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ORIGINAL ARTICLE

Weight gain related to treatment with atypical antipsychotics is due to activation of PKC-β

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Atypical antipsychotics (APDs) are currently used in clinical practice for a variety of mental disorders such as schizophrenia, bipolar disorder and severe behavioral disturbances. A well-known disadvantage of using these compounds is a propensity for weight gain, resulting frequently in obesity. The mechanisms underlying pharmacologically induced weight gain are still controversial. The objective of this study was to evaluate *in vitro* the effects of different APDs on adipogenic events in cultured human pre-adipocytes and in rat muscle-derived stem cells (MDSCs), aiming to identify a common intracellular event contributable to these drugs. Culture behavior was evaluated in terms of cell proliferation, lipid accumulation, gene expression and morphological features. Results indicate that APDs influence adipogenic events through changes in the differentiation and proliferation of pre-adipocytes and MDSCs that are brought on by protein kinase C-β (PKC-β) activation. These data identify a signaling route that could be a potential target of pharmacological approaches for preventing the weight gain associated with APD treatment.
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Introduction

Atypical antipsychotics (APDs) are currently used in clinical practice for a variety of mental disorders such as schizophrenia, bipolar disorder and severe behavioral disturbances.[1–5] Currently, they are also being investigated for use in more mild pathologies such as borderline personality disorders associated with impulsivity, anger, hostility[6] and depression.[7] A well-known disadvantage of APD use is weight gain, obesity and metabolic side effects, including lipid abnormalities and diabetes.[2,8,9]

The mechanisms underlying this pharmacologically activated weight gain are still controversial. Several hypothesis have been explored, such as genetic factors,[8] association with central histamine H1 antagonism resulting in appetite increase,[10] modulation of serotoninergic/noradrenergic pathways in the central nervous system and/or involvement of leptin, gherlin and adiponectin hormones.[2]

Many studies have explored the mechanisms of change related to this adipogenesis. APDs were shown to directly induce insulin resistance and alter lipogenesis and lipolysis in favor of progressive lipid accumulation and adipocyte enlargement in rat adipocyte cultures. Clozapine was shown to enhance differentiation of adipose tissue precursor cells to mature adipocytes.[11] Similarly, olanzapine induced adipogenesis in 3T3-L1 preadipocytes, probably through

activation of the sterol regulatory element-binding protein-1 regulatory pathway.[12] A recent study, in which olanzapine, ziprasidone and haloperidol were tested on animal adipocytes, showed that only olanzapine administration decreased lipolysis in adipocytes, as well as induced a gene fatty acid synthase overexpression and a gene hormone-sensitive lipase repression that resulted in a significant fat accumulation in rats.[13]

In light of these considerations, it could be concluded that obesity event associated with administration of different APDs could be enhanced by several pathways. To our knowledge, no study has been performed to test different classes of atypical neuroleptics on human adipocytes. The objective of this study was to compare the *in vitro* effects of different APDs (clozapine, olanzapine, quetiapine, risperidone and aripiprazole) on adipogenic events in cultured human adipocyte-derived stem cells (ADSCs). Moreover, we analyzed whether the APDs influence the differentiation of uncommitted mesenchimal precursors or even the transdifferentiation of stem cells of other mesenchimal tissues, such as the muscle-derived stem cells (MDSCs) derived from neonatal rats. The MDSCs include the so-called satellite cells, that is, the precursor cells that are capable of differentiating *in vivo* to mature muscle fibers but that retain broad differentiation capacity, including the generation of adipocytes.[14] The effects of APDs on cellular proliferation, lipid production and gene expression were followed in both preadipocytes derived from ADSCs and in MDSC. As we have previously reported,[14] a high-glucose state drives stem cells from both adipose tissue and skeletal muscle toward an adipogenic potential, providing some insight into the signals that underlie this process. With regard to the signals driving fat accumulation, a crucial role of protein kinase C-β (PKC-β) was revealed by its strongly inhibitory effect on small interfering RNA (siRNA) in the neo-formation of adipose cells. With this in mind, we focused on the possible effects of these APDs on PKC-β activation of adipogenic events.

Materials and methods

Cell cultures: MDSCs

Primary cultures of MDSCs were prepared from newborn rats (2–3 days) as described in Brini *et al*.[15] Adipogenic conversion was observed both in the first preplating (15 min) and in the replating of non-rapidly adherent cells. For this reason, the first pool of adherent cells was used for all experiments. The viable cells obtained were counted using the Trypan blue exclusion assay and were seeded at a density of 10×10^5 cells per square centimeter for *in vitro* expansion in Dulbecco's modified Eagle's medium (DMEM) supplemented with glucose 25 mM for 5 days. At day 5, APDs (clozapine, olanzapine; quetiapine, risperidone and aripiprazole (250 mM)) were added for the following 3 days. The DMEM without the addition of high glucose (low glucose) was used as a negative control.

