the compound C2, as well as by DPCPX (Figure 8).



(**A**)

Figure 6. *Cont.*



(**B**)

Figure 6. Effect of A_1R ligands on the adipogenic differentiation of ASCs of normal-weight and obese subjects. ASCs were grown in adipogenic medium for 28 DIV and exposed to the indicated pharmacological treatments during this period. Each drug was added to the cultures at each medium change, that is every 7 days. (**A**,**B**) The panels are representative images showing ASCs of normal-weight (panel **A**) and obese (panel **B**) subjects stained by Oil Red O (ORO) at 28 DIV. Cells were photographed (40× magnification; scale bar: 100 μm) by using a Cool-SNAPcf digital CCD camera (PhotoMetrics, Huntington Beach, CA, USA). The bar graphs, related to the quantitation of the ORO staining by spectrophotometer reading of the isopropanol extracts at 500 nm, show units of optical density (OD). Results are the mean ± SD of four independent experiments, with cells from four different donors. *** $p < 0.001$: statistical significance of differentiated vs. undifferentiated ASCs not exposed to pharmacological treatments (one-way ANOVA plus Dunnett's test); # $p < 0.05$, ## $p < 0.01$: statistical significance of values obtained in differentiated ASCs exposed to pharmacological treatments vs. the same cells not exposed to drugs; § $p < 0.05$: statistical significance of values obtained in differentiated ASCs exposed to A_1R full or partial agonists plus DPCPX vs. the differentiated ASCs exposed to the same pharmacological treatments without the presence of DPCPX (one-way ANOVA plus Dunnett's test).

