Atypical antipsychotics alter cholesterol and fatty acid metabolism in vitro

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Abstract

Haloperidol, a typical antipsychotic, has been shown to inhibit cholesterol biosynthesis by affecting Δ7-reductase, Δ8-7-isomerase, and Δ14-reductase activities, which results in the accumulation of different sterol intermediates. In the present work, we investigated the effects of atypical or second-generation antipsychotics (SGA), such as clozapine, risperidone, and ziprasidone, on intracellular lipid metabolism in different cell lines. All the SGAs tested inhibited cholesterol biosynthesis. Ziprasidone and risperidone had the same targets as haloperidol at inhibiting cholesterol biosynthesis, although with different relative activities (ziprasidone > haloperidol > risperidone). In contrast, clozapine mainly affected Δ24-reductase and Δ8-7-isomerase activities. These amphiphilic drugs also interfered with the LDL-derived cholesterol egress from the endosome/lysosome compartment, thus further reducing the cholesterol content in the endoplasmic reticulum. This triggered a homeostatic response with the stimulation of sterol regulatory element-binding protein (SREBP)-regulated gene expression. Treatment with SGAs also increased the synthesis of complex lipids (phospholipids and triacylglycerides). Once the antipsychotics were removed from the medium, a rebound in the cholesterol biosynthesis rate was detected, and the complex-lipid synthesis further increased. In this condition, apolipoprotein B secretion was also stimulated as demonstrated in HepG2 cells. These effects of SGAs on lipid homeostasis may be relevant in the metabolic side effects of antipsychotics, especially hypertriglyceridemia.

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The antipsychotic actions of classic neuroleptics (typical or first-generation antipsychotics, FGA) revolutionized the therapy of schizophrenia, but their extensive use has been impeded by side effects, such as extrapyramidal symptoms, and a high incidence of nonresponders. FGAs, such as haloperidol, act predominantly by blocking dopamine D2 receptors (1). Atypical or second-generation antipsychotics (SGA) display relatively weaker antagonism of dopamine D2 receptors but potent antagonism to serotonin 5-HT2A receptors (1). The therapeutic use of SGAs has reduced concern on neurological side effects, but they are not free of metabolically adverse effects. An increase in body weight is fairly rapid after initiating treatment with these drugs. At long term, this may result in overt obesity, along with dyslipidemia, insulin resistance, abnormal glucose tolerance, and diabetes (2–4). Dyslipidemia is commonly manifested as an increase in total triglyceride and a decrease of high-density lipoprotein (HDL)-cholesterol plasma.

Abbreviations: CE, cholesteryl ester; DiI, 1,1dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate; ER, endoplasmic reticulum; FASN, fatty acid synthase; FF-MAS, Δ5,12,24-dimethylcholestatrienol; FGA, first-generation antipsychotic; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, LDL receptor; LPDS, lipoprotein-deficient serum; MAS-412, Δ5,12,24-dimethylcholestanol; MAS-414, Δ5-dimethylcholestadienol; MTP, microsomal triglyceride transfer protein; PL, phospholipid; SCD, stearoyl-coenzyme A desaturase; SGA, second-generation antipsychotic; SREBP,sterol regulatory element-binding protein; T-MAS, Δ5,24-dimethylcholestenol; 7DH, 7-dehydrocholesterol; 7DH, 7-dehydrocholesterol.

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concentrations (5). However, important differences among the different antipsychotics exist, more frequently with clozapine than ziprasidone (5–7). More controversial are the potential effects of antipsychotics on blood cholesterol (8). In adult patients with schizophrenia, treatment with clozapine or olanzapine but not haloperidol or risperidone was found to increase total cholesterol levels (9). In youth patients on first medication with SGA, an increase in total and low-density lipoprotein (LDL)-cholesterol levels were observed with olanzapine but not aripiprazole, quetiapine, or risperidone (10). These measurements were performed in samples obtained on fasting conditions. A fatty meal test revealed a significantly greater increase in very low density lipoprotein (VLDL) levels, both triglyceride and cholesterol, in olanzapine-treated compared with risperidone-treated subjects, demonstrating the alteration in VLDL metabolism (11). Finally, in a study directed to examine gene expression in whole blood, fatty acid biosynthesis genes FASN (fatty acid synthase) and SCD (stearoyl-CoA desaturase) were overexpressed in olanzapine-treated compared with unmedicated patients (12). It may be relevant to consider whether these metabolic effects are a consequence of weight gain or whether they are independent. In this regard, recent studies in rats have shown that subchronic administration of olanzapine elevates serum TG levels and upregulates the expression of lipogenic SREBP-1-controlled genes independently of weight gain (13).

Cholesterol biosynthesis from acetyl-CoA is a multistep pathway involving upwards of 20 enzymatic activities (supplemental Fig. I). There are few data on the effects of antipsychotics on cholesterol biosynthesis. In 1965, Summener and Yardley were the first to demonstrate that haloperidol inhibits cholesterol biosynthesis in rat skin (14). We previously reported that in both neuroblastoma SH-SY5Y and promyelocytic HL-60 human cell lines, haloperidol inhibited cholesterol biosynthesis, resulting in a decrease in the cell cholesterol content and the accumulation of different sterol intermediates [7-dehydrocholesterol (7DHC), zymostenol, and cholesta-8,14-dien-3β-ol] depending on the dose of the drug, suggesting the inhibition of Δ7-reductase > Δ12-isomerase > Δ19-reductase enzyme activities in this order (15, 16). By determining the incorporation of radioactive acetate into cholesterol, Kristiana et al. (17) confirmed this effect of haloperidol and reported that SGAs, such as clozapine, quetiapine, olanzapine, risperidone, and ziprasidone, have the ability to inhibit cholesterol biosynthesis, although the affected steps were not elucidated. In contrast, Laurencergues et al. reported that the SGAs clozapine and olanzapine (18) as well as risperidone (19) increased cholesterol biosynthesis in primary cultures of rat hepatocytes, whereas other antipsychotics, such as haloperidol, quetiapine, and aripiprazole, did not affect this pathway (18).

Most antipsychotics are cationic amphiphiles, which are positively charged by virtue of an amine group that can be protonated, and display both hydrophilic and hydrophobic properties (supplemental Fig. II). Interestingly, other cationic amphiphiles, such as U18666A (20) and tamoxifen (21, 22), have been shown to inhibit several enzymes involved in cholesterol biosynthesis and to affect the LDL endocytotic trafficking to the endoplasmic reticulum (ER). We previously reported that haloperidol interfered with free cholesterol egress from this intracellular compartment to the ER, producing an accumulation of free cholesterol in endosome/lysosome vesicles (16). This effect could be responsible for the upregulation of SREBP and its target genes in response to antipsychotic treatment as observed by others (23–25).

In this study, we analyzed the effects of SGAs on different aspects of intracellular cholesterol homeostasis, including cholesterol biosynthesis and intracellular cholesterol traffic, as well as on fatty acid synthesis and apolipoprotein B100 (apoB100) secretion to elucidate their actions on lipid metabolism.

METHODS

All chemicals, unless otherwise stated, were purchased from Sigma (Sigma-Aldrich Química, S.A., Tres Cantos, Madrid, Spain). The antipsychotics used were clozapine free base (Sigma), haloperidol free base (Sigma), risperidone free base (Sigma), and ziprasidone hydrochloride (Tocris).