Adipose-derived stem cells

ADSCs were extracted from human adipose tissues of five healthy female patients undergoing cosmetic surgery procedures, following guidelines from the Clinic of Plastic Surgery, University of Padova. Adipose tissues were digested with 0.075% collagenase (type 1A; Sigma–Aldrich, Milan, Italy) in modified Krebs–Ringer buffer for 60 min at 37 °C followed by 10 min with 0.25% trypsin. The viable cells obtained were counted using the Trypan blue exclusion assay and seeded at a density of 1×10^6 cells per square centimeter for *in vitro* expansion in DMEM supplemented with glucose 25 mM after 5 days of expansion. At day 5, APDs were added for the following 3 days. DMEM without the addition of high glucose (low glucose) was used as a negative control.

For experiments relative to the effects of APDs on PKC-β inhibition, hispidine or lentiviral siRNA (see above) were added simultaneously with APDs.

Cell proliferation determination (MTT assay)

Cell proliferation rates were determined by the 3-4,5-dimethylthiazol-2yl-2,5-diphenyltetrazolium bromide (MTT)-based cytotoxicity test using the Denizot and Lang method with minor modifications.[16]

DNA content

DNA content was determined using a DNeasy kit (Qiagen, Jesi-Ancona, Italy) to isolate total DNA from cell cultures following the manufacturer's protocol for tissue isolation, using overnight incubation in proteinase K (Qiagen). The concentration of DNA was detected by measuring the absorbance at 260 nm in a spectrophotometer.

Real-time reverse transcriptase-PCR

For each target gene, primers and probes were selected using Primer3 software (White Head Institute, Cambridge, MA, USA). Gene expression was measured using real-time quantitative PCR on a Rotor-GeneTM 3500 (Corbett Research, Jesi-Ancona, Italy). PCR reactions were carried out using the primers at 300 nM and the SYBR Green I (Invitrogen, San Giuliano Milanese, Milan, Italy). All complementary DNA samples were analyzed in duplicate. For each complementary DNA sample, the Ct value of the reference gene β-actin was subtracted from the Ct value of the target sequence to obtain the ΔCt. Relative quantification of marker gene expression is given as a percentage of the β-actin product. Experiments were performed with three different cell preparations and repeated at least thrice.

Adipocyte differentiation detection

Oil red (Sigma–Aldrich) staining of the cytoplasmic droplets of neutral lipids was performed according to the modification from Ramirez-Zacarias *et al*.[17]

PKC-β silencing

PKC silencing was achieved by infecting cells the day after seeding with a commercially available lentiviral siRNA for PKC-β (Sigma–Aldrich), at a viral titol of 3.5 TU ml^{-1}. As a control, an unrelated gene (*FHIT*) was silenced (data

not showed), as an siRNA scrambled mixture was shown[18] and confirmed in our studies to enhance adipocyte differentiation.

Microscopic analysis of PKC translocation

The cells were seeded before transduction onto 24 mm glass coverslips and allowed to grow to 50% confluence. At this stage, images of PKC-β translocation were obtained with digital imaging system, at 24 h after infection with adenovirus PKC-β–green fluorescent protein chimera. At 24 h after infection, the medium was changed from DMEM and 10% fetal calf serum to Krebs–Ringer buffer. Images were recorded using a digital imaging system based on a Zeiss Axiovert 200 (Arese, Milan, Italy) fluorescence microscope equipped with a back-illuminated charge coupled device camera (Roper Scientific, Trenton, NJ, USA), excitation and emission filterwheels (Sutter Instrument Company, Novato, CA, USA) and piezoelectric motoring of the *z*-stage (Physik Instrumente, GmbH & Co., Germany) for rapid focusing in the *z*-plane. The data were acquired and processed using the MetaMorph analyzing program (Universal Imaging Corporation, Downington, PA, USA). This allows the direct monitoring of fluorescence intensity. A high-resolution, three-dimensional reconstruction of the distribution of a green fluorescent protein chimera can be obtained with the technique of digital image restoration, also called deconvolution or deblurring. The graph indicates the plasma membrane of PKC-β–green fluorescent protein expressed as the increase in fluorescence ratio with respect to time zero (calculated as a ratio of plasma membrane/cytosol average fluorescence). Averaging results are representative of at least three independent experiments.

Results

APD effects on ADSCs

The ability of APDs to increase total lipid production on ADSCs was first established. To this end, ADSCs, previously committed in mature adipocytes by culturing them in the presence of high glucose,[14] were treated for 3 days with different APDs (see Material and Methods section for details).

