Cholesterol Esterification by Host and Parasite Is Essential for Optimal Proliferation of Toxoplasma gondii*

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Upon invasion of a host cell, the apicomplexan parasite Toxoplasma gondii resides in a specialized compartment termed the parasitophorous vacuole (PV). The PV is unique because it is derived from the host cell membrane but modified by the parasite. Despite the segregation of the parasitophorous vacuole from the host endocytic network, the intravacuolar parasite has been shown to acquire cholesterol from the host cell. In order to characterize further the role of sterol metabolism in T. gondii biology, we focused our studies on the activity of acyl-CoA:cholesterol acyltransferase (ACAT), a key enzyme for maintaining the intravacuolar homeostasis of cholesterol through the formation of cholesterol esters. In this study, we demonstrate that ACAT and cholesterol esters play a crucial role in the optimal replication of T. gondii. Moreover, we identified ACAT activity in T. gondii that can be modulated by pharmacological ACAT inhibitors with a consequent detrimental effect on parasite replication.

Upon invasion of a host cell, the apicomplexan parasite Toxoplasma gondii resides in a specialized compartment, the parasitophorous vacuole (PV). The PV is unique because it is predominantly derived from the host cell plasma membrane but is devoid of host cell transmembrane proteins (2, 3) and does not fuse with endocytic or exocytotic vesicles of the host cell (4–7). After invasion, T. gondii rapidly modifies the PV membrane by secreting proteins stored in rhoptries and dense granules (7–13).

Despite the apparent segregation of the PV from the host cell endocytic network, metabolites essential for the parasite are known to exchange with the intravacuolar space. Aqueous soluble molecules of less than 1300 daltons enter the vacuole through non-selective pores in the PV membrane, thus satisfying the parasite’s auxotrophy for purines and tryptophan (14, 15).

Much less is known about the uptake and metabolism of lipids by T. gondii. Since proteins involved in lipid biosynthesis such as acyl carrier proteins and subunits of type II fatty-acyl synthase have been shown to localize in the apicoplasts, the plastid organelle may play a role in the lipid metabolism of T. gondii (16). T. gondii membranes contain cholesterol and are characterized by a low cholesterol/phospholipid ratio and consequent high level of fluidity (17, 18). It has been shown recently that the parasite depends on host cell cholesterol derived from endocytosed LDL and not from endogenous synthesis (19). However, the exact mechanism of cholesterol uptake by intravacuolar parasites has not been completely elucidated.

An essential enzyme for the regulation of intracellular cholesterol homeostasis is acyl-CoA:cholesterol acyltransferase 1 (ACAT-1). ACAT-1 covalently joins excess free cholesterol (FC) with fatty acyl-CoA to form cholesterol esters (CE), which can then be stored in the form of cytoplasmic lipid droplets. The enzyme is a transmembrane protein located mainly in the endoplasmic reticulum and perinuclear region (20) and functions as a homotramer (21) with the active site apparently oriented toward the cytoplasm (22). Whereas ACAT-1 is expressed ubiquitously in mammalian tissues (23, 24), a related enzyme, ACAT-2, is expressed in the liver and small intestine and is involved in absorption of dietary cholesterol (25, 26).

Because the parasite depends on cholesterol acquired by host cell endocytosis but inhabits a vacuole that does not interact with host cell endocytic vesicles, one or more intermediate steps of cholesterol uptake by the parasite must exist and remain to be identified. Because cholesterol esters formed by ACAT are an effective way to store cholesterol in the cytoplasm, we reasoned that ACAT activity with its role in cholesterol ester formation and cellular cholesterol homeostasis plays a role in the optimal parasite proliferation. To test this hypothesis, we analyzed T. gondii replication in cells where ACAT enzyme activity was absent through either genetic deletion or pharmacologic inhibition.