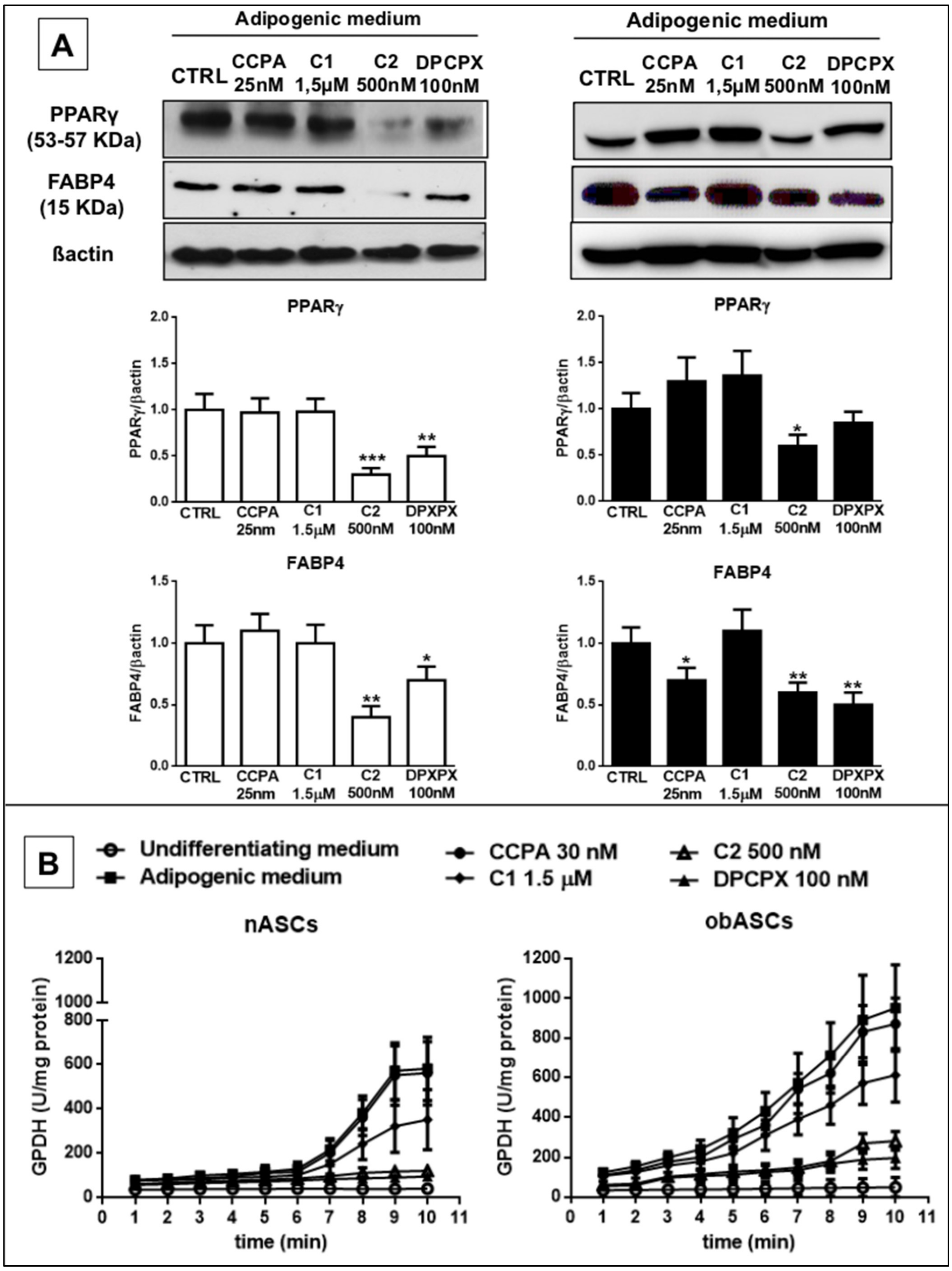


Figure 7. Effect of A_1R ligands on the expression of adipogenic markers in ASCs of normal weight and obese individuals. ASCs growing in adipogenic medium for 10 DIV were exposed to CCPA, compounds C1 and C2 or DPCPX at the indicated concentrations. Drugs were added every 7 DIV, at each medium change. (**A**) Levels of PPARγ and FABP4 were determined by Western blot analysis (50–60µg of proteins were loaded per lane). Immunoblots, re-probed with antibody against β actin, to assure equal sample loading, were quantified by densitometric analysis, the values of which, normalized to β actin, are reported in the histograms. Statistical analysis: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significantly different from untreated ASCs submitted to adipogenic differentiation for 10 DIV (CTRL, control) (one-way ANOVA plus Dunnett's test). (**B**) The activity of glycerol-3-phosphate dehydrogenase (GPDH) was determined in ASCs growing in adipogenic medium for 14 DIV and exposed during this period to the pharmacological treatments indicated above using a commercially available kit. The values are expressed as units per mg of proteins. In both panels the values are the mean ± SD of three independent experiments for each type of cells. Statistical analysis: data in the curves related to the effects caused by C1 compound, C2 compound and DPCPX either in ASCs of normal-weight subjects (C1 compound: $p < 0.05$; C2 compound and DPCPX: $p < 0.01$) or ASCs of obese individuals (C1 compound: $p < 0.01$; C2 compound and DPCPX: $p < 0.001$) were significantly different from those evaluated in both types of ASCs grown in adipogenic medium without exposure to any pharmacological treatment (one-way ANOVA plus Dunnett's test).