Culture of cells

For this study, three human cell lines were selected. The hepatoma cell line HepG2 (ATCC HB-8065) (Rockville, MD) was chosen because of the intense lipid metabolism of this tissue and its involvement in the metabolism of antipsychotics. The neuroblastoma SH-SY5Y cell line (ATCC CRL-2266) was selected because neurons, from the therapeutic view point, are the main target cells of antipsychotics. Finally, HL-60 promyelocytic cells (ECACC 98070106) are very useful to study cholesterol biosynthesis due to their high proliferation rate. HepG2 cells were cultured in DMEM high glucose (Gibco BRL Invitrogen S.A., Barcelona, Spain) supplemented with MEM nonessential amino acids, 10% fetal bovine serum (FBS), and antibiotics (Gibco BRL) at 37°C in a 5% CO₂ atmosphere. SH-SY5Y cells were cultured in RPMI 1640 (Gibco BRL) medium with L-glutamine supplemented with MEM nonessential amino acids, 10% FBS, and antibiotics. HL-60 leukemia cells were cultured in cholesterol-free ITS+ medium with the following composition: RPMI 1640 supplemented with 625 μg/ml transferrin (Roche, Basel, Switzerland), 625 μg/ml insulin, 535 μg/ml linoleic acid-BSA, 625 ng/ml sodium selenite, 125 μg/ml human serum albumin (Gri Isco 20%, Barcelona, Spain), and antibiotics. For the experiments, HepG2 and SH-SY5Y cells were cultured in medium with 10% lipoprotein-deficient serum (LPDS) and HL-60 cells in serum-free medium and treated with clozapine, haloperidol, risperidone, or ziprasidone. The antipsychotics were dissolved in DMSO. The final concentration of DMSO in the medium was 0.044%.

Immunofluorescence microscopy

Human LDL was labeled with the fluorescent probe Dil (1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanineperchlorate,
Life Technologies Corporation, Madrid, Spain) as described elsewhere (27). HepG2 cells were cultured on glass coverslips and fixed with 4% paraformaldehyde/PBS for 5 min. Next, the cells were permeabilized in 0.1% Triton X-100/PBS for 5 min and incubated with 2% BSA (BSA) in PBS for 45 min. For free cholesterol staining, cells were exposed to filipin (50 mg/1 in PBS) for 45 min. Anti-Lamp2 (Abcam, Cambridge, UK) or anti-CD63 (BD Biosciences) were used at a 1:200 dilution for 45 min. Following incubation with Alexa fluor 488-conjugated antimouse IgG (Molecular Probes, Invitrogen S.A.) in PBS at a 1:500 dilution for 45 min. Cells were mounted for microscopy and examined on a Nikon D- Eclipse CI confocal microscope.

Analysis of sterol biosynthesis by HPLC

The cells (HL-60 incubated in cholesterol-free ITS+ medium and HepG2 incubated in DMEM medium containing 10% LPDS) were preincubated for 2 h in the absence (control) or the presence of different antipsychotics at the indicated concentrations, and then the medium was supplemented with 40 µCi of 14C-acetate and incubated for a further 8 h. At the end of the incubation period, the cells were washed twice with ice-cold PBS and lysed with 10% (w/v) KOH. The non-saponifiable lipids were extracted and used for sterol separation by reverse-phase HPLC, as previously described (28), using a Luna-Pack 5 µm pore size C18 (250 mm × 4.6 mm; Phenomenex) column for HepG2 cells or a Mediterranea SEA 18 5 µm pore size C18 (250 × 4.6 mm, Teknokroma) for HL-60 cells. Lipids were eluted with acetonitrile/water (95:5 v/v) for HepG2 cells or with acetonitrile/water (95:3.5 v/v) for HL-60 cells at a flow rate of 1.2 ml/min. The eluent was monitored simultaneously for UV absorption (Diodo Array 168 detector, Beckman Coulter) and online radioactivity counting (Radioactivity detector LB 509, Berthold Technologies). The eluting sterols were identified by comparison of the retention time and the UV spectrum with those of pure standards. The HPLC method could not resolve zymostenol from cholesterol or zymosterol from desmosterol.

In other experiments, in order to elucidate the cellular response to the removal of the antipsychotic drugs from the medium, HepG2 cells were incubated in DMEM medium containing 10% LPDS in the absence (control) or the presence of the different antipsychotics at 10 µM for 16 h. Then the medium was removed and the cells were washed with PBS, and finally, medium without the drugs was added and supplemented with 40 µCi of 14C-acetate. Cells were incubated for a further 8 h and processed as before for the analysis of radioactivity incorporation into sterols.

Analysis of sterols by GC/MS

Extracted sterols were analyzed as previously described (16). Briefly, total sterols were dissolved in tert-butylmethylether (TBME) and derivatized with silylation reagent (N-methyl-Ntrimethylsilyl-trifluoroacetamide/trimethylsilylimidazole 9:1, v/v). Sterols were analyzed using an Agilent 6890N GC and an Agilent 5975 MS detectors (Agilent Technologies) with an Agilent DB-5 ms, 30 m × 0.25 mm × 0.1 µm. Helium was used as carrier gas at a flow rate of 1.0 ml/min and variable pressure according to retention time locked for 5a-cholestan. The inlet temperature was maintained at 260°C. The oven temperature was initially held at 55°C for two minutes and was increased to 240°C at a rate of 55°C/min, held 10 min, and then increased to 310°C at a rate of 7°C/min. The injector was settled to splitless (injection volume 2 µl). GC/MS was carried out using electron ionization at 70eV. For quantitation purposes, the MS detector was operated in selective ion monitoring (SIM) mode following at least one quantifier and two qualifying ions for each sterol. Peak identification was achieved both by comparison with known standards and by monitoring characteristic ions as described elsewhere (29).

RNA isolation and real-time qRT-PCR

Total RNA from HepG2 cells was extracted using TriReagent according to the manufacturer’s recommendations and reverse-transcribed using M-MLV reverse transcriptase enzyme (Promega, WI) in the presence of the RNase inhibitor RNAsin (2 U/µl) (Promega). Real-time PCR amplification was performed on a LightCycler 480 using the SYBR Green I Master kit (Roche Applied Science). The thermocycle protocol consisted of an initial denaturation step of 95°C for 5 min, followed by 45 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 10 s. Melting curves were evaluated for each gene, and the PCR reaction products were separated on 2% agarose gels to confirm the presence of a single product. The efficiency of the reaction was evaluated by amplifying serial dilutions of cDNA (1:10, 1:100, 1:1000, and 1:10,000). We ensured that the relationship between the threshold cycle (Ct) and the log(RNA) was linear (−3.6 < slope < 3.2). All analyses were performed in triplicate, and the target gene copy number was normalized against the housekeeping gene Rplp0 (encoding ribosomal protein large P0). The primers used in the real-time PCRs are shown in supplemental Table I.

Lipid synthesis assay

HepG2 cells were incubated in the absence (control) or the presence of the different drugs at 10 µM. After 20 h, 14C-acetate (2 µCi/ml per well) was added, and incubation was continued for additional 4 h. In other experiments, the cells were incubated in the presence of the drugs or control conditions for 20 h, then the cells were washed twice with PBS and incubated for additional 4 h in plain medium containing 14C-acetate (2 µCi/ml per well). In all cases, at the end of the incubation, cells were washed, and lipids were extracted by the Folch procedure. Lipid extracts were dried and separated into major lipid classes on TLC plates Silica Gel F254 with a mobile phase of hexane/diethyl ether/glacial acetic acid (90:30:1, v/v/v). The lipids bands corresponding to phospholipids (PL), free fatty acid (FFA), triacylglycerides (TG), and cholesterol ester (CE) were cut into vials, and radioactivity was counted by liquid scintillation.

