Selective Disruption of Phosphatidylcholine Metabolism of the Intracellular Parasite Toxoplasma gondii Arrests Its Growth*

Nishith Gupta‡, Matthew M. Zahn‡, Isabelle Coppens§, Keith A. Joiner¶, and Dennis R. Voelker‡*

Toxoplasma gondii is an intracellular protozoan parasite capable of causing devastating infections in immunocompromised and immunologically immature individuals. In this report, we demonstrate the relative independence of T. gondii from its host cell for amnoglycerophospholipid synthesis. The parasite can acquire the lipid precursors serine, ethanolamine, and choline from its environment and use them for the synthesis of its major lipids, phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn), and phosphatidylcholine (PtdCho), respectively. Dimethylethanolamine (Etn(Me)₂), a choline analog, dramatically interfered with the PtdCho metabolism of T. gondii and caused a marked inhibition of its growth within human foreskin fibroblasts. In tissue culture medium supplemented with 2 mM Etn(Me)₂, the parasite-induced lysis of the host cells was dramatically attenuated, and the production of parasites was inhibited by more than 99%. The disruption of parasite growth was paralleled by structural abnormalities in its membranes. In contrast, no negative effect on host cell growth and morphology was observed. The data also reveal that the Etn(Me)₂-supplemented parasite had a time-dependent decrease in its PtdCho content and an equivalent increase in phosphatidyldimethylethanolamine, whereas other major lipids, PtdSer, PtdEtn, and PtdIns, remained largely unchanged. Relative to host cells, the parasites incorporated more than 7 times as much Etn(Me)₂ into their phospholipid. These findings reveal that Etn(Me)₂ selectively alters parasite lipid metabolism and demonstrate how selective inhibition of PtdCho synthesis is a powerful approach to arresting parasite growth.

Toxoplasma gondii is a ubiquitous, obligate intracellular protozoan parasite capable of infecting virtually all types of nucleated mammalian and avian cells (1). As an opportunistic human pathogen, T. gondii is an important cause of disease in immunocompromised individuals (2) and in neonates following congenital infection (3). Upon invasion of a host cell, the parasite resides in a specialized compartment, the parasitophorous vacuole (PV),¹ a unique and dynamic nonfusogenic membrane organelle (4, 5). Successful replication of T. gondii within its PV requires a substantial increase in membrane biogenesis. Despite the apparent segregation of the PV from the host cell endocytic network, metabolites essential for the parasite are known to exchange with the intracellular space. Shortly after infection, the PV membrane quickly becomes physically associated with sites of host cell lipid biosynthesis, the endoplasmic reticulum and mitochondria (5, 6). Therefore, these organelles might function as the donors of essential lipids to the growing parasite. Another possibility is that like Plasmodium falciparum (7), a related apicomplexan parasite, T. gondii is independent of its host regarding its lipid requirement and harbors its own lipid biosynthetic machinery. Currently there is a paucity of information about the lipid metabolism of T. gondii. A study by Charron and Sibley (8) using fluorescent lipids and radioactive precursors suggested that T. gondii is capable of both autonomous phospholipid synthesis and scavenging of phosphatidylcholine (PtdCho) from the host cell, but no quantitative measurements were made.

In this study, we investigated the phospholipid metabolism of the free T. gondii to gauge its capacity for membrane biogenesis independent of the host cell. We focused on the quantitative analysis of the amnoglycerophospholipid synthetic pathways that produce phosphatidylserine (PtdSer), phosphatidylethanolamine (PtdEtn), and PtdCho. In many eukaryotes the metabolism of these three lipids is intimately interconnected with the decarboxylation of PtdSer producing PtdEtn and the methylation of PtdEtn producing PtdCho (9). Eukaryotes also possess pathways for PtdEtn and PtdCho synthesis via the Kennedy pathways using phospho-Etn/Cho and CDP-Etn/Cho intermediates (9). We also investigated whether the ability of the parasite to autonomously synthesize phospholipid rendered it uniquely susceptible to modifiers of phospholipid metabolism. We specifically focused upon Etn(Me)₂, which is known to alter the phospholipid composition of eukaryotic cells (10, 11). Our findings reveal that the parasite has a high capacity for independent phospholipid synthesis and that its PtdCho metabolism is markedly altered by Etn(Me)₂, leading to a dramatic arrest of replication.