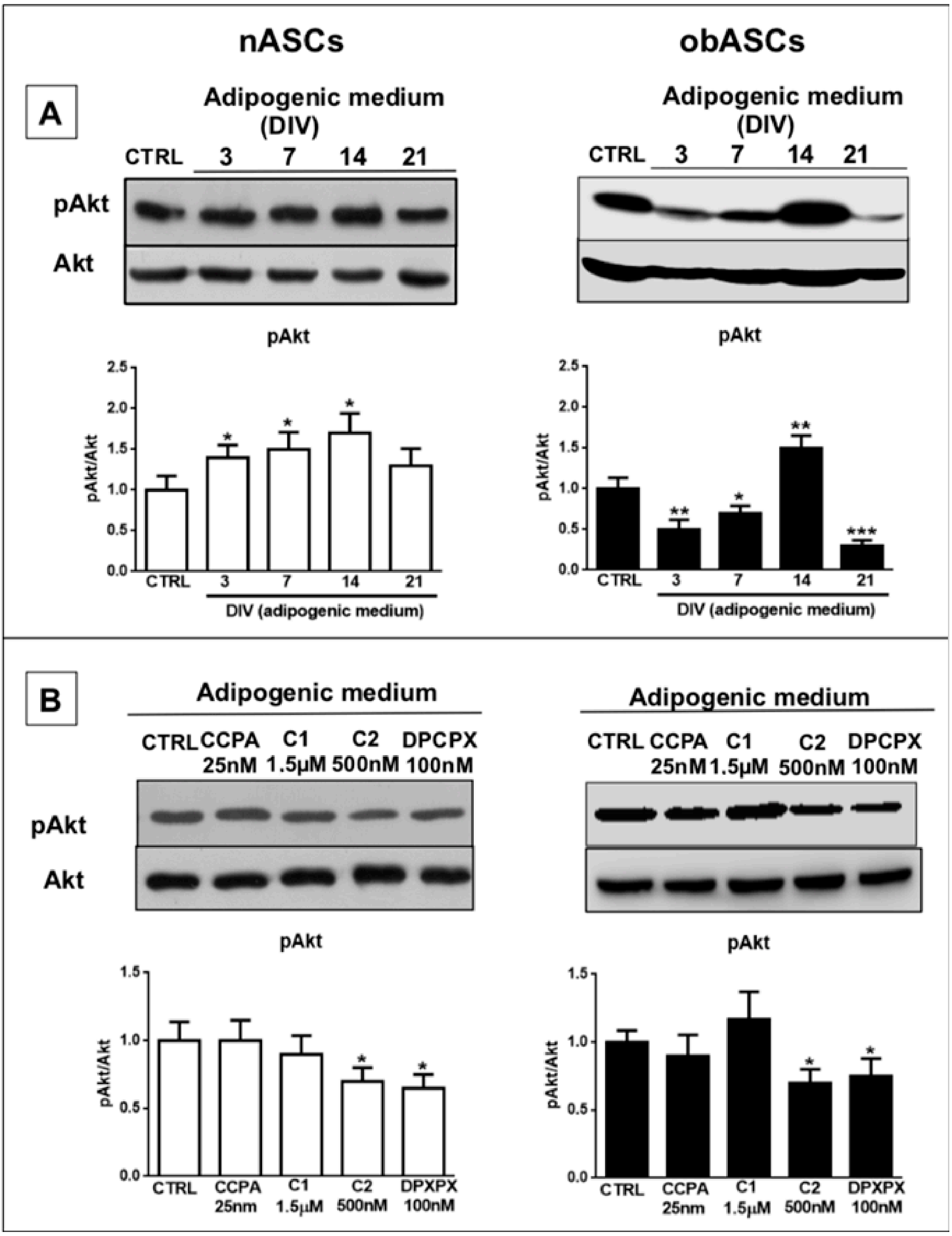


Figure 8. Effect of A_1R ligands on the phosphorylation of Akt evaluated by Western blot analysis in ASCs of normal and obese subjects. (**A**) ASCs of normal-weight (left panel) and obese (right panel) individuals were induced towards adipogenic differentiation for 3 up to 21 DIV. CTRL (control): Akt phosphorylation revealed in undifferentiated cells. (**B**) ASCs of normal-weight (left panel) or obese (right panel) subjects committed towards differentiation were exposed to A_1R ligands at the indicated concentrations for 14 DIV (culture medium and drugs were renewed every 7 days). In A and B panels, Akt phosphorylation was determined by Western blot analysis (50–60µg of proteins were loaded per lane). Immunoblots, re-probed with an antibody against nonphosphorylated Akt to assure equal sample loading, were quantified by densitometric analysis, the values of which, normalized to Akt, are reported in the histograms. The images are representative of blots obtained using different ASCs and the values in the graphs are the mean ± SD of three independent experiments for each type of cells. Statistical analysis: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$: significantly different from undifferentiated (panel A) or untreated ASCs submitted to adipogenic differentiation for 14 DIV (panel B) (one-way ANOVA plus Dunnett's test).