As shown in Figure 1a, a clear difference in terms of lipid production is present in ADSCs treated with quetiapina, olanzapine and risperidone. On the contrary, the pharmacological principle aripiprazolo induces lipid production comparable to that observed in untreated cells.

This difference in lipid production induced by APDs can be easily appreciated through morphological analysis of lipid drops stained with oil red, as shown in Figure 2. Indeed, incubation of ADSCs with aripiprazole resulted in a markedly less diffuse pattern of cytoplasmic staining than in cells treated with quetiapine.

We then evaluated whether the increased lipid production observed was due to an enhanced ADSC proliferation or an increased gene expression of transcripts involved in adipogenesis.

Results on proliferative events are reported in terms of increase in cell number (through DNA content, Figure 1b) and vitality (MTT test, Figure 1c) in ADSCs cultured in the same conditions of Figure 1a. The major stimulatory effects were detected in the presence of quetiapine, olanzapine and risperidone. Clozapine and aripiprazole induced only a small (clozapine) or no (aripiprazole) effect when compared with the control. Thus, clozapine showed a significant increase in lipid accumulation that was not related to a proportional increase in cell number.

Gene expression related to lipid production (expression of lipoprotein lipase and glucose transporter-4) confirmed this tendency, as reported in Figure 1d.

APD effects on MDSCs

The potential adipose commitment of MDSCs due to APD administration has then been analyzed. Recently, we have shown that a clear induction of adipogenic transformation in MDSC was observed upon the application of high-glucose culture conditions.

MDSC cells were thus treated as previously described for ADSC and the MTT test, and the DNA content, oil red staining and gene expression were evaluated. When MDSCs were cultured with APDs in a low-glucose environment, no differences in cell proliferation or lipid production were detected (data not shown). In contrast, the addition of APDs in a high-glucose environment revealed a completely different MDSC behavior, particularly with regard to adipogenesis.

In addition, for ADSCs also, a well-defined difference in lipid production was observed (Figure 1e). All drugs induced a notable accumulation of adipose products, with the exception of aripiprazole, which once again resulted in the smallest increase, comparable to control levels.

In Figures 1f and g, proliferative events are reported in terms of DNA content (that is, cell number, Figure 1f) and vitality (Figure 1g). No significant effect on MDSC proliferation was detected, with the major reducing effect attributed to aripiprazole compared with the control group.

A parallel culture of MDSCs in a non-adipogenic low-glucose environment was also carried out. As reported in Figure 1, proliferation (Figures 1f and g) was the same in a low- versus high-glucose environment, yet lipid accumulation was not observed under low-glucose conditions. Thus, only adverse metabolic stimuli, such as the presence of high glucose or APD or both, resulted in cytoplasmic lipid accumulation. Gene expression related to lipid production (expression of lipoprotein lipase and sterol regulatory element binding protein) confirmed this tendency, as reported in Figure 1d, in which only aripiprazole resulted in a downregulation of gene expression when compared with controls.

Effects of PKC-β inhibition on APD-promoting adipogenesis of ADSCs and MDSCs

ADSCs and MDSCs were cultured in the presence of two different PKC-β isoform-specific inhibitors: a pharmacological inhibitor, that is, hispidin and a molecular inhibitor, that is, siRNA for PKC-β mRNA.