¹ The abbreviations used are: PV, parasitophorous vacuole; PtdSer, phosphatidylserine; PtdEtn, phosphatidylethanolamine; PtdCho, phosphatidylcholine; Etn(Me)₂, N,N-dimethylethanolamine; PtdEtn(Me)₂, phosphatidyldimethylethanolamine; MEM, minimal essential medium; HFF, human foreskin fibroblast; Etn, ethanolamine; Cho, choline; TLC, thin layer chromatography; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; EST, expressed sequence tag.

© 2005 by The American Society for Biochemistry and Molecular Biology, Inc.
Published, JBC Papers in Press, February 11, 2005, DOI 10.1074/jbc.M501523200

This paper is available on line at http://www.jbc.org
Disruption of T. gondii PtdCho Metabolism

EXPERIMENTAL PROCEDURES

Chemicals—Dulbecco's modified Eagle's medium and MEM amino acids and vitamins were purchased from Invitrogen. The [3H]serine and [3H]ethanolamine were from Amersham Biosciences. The [3H]choline, [3H]leucine, and [3H]PO4 were obtained from PerkinElmer Life Sciences. Purine and CDP-[1,2-3H]ethanolamine (9.1, v/v) were obtained from American Radiolabeled Chemicals Inc. and [1-14C]serine was from ICN Radiochemicals. The N,N-dimethylethanolamine (EtMe2) was purchased from Aldrich. All lipids were obtained from Avanti Polar Lipids. Silica gel 60 and H plates for thin layer chromatography were obtained from Merck and Analtech, respectively. Stock solutions of EtMe2 were prepared at a concentration of 5 mM in 5 M HCl and were freshly diluted to the concentrations required for each experiment.

Cell and Parasite Culture—Human foreskin fibroblasts (HFFs) obtained from American Type Culture Collection were routinely cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were passaged by trypsinization at least once a week, and 9-cm culture dishes or 100 mm borosilicate glass tubes were used in the experiments described in this report. To prepare the parasites, supernatants from freshly lysed monolayers were collected and centrifuged followed by three subsequent washings with phosphate-buffered saline (PBS). The parasites were routinely used in the early stages of these studies and were suspended in 50–100 ml of Dulbecco's modified Eagle's medium and MEM amino acids, and serine-free non-essential amino acids, pH 7.4. The extracellular type medium contained 20 mM HEPES, 140 mM KC1, 10 mM NaCl, 2.5 mM MgCl2, 5 mM glucose, 0.1 mM CaCl2, 1 mM sodium pyruvate, MEM vitamin solution lacking choline, MEM amino acids, and serine-free non-essential amino acids, pH 7.4. The serine-free non-essential amino acid solution contained 10 mM each of Ala, Asp, Glu, Gly, Pro, and Asn. ATP was freshly prepared and added to a final concentration of 1 mM along with 1 mM MgCl2 just prior to starting the reaction. The reaction was terminated by addition of 1.8 ml of methanol:PBS:chloroform (10:9:1, v/v) and in chloroform:methanol:acetic acid:methanol (65:35:5, v/v) and in chloroform:acetic acid:methanol:water (75:25:5:2, v/v). Phospholipids were visualized by staining with iodine vapor and were replaced with a solution containing 1 mM defatted BSA, 50 mM sodium succinic acid and exposure to ultraviolet light. All lipids were identified based on their co-migration with authentic standards.

Preparation of Parasite Homogenates—A suspension containing 2 × 108 T. gondii tachyzoites/ml in buffer (described below for different enzyme assays) was probe sonicated at 0 °C using five 30-s bursts at 50 watts with 30-s cooling intervals between bursts. The homogenates were kept on ice prior to initiating enzyme reactions. The enzyme reactions were routinely performed by premixing all assay components at 0 °C and then shifting to 37 °C. All assays were performed in 16 × 100-mm tubes at 37 °C for 45 min to 1 h. Heat-inactivated (85 °C for 30 min) enzyme extract was included as a negative control in each assay. Unless stated otherwise, the reactions were terminated with 1.5 ml of CH3OH:CHCl3 (2:1, v/v) followed by addition of 0.5 ml of CHCl3 and 0.7 ml of PBS. The resultant chloroform phase was backwashed three times with 1.8 ml of CH3OH:CHCl3 (0.2 ml CHCl3:10:9:1, v/v). The final chloroform phase was dried overnight in scintillation vials, and the radioactivity was quantified by liquid scintillation spectrometry.