4. Discussion

In our study, we first compared some biological properties of the ASCs of normal-weight and obese subjects related to their proliferation and adipogenic differentiation ability, observing that the latter showed a lower proliferation rate, both when undifferentiated or induced towards adipogenic differentiation, as well as a greater propensity to differentiate into adipocytes than the former. These results are in partial agreement with previous data in which a decrease in both proliferation and adipogenic differentiation of ASCs of obese subjects compared to those from normal weight individuals was demonstrated [24]. However, in that study, the adipogenic differentiation was determined in ASCs exposed for 21 days to hypoxic conditions, representing for the authors the in vivo physiological environment for mesenchymal cell niches [25]. In contrast, we maintained all our cells in normoxic conditions; it is therefore possible that, in our case, ASCs of obese individuals are more likely to differentiate into adipocytes than those from normal-weight subjects, confirming the evidence that ASCs deriving from the SAT of overweight subjects usually offer an adequate support for the expansion of fat mass [26].

Our results also showed that both cell types were endowed with all adenosine receptors, the expression of which has previously been reported in several human and rodent mesenchymal stem cells, including ASCs, with the highest expression of adenosine A_{2B} receptors [27]. In relation to A_1R, on which we focused our study, in a previous study we pointed out its expression also in ASCs from normal subjects induced towards osteogenesis, showing that A_1R stimulation by the agonist CCPA enhanced their osteogenic differentiation, an effect abolished by cell pretreatment with another A_1R selective antagonist, 1-butyl-8-(hexahydro-2,5-methanopentalen-3a(1H)-yl)-3,7-dihydro-3-(3-hy-droxypropyl)-1H-purine-2,6-dione (PSB36) [22]. Here, we followed the trend of A_1R expression along the process of the adipogenic differentiation of ASCs and, as far as we know, this is the first time that this aspect has been evaluated in primary cultures of ASCs from obese individuals. A_1R expression was fairly stable in cells of normal-weight subjects, while it tended to decrease in those of obese individuals, resulting even lower than that observed in the undifferentiated ASCs from the same source at 21 DIV. These findings seem not to be in complete agreement with the current literature. Indeed, it has been reported that A_1R expression generally increases during the differentiation of adipose cells, reaching the highest values in mature adipocytes, as reviewed in [27]. However, for most experiments the mesenchymal stem cells of rodents were used [28–30]. In this respect, it should be emphasized that the expression of A_1R, and more generally of most receptors, usually depends on several factors including diseases, age or race [31–34], as well as cell biology or molecular pathways related to signal transduction [35,36]. Instead, in line with our observations, it depends on the reduced number of A_1R in the adipocyte membranes from obese humans as previously reported [37]; moreover, a decreased expression of A_1R was found in the visceral adipose tissue of Afro-American rather than Caucasian women and this could support the observation that the former showed greater difficulty than the latter in losing weight [35].

Since A_1R activation has long been known to inhibit lipolysis in adipose tissue/mature adipocytes [15,38], we initially hypothesized that A_1R stimulation could also induce the commitment of ASCs towards adipogenesis, favoring the accumulation of fat in the vacuoles. The findings reported here showed another picture. In fact, the activation of A_1R by the full agonist CCPA did not substantially modify the adipogenic differentiation of ASCs deriving from normal weight or obese subjects, at least in vitro. In contrast, the two A_1R partial agonists analyzed, mainly the compound C2, as well as the selective A_1R antagonist DPCPX limited the differentiation process and this effect was not due to a reduction in cell viability or an increase in apoptotic or necrotic cell death, either when drugs were used alone or in combination and even for a long period, i.e., up to 28 DIV.