**Experimental Procedures**

**Chemicals—**Unless otherwise stated, all reagents were purchased from Sigma. The acyl amide ACAT inhibitors SnH 58-035 and CI 976 were obtained from Sandoz Inc., East Hanover, NJ, and Parke-Davis, respectively. Stock solutions of inhibitors were prepared at a concentration of 2 mM in Me2SO and were freshly diluted to the concentrations required for each experiment.

Radilabeled lipids were purchased from Amersham Pharmacia Biotech.

**Cell Types, Parasite Culture, and Purification—**The cell types used for infection assays with T. gondii were human foreskin fibroblasts

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The abbreviations used are: PV, parasitophorous vacuole; ACAT, acyl-CoA:cholesterol acyltransferase; CE, cholesterol esters; FC, free cholesterol; HFF, human foreskin fibroblasts; LDL, low density lipoprotein; MEF, murine embryonic fibroblasts; m.o.l., multiplicity of infection; MFP, murine peritoneal fibroblasts; PFA, paraformaldehyde; PBS, phosphate-buffered saline.

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Different concentrations of SaH 58-035 and CI 976 or solvent alone were added to the parasites during the ACAT stimulation with 25-
hydroxycholesterol and cholesterol; the total time of inhibitor treatment of T. gondii tachyzoites was limited to 7 h to maintain the viability of extracellular parasites.

**HeLa Cell Viability Assay**—Cell viability was tested using the CellTiter 96®Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Briefly, HFF were grown to confluence in 96-well plates, treated with different concentrations of SaH 58-035, CI 976, or solvent alone for 72 h, and processed according to the instructions provided by the manufacturer. Absorbance was measured at wavelength of 490 nm using the microplate reader described previously.

**Determination of ATP Content**—ACAT-1−1/2 MEF, ACAT-1−1/2 MEF, and HFF were grown to confluence in 96-well plates, and HFF were treated with different concentrations of SaH 58-035, CI 976, or solvent alone for 72 h. Cells were washed in PBS and lysed for 30 min at 4°C in 75 μl of lysis buffer (0.1 M potassium phosphate, pH 7.6, 1% Triton X-100, 1 mM dithiothreitol, 2 mM EDTA). After centrifugation at 2600 × g for 20 min, 20 μl of supernatant was collected and added to 100 μl of firefly luciferase/luciferin solution (Sigma). Bioluminescence was detected with a TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA), and ATP concentration of the samples was calculated by comparison with an ATP standard curve.

**Amino Acid Incorporation For Cholesterol and Cholesterol Ester Content**—ACAT-1−/− MEF, ACAT-1−/− MEF, and HFF were grown to confluence in 12-well plates, and HFF were treated for 72 h with different concentrations of SaH 58-035, CI 976, or solvent alone. After washing in 0.2% albumin in PBS, cellular lipids were extracted and separated by TLC using hexane/ethyl ether/acetic acid (80:20:1) or chloroform/methanol (40:60) for developing FC and CE, respectively. Bands of lipids were visualized using a solution of 3% cupric acetate in 15% aqueous phosphoric acid followed by charring of the plates at 120°C for 15 min. The intensity of the bands was analyzed with an Alpha Imager 2000 (Alpha Innotech Corporation, San Leandro, CA), and the FC and CE amounts were determined by comparison with standard curves.

**LDL Receptor Assay**—For immunoblot analysis, total extracts of ACAT-1−/− MEF, ACAT-1−/− MEF, and ACAT inhibitor-treated HFF were obtained by trypsinizing and resuspending confluent monolayers in SDS sample buffer in the absence of reducing agents. Proteins (5 μg) from total extracts were separated by SDS-polyacrylamide gel electrophoresis in a precast gradient gel (4–15% acrylamide, Bio-Rad) and transferred to nitrocellulose filters. After blocking of unspecific binding sites with TBS containing 5% skim milk and 0.3% Tween 20, bands were incubated with a rabbit anti-serum recognizing the LDL receptor (32) and visualized using horseradish peroxidase-conjugated anti-rabbit antibodies and the Renaissance®Chimiluminescence Reagent Plus (PerkinElmer Life Sciences). Polyclonal antibodies against annexin I were used to normalize the amount of proteins present in the gel (33).