Immunoprecipitation of apoB from culture medium or cell lysate

We followed the method published by Meex and coworkers (30). The cells were metabolically labeled with [35S]methionine (Hartmann Analytic, Germany) during 15 min, then the medium was removed and the cells were incubated with chase medium supplemented with unlabeled methionine. After 180 min, newly synthesized apoB was immunoprecipitated from culture medium as described in Ref. 30. Quantitation of labeled apoB was performed by SDS-PAGE and radioactivity detection using a Typhoon 9400 PhosphoImager. Total protein synthesis was measured by determination of trichloroacetic acid precipitable radioactivity in aliquots of the medium. Microsomal triglyceride transfer protein (MTP) inhibitor CP-346086 was used at 10 µM.

Statistical analysis

Data were presented as mean ± SEM. Statistical analyses were performed using a 2-tailed, unpaired Student’s t-test. The difference between two sets of values was considered significant if the P value was less than 0.05.
RESULTS

Effects of second-generation antipsychotics on cholesterol biosynthesis

The effects of SGAs (clozapine, risperidone, and ziprasidone) compared with haloperidol on cholesterol biosynthesis were studied. Cells were incubated in the absence (control) or presence of different concentrations of the drugs for a total of 10 h, and the incorporation of $^{14}$C-ol into sterols during the last 8 h was determined. In HepG2 cells incubated in control conditions, radioactivity mainly accumulated in cholesterol (peak 1) and desmosterol (peak 2) (Fig. 1A). As expected, treatment with haloperidol resulted in a decrease in radioactivity incorporation into cholesterol (less than 10% of the control) and an increase in the biosynthetic precursors 7-DHC, zymosterol, cholesta-8,14-dien-3β-ol and cholesta-8,14,24-trien-3β-ol in a dose-dependent manner (Fig. 1). This confirmed our previous results, using different cell lines, that haloperidol inhibits the cholesterol biosynthetic reactions catalyzed by Δ7-reductase, Δ8,7-isomerase, and Δ14-reductase, respectively (15, 16). Treatment with clozapine resulted in a smaller decrease in radioactivity incorporation into cholesterol (roughly 15% of the control) and an increase in radioactivity incorporation into desmosterol/zymosterol, which was indicative of the inhibition of the Δ24-reductase and/or Δ8,7-isomerase reaction (Fig. 1). Treatment with risperidone resulted in a decrease in radioactivity incorporation into cholesterol (65–90% of the control) and an increase in 7-DHC in a dose-dependent manner. At the highest dose, lanosterol and cholesta-8,14,24-trien-3β-ol were also increased. These results are compatible with the inhibition of reactions catalyzed by Δ7-reductase, Δ14-reductase, and 1α-demethylase due to the effect of risperidone. Finally, ziprasidone even at the lowest dose (5 µM) completely blocked radioactivity incorporation into cholesterol (Fig. 1), and radioactivity accumulated in 7-DHC (compound #3, which was clearly distinguished from compound #11 in the UV-absorption spectra, Fig. 1B), cholesta-8,14-dien-3β-ol, and cholesta-8,14,24-trien-3β-ol, which was suggestive of the inhibition of the reactions catalyzed by Δ7- and Δ14-reductases, respectively.

The study was extended to HL-60 cells (Table 1 and supplemental Fig. III). In control conditions, these cells accumulated $^{14}$C-radioactivity only in cholesterol. Treatment of cells with haloperidol resulted in a profound decrease in radioactivity incorporation into cholesterol, whereas an increase in the precursors 7-DHC, 7-dehydrodesmosterol (7DHD) and cholesta-8,14-dien-3β-ol was observed. Treatment with risperidone resulted in a decrease in radioactivity incorporation into cholesterol and an increase in 7-DHC in a dose-dependent manner, suggesting inhibition of the reaction catalyzed by Δ7-reductase. Ziprasidone resulted in an accumulation of radioactivity in 7-DHC, cholesta-8,14-dien-3β-ol, cholesta-8,14,24-trien-3β-ol, and lanosterol, which was suggestive of the inhibition of the reactions catalyzed by Δ7- and Δ14-reductases and 1α-demethylase, respectively. Clozapine was less potent in inhibiting cholesterol biosynthesis; increments of $^{14}$C-radioactivity in 7-DHC, 7DHD, and lathosterol were observed with the highest dose used (Table 1 and supplemental Fig. III).

To quantify the different sterols, HPLC has some limitations due to the poor resolution between cholesterol/zymostenol and desmosterol/zymosterol. Thus, to determine how the inhibition of cholesterol biosynthesis elicited by SGAs affects the sterol composition of cells, we used GC/MS. For GC/MS experiments, the cells were treated in the absence (control) or presence of different concentrations of the antipsychotic for 24 h and then analyzed. As shown in Table 2, in control conditions, HepG2 cells contained, in addition to cholesterol, small amounts of zymostenol, lathosterol, and desmosterol. All the antipsychotics tested decreased the cholesterol content in a dose-dependent manner, and the concentrations of several cholesterol precursor sterols were increased. In cells treated with haloperidol, 7-DHC, zymosterol, cholesta-8,14-dien-3β-ol, and cholesta-8,14,24-trien-3β-ol were greatly increased, as well as FF-MAS (Δ8,14,24-dimethylcholestatrienol) and zymosterol at the highest dose used. Treatment with clozapine increased zymostenol, 8-dehydrocholesterol, and desmosterol. Risperidone increased 7-DHC, zymosterol, 8-dehydrocholesterol, lathosterol, and desmosterol. Cells treated with ziprasidone showed an increase in 7-DHC and, particularly, cholesta-8,14-dien-3β-ol and cholesta-8,14,24-trien-3β-ol. Taken together, these results suggest that in HepG2 cells, haloperidol inhibits the activities of Δ-reductase, Δ8,7-isomerase, and Δ14-reductase (Table 2). Clozapine inhibits Δ8,7-isomerase and Δ24-reductase; risperidone inhibits Δ7-reductase, Δ8,7-isomerase, Δ5-desaturase, and Δ24-reductase; and ziprasidone inhibits Δ14-reductase and Δ7-reductase.