In relation to the different activity exhibited by the two A_1R agonists, recent studies, in which CCPA was used as a reference full agonist, have demonstrated that the compound C1 behaved as a partial agonist on A_1R in functional studies aimed at evaluating its ability to inhibit cAMP production in CHO cells, stably transfected with hA_1R, contraction of the smooth muscle of mouse ileum or heart rate (α = 0.70, 0.75 and 0.32, respectively) [20]. On the contrary, the compound C2 behaved as a full agonist in CHO cells and as partial agonist in the aforementioned mouse ileum or heart rate (α = 1 and 0.78 or 0.42, respectively). However, it is worthwhile that the first type of experiments accounts for the early phases following activation of A_1R, while the second ones evaluate the final tissue effect, which may also involve other intracellular pathways. Therefore, the latter effects seem to be more significant in defining the efficacy of such compounds, which behaved as partial agonists in the whole tissue. In this respect, it is now recognized that the affinity of a ligand towards a receptor and the signals activated downstream can depend on a variety of factors, including variations in the receptor expression in different tissues and/or differential complexation of the receptor to the microenvironment of membrane [39].

Therefore, to interpret our results, we propose the following explanation: in vitro adipogenic differentiation of ASCs is usually induced by means of conditioned media containing factors that favor this process. More specifically, the "differentiation" medium (commercially available), we used for the first week of induction of ASCs to adipogenesis, contained insulin, dexamethasone and 3-isobuyl-1-methylxanthine (IBMX) at defined concentrations, while for the next period of 21 DIV (i.e., up to 28 DIV), we used a "maintenance" medium obtained from the same manufacturer without IBMX. The presence of this agent, known as an inhibitor of phosphodiesterases, the enzymes responsible for the inactivation of cAMP, is crucial in the first phase of the adipogenic differentiation. In fact, an increase in intracellular levels of cAMP is essential to initiate the differentiation process, while it can be harmful if present in the subsequent period [40], when insulin, whose pro-adipogenic activity is mainly linked to the activation of the PI3K pathway, becomes more important, being crucial mainly in the late part of the process leading to a complete adipogenic differentiation of ASCs [23]. On the other hand, it is known that the stimulation of A_1R is generally linked to the inhibition of cAMP formation through the interaction with an alpha subunit of the Gi protein, whereas the activity of the beta and gamma subunits of the Gi protein coupled to A_1R could cause the activation of other molecular pathways, including that of PI3K leading to Akt phosphorylation [41–43]. Thus, the full agonist CCPA, causing the activation of Gi proteins, would inhibit the formation of cAMP, at the same time stimulating the PI3K pathway.

On this basis, it could be expected that CCPA administration caused a decrease in ASC differentiation after 7 DIV, while increasing this process during and/or at the end of a longer period of additional 21 DIV. However, in our experiments, CCPA did not substantially alter the ASC adipogenic differentiation, apart from causing decreased expression of the adipogenic marker FABP4 only in the ASCs of obese individuals, which was not sufficient to restrain the adipogenic differentiation of these cells. Thus, we think that CCPA, at the doses used in our experiments, was unable to affect the early phase as well as to increase the final differentiation process of ASCs, given the presence of a large amount of factors in the culture media favoring adipogenesis, including insulin.

As for the effects provoked by DPCPX, they should be substantially linked to the A_1R antagonism that usually produces lipolysis, an event linked to the inhibition of Gi protein activity which in turn prevents both the decrease in cAMP and the activation of molecular pathways including that of PI3K [44,45]. Finally, as for the effect promoted by the partial agonists, it is reasonable to interpret the reduction of the final adipogenic differentiation of ASCs as linked to the partial affinity/antagonism of these compounds to A_1R with a consequent partial inhibition of the A_1R-linked Gi protein activities. Accordingly, the addition of DPCPX to the compounds C1 or C2 further decreased the adipogenic differentiation of ASCs. The fact that the compound C1 did not show a decrease in the expression of adipogenic differentiation markers (PPARγ and FABP4) or Akt phosphorylation could be due to the inhibitory effect of this drug on the ASC adipogenic differentiation becoming evident from 14 DIV onwards, likely indicating that it requires a more prolonged exposure.