For flow cytometry analysis, confluent monolayers of ACAT-1−/− MEF, ACAT-1−/− MEF, and ACAT inhibitor-treated HFF were trypsinized, washed in chilled PBS, and resuspended in PBS with 0.02% bovine serum albumin. Aliquots of 106 cells were incubated on ice with the previously described anti-LDL receptor antibodies, followed by incubation with fluorescein isothiocyanate-conjugated anti-rabbit antibodies. Five thousand cells were analyzed for LDL receptor expression on a FACSsort flow cytometer with CellQuest software (Becton Dickinson, Mountain View, CA). Background fluorescence of the secondary antibody alone was subtracted using the CellQuest software, from sample fluorescence before analyzing the mean fluorescence.

**Lipid Staining and Fluorescence Analysis**—For staining of neutral lipids in T. gondii, purified tachyzoites were applied to poly-lysine-coated glass coverslips, fixed in 75% ethanol for 5 min, and grown to confluence in 75-cm2 tissue culture flasks in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C, 5% CO2. MEF cultures were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) with the same supplements described above.

Cultures were passaged by trypsinization at least once a week, and cells were used up to the 20th passage.

**T. gondii tachyzoites of the βRH strain were used for infection. This strain expresses Escherichia coli β-galactosidase under control of the T. gondii sag1 promoter (28) and has been used previously to assay parasite proliferation (29) Parasites were harvested from host cells, passed 3 times, and used for the standard curve.**

**Determination of Parasite Proliferation**—T. gondii was quantitated in infected cells by colorimetric detection of β-galactosidase activity expressed by the parasite strain βRH as described (29). Briefly, either ACAT-1−/− MEF, ACAT-1−/− MEF or HFF were grown to confluence in 96-well plates in medium lacking phenol red, and HFF were treated for 12 h with different concentrations of SaH 58-035, CI 976, or solvent alone. Cells were then infected with purified parasites at m.o.i. 0.1. After 72 h, 10 μl of the β-galactosidase substrate chlorophenol red β-galactopyranoside (Roche Molecular Biochemicals) was added to the culture medium to give a final concentration of 100 μM. Following an additional incubation at 37°C, 5% CO2 for 1–4 h until color development, plates were read at a dual wavelength of 570/630 nm using a Spectra MAX 250 microplate reader ( Molecular Devices, Sunnyvale, CA). In some experiments, parasites were directly counted following nuclear staining using a Leica DMRB fluorescence microscope (Deerfield, IL). Consistent results of parasite quantitation were obtained by using both the colorimetric and the direct counting methods.

**T. gondii Invasion Assay**—ACAT-1−/− MEF and HFF were grown to confluence on polylisine-coated glass coverslips, and HFF were treated with 40 μM SaH 58-035, CI 976, or solvent alone for 12 h. Purified T. gondii tachyzoites were allowed to infect the cells for 1 h at m.o.i. of 0.1. Extracellular parasites were removed by washing the coverslips in PBS, and the remaining tissue was finely minced. The entire mixture was then transferred to a Petri dish containing a solution of 0.02% collagenase (40:60) for developing FC and CE, respectively. Bands of lipids were visualized using a solution of 3% cupric acetate in 15% aqueous phosphoric acid followed by charring of the plates at 120°C for 15 min. The intensity of the bands was analyzed with an Alpha Imager 2000 (Alpha Innotech Corporation, San Leandro, CA), and the FC and CE amounts were determined by comparison with standard curves.

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**Lipid Staining and Fluorescence Analysis**—For staining of neutral lipids in T. gondii, purified tachyzoites were applied to poly-lysine-coated glass coverslips, fixed in 4% PFA, and incubated at room temperature for 30 min in 5 μl of Nile Red in PBS (stock solution 0.5 mg/ml in acetone). Coverslips were then rinsed in PBS and mounted in glycerol for fluorescence microscopy employing a filter set for fluorescein (450–500 nm band pass excitation filter) for yellow-gold fluorescence. A similar procedure was used for cytochemical staining of cellular free cholesterol with the antibiotic filipin at concentration of 50 μg/ml in PBS (stock solution 2.5 mg/ml in dimethyl formamide), using an excitation filter of 350–410 nm.