The sterol content in HL-60 cells was quite distinct (Table 3). On the one hand, the cholesterol content was about 10 µg/mg cell protein, three times less than in HepG2 cells. On the other hand, HL-60 cells contained some 7-DHC, in contrast to HepG2 cells, which contained desmosterol. These data suggest that the limiting enzyme in HL-60 cells is Δ7-reductase, whereas in HepG2 cells, the limiting enzyme is Δ24-reductase. With regard to the changes produced by the antipsychotics, with the exception of clozapine, all of them significantly decreased the content of cholesterol. Haloperidol, even at the lowest concentration used, highly increased the cell content of 7-DHC; at increasing drug concentrations, lathosterol, 8-dehydrocholesterol, and zymosterol, as well as cholesta-8,14-dien-3β-ol and cholesta-8,14,24-trien-3β-ol, were also increased. These same cholesterol pathway intermediaries were also affected by treatment with risperidone and ziprasidone but at different intensities. Risperidone greatly increased the content of 7-DHC in a dose-dependent manner; at the highest dose, it also increased zymostenol and cholesta-8,14-dienol, substrates of Δ8,7-isomerase and Δ14-reductase, respectively. Finally, treatment with ziprasidone resulted in marked increases in cholesta-8,14-dienol and cholesta-8,14,24-dienol (substrates of Δ14-reductase) and in 7-DHC (substrate of Δ7-reductase), as well as a small increase in zymostenol. In HL-60 cells, an increase in lathosterol due to the antipsychotics haloperidol, clozapine, and
Fig. 1. A: Effects of antipsychotics on 14C-acetate incorporation into sterols in HepG2 cells. The cells were treated with haloperidol, clozapine, risperidone, or ziprasidone at the indicated concentrations or vehicle (control) and 14C-acetate for 8 h, and radioactivity incorporation into the sterols was determined by HPLC and on-line radioactivity detection. B: UV spectra of eluting sterols in the cells not treated (control) or treated with 25 µM of clozapine, haloperidol, risperidone, or ziprasidone. Results from a representative experiment are shown. The numbers are the following: 1, cholesterol; 2, desmosterol; 3, 7DHC; 4, 7DHD; 8, zymosterol; 11, cholesta-8,14-dien-3β-ol; 12, cholesta-8,14,24-trien-3β-ol; and 14, lanosterol. The HPLC method did not resolve between cholesterol/zymostenol and desmosterol/zymosterol. 7DHC (peak 3) was distinguished from cholesta-8,14-dien-3β-ol (peak 11) and 7DHD (peak 4) from cholesta-8,14,24-trien-3β-ol (peak 12) by their distinct UV-absorption spectra.
The present results show that exposure of cells to different antipsychotic drugs inhibited cholesterol biosynthesis and decreased the cholesterol content in cells but increased that of several cholesterol precursors and unphysiological sterols. Other authors reported that pretreatment of rat hepatocytes with both clozapine (18) and risperidone (19) increased the incorporation of $^{14}$C-acetate into free cholesterol when studied 4 h after removing the antipsychotics from the culture medium. Thus, we directly determined the effect of removal of the antipsychotics from the incubation medium on the rate of cholesterol biosynthesis. For this, HepG2 cells were incubated for 16 h in the presence of haloperidol, risperidone, or ziprasidone. Then one set of cells was exposed to the drugs for an additional 8 h. In another set, the medium was removed, the cells were washed, and new medium without drugs was added. During the last 8 h of incubation, the incorporation of $^{14}$C-acetate into sterols was determined. As shown in Fig. 2, in all cases, drug removal resulted in a marked stimulation of radioactivity incorporation into sterols, including cholesterol, compared with cells continuously exposed to the drugs. These results firmly indicate that cholesterol biosynthesis was expressed as a percentage considering the cholesterol area as 100% (shown in parentheses). Results are means of two experiments.

The HPLC method did not resolve between cholesterol/zymostenol and desmosterol/zymosterol. 7-Dehydrocholesterol was distinguished from 7DHC – 27651 36432 – 19145 – 31846 15031 16695 23690 – – –. Zymostenol, 14C-acetate for 8 h, and radioactivity incorporation into the sterols was determined by HPLC. Results from a representative experiment are shown.

The cells were treated with haloperidol, clozapine, risperidone, or ziprasidone at the indicated concentrations or vehicle (control) and $^{14}$C-acetate for 8 h, and radioactivity incorporation into the sterols was determined by HPLC. Results from a representative experiment are shown.

**TABLE 1. Effects of antipsychotics on $^{14}$C-acetate incorporation into sterols in HL-60 cells**

| Sterol (dpm/mg protein) | Control | Haloperidol | Clozapine | Risperidone | Ziprasidone |
|-------------------------|---------|-------------|-----------|-------------|-------------|
|                         | 5 µM    | 10 µM       | 25 µM     | 5 µM        | 10 µM       | 25 µM       |
| Lanosterol              | –       | –           | –         | –           | –           | –           |
| Cholesta-8,14,24-trienol| –       | –           | –         | –           | –           | –           |
| Cholesta-8,14-dienol    | –       | –           | –         | –           | –           | –           |
| Lathosterol             | –       | –           | –         | –           | –           | –           |
| 7DHD                    | –       | –           | –         | –           | –           | –           |
| 7DHC                    | 42358   | 132199      | 108725    | 57601       | 28039       | 35540       |
| Cholesterol             | 321658  | 46990       | 56105     | 51693       | 102550      | 136253      |

**TABLE 2. Effects of antipsychotics on the sterol content of the cell line HepG2**

| Sterol (ng/mg protein) | Control | Haloperidol | Clozapine | Risperidone | Ziprasidone |
|-------------------------|---------|-------------|-----------|-------------|-------------|
|                         | 5 µM    | 10 µM       | 25 µM     | 5 µM        | 10 µM       | 25 µM       |
| Lanosterol              | 23      | 22          | 27        | 59          | 14          | 25          | 22          | –           | –           | –           | –           |
| FF-MAS                  | –       | –           | 39        | 134         | –           | –           | –           | –           | –           | –           | –           |
| Cholesta-8,14,24-trienol| –       | (0.33)      | (0.60)    | (1.60)      | –           | –           | –           | –           | –           | –           | –           | (1.10)      | (1.08)      | (1.02)      |
| Cholesta-8,14-dienol    | –       | 97          | 263       | 2010        | –           | –           | –           | –           | –           | –           | –           | –           | –           | –           | –           |
| Zymosterol              | –       | –           | –         | (0.11)      | –           | –           | –           | –           | –           | –           | –           | –           | 3381        | 3172        | 2802        |
| Zymostenol              | 205     | 159         | 271       | 732         | 257         | 249         | 246         | 206         | 218         | 172         | –           | –           | –           | –           | –           |
| 8-Dehydrocholesterol    | 29      | –           | –         | 32          | 86          | –           | –           | 54          | 41          | 31          | –           | –           | –           | –           | –           |
| Δ$^{14}$-cholestadienol | –       | –           | –         | –           | –           | –           | –           | –           | –           | –           | –           | –           | Δ$^{14}$-cholestadienol |
| Lathosterol             | 142     | 40          | –         | –           | 105         | 177         | –           | 277         | –           | –           | –           | –           | –           | –           | –           |
| 7DHC                    | –       | 1149        | 1565      | 1935        | –           | –           | –           | 566         | 883         | 1316        | 631         | –           | –           | –           | –           |
| Desmosterol             | 50      | –           | –         | 69          | 238         | 70          | –           | 115         | 124         | –           | –           | –           | –           | –           | –           |
| Cholesterol             | 56476   | 28156       | 24475     | 22919       | 28158       | 27893       | 25382       | 30606       | 29364       | 24710       | 22263       | 21640       | 21720       | –           | –           |
| Total sterols           | 36934   | 29623       | 26640     | 27789       | 28155       | 28668       | 25773       | 31547       | 30630       | 26506       | 20275       | 24812       | 24522       | –           | –           |

HepG2 cells were left untreated (control) or were treated for 24 h with 5, 10, or 25 µM of the antipsychotic (clozapine, haloperidol, risperidone, or ziprasidone). Lipids were extracted and analyzed by GC/MS. Data are shown as nanograms of each sterol species per milligram of protein, except for those sterol species that were detectable but could not be quantified for lack of standard [in this case, concentration was expressed as a percentage considering the cholesterol area as 100% (shown in parentheses)]. Results are means of two experiments. ‘‘Total sterols’’ was calculated without considering the sterol species that could not be quantified. –, not detectable; FF-MAS, Δ$^{14}$-dimehtylcholestadienol; MAS-412, Δ$^{14}$-dimehtylcholestadienol; MAS-414, Δ$^{14}$-dimehtylcholesterol; T-MAS, Δ$^{14}$-dimethylcholesterol.
Effects of antipsychotics on fatty acid and derived lipids

To investigate whether antipsychotics could affect de novo synthesis of lipids, we determined the levels of labeled