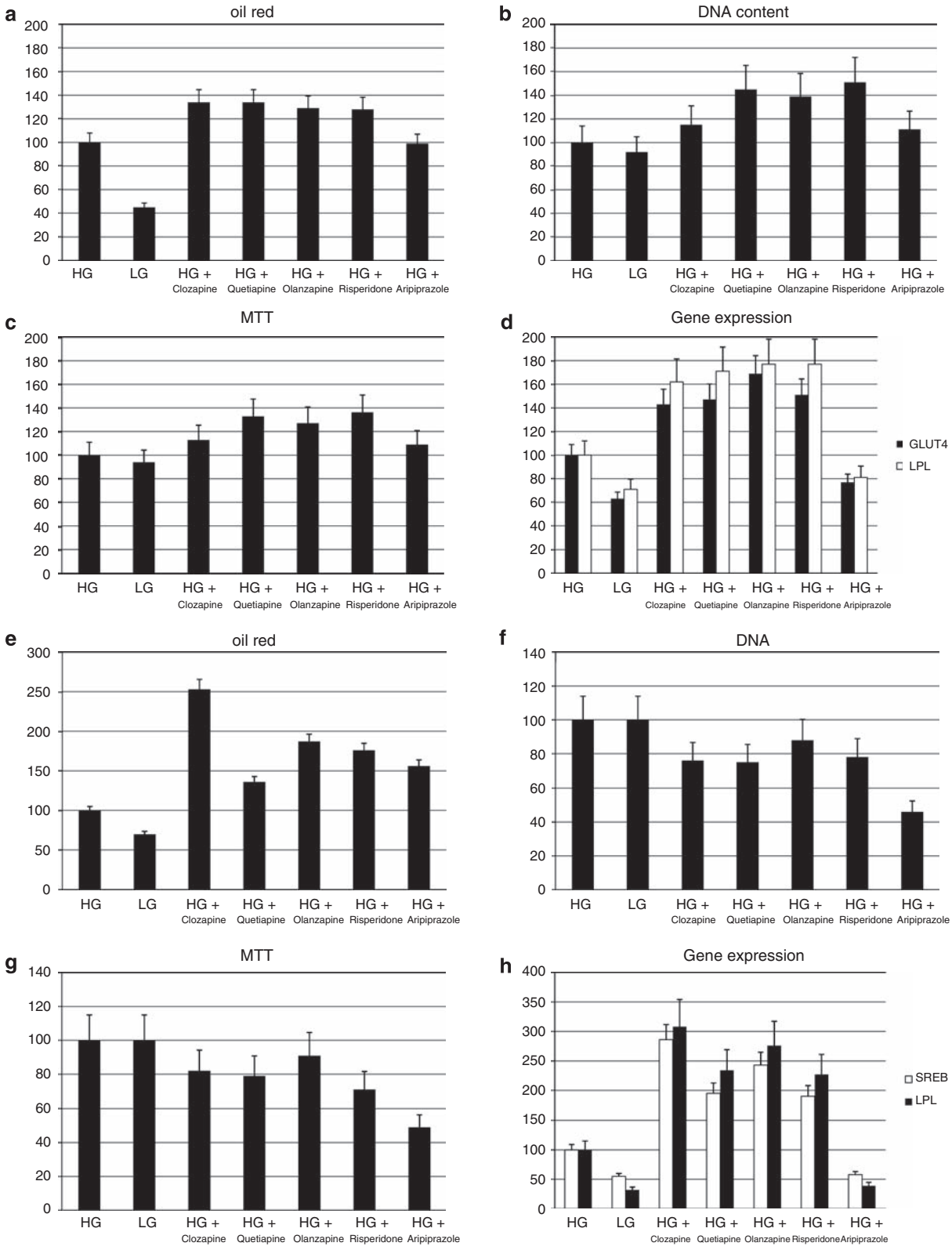

Figure 1 Adipose-derived stem cells (**a**–**d**) and muscle-derived stem cells (**e**–**h**) cultured with high-glucose medium (H); low-glucose medium (LG); and aripiprazole, clozapine, olanzapine; quetiapine and risperidone in high-glucose medium. Cells have been evaluated in terms of lipid production (oil red quantification (**a**, **e**); DNA content (**b**, **f**); MTT (**c**, **g**); and gene expression (**d**, **h**). Results are reported in term of percentage variation compared with the high-glucose medium that has been set to 100%.

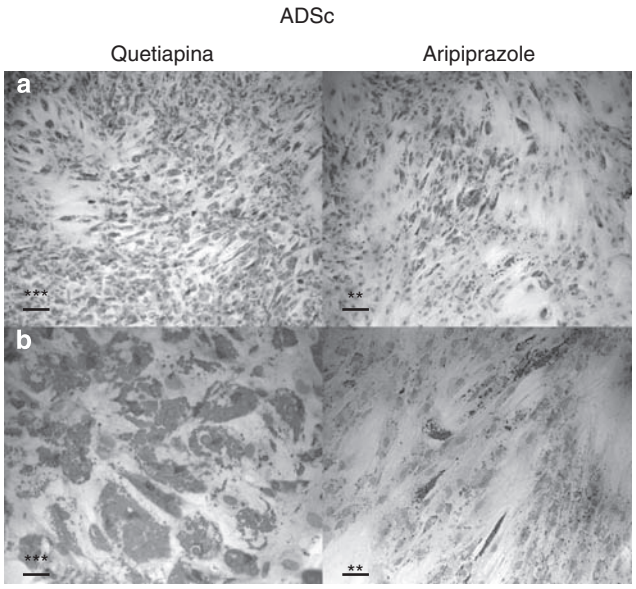

Figure 2 Lipid drops presence (in red) in cytoplasm of ADSCs cultured with quetiapine and aripiprazole. (**a** ×20 bar: 80 μm; **b** ×40; bar = 40 μm). Analysis of variance (ANOVA) test: *$P<0.05$; **$P<0.01$; ***$P<0.001$. The asterisks are added when the differences in terms of lipid drops from the control (high-glucose condition) are statistically significant. The color reproduction of this figure is available on the html full text version of the manuscript.