**Determination of Protein Concentration**—Protein content was determined using the BCA Protein Assay Kit (Pierce) according to the instructions provided by the manufacturer. Bovine serum albumin was used for the standard curve.
but it did not kill the parasites. In both ACAT-1−/− lack of cholesterol esterification impaired parasite replication, suggesting that an aberrant initial formation of the PV is not responsible for the anti-T. gondii effect observed in the absence of cholesterol esterification could be a consequence of decreased invasion efficiency, decreased parasite replication, or both. In order to assess whether the alteration in cholesterol ester metabolism compromises the ability of T. gondii to enter host cells, an invasion assay was performed. The number of intracellular parasites in both ACAT-1−/− MEF (Fig. 2A) and inhibitor-treated HFF (Fig. 2B) was comparable to the number of parasites in control cells at 1 h postinfection but was significantly decreased at 24 h postinfection. Therefore, it appears that ACAT activity is not required for efficient invasion of host cells by T. gondii but for subsequent intracellular replication. Deficiency or inhibition of ACAT results in an increase in the doubling time of intracellular tachyzoites from between 6 and 8 h to over 10 h (data not shown).

To determine whether the proliferation of parasites in cells with compromised cholesterol esterification impairs the ability of T. gondii to accomplish additional cycles of invasion, parasites were isolated from ACAT-1−/− MEF or inhibitor-treated HFF after two lysis passages and allowed to invade and proliferate in untreated HFF. We found that parasites isolated from cells with compromised cholesterol esterification were as efficient at invading and replicating in HFF as were parasites isolated from control cells (not shown). Therefore, alteration of cholesterol esterification does not permanently impair intracellular cycles of T. gondii.

SaH 58-035 and CI 976 Inhibit ACAT in HFF Without Host Cell Toxicity—While the impairment of T. gondii replication in both ACAT-1−/− MEF and in ACAT inhibitor-treated HFF implied that the lack of ACAT activity was responsible for the anti-T. gondii effect, it was necessary to obtain direct evidence in support of this mechanism. Therefore, we examined the ACAT enzymatic activity in these cells, measured as incorporation of radiolabeled fatty acid into cholesterol esters. The amount of newly formed cholesterol esters was decreased by ≥95% in ACAT-1−/− MEF compared with control ACAT-1+/+ MEF. Similarly, the formation of new cholesterol esters was inhibited by ≥95% in HFF treated with 1.0 μM or higher concentrations of either SaH 58-035 or CI 976 (Fig. 3). This finding is consistent with the published observation that SaH 58-035 and CI 976 inhibit cholesterol esterification in other cell types with IC50 values of average 0.5 μM (34, 35).

The observation that the anti-T. gondii effect of ACAT inhibitors required significantly higher concentrations of the inhibitors than those required for maximal inhibition of cellular ACAT activity raised the possibility that the ACAT inhibitors exerted their effects on parasite replication through nonspecific host cell toxicity. Although neither visual inspection of the morphology nor trypan blue staining of HFF indicated obvious host cell toxicity at concentrations of inhibitors that slowed T. gondii replication, we further examined host cell toxicity using two distinct biochemical assays. First, we used a colorimetric assay of cell viability based on functionality of dehydrogenase enzymes found in metabolically active cells. This revealed that host cell metabolism was not affected by the presence of the inhibitors except at the highest dose (100 μM) of CI 976, a concentration much higher than that required to reduce T. gondii replication (data not shown). Some morphologic alteration of the cells and loss of adherence were also observed at this concentration of CI 976. Second, we assayed the ATP content of host cells to determine whether ACAT inhibitors reduced the proliferation of T. gondii by blocking the production of this host cell metabolite on which T. gondii is known to depend (15, 36). ATP levels were not reduced in ACAT-1−/− MEF or inhibitor-treated HFF, indicating that ATP depletion did not account for the anti-T. gondii effect (data not shown).