### TABLE 3. Effects of antipsychotics on the sterol content of the cell line HL-60

| Sterol (ng/mg protein) | Control | 5 µM | 10 µM | 25 µM | 5 µM | 10 µM | 25 µM | 5 µM | 10 µM | 25 µM | 5 µM | 10 µM | 25 µM |
|------------------------|---------|------|-------|-------|------|-------|-------|------|-------|-------|------|-------|-------|
| Lanosterol             | 55      | 53   | 72    | 84    | 71   | 47    | 55    | 72   | 83    | 72    | 84   | 123   | 175   |
| Dihydrolanosterol      | 37      | 19   | –     | 23    | 29   | 16    | 14    | 22   | –     | –     | 23   | 20    | 33    |
| FF-MAS                 | –       | –    | –     | 52    | –    | –     | –     | –    | –     | –     | –    | 60    | 86    |
| MAS-412                | –       | –    | –     | –     | –    | –     | –     | –    | –     | –     | –    | –     | 125   |
| Cholesta-8,14,24-trienol| –       | –    | (0.87)| –     | –    | –     | –     | –    | (1.37)| (2.29)| (2.02)|
| Cholesta-8,14-dienol   | 74      | 206  | 1122  | –     | –    | –     | –     | 206  | 4177  | 4407  | 3764 |
| T-MAS                  | 12      | 9    | 10    | 10    | 13   | 17    | 8     | 26   | 10    | –     | –    | –     | –     |
| MAS-114                | –       | –    | –     | 18    | –    | –     | –     | –    | –     | –     | –    | –     | –     |
| Zymosterol             | 27      | 200  | 371   | 1051  | 49   | 55    | 70    | –    | –     | 371   | 142  | 82    | 43    |
| 8-Dehydrocholesterol   | 31      | 111  | 106   | 165   | 40   | 38    | 41    | 78   | 153   | 106   | –    | –     | –     |
| Δ7,24-Cholestadienol   | –       | –    | –     | –     | (0.07)| (1.54)| –     | –    | –     | –     | –    | –     | –     |
| Lathosterol            | 143     | 276  | 271   | 291   | 171  | 142   | 127   | 172  | 106   | 271   | 159  | 82    | 65    |
| 7DHC                   | 204     | 12044| 11800 | 7352  | 347  | 253   | 317   | 1318 | 4253  | 11800 | 5454 | 1977  | 1239  |
| Desmosterol            | –       | –    | –     | 22    | 24   | 77    | –     | –    | –     | –     | –    | –     | –     |
| Cholesterol            | 10747   | 5038 | 4964  | 5587  | 11869| 10606 | 9903  | 10796| 7854  | 4964  | 5628 | 5813  | 6189  |
| Total sterols          | 11256   | 17824| 17800 | 15677 | 12611| 11199 | 10619 | 12466| 12475 | 17800 | 13707| 12590 | 11721 |

HL-60 cells were left untreated (control) or were treated for 24 h with 5, 10, or 25 µM of the antipsychotic (haloperidol, clozapine, risperidone, or ziprasidone). Lipids were extracted and analyzed by GC/MS. Data are shown as nanograms of each sterol species per milligram of protein, except for those sterol species that were detectable but could not be quantified for lack of standard [in this case, the concentration was expressed as a percentage considering the cholesterol area as 100% (shown in parentheses)]. Results are means of two experiments. “Total sterols” was calculated without considering the sterol species that could not be quantified. 7-dehydrodesmosterol was not detectable in the HL-60 samples. –, not detectable; FF-MAS, Δ7,14,24-dimethylcholestatrienol; MAS-412, Δ7,14-dimethylcholestadienol; MAS-414, Δ7-dimethylcholestanol; T-MAS, Δ7,14-dimethylcholestanol.

is inhibited when drugs are present in the medium and that thereafter, probably as a result of both the alleviation of enzyme inhibition and enzyme induction, cholesterol biosynthesis is activated.

**Fig. 2.** Effects of the removal of antipsychotics from the medium on 14C-acetate incorporation into sterols. HepG2 cells were treated with haloperidol, risperidone, or ziprasidone at 10 µM concentrations or vehicle (control) for 16 h, and then 14C-acetate for 8 h in the absence (washout) or the presence of the antipsychotics. Radioactivity incorporation into the sterols was determined by HPLC and on-line radioactivity detection. Results from a representative experiment are shown. The numbers are the following: 1, cholesterol; 2, desmosterol; 3, 7DHC; 4, 7DHD; 11, cholesta-8,14-dien-3β-ol; and 12, cholesta-8,14,24-trien-3β-ol.
FFA, PL, TG, and CE produced from \(^{14}\)C-acetate in HepG2 cells. Two settings were used. In the first, \(^{14}\)C-acetate was added to cells while antipsychotics (10 \(\mu\)M) were present in the medium. In the second, cells were pretreated with the antipsychotic, then it was removed, and the tracer was added in the washout period.

When lipid synthesis was analyzed in cells currently treated with the antipsychotics, risperidone and ziprasidone increased \(^{14}\)C-radioactivity incorporation into both PL and TG, the major lipids in cells, whereas clozapine increased TG synthesis only (Fig. 3). Ziprasidone also increased FFA synthesis compared with the control (not treated with antipsychotics). On the contrary, CE synthesis was decreased by effect of haloperidol and risperidone (Fig. 3).

When lipid synthesis was analyzed in the washout period, pretreatment with any of the antipsychotic used, either FGA (haloperidol) or SGA (clozapine, risperidone, and ziprasidone), resulted in a significant increase in radioactivity incorporation into both PL and TG compared with the control (Fig. 3). Clozapine also increased FFA and CE synthesis (128 \(\pm\) 0.5% and 221 \(\pm\) 4.8%, respectively), whereas risperidone and ziprasidone decreased these parameters (approximately 10–20% with respect to the control) (Fig. 3). In general, the most active antipsychotics stimulating lipid synthesis were ziprasidone during the treatment period and clozapine during the washout period.

**Effects of antipsychotics on gene expression**

We hypothesized that the inhibition of cholesterol biosynthesis exerted by the antipsychotics would result in the activation of SREBP transcription factors, which ultimately could lead to a transitory stimulation of lipid biosynthesis right after the removal of the drugs from the medium. To test this hypothesis, we first measured the mRNA levels of SREBF2, HMGR (3-hydroxy-3-methyl-glutaryl-CoA reductase), and LDLR (LDL receptor), which are regulated by active SREBP-2 (31). HepG2 cells were treated with the antipsychotics (10 \(\mu\)M) for 2, 4, or 16 h, or for 16 h plus additional 8 h after removing the medium and substitution by medium without drugs (16 h + 8 h) (washout). In HepG2 cells incubated in control conditions, a decline in the expression of these genes was observed (Table 4), which is attributed to the presence of LDL in the incubation medium. In cells treated with antipsychotics, an increase in gene expression was observed, especially at shorter incubation times. At 16 h of incubation, the stimulatory effect persisted for some antipsychotics (Table 4).

In the washout period (16 h + 8 h), likely as a result of the removal of LDL from the incubation medium, the expression of both SREBF2 and LDLR tended to increase in
all conditions, including the control (Table 4). In general, this rebound effect was more evident in cells pretreated with antipsychotics. Thus, at the end of the washout period, mRNA levels of SREBF1, HMGR, and LDLR in cells pretreated with clozapine were significantly higher than in control cells. Risperidone and ziprasidone also induced a strong rebound, although mRNA levels reached were similar to those found in the control (Table 4). Finally, HMGR mRNA levels were more resistant to increase after LDL removal, and in cases where expression levels were relatively high at 16 h (haloperidol, clozapine, and ziprasidone), they even decreased during the washout period.