Figure 3 reports the effects of these inhibitors on ADSC proliferation (detected using the MTT test) and on lipidic production (detected using oil red staining). As reported in Figure 3, the presence of hispidin or siRNA did not affect the proliferative capacity of cells. The results are indeed the same obtained without siRNA/hispidine treatment in which MTT test showed that quetiapine, olanzapine and risperidone increased ADSC proliferation, whereas aripiprazole and clozapine treatments did not. In contrast, a remarkable reduction in lipid metabolism was showed by oil staining (Figures 3b and d). Both hispidine and siRNA treatment, although the latter to a lesser degree, resulted in a dramatic reduction in lipid accumulation when compared with the conditions in which PKC-β was neither inhibited nor downregulated (Figure 1). Addition of high glucose and APD to the culture medium in the presence of a PKC-β inhibitor resulted in a very low level of oil red staining. The most dramatic effects were obtained with hispidine, which halved oil red staining, resulting in levels significantly lower to those observed with low glucose. Similarly, in the presence of the molecular inhibitor, all antipsychotic treatments resulted in lipid accumulation comparable to that observed under low-glucose conditions.

Similar findings were observed with MDSCs. Although no effect on proliferation was detectable (Figures 3e and g) in the presence of the molecular PKC-β inhibitor, important changes did occur in lipid production (Figures 3f and h). In addition, in this case, hispidine induced a dramatic reduction in oil red staining. In the presence of the molecular PKC-β inhibitor, incubation with quetiapine or olanzapine under high-glucose culture conditions resulted in a scarce lipid accumulation comparable to that observed under low-glucose conditions. Clozapine and risperidone reduced lipid accumulation by half, whereas aripiprazole treatment resulted in lipid accumulation similar to that observed when incubated with the molecular PKC-β inhibitor.

Analysis of PKC-β localization in APD-treated cells

Endogenous PKC-β revealed using immunofluorescence was mostly cytosolic in ADSCs maintained under low-glucose DMEM conditions (Figure 4, time 0). Incubation for 1 h with APDs induced a notable translocation of kinase into the plasmamembrane (that is, its typical localization after activation). The ratio of signal detected in cytoplasm when compared with plasmamembrane showed that the translocation kinetics was directly related to APD treatment.

The highest level of translocation, rising fourfold, occurred with the addition of clozapine. Quetiapine also caused a significant twofold rise in translocation, whereas aripiprazole and risperidone treatment induced an increase of only 30%.

Discussion

A common side effect of antipsychotic treatment with atypical neuroleptics is significant weight gain (Table 1).[8,19–28] Evidence from large clinical samples indicates a high prevalence of metabolic dysfunction in people with serious mental illness, particularly in schizophrenic patients with nearly twice the normal risk of dying from cardiovascular disease.[29]

High levels of medication non-compliance are present in a variety of psychiatric disorders, and metabolic side effects, in particular weight gain, could contribute in discontinuing antipsychotic treatment.[30–32] This may not be surprising in a society in which thinness is observed as desirable and in which self-esteem is also built on body image. In clinical practice this is a great problem and it is important to better understand the underlying mechanisms and possibly find a solution to help psychiatric patients who need these types of medication to improve their quality of life.

As showed in this article, the possibility of antagonizing weight gain in introducing a molecular inhibitor is an important finding.

Assuming that the rise of body fat could involve both an increase in lipid accumulation and an enhancement of preadipocyte differentiation, we aimed to analyze whether PKC-β was involved in these APD-related adipogenic events.

In our experimental design phase we assumed that the increase in body fat could involve both the increase in lipid accumulation and the enhancing of preadipocytes differentiation. We also ascertained whether, in this contest, PKC-β could carry out a role of the real effectors.

First, we defined the pathways activated by the APDs most frequently used in clinical practice, such as aripiprazole, clozapine, quetiapine and risperidone. In this study they were compared in term of the effects on proliferation and