Increases in Intracellular Free Cholesterol Content Are Not Responsible for the Anti-T. gondii Effect—The anti-T. gondii effect observed in the absence of cholesterol esterification could have resulted from accumulation of free cholesterol that is known to be toxic to mammalian cells (37). Therefore, we examined the free cholesterol content in both ACAT-1−/− MEF and ACAT inhibitor-treated HFF. Quantitative analysis of free cholesterol by TLC showed that the free cholesterol content did

**RESULTS**

Absence or Inhibition of ACAT Activity Decreases Parasite Replication—To determine whether T. gondii replication is affected by alteration in cholesterol ester formation, tachyzoites were used to infect either MEF deficient for ACAT-1 or HFF treated with ACAT inhibitors SaH 58-035 and CI 976 (34) (Fig. 1A). In ACAT-1−/− MEF, parasite replication was reduced by 60% compared with that in ACAT-1+/+ MEF. In addition, the two structurally distinct pharmacological ACAT inhibitors, SaH 58-035 and CI 976, exerted a dose-dependent inhibition of T. gondii replication in HFF. This anti-T. gondii effect was also present when the ACAT inhibitor treatment followed the T. gondii infection of the host cells (Fig. 1B), suggesting that an aberrant initial formation of the PV is not the cause of the observed reduction in parasite replication. The lack of cholesterol esterification impaired parasite replication, but it did not kill the parasites. In both ACAT-1−/− and inhibitor-treated cells, T. gondii tachyzoites were able to induce host cell lysis. However, this process was delayed by an average of 48 h compared with that in cells with normal ACAT activity.

Absence or Inhibition of ACAT Activity Does Not Affect Parasite Invasion—The reduction in parasite number seen in cells lacking ACAT activity could have been a consequence of decreased invasion efficiency, decreased parasite replication, or both. In order to assess whether the alteration in cholesterol ester metabolism compromises the ability of T. gondii to enter host cells, an invasion assay was performed. The number of intracellular parasites in both ACAT-1−/− MEF (Fig. 2A) and inhibitor-treated HFF (Fig. 2B) was comparable to the number...
not increase in the absence of ACAT activity (data not shown). In addition, cells were stained for free cholesterol using the fluorescent polyene antibiotic filipin and examined by fluorescence microscopy. Fluorescence did not increase in cells with compromised ACAT activity compared with control cells (not shown). This indicates that homeostatic mechanisms used by mammalian cells to maintain the cholesterol at physiological levels are effective when ACAT activity is reduced in cultured cells and provide evidence that the anti-*T. gondii* effect of deficiency of ACAT is not mediated by toxicity of excess free cholesterol (38, 39).

**Down-regulation of LDL Receptor Is Not Responsible for the Anti-*T. gondii* Effect**—Possible cellular mechanisms to maintain the free cholesterol at physiological levels are efflux of free cholesterol (40, 41) and regulation of the enzymes involved in cholesterol and fatty acid synthesis, as well as LDL uptake (42, 43).

Since it has been shown that receptor-mediated endocytosis of plasma LDL in the host cell is a key element for intravacuolar proliferation of *T. gondii* (19), we asked whether the inhibition of the enzymatic ACAT activity might cause a decrease in LDL endocytosis through down-regulation of LDL receptors and consequent decrease in parasite proliferation. To examine this possibility, the presence of LDL receptor was analyzed by Western blot in ACAT-1 knockout mice (Fig. 4A) and inhibitor-treated HFF (Fig. 4B). The expression of the LDL receptor did not significantly decrease in cells lacking ACAT activity compared with the amount observed in control cells. To assess the amount of LDL receptor present on the surface of the host cells, inhibitor-treated HFF were incubated at 4 °C with an antibody recognizing the LDL receptor and subsequently analyzed by flow cytometry. Mean fluorescence is expressed as percent of control (Me2SO-treated HFF) ± S.E. from two experiments done in duplicate.