Once we demonstrated that fatty acids and complex lipid synthesis was increased by FGA and SGA, we analyzed the mRNA expression of SREBF1 and its associated target gene FASN. In control conditions, cells responded to LDL by decreasing the expression of both SREBF1 and FASN, without appreciable changes after the removal of LDL (16 h + 8 h). In cells treated with antipsychotics, higher expression levels of both SREBF1 and FASN were observed compared with the control, especially at shorter incubation times (<4 h) (Table 4). In the washout period (16 h + 8 h), compared with the preceding time studied (16 h), no significant changes in the expression of SREBF1 were observed in cells treated with haloperidol and clozapine, whereas a further decline in gene expression was observed in cells treated with risperidone and ziprasidone. Regarding FASN, expression levels further decreased in the washout period with all drugs tested except risperidone (Table 4).

Finally, we measured expression levels of ABCA1, which is mainly governed by LXR. In cells incubated in control conditions, no appreciable changes in ABCA1 mRNA levels were observed in response to LDL (times 0–16 h) and after its removal (16 h + 8 h) (Table 4). In cells treated with antipsychotic drugs, no major changes in ABCA1 mRNA levels were observed compared with the control, except a slight decrease at time 4 h (Table 4).

Effects of antipsychotics on apoB secretion

The HepG2 cell line has been shown to be model of choice for studying human apoB synthesis and secretion (30). Once we demonstrated that lipid synthesis was increased by effect of antipsychotics and, in some cases, especially after their removal, we sought to determine whether antipsychotics affect apoB secretion. In previous experiments, we confirmed that apoB100 secretion was specific, as the MTP inhibitor CP-346086 (10 µM) almost completely abolished the appearance of apoB100 in the medium (data not show).

### Table 4. Effects of antipsychotics on lipid-metabolism-related gene expression

| Gene | Control | Haloperidol | Clozapine | Risperidone | Ziprasidone |
|------|---------|-------------|-----------|-------------|-------------|
| SREBF2 | 1.000 ± 0.038 | 1.045 ± 0.038* | 1.254 ± 0.062** | 1.045 ± 0.038* | 1.192 ± 0.070** |
| 2 h | 0.823 ± 0.069 | 1.124 ± 0.074** | 1.075 ± 0.041** | 1.118 ± 0.065** |
| 4 h | 0.911 ± 0.061 | 1.460 ± 0.101** | 1.081 ± 0.041* | 1.041 ± 0.108 |
| 16 h | 0.517 ± 0.055 | 0.854 ± 0.059** | 0.743 ± 0.099* | 0.545 ± 0.071 |
| 16 h+8 h | 0.734 ± 0.040** | 0.958 ± 0.084* | 0.869 ± 0.024** | 0.695 ± 0.029* |
| HMGR | 1.000 ± 0.052 | | | | |
| 2 h | 0.806 ± 0.061 | 1.086 ± 0.082** | 1.209 ± 0.029*** | 1.048 ± 0.054** |
| 4 h | 0.659 ± 0.050 | 1.294 ± 0.101*** | 1.206 ± 0.051*** | 0.814 ± 0.020** |
| 16 h | 0.263 ± 0.014 | 0.525 ± 0.038*** | 0.746 ± 0.037*** | 0.245 ± 0.011 |
| 16 h+8 h | 0.300 ± 0.020 | 0.354 ± 0.019*** | 0.659 ± 0.014*** | 0.369 ± 0.012*** |
| LDLR | 1.000 ± 0.026 | | | | |
| 2 h | 0.748 ± 0.035 | 0.978 ± 0.046** | 1.355 ± 0.078*** | 1.022 ± 0.046** |
| 4 h | 0.548 ± 0.022 | 1.104 ± 0.081*** | 1.313 ± 0.035*** | 0.708 ± 0.012*** |
| 16 h | 0.448 ± 0.020 | 0.474 ± 0.044 | 0.685 ± 0.032*** | 0.337 ± 0.021*** |
| 16 h+8 h | 0.600 ± 0.059* | 0.552 ± 0.020* | 0.869 ± 0.024*** | 0.715 ± 0.051*** |
| SREBF1 | 1.000 ± 0.015 | | | | |
| 2 h | 1.021 ± 0.017 | 1.228 ± 0.044*** | 0.964 ± 0.032 | 0.962 ± 0.011 |
| 4 h | 0.737 ± 0.027 | 0.930 ± 0.029** | 1.474 ± 0.075*** | 1.150 ± 0.028*** |
| 16 h | 0.840 ± 0.034 | 0.741 ± 0.021* | 0.928 ± 0.028* | 0.876 ± 0.014 |
| 16 h+8 h | 0.882 ± 0.023 | 0.715 ± 0.015*** | 0.914 ± 0.013 | 0.776 ± 0.028*** |
| FASN | 1.000 ± 0.018 | | | | |
| 2 h | 0.933 ± 0.023 | 1.200 ± 0.044*** | 1.055 ± 0.012** | 0.918 ± 0.033 |
| 4 h | 0.704 ± 0.022 | 1.357 ± 0.135*** | 1.026 ± 0.057*** | 0.909 ± 0.018*** |
| 16 h | 0.476 ± 0.021 | 0.827 ± 0.046*** | 0.744 ± 0.035*** | 0.462 ± 0.016 |
| 16 h+8 h | 0.451 ± 0.020 | 0.457 ± 0.016*** | 0.580 ± 0.024*** | 0.468 ± 0.019 |
| ABCA1 | 1.000 ± 0.050 | | | | |
| 2 h | 1.026 ± 0.054 | 1.200 ± 0.031** | 1.013 ± 0.048 | 1.048 ± 0.053 |
| 4 h | 1.130 ± 0.039 | 0.986 ± 0.040* | 1.111 ± 0.065 | 1.017 ± 0.107* |
| 16 h | 0.938 ± 0.061 | 0.955 ± 0.030 | 0.914 ± 0.060 | 0.888 ± 0.052 |
| 16 h+8 h | 1.007 ± 0.045 | 1.051 ± 0.049 | 1.047 ± 0.055 | 0.931 ± 0.035 |

HepG2 cells were cultured in lipoprotein-deficient serum-containing medium. At time 0, the medium was supplemented with LDL (30 µg/ml of cholesterol) and the different antipsychotics or placebo (control). The cells were incubated for 2, 4, or 16 h or for 16 h plus an additional 8 h (16 h + 8 h) after the medium was replaced by plain medium. At the indicated times, cells were collected, total mRNA was extracted, and individual mRNA species were quantified by qRT-PCR. Expression levels were normalized to Rplp0 mRNA. Expression is presented as means ± SEM of three independent experiments performed in triplicate. Statistical comparisons are shown versus control (*P<0.05, **P<0.01, ***P<0.001) or each 16 h antipsychotic treatment plus 8 h of washout versus antipsychotic 16 h (P<0.05, **P<0.01, ***P<0.001).
To investigate the effects of drug removal, we choose clozapine for its potent effect on lipid synthesis. For this, HepG2 cells were treated with clozapine (5, 10, and 25 µM) for 20 h. Then the medium was replaced, one set of cells continued to be exposed to clozapine (present), and the other set was incubated in medium containing no antipsychotic (washout) and [35S]-methionine was added for 15 min, followed by 3 h chase. In cells continuously exposed to clozapine, at any dose, no significant changes in apoB100 secretion were observed compared with the control (Fig. 4A). However, when apoB100 secretion was measured after drug removal (washout), a significant increase was observed in cells pretreated with 10 and 25 µM clozapine (Fig. 4A). No changes in the secretion of albumin, a control secretory protein, were observed among the different conditions.