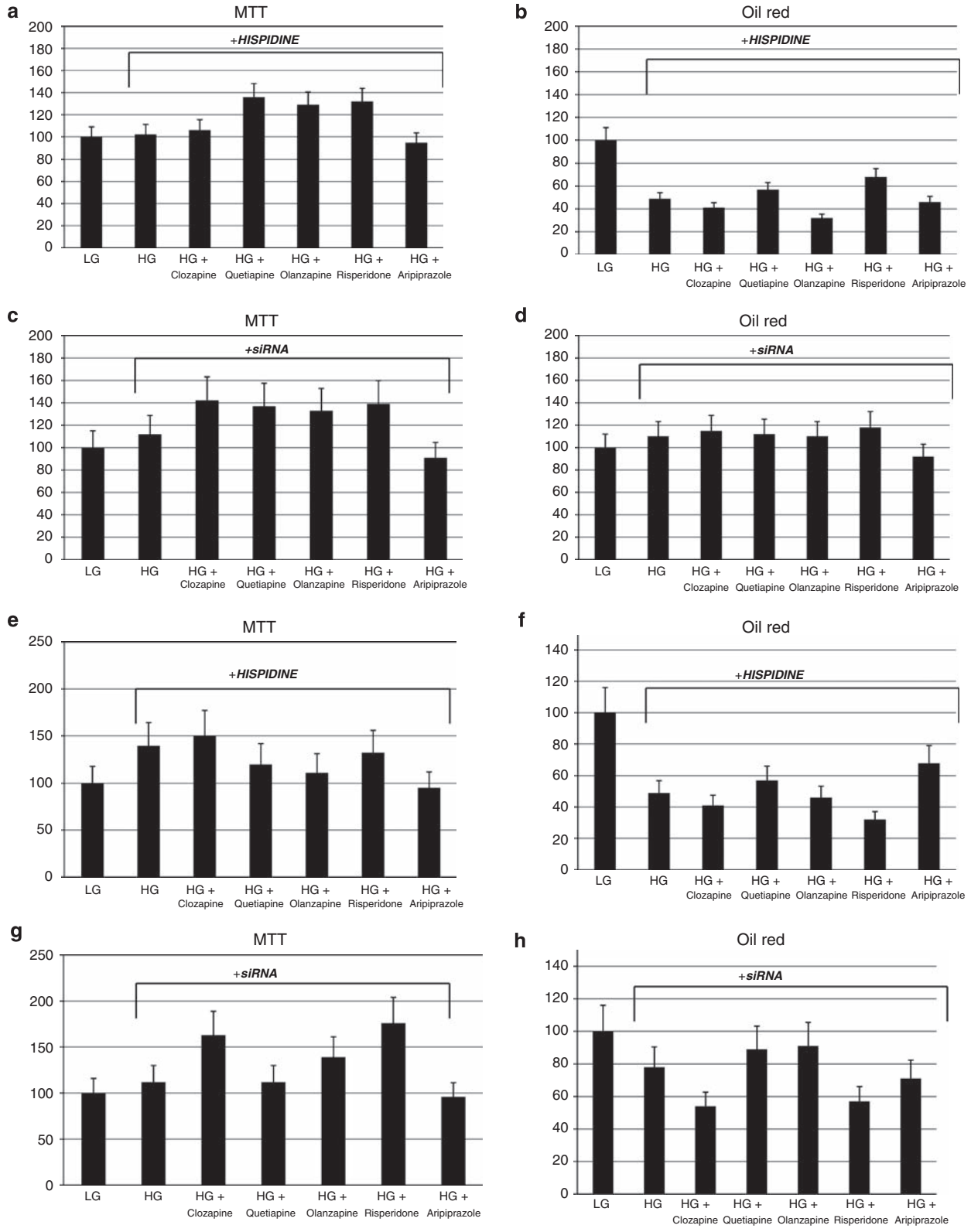

Figure 3 Adipose-derived stem cells (**a–d**) and muscle-derived stem cells (**e–h**) cultured with aripiprazole, clozapine, olanzapine and quetiapine and risperidone in high-glucose medium and in the presence of PKC-β inhibitors: hispidine (**a**, **b**, **e**, **f**) and siRNA (**c**, **d**, **g**, **h**). Cells have been evaluated in terms of lipid production (oil red quantification **a**, **c**, **e**, **g**) and gene expression (**b**, **d**, **f**, **h**). Results are reported in terms of percentage variation compared with the low-glucose medium (LG) that has been set to 100%.