Phosphatidylethanolamine Decarboxylase Assay—Phosphatidylethanolamine decarboxylase activity was measured by trapping 14CO2 released from Ptd- [1-14C]ethanolamine by using clips that were added to the assay tubes after the reaction had been terminated. The PtdSer synthase was purified from Escherichia coli strain JD200 harboring the plasmid pCT155, which caused 100-fold overproduction of the enzyme (15). The reactions were performed in 16 × 100-mm borosilicate glass tubes sealed with an air-tight rubber septum to which was attached a well holding the base-saturated filter paper. The parasite extract was prepared in 50 mM potassium phosphate buffer (pH 6.8), 0.25 mM sucrose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 3 mM EDTA. The 0.8-ml assay mixture contained 60 mM potassium phosphate (pH 6.8), 0.25 mM magnesium chloride, 0.35 mM PMN, 0.325 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.5 mM dioleoylphosphatidyl-1-[1-14C]serine (0.14 Ci/mmol), 0.1% (w/v) Triton X-100, and 0.2 ml of parasite extract. The reaction was terminated after 45 min by the addition of 0.5 ml of 0.25 M H2SO4 introduced through the rubber septum using a hypodermic needle. The radioactive CO2 evolved was trapped over a period of 30 min prior to recovering the filter paper for liquid scintillation spectrometry.
The parasite RNA metabolism was examined by measuring the incorporation of \(^{3}H\)uracil into nucleic acids. The HDF cells were grown in 24-well plates until reaching confluence and then infected with parasites at a multiplicity of 2. The cells were treated with 2 mM Etn(Me)_2, at the time of infection as described in the figure legends. After 6 h of incubation the non-invading parasites were removed by washing the monolayer twice with PBS. The cultures were shifted to complete medium supplemented with 5 \(\mu\)Ci/well \(^{3}H\)uracil. After 16 h in the presence of radioisotope the wells were washed with cold PBS and then treated with 10% trichloroacetic acid for 15 min on ice to precipitate macromolecules. Subsequently the precipitate was washed with cold PBS and neutralized with 200 \(\mu\)l of 0.2 M NaOH. The solubilized macromolecules were recovered after shaking for 15 min at 37 °C. The radioactivity was quantified by liquid scintillation spectrometry.

**RESULTS**

**Toxoplasma Can Metabolize Free Serine into PtdSer and PtdEtn**—T. gondii is an obligate intracellular parasite with only a brief extracellular phase when propagated in vitro. The viability of the parasite declines with a \(t_1/2\) of 10 h outside the host cell (19). We examined phospholipid synthesis in extracellular parasites to assess the capacity of the organism for autonomous membrane biogenesis. The aminoglycerophospholipids PtdCho, PtdEtn, and PtdSer constitute the bulk of membrane polar lipids in most animal eukaryotes. Initially we examined the metabolism of \(^{3}H\)Ser and found that this precursor was readily incorporated into the parasite lipid pool (Fig. 1A). The lipid synthesis was nearly linear for the first 2 h and then slowed progressively over the ensuing 4 h of incubation. The two major lipids synthesized from \(^{3}H\)Ser were PtdSer and PtdEtn as determined by thin layer chromatography shown in Fig. 2. Two minor lipids co-migrated with PtdCho and PtdIns regions of thin layer plates. However, unlike PtdCho and PtdIns, these lipids were stable against alkaline deacylation suggesting they are sphingolipids that are expected to also be labeled with \(^{3}H\)Ser. Further identification of these minor lipids was not undertaken in this study.

Initially \(^{3}H\)Ser was metabolized to PtdSer that was rapidly decarboxylated to PtdEtn (Fig. 2). We failed to detect any evidence of significant methylation of nascent PtdEtn to form PtdCho (see also \(^{3}H\)Etn labeling studies described below). We also considered that the parasite might require exogenous \(S\)-adenosylmethionine, the methyl group donor for PtdEtn methylation in eukaryotes. We performed the same experiment with \(^{3}H\)Ser labeling in the presence of 1 mM \(S\)-adenosylmethionine but failed to detect any significant PtdCho synthesis or any qualitative or quantitative change in the overall lipid biosynthesis (data not shown). We also carried out this experiment in intracellular type (high \(K_+\)) and extracellular type (low \(K_+\)) media as indicated. Lipids were extracted and quantified by liquid scintillation spectrometry. Values are means \(\pm\) S.E. for three experiments.