In the next experiments, the effects of the other antipsychotics were investigated in the washout period. As shown in Fig. 4B, pretreatment with haloperidol (FGA) and ziprasidone and risperidone (SGAs) increased apoB100 secretion into the medium compared with the control, the differences being statistically significant even at the lowest studied dose (5 µM) for ziprasidone and haloperidol.

**Effects of SGAs on the intracellular traffic of LDL-cholesterol**

Antipsychotics are cationic amphiphiles with the potential to interfere with the endosomal/lysosomal pathway (16, 17).

**DISCUSSION**

We studied the effects of SGAs on different aspects of intracellular lipid homeostasis and lipid secretion. These neuroleptics are widely used for the treatment of schizophrenia and other mental disorders, but they are not free of side effects, such as weight gain, hyperglycemia, and hypertriglyceridemia. In this context, to determine whether SGAs affect lipid metabolism, intracellular cholesterol...
traffic, or lipid secretion might help to define the consequences of antipsychotic treatment on cell physiology.

Since the pioneering observations by Summerly and Yardley (14), who demonstrated that haloperidol administration in rats resulted in the inhibition of incorporation of radiolabeled acetate into cholesterol in rat skin, interest in this field has been reborn with the introduction of SGAs, and some studies have been published recently that report seemingly controversial results on the effects of antipsychotics on cholesterol biosynthesis (15–19). We report here that SGAs (clozapine, risperidone, and ziprasidone) inhibit cholesterol biosynthesis and produce changes in the sterol composition of cells. Assuming that feedback mechanisms are triggered by the decrease in intracellular cholesterol levels, these results are in line with the reported activation of SREBP-2 processing and increased expression of genes involved in cholesterol biosynthesis induced by SGAs (present results and Refs. 23–25).

In both HepG2 and HL-60 cell lines, we found that several SGAs reduced the incorporation of 14C-acetate radioactivity into cholesterol and increased it in some cholesterol precursor sterols. Parallel changes in cell sterol composition as analyzed by GC/MS were observed. These effects varied widely depending on the drug and concentration used. Based on these changes, the affected enzymes could be estimated. In hepatoma HepG2 cells, clozapine mainly affected the reactions catalyzed by Δ24-reductase and Δ8,7-isomerase; risperidone affected the reactions catalyzed by Δ7-reductase and Δ8,7-isomerase; and ziprasidone affected the reactions catalyzed by Δ7- and Δ14-reductases. In promyelocytic HL-60 and neuroblastoma SH-SY5Y cells, the effects of the studied SGAs were quite similar to those observed in HepG2 cells. Clozapine was less active in inhibiting cholesterol biosynthesis and affected both Δ24-reductase and Δ8,7-isomerase, based on the increases in the C24-unsaturated sterols, zymosterol and zymostenol (substrates of Δ8,7-isomerase), respectively. Risperidone clearly inhibited Δ7-reductase and less strongly inhibited Δ14-reductase and ΔΔ7-isomerase. In addition, ziprasidone strongly inhibited Δ14-reductase and Δ7-reductase, and it inhibited Δ8,7-isomerase less.

In light of these changes in intermediary sterols, ziprasidone and risperidone, two of the SGAs studied, affected Δ7-reductase > Δ8,7-isomerase = C5-desaturase > Δ14-reductase, which is similar to haloperidol, used as a reference FGA. Thus, these SGAs apparently have the same targets as haloperidol in the inhibition of cholesterol biosynthesis, although with different relative activities: ziprasidone > haloperidol > risperidone. In contrast, clozapine mainly increased C24-unsaturated sterols (desmosterol, Δ7,24-cholestadienol, and zymosterol), indicative of Δ24-reductase inhibition, and to a minor extent, it also affected Δ8,7-isomerase.

Differences in the cholesterol biosynthesis activity among the different cell lines used merit some comment. HepG2 and SH-SY5Y cells had a relatively high content of cholesterol, about 36–37 g/mg cell protein, whereas in HL-60 cholesterol content is just 11 g/mg cell protein. Regarding intermediates, HL-60 and SH-SY5Y had appreciable 7DHC, whereas HepG2 had undetectable 7DHC but contained some desmosterol. These profiles suggest that Δ7-reductase activity may be limiting in HL-60 and SH-SY5Y cells but not in HepG2 cells. Treatment with 5 µM haloperidol strongly inhibited Δ7-reductase activity in all cell lines,

Fig. 5. Effects of antipsychotics on endosome/lysosome accumulation of LDL. Untreated HepG2 cells (control) and cells treated for 24 h with haloperidol, clozapine, risperidone, and ziprasidone 10 µM were incubated with Dil-LDL (30 µg/ml of cholesterol). A: At the end of the incubation period, cells were fixed and stained with filipin and LAMP2 and then photographed using a confocal microscope. B: At the end of the incubation period, cells were fixed and stained with filipin and CD63 and then photographed using a confocal microscope. Results from a representative experiment of three independent experiments are shown.
but the levels 7DHC differed greatly among the cell lines: in HL-60 and SH-SY5Y, they reached 12–14 μg/mg, whereas in HepG2, only 1 μg/mg cell protein. Moreover, in the former cell type, the increase in 7DHC was such that the total sterol content increased over the control, even though the cholesterol content decreased. In HepG2 cells, a minor amount of sterol intermediates accumulated; therefore, the total sterol content was reduced by effect of the antipsychotic. These results illustrate that the quantitative effects of antipsychotics on the total sterol content varied among tissues, depending on the relative activity of the different enzymes involved in cholesterol biosynthesis.

Results of the effects of antipsychotics on cholesterol biosynthesis are controversial. Silve et al. demonstrated that haloperidol binds to EBP and inhibits Δ7-7-isomerase activity in yeast (32). Kelley and Hennekam proposed, based on an increase in 7DHC plasma concentration in patients treated with haloperidol, that this antipsychotic inhibited Δ-reductase (33). More recently, Kristiana et al. confirmed that both haloperidol and a panel of SGAs inhibited cholesterol biosynthesis, although the sterol intermediates accumulated and the affected enzymes were not elucidated (17). In sharp contrast, Lauresergues et al., who determined 14C-acetate incorporation into cholesterol in cells 4 h after removing the antipsychotics from the culture medium, found that pretreatment with SGAs clozapine, olanzapine (18), and risperidone (19) increased rather than decreased the incorporation of 14C-acetate into cholesterol, whereas other antipsychotics, such as haloperidol, quetiapine, and aripiprazole, had no effect (18). We addressed these seemingly contradictory results by analyzing cholesterol biosynthesis in cells during exposure to the antipsychotics and after drug withdrawal (washout). The results showed that shortly after antipsychotic withdrawal, the radioactivity incorporation into sterol intermediates sharply decreased while cholesterol increased, reaching values close to those in control cells not treated with the drugs. This indicates that, depending on the design of the experiment, either an inhibition or an activation of the cholesterol biosynthesis may be found.

The inhibition of both the cholesterol biosynthesis and cholesterol egress from the endosomal/lysosomal pathway likely contributed to the observed activation of SREBP-2 and subsequent overexpression of its target genes, including those involved in cholesterol biosynthesis (present results and Refs. 23–25). The increase in enzyme protein levels that would eventually take place in response to SREBP-2, however, might be counterbalanced by the inhibitory effect of these drugs on enzyme action. Hence, as long the antipsychotics are present within the cells, cholesterol biosynthesis will be inhibited. Shortly after the removal of antipsychotics, cholesterol biosynthesis is activated, likely due to both the alleviation of enzyme activity inhibition and the increased expression of enzyme proteins induced by SREBP-2. How this dual effect observed in vitro translates into the in vivo situation is not evident. More studies are required to directly examine the correspondence between the intracellular cholesterol homeostasis in relevant tissues and the eventual changes in plasma lipids in animals administered with antipsychotics.