**Alteration in Cholesterol Ester Content in Cells Lacking ACAT Activity**—Previously we demonstrated that the treatment with ACAT inhibitors causes an efficient block in the formation of new cholesterol esters in the host cell (Fig. 3). However, it is possible that even in the presence of ACAT inhibitors, existing cholesterol esters are hydrolyzed by lipoprotein lipase (LPL) and are released to the circulation. Thus, in addition to changes in ACAT activity, there may be changes in the total pool of cholesterol esters in the host cell. To confirm the loss of cholesterol esters in the absence of ACAT, MEF were pulsed for 2 h with [14C]oleic acid and the lipids extracted. After TLC separation, bands corresponding to CE were counted for the presence of radioactive [14C]. HFF were treated for 72 h with ACAT inhibitors at the indicated concentrations and similarly analyzed. Data are expressed as percent of control (ACAT-1+/+ MEF or Me2SO-treated HFF) ± S.E. from three separate experiments done in duplicate.
Role of ACAT in T. gondii Proliferation

Inhibitors residual stores of cholesterol esters are still present in the host cytoplasm in form of lipid droplets. To determine whether 72 h of inhibitor treatment was sufficient to deplete the host cells of cholesterol esters, we performed a direct quantitation of CE. As shown in Fig. 5, the amount of CE was virtually absent as expected in ACAT-1−/− MEF, whereas it decreased but was still detectable in inhibitor-treated HFF. This suggested that the absence of cholesterol esters in the host cell is likely to be the direct cause of the anti-T. gondii effect in ACAT-1−/− MEF, but additional mechanisms might be required for the anti-T. gondii effect in inhibitor-treated HFF cells.

Evidence of Endogenous ACAT Activity in T. gondii—The presence of an ACAT homologue in T. gondii with a lower sensitivity to the ACAT inhibitors might explain the difference in the dose-response of the anti-T. gondii effect of the inhibitors and their potency for inhibiting ACAT activity in HFF. To determine whether T. gondii tachyzoites are actually capable of esterifying cholesterol, we monitored the formation of new cholesterol esters by incubating cell-free tachyzoites with radiolabeled oleic acid, followed by lipid extraction and TLC analysis. Purified parasites were also treated with ACAT inhibitors and tested for decreases in cholesterol esterification. For this experiment, T. gondii tachyzoites were isolated from ACAT-1−/− MEF in order to exclude contamination with ACAT activity derived from the host cell. As shown in Fig. 6, free T. gondii tachyzoites were clearly able to incorporate radiolabeled oleic acid into cholesterol esters. Moreover, cholesterol esterification by free tachyzoites was sensitive to inhibition by SaH 58-035 and CI 976, showing a dose-response closely resembling the anti-T. gondii effect observed in HFF. The sensitivity to ACAT inhibitors was strikingly lower in T. gondii tachyzoites than in HFF cells, whereas inhibitor concentrations ≥10 μM were required to reduce cholesterol esterification by the parasites (Fig. 6A). Inhibitor concentration of 1 μM was sufficient to inhibit ACAT activity of HFF (Fig. 6B). To test the possibility that ACAT inhibitors impair parasite replication by inhibiting the parasite ACAT, we treated peritoneal fibroblasts derived from ACAT-1−/− mice with different concentrations of SaH 58-035 and CI 976. The presence of ACAT inhibitors further decreased the parasite proliferation in host cells deficient for ACAT activity, indicating the essential role of the T. gondii enzyme for the optimal replication of the parasite (Fig. 6C).

Moreover, T. gondii tachyzoites were purified from control untreated HFF, inhibitor-treated HFF, and ACAT-1−/− MEF and analyzed for fluorescent intracellular lipid droplets after staining with Nile Red. Neutral lipid droplets were visualized using a filter set for fluorescein.