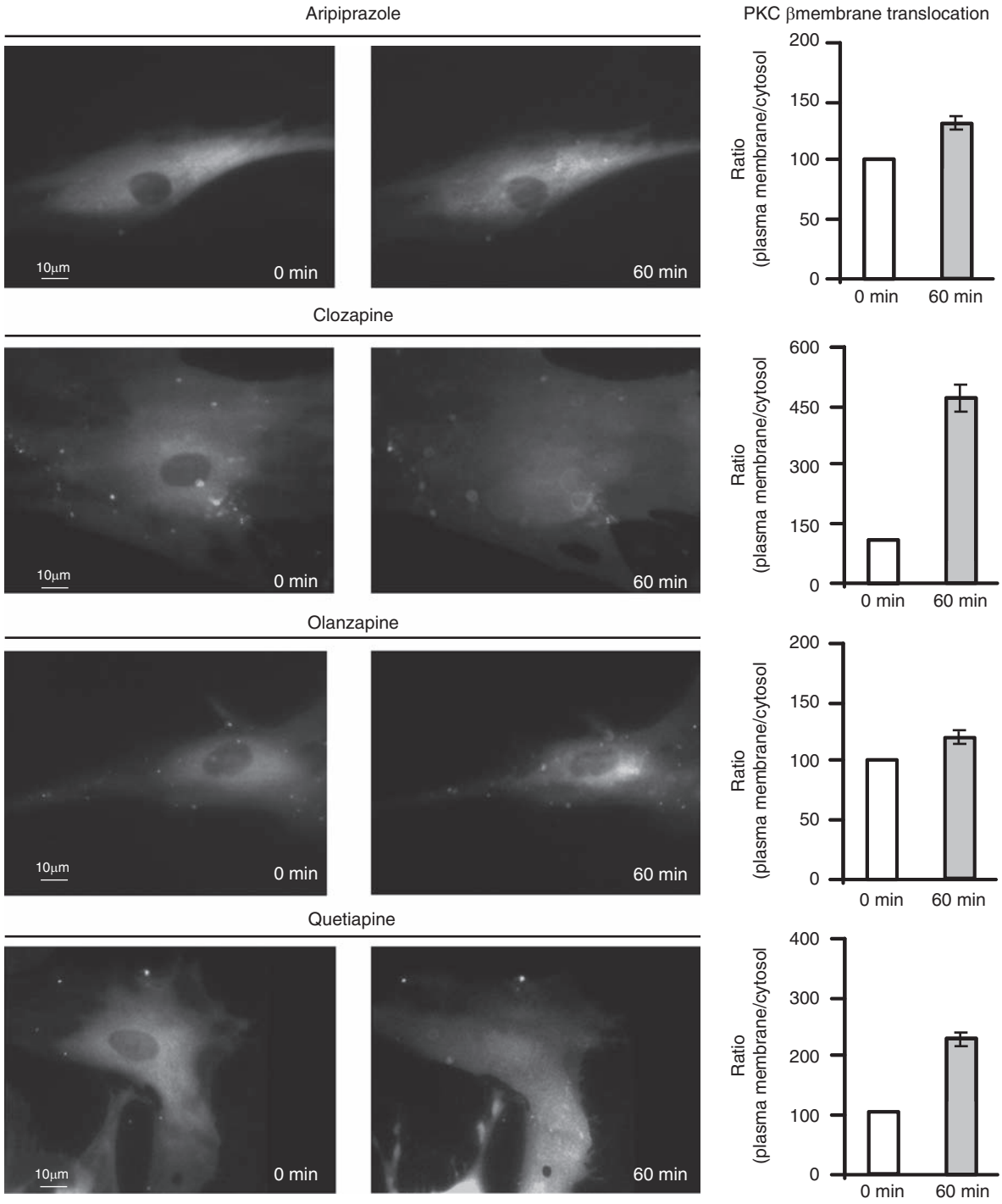

Figure 4 PKC-β membrane translocation, as revealed using immunofluorescence microscopy. Representative images show cells maintained in LG-DMEM (time 0) and incubated for 1 h with APD. The graphs show the quantitation of PKC-β fluorescence intensity along a line crossing the cell.

differentiation of preadipocytes derived from both adipose tissue and mesenchymal stem cells.

Following our previous study,[14] in which we showed that high glucose by means of PKC-β activation drove stem cells from both adipose tissue and skeletal muscle toward an adipogenic potential, in this study also we have blocked its activities in the presence of APDs, provided that is involved into the signals that underlie this process.