The mechanism by which the antipsychotics affect enzyme activity is not known. The relative lack of selectivity of haloperidol, ziprasidone, and risperidone on enzyme activities is similar to that of another lipophilic, cationic amine A9944 (supplemental Fig. II), which also inhibits Δ7-reductase, Δ7-7-isomerase, and Δ5-reductase (28, 34). Enzymes acting in the last part of the cholesterol biosynthesis pathway exhibit high affinity binding for lipophilic drugs, such as A9944, which may mimic the carbocationic high-energy intermediates involved in the reactions catalyzed by these enzymes (35). Thus, it is possible that the above-mentioned antipsychotics that are protonated under physiological conditions act similarly by mimicking the carbocationic intermediates. The causes of the differences in enzyme inhibition exhibited by clozapine are unclear. Another possibility is that antipsychotics exert their effects by altering membrane properties. Antipsychotics interact with lipids and show higher affinity for sphingomyelin than for phosphatidylcholine, and they alter liquid-ordered domain formation and bilayer thickness (36). Moreover, antipsychotics have been shown to intercalate among anionic glycerophospholipids in cellular membranes and to alter the relative spatial positioning of PLs, affecting the activity of membrane-bound proteins (37, 38).

Based on the well-known inverse relationship between cholesterol levels in the ER and SREBP-2 activation, we expected that the different antipsychotics stimulated gene expression according with their inhibition of cholesterol biosynthesis. In general, this actually was the case as ziprasidone and haloperidol, which decreased the cholesterol content most (Table 2), exhibited higher SREBF2, HMGCR, and LDLR-mRNA levels at short incubation times than did risperidone (Table 4). This relationship, however, does not stand for clozapine. It is worth mentioning that clozapine targets different enzymes on the cholesterol biosynthesis pathway than the other antipsychotics. Moreover, sterol intermediates also modulate SREBP-2 activation (39, 40). Finally, the sterol content in cell lysates as measured here may not necessarily reflect the regulatory cholesterol pool in the ER, particularly if the intracellular trafficking is impaired as in cells treated with antipsychotics.

It has been shown by others that antipsychotics, besides stimulating SREBP-2 expression, also upregulate SREBP-1 and its associated targets (FAS, SCAD1, and ACC1) (17-19, 23, 41). Very recently, hypertriglyceridermia, which is independent of weight gain, induced in rats by olanzapine has been associated with the upregulation of SREBP-1-controlled lipogenic gene expression in adipose tissue (15). We thus determined mRNA levels of SREBF1 and FASN in HepG2, and the results confirmed the increased expression of both genes by effect of all studied FGA and SGAs. In line with the gene expression data, a number of antipsychotics have been shown to increase the synthesis of PL, TG, and FFA both in primary hepatocytes in culture (18, 19). Moreover, acute exposure of rats to clozapine induced a pronounced increase in the content of TG, PL, and cholesterol in the liver (41). We studied the effects of antipsychotics in HepG2 cells, and the results confirm the previously published data for haloperidol, risperidone, and clozapine, and...
chotics has not been studied thus far. Several studies have shown decreased plasma concentration of HDL-cholesterol in subjects treated with SGAs for a long term (10, 46, 47). Whether the change in $ABCA1$ gene expression induced by antipsychotics contributes to the reduction of HDL-cholesterol levels is not known; more detailed studies are required to elucidate this important issue. Another important action of antipsychotics related to intracellular lipid homeostasis is the inhibition of cholesterol egress from the late endosome/lysosome compartment. In the present study, we showed that the SGAs clozapine, risperidone, and ziprasidone, like haloperidol, all induced the accumulation of free cholesterol in endosome/lysosome vesicles, indicative of the retention of LDL-derived free cholesterol in this subcellular compartment. These results confirmed our previous findings on haloperidol (16) and extend the observations by Kristiana et al. with different atypical antipsychotics (17). Antipsychotics can be classified as hydrophobic amine or cationic amphiphile/amphipath drugs, as they are positively charged by virtue of an amine group that can be protonated, and they display both hydrophilic and hydrophobic properties. It is well recognized that many amines have a strong propensity to specifically and substantially accumulate in highly acidic intracellular compartments, such as lysosomes, through a mechanism referred to as ion trapping (48). In their unionized form, weak basic amines are relatively membrane permeable, but they are membrane impermeable in their ionized conjugate acidic state (48). Some antipsychotic and antidepressant drugs have been shown to extensively accumulate in lysosomes (49, 50).

Fig. 6. Scheme summarizing the changes in lipid homeostasis in cells exposed to antipsychotics (left panel, A) and after antipsychotics withdrawal (right panel, B). A: Antipsychotics inhibit the cholesterol biosynthesis and reduce the lipoprotein-derived cholesterol trafficking to the cholesterol-regulated machinery in the ER, which induces the transcription of lipogenic genes. B: Once the antipsychotics are removed from the medium and their direct actions on cholesterol biosynthesis enzymes and late-endosome cholesterol egress are relieved, the homeostatic response prevails, highly increasing the synthesis of both cholesterol and complex lipids, as well as apoB100 secretion.
Many cationic amphiphilic compounds, not antipsychotics, have been reported to inhibit intracellular cholesterol trafficking, such as U18666A (51–53). This effect of U18666A appears to be independent of protein synthesis, integrity of the cytoskeleton, energy availability, and pH of the lysosome (52, 54). This compound binds with high specificity to the cell membrane, and this binding can alter the organization of membrane lipids (55, 56). Interestingly, U18666A has also been shown to inhibit 2,3-oxidosqualene cyclase, $\Delta^{7}$-isomerase, and $\Delta^{24}$-reductase by a noncompetitive mechanism (56–58). Tamoxifen, another cationic amphiphilic drug, was shown to inhibit both LDL endocytic trafficking and the activities of sterol $\Delta^{7}$-isomerase and $\Delta^{24}$-reductase (21, 22). How these compounds affect the activity of enzymes located in the ER is unknown. It may be speculated that amine amphiphiles, including the antipsychotics, by intercalating in intracellular membranes, may alter the conformation of resident proteins (either enzymes or transporters), affecting their function. Further studies are needed to clarify this hypothesis.

The results of the present work and those previously reported by us and other investigators allow us to speculate on the mechanism by which cholesterol synthesis inhibition produced by antipsychotics may lead to the hypertriglyceridemia often exhibited by subjects medicated with these drugs. A scheme is shown in Fig. 6. As a result of the inhibition of several enzymes involved in cholesterol biosynthesis, antipsychotics reduce the cholesterol cell content and increase that of different intermediate sterols (present results and Refs. 15–17). Antipsychotics also inhibit the egress of lipoprotein-derivated cholesterol from the endosome/lysosome compartment to the ER, further decreasing the cholesterol content in this regulatory compartment (present results and Refs. 16, 17). As a consequence, SREBP-1 and SREBP-2 are activated (17–19, 23–25, 41), and the expression of genes involved in cholesterol and fatty acid metabolism are stimulated (present results and Refs. 17–19, 23–25, 41). As long antipsychotics are present within the cell, cholesterol biosynthesis is inhibited (present results). Once antipsychotics are removed from the medium (B), their direct actions on cholesterol biosynthesis and lipid trafficking are relieved, and the SREBP-driven homeostatic response is maximally expressed, resulting in increased synthesis of cholesterol, fatty acid, and complex lipids (present results and Refs. 18, 19). Finally, the augmented accretion of lipids may stimulate apoB100 secretion, as reported here. These effects may be relevant in the metabolic side effects of antipsychotics.

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