FIG. 5. Cholesterol ester content in cells lacking ACAT activity. Cellular lipids were extracted from ACAT-1−/− MEF and ACAT inhibitor-treated HFF, separated on TLC plates, and stained with a solution of cupric acetate in 15% aqueous phosphoric acid. After incubation of the plates at 125 °C the bands co-migrating with standard CE were densitometrically quantified. Data are expressed as percent of control (ACAT-1−/− MEF or Me2SO-treated HFF) ± S.E. from two experiments done in duplicate.

FIG. 6. Endogenous ACAT activity in T. gondii tachyzoites. A, parasites were harvested from ACAT-1−/− MEF and treated with the indicated concentrations of inhibitors for 7 h. Extracted lipids were separated on TLC plates and bands corresponding to CE counted for the presence of radioactive 14C. B, for comparison, HFF cells were similarly treated with inhibitors for 7 h and analyzed for newly esterified cholesterol. Data are expressed as percent of control (Me2SO-treated parasites) ± S.E. from three separate experiments done in duplicate. C, MPF from ACAT-1−/− mice were treated for 12 h with the indicated concentrations of ACAT inhibitors SaH 58-035 or CI 976 and then infected with T. gondii tachyzoites of the βRH strain at an m.o.i. of 0.1. After 72 h, live parasites were quantitated by measuring the parasite β-galactosidase activity. Data are expressed as a percent of control (Me2SO-treated MPF) ± S.E. from two experiments done in triplicate.

FIG. 7. Presence of neutral lipid droplets in T. gondii tachyzoites. Parasites were harvested from HFF (A), HFF-treated with 40 μM CI 976 (B), or ACAT-1−/− MEF (C), fixed and stained with Nile Red. Neutral lipid droplets were visualized using a filter set for fluorescein.
ACAT is a key element in the intracellular homeostasis of cholesterol. This enzyme mediates esterification of cholesterol and fatty acids and allows the cells to store cholesterol esters in the cytosol. In the present work, we demonstrated that ACAT plays an essential role in the intracellular proliferation of *T. gondii*, using as a model system host cells lacking ACAT activity through either gene disruption or pharmacological inhibition. We found that the lack of ACAT activity in the host cell does not affect the parasite processes of host recognition, adhesion, and penetration with formation of the PV. However, once inside the host cell, *T. gondii* tachyzoites showed an average reduction in proliferation rate of 60% in the complete absence of host ACAT and a dose-dependent decrease in replication in host cells treated with ACAT inhibitors. The anti-*T. gondii* effect of the ACAT inhibitors was not caused by host cell toxicity, as cell morphology, viability, and cellular ATP content were unaffected at the concentrations of inhibitors used.

In the absence of ACAT activity, both intracellular free cholesterol content and LDL receptor expression remained unaltered. Thus it appears that neither a toxic increase in free cholesterol nor a down-regulation of LDL uptake is responsible for the anti-*T. gondii* effect observed. These results also show that in our experimental conditions where host cells were not loaded with high doses of exogenous cholesterol, inhibitor treatment does not decrease LDL receptor expression and that other compensatory mechanisms regulate the intracellular cholesterol homeostasis in the absence of ACAT activity.

Surprisingly, the importance of ACAT activity is not restricted to the host cell enzyme. The most important indication derives from the fact that the concentrations of ACAT inhibitors required for the observed anti-*T. gondii* effect are significantly higher than those required for inhibition of host cell ACAT. Although this could indicate that the anti-*T. gondii* effect of ACAT inhibitors is due to an unrelated activity of the inhibitors, additional results imply that this is due to the presence of an endogenous ACAT activity in the parasite. Evidence of esterified lipids in *T. gondii* tachyzoites had been restricted so far to the visualization of intracellular lipid droplets upon staining with the neutral lipid specific dye Nile Red (19). Our result showed that (i) an ACAT activity can be effectively detected in *T. gondii* tachyzoites and that (ii) this activity can be modulated by treatment with ACAT inhibitors thus affecting the parasite proliferation. The effect of SaH 58-035 and CI 976 on *T. gondii* ACAT does not reach the level of inhibition that was observed on host cell ACAT, suggesting a lower efficiency of the pharmacological compounds on the parasite enzyme. A similar decrease in efficiency has been demonstrated for CI 976 on the ACAT homologue SAT1 in the budding yeast *Saccharomyces cerevisiae* where a 95% inhibition of ergosterol esterification could be obtained only in presence of 200 μM inhibitor (44). Interestingly, null mutations in the *S. cerevisiae* SAT1 do not affect the growth phenotype of the vegetative form of the yeast but decrease the efficiency of budding sporulation in the diploid cells (44). Considering our results as a whole, the observed reduction in *T. gondii* replication in either ACAT−/− MEF or ACAT inhibitor-treated HFF is likely to be mediated by at least two distinct mechanisms.