**Toxoplasma Can Metabolize Etn into PtdEtn but Does Not Methyle the Lipid**—We next examined the metabolism of \(^{3}H\)Etn by the free parasite. As shown in Fig. 1A, T. gondii could also acquire Etn from its environment and readily incorporate it into polar lipids. Like Ser, the metabolism of Etn also showed a time-dependent increase in lipid synthesis that progressively slowed over a 6-h period. The major lipid produced was PtdEtn (Fig. 3). Other minor lipids co-migrating with PtdSer, PtdCho, and PtdIns were also observed, but their identities are not known. Similar to experiments with \(^{3}H\)Ser, we did not find evidence for any significant conversion of PtdEtn to PtdCho. In addition, supplementation of cultures with 1 mM \(S\)-adenosylmethionine also failed to produce any PtdCho from \(^{3}H\)Etn. From these studies we conclude that T. gondii can readily synthesize PtdEtn from Etn and that the resultant pool of phospholipid is not converted to PtdCho.

**Toxoplasma Can Metabolize Choline into PtdCho**—The incorporation of \(^{3}H\)Cho into the polar lipids of T. gondii was also examined. The extracellular T. gondii could readily acquire Cho from the medium (Fig. 1B) and metabolized it into PtdCho.

**Fig. 1.** T. gondii synthesizes aminoglycerophospholipids from serine, ethanolamine, and choline. A, \(^{3}H\)Ser and \(^{3}H\)Etn (10 \(\mu\)Ci, 25 \(\mu\)M) were incubated with purified T. gondii tachyzoites of the RH strain (10^6) at 37 °C in extracellular type medium. B, \(^{3}H\)Cho (10 \(\mu\)Ci, 25 \(\mu\)M) was incubated with purified T. gondii tachyzoites of the RH strain (10^6) in extracellular type (ECM) and intracellular type (ICM) media as indicated. Lipids were extracted and quantified by liquid scintillation spectrometry. Values are means \(\pm\) S.E. for three experiments.

**Fig. 2.** T. gondii can metabolize serine to synthesize PtdSer and PtdEtn. Purified T. gondii tachyzoites of the RH strain (10^6) were incubated with \(^{3}H\)Ser (10 \(\mu\)Ci, 25 \(\mu\)M) at 37 °C in extracellular type medium followed by lipid extraction. The lipids were separated on a silica gel H plate using chloroform:methanol:2-propanol:KCl (0:25% in water:triethylamine (90:28:75:18:54, v/v). The phospholipid bands were visualized under UV light after spraying with 8-anilino-1-naphthalene sulfonic acid (0.2%, w/v). The bands were scraped, and the radioactivity was quantified by liquid scintillation spectrometry. Values are means \(\pm\) S.E. for three experiments.
Disruption of T. gondii PtdCho Metabolism

FIG. 3. T. gondii can synthesize PtdEtn but not PtdCho from ethanolamine. Purified T. gondii tachyzoites of the RH strain (10⁸) were incubated with [³H]Etn (10 μCi, 25 μM) at 37 °C in extracellular type medium followed by lipid extraction. Phospholipids were separated by thin layer chromatography and quantified by liquid scintillation spectrometry. Values are means ± S.E. for three experiments.

FIG. 4. T. gondii can metabolize choline to synthesize PtdCho. Purified T. gondii tachyzoites of the RH strain (10⁸) were incubated with [³H]Cho (10 μCi, 25 μM) at 37 °C in extracellular type medium followed by lipid extraction. Phospholipids were separated by thin layer chromatography and quantified by liquid scintillation spectrometry. Values are means ± S.E. for three experiments.

FIG. 5. Aminoglycerophospholipid synthesis increases with water-soluble precursor concentration. Purified T. gondii tachyzoites (10⁸) were incubated with [³H]Ser, [³H]Etn, and [³H]Cho in the intracellular type medium for 4 h at 37 °C, and the incorporation of the precursors into lipid was quantified by lipid extraction and liquid scintillation spectrometry. Values are means ± S.E. for three experiments.