In summary, S-ASCs from normal weight or obese subjects express A_1R during their adipogenic differentiation, although with some differences. In comparison with CCPA, the full A_1R agonist, the partial A_1R agonists, such as the A_1R antagonist, were able to reduce the adipogenic differentiation of ASCs without altering their growth/viability. Drug effects were not substantially affected by the source from which the ASCs were obtained, that is from the subcutaneous tissue of normal weight or obese subjects.

At this point, a crucial question arises: given the invoked anti-lipolytic effect by A_1R agonists and their supposed benefits in obesity, especially when linked to diabetes, and the parallel evidence that the use of full A_1R agonists may provoke unwanted side-effects [17], is the use of A_1R partial agonists advisable to help obese subjects in the control of weight, given the related harmful consequences?

To correctly answer this question, it would be necessary to evaluate first the activity of these compounds on mature adipocytes in order to assess their effect on lipogenesis/lipolysis and on animals in vivo to verify whether the drug-induced decrease in the number of cells differentiating into preadipocytes also occurs in these conditions. If data in vitro were confirmed, then the use of A_1R partial agonists like those we have examined should be, in our opinion, examined in animals/humans submitted to a balanced caloric restriction for the treatment of obesity, to avoid fat accumulation around vital organs (heart, liver, etc.) and the detrimental consequences of the metabolic syndrome. Indeed, since the enlargement of fat mass is likely associated with proliferation of cells mainly deriving from the subcutaneous district (i.e., ASCs), which would assure the storage of lipids in excess protecting individuals from the negative consequences of metabolic disease, the use of partial A_1R agonists would not limit proliferation of ASCs but could contrast their differentiation, thus impairing the task of accumulating fat in excess.

However, data so far obtained only in animals on A_1R and obesity are contrasting. Indeed, while no difference in body weight was noticed in A_1R knock-out (KO) or wild type mice grown on regular chow, a higher body weight was observed in aged A_1R KO rodents compared to wild type animals, that was ascribed to higher food intake. In contrast, a reduction in fat accumulation was also demonstrated in A_1R KO animals, probably due to a reduction in the development of the inflammatory environment often observed in obesity, as reviewed in [15,46].

It would be interesting to investigate whether treatment with A_1R partial agonists in obese animals/subjects performing physical exercise associated to a diet may produce further beneficial effects.

5. Conclusions

In conclusion, the use of A_1R full agonists in obesity would be more advantageous than that of partial A_1R agonists, since they reduce lipolysis in mature adipocytes and, at least in vitro, drugs like CCPA do not limit the proliferation and adipogenic differentiation of ASCs, reputed to increase the number of adipocytes to accumulate fat in excess. However, an excessive stimulation of A_1R could also lead to the development of obesity due to lipolysis inhibition [47]. Moreover, the problem of the negative side-effects caused by A_1R full agonists remains. Thus, the adoption of A_1R partial agonists would seem appealing. One of these (CVT-3619) could be particularly interesting, since it significantly reduced plasma free fatty acid levels in rats, without eliciting cardiovascular side effects; it also lowered plasma free fatty acids and triglycerides, improving insulin sensitivity and glucose clearance in diabetic animals. Moreover, when tested in healthy non-obese and obese subjects in early phase trials, showed to be a promising therapy for type 2 diabetes and dyslipidemia, reviewed in [48]. However, no clinical trial has proceeded to the next experimental phases. In the light of the results we have obtained, it is possible that partial A_1R agonists have failed as safe anti-obesity drugs due to the fact, among others, that, despite positive anti-lipolytic activity on mature adipocytes, they might counteract a safeguard mechanism in obesity, that is the differentiation of the ASCs in adipocytes necessary for storing circulating lipids, as we have demonstrated in our experiments.

Therefore, in our opinion, further studies on these and other agents should be re-thought, taking into account also the function function of ASCs in the control of obesity.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10123560/s1, Figure S1: Expression of adenosine receptors in ASCs of normal weight (nASCs) and obese (obASCs) subjects grown in undifferentiating or adipogenic medium for different time periods (7 and 21 days in vitro, DIV).

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