In the first case, the absence of ACAT enzyme causes a permanent deficiency of intracellular cholesterol esters. If host cholesterol esters were used by the parasite as source of cholesterol and/or fatty acids, the absence of such esters would create a situation of lipid starvation for *T. gondii* with consequent reduction in parasite replication. This model of cholesterol esters as lipid shuttle from the host cytoplasm to the PV is supported by the following: (i) the PV is located in strict proximity to the host cell endoplasmic reticulum which is the location of ACAT; (ii) cholesterol esters are an efficient way to incorporate a hydrophobic and membrane-bound element such as cholesterol in structures that are soluble in the cytoplasm; (iii) the intracellular movement of such lipid droplets does not require vesicular trafficking, which is consistent with the observation that the PV acquires host cell cholesterol without involving the host cell vesicular machinery (19).

In the second case, the presence of ACAT inhibitors can affect *T. gondii* proliferation by another mechanism. In this situation, despite the complete blockade of new cholesterol esterification by host ACAT, residual stores of lipid droplets containing cholesterol esters are still present in the host cytoplasm, thus providing a lipid source for the parasite. However, the additional reduction in the activity of the ACAT homologue in *T. gondii* may still exert a detrimental effect on parasite proliferation due to the consequent defect in cholesterol ester storage and/or capacity of free cholesterol detoxification. In this case the dose-dependent inhibition of *T. gondii* ACAT could account for the similar dose-dependent reduction in parasite proliferation even in the presence of a complete blockade of host cell ACAT. Another possibility is that the inhibition of the ACAT activity in both host cells and parasites could result in an alteration of the lipid composition of the growing PV, thus affecting the parasite proliferation.

The composition of cytosolic lipid droplets suggests possible mechanisms for the acquisition of cholesterol by the vacuole resident *T. gondii*. The surface of lipid droplets is composed of a phospholipid monolayer with additional proteins (45–47) and unesterified cholesterol components (48, 49). The cytosolic side of the PV might contain receptors able to recognize the proteins on the lipid droplets, thus mediating the docking and the formation of an interfacial membrane continuum between the lipid droplet monolayer and the surface of the parasite vacuole. This could allow the movement of lipids in a way similar to that proposed for the intracellular diffusion of free cholesterol in adipocytes (49). Alternatively, the lipid monolayer of the lipid droplets could fuse with or bud through the PV membrane with consequent budding in the intravacuolar space in a process similar to the release of lipoproteins in the extracellular medium. As a third possibility, lipids, including cholesterol esters, could be shuttled from lipid droplets to the PV as monomers or oligomers by carrier proteins, without direct contact between lipid droplets and PV.

In summary our studies provide evidence that cholesterol esterification mediated by the ACAT enzyme is a crucial element for optimal intracellular replication of *T. gondii*. In addition, our results imply that cholesterol esters not only constitute a form of lipid storage but could have a role in dynamic processes of lipid transport inside the cell. Moreover, the finding that an ACAT homologue is active in this apicomplexan parasite gives new insights on the incompletely understood regulation of lipid metabolism in *T. gondii*. ACAT inhibitors, by reducing cellular cholesterol esters, may warrant consideration as antitoxoplasmosis agents.

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