Table 1 Summary of published studies on APDs

Authors	*Sample size (N patients/drugs)*	*Methodology*	*Results*
Birkás Kováts *et al.*[19]	19/olanzapine 15/risperidone	Cross-sectional study	Significant lower ghrelin levels in antipsychotic groups compared with controls. No BMI differences among three groups.
Gorobets[20]	88/risperidone 84/olanzapine 61/clozapine 68/quetiapine 45/amilsulpiride	Prospective study	Long-term therapy with olanzapine, clozapine and risperidone exerts a more marked influence on the body mass when compared with quetiapine and amisulpiride regardless of the patient's sex.
Esen-Danaci *et al.*[21]	20/clozapine 28/olanzapine 22/risperidone 20/quetiapine 22/amilsupride	Cross-sectional study	Weight gain induced by atypical antipsychotics could be associated with the orexigenic effect of elevated serum ghrelin rather than leptin deficit. Only quetiapine does not induce an elevation on the ghrelin level.
Kolotkin *et al.*[22]	555/aripiprazole	Randomized study	Patients treated with aripiprazolo experienced decreased weight and improved weight-related quality of life over 26 weeks.
Khazaal *et al.*[23]	22/olanzapine, clozapine , quetiapine, risperidone	A multivariate regression of the data obtained from the entire sample was performed to establish the relationship between alliesthesia-dependent variables and weight gain as a predictor	Atypical antipsychotic drug-induced weight gain seems to result from a raised body weight set-point.
Musil *et al.*[24]	162/atypical antipsychotics 312/healthy control subjects	Case–control study three polymorphisms in the SNAP-25 gene using PCR, PANSS scores were investigated	Impact of SNAP-25 gene polymorphisms on weight gain during antipsychotic treatment.
Le Hellard *et al.*[25]	160/atypical antipsychotics	Genetic study	A strong association between three markers localized within or near the INSIG2 gene (rs17587100, rs10490624 and rs17047764) and antipsychotic-related weight gain was found.
Albert *et al.*[26]	154/atypical antipsychotics	Retrospective chart review	No evidence that short-term use of atypical antipsychotics is associated with the onset or worsening of diabetes mellitus.
Gregoor *et al.*[27]	200/atypical antipsychotics	Cross-sectional study	LEPR Q223R polymorphism may be associated with obesity in women treated with atypical antipsychotics.
Seo *et al.*[28]	100/atypical antipsychotics and antidepressants 172/antidepressants	Case–control study	Subjects treated with SSRIs and olanzapine had the greatest weight gain.
Gebhardt *et al.*[8]	65/clozapine, olanzapine and/or risperidone	Retrospective cross-sectional study	The study indicates increased parents' BMI and patients' premorbid BMI, female gender, younger age and, as a trend, the diagnosis of a schizophrenia-spectrum disorder to be predictors for antipsychotic-induced body weight gain involving atypical antipsychotics.

Abbreviations: APD, atypical antipsychotic; BMI, body mass index; PANSS, positive and negative syndrome scale; SSRI, selective serotonin reuptake inhibitor. A common side effect of antipsychotic treatment with atypical neuroleptics is a significant weight gain. Evidence from large clinical samples indicates a high prevalence of metabolic syndrome and all of its components in people with severe mental illness, particularly in patients with schizophrenia with nearly twice the normal risk of dying from cardiovascular disease.

In preadipocytes, the quetiapine, olanzapine and risperidone treatment induced an increase in both cell number (Figures 1b and c) and in adipose metabolism (detected with oil red staining and gene expression for glucose transporter-4 and lipoprotein lipase marker). Clozapine treatment induced only an increase in lipid production and not in cell number, whereas aripiprazole was shown to have no effect on either proliferation or metabolism.

Subsequently, attention was focused on the transdifferentiation of muscle progenitors to an adipogenic lineage, given that

myopathic skeletal muscle is characterized by the replacement of myofibers by adipose tissue.[33] As in hyperglycemia, high-glucose concentrations upregulate sterol regulatory element binding protein 1c, resulting in *de novo* lipogenesis and intracellular lipid accumulation in contracting myotubes.[34]

In this study we show that when MDSCs are exposed to external stimuli of an adipogenic lineage, such as the presence of a high-glucose environment, the APD exposure induces a remarkable increase in adipogenic transformation. Interestingly, APD treatment affected only transdifferentional events of MDSCs and not cell number. This APD-activated adipogenic differentiation program leading to a conversion of muscle stem cells into adipocytes, as verified by molecular criteria, has been never described.

Regarding the signals driving this differentiation program, our data suggest a crucial role for PKC-β, as revealed by the strongly inhibitory effect of hispidine and siRNA leading to the neo-formation of adipose cells through metabolic pathways and not in cell proliferation. This event was confirmed by morphological evaluation in which a clear translocation of PKC-β from cytosol to plasma membrane, in which it is then activated,[35] occurred only in the presence of APDs (Figure 4). This event was strongly related to the well-known cellular response to high glucose that induces an increase in reactive oxygen species production. In fact, reactive oxygen species, through downstream effectors and in particularly through PKC-β, lead to the neo-formation of adipose cells.[36]

In the end, in light of the results presented in this study we can conclude that:

(a) Clozapine induces a strong lipid increase activating adipogenesis through PKC-β activation and then through reactive oxygen species;
(b) Olanzapine, quetiapine and risperidone induce weight gain by means of both activation of adipocyted proliferation and adipogenesis activation (also in this case by PKC-β-reactive oxygen species);
(c) Aripiprazolo is the APD showing less activation on adipogenic event; and
(d) The adipogenic event in MDSCs is strongly boosted by APD supplementation.

It could then inquire that the parallel administration of PKC-β inhibitor to APDs could fight the related weight gain. Moreover, our data indicate that the weight gain associated with APDs involved not only preadipocytes derived from adipose tissue but also the commitment in adipogenic feature of stem cells derived from muscle tissue.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

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