TABLE I

Activities of key enzymes in aminoglycerophospholipid synthesis in T. gondii

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activitya (nmol/h/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphatidylserine decarboxylase</td>
<td>214 ± 2.8</td>
</tr>
<tr>
<td>Choline phosphotransferase</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Ethanolamine phosphotransferase</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>Base exchange-dependent</td>
<td></td>
</tr>
<tr>
<td>phosphatidylserine synthase</td>
<td>0.10 ± 0.02</td>
</tr>
</tbody>
</table>

a Values are means ± S.E. of three determinations.

The Cho metabolism was linear for 6 h in extracellular type medium (high Na⁺/K⁺), although it showed a very high interexperimental variation for unknown reasons. The major lipid synthesized was PtdCho, and no other lipid classes were significantly labeled under our experimental conditions. When labeling experiments were conducted in intracellular type medium (high K⁺/Na⁺), we typically observed a doubling of the amount of PtdCho synthesized as compared with extracellular type medium.

Quantification of the phospholipid profile of the free parasite by chemical measurement of phosphorus demonstrated that PtdCho was the most prevalent lipid and accounted for about 75% of the total phospholipid. The next most abundant lipids were PtdEtn (10%), PtdIns (7.5%), PtdSer (6%), and PtdOH (1%). The parasite phospholipid class distribution was significantly different from that of the host cell, described later in Fig. 9. Comparing the uptake of all lipid precursors tested in this study, Etn showed the fastest rate of metabolism under the extracellular ionic conditions followed by Cho and Ser. In the intracellular type medium, the metabolism of Ser and Etn was largely unaffected but that of Cho was increased about 2-fold (see Fig. 1B), suggesting that Cho metabolism may be up-regulated in response to parasitic invasion of host cells. The lipid precursors described in the preceding experiments were used at concentrations within the range of tissue culture media. We conducted additional experiments using precursor concentrations that gave maximal rates of incorporation into lipid. The results of these experiments are shown in Fig. 5. The maximum rates of PtdSer, PtdEtn, and PtdCho synthesis were 4.2, 12.4, and 8.2 nmol/8 h, respectively, for aliquots of 10⁸ cells. These rates of phospholipid synthesis were sufficient for a cell doubling for PtdEtn, 50% of that required for PtdSer, and about 9% of that required for a cell doubling for PtdCho. These estimates were based upon a phospholipid content of T. gondii of 130 nmol/10⁸ cells as determined by measurement of lipid phosphorus.

Lipid Enzymology Identifies the Major Pathways for Aminoglycerophospholipid Synthesis—In conjunction with the in vivo labeling of T. gondii, we also measured the activities of key enzymes in phospholipid biosynthesis in extracts prepared from isolated parasites (Table I). As anticipated from the [³H]Etn and [³H]Cho incorporation data, the terminal enzymes of the Kennedy pathways for PtdEtn and PtdCho synthesis (20) were measurable in homogenates prepared from the parasites. Both Cho phosphotransferase and Etn phosphotransferase activities increased 2-fold in the presence of exogenous 1,2-diacylglycerol. The Cho phosphotransferase activity was more than twice the rate of Etn phosphotransferase activity as shown in Table I. PtdSer synthase was measured using methods for the base exchange activity and the CDP-diacylglycerol-dependent activity. The base exchange activity was Ca²⁺- and phospholipid-dependent. Chelation of Ca²⁺ with EDTA eliminated catalytic activity. The inclusion of PtdEtn in the reaction, as a donor of the phosphatidyl moiety, increased the catalytic activity 6–8-fold. Low activity for PtdSer synthesis was observed using conditions for the CDP-diacylglycerol-dependent reaction. We were unable to demonstrate any dependence of PtdSer synthesis upon the addition of CDP-diacylglycerol. The PtdSer decarboxylase activity (214 nmol/h/mg protein) was 200-fold higher than that of the other lipid synthetic enzymes measured. The activity of the parasite PtdSer decarboxylase was also about 10-fold higher than that observed in extracts prepared from other eukaryotes including yeast (21) and mammalian cells (22). These enzymatic data complement the precursor utilization studies and identify the Kennedy pathways for PtdEtn and PtdCho synthesis, the base exchange pathway for PtdSer synthesis, and the PtdSer decarboxylase pathway for PtdEtn synthesis as active in T. gondii tachyzoites.
The Choline Analog Etn(Me)$_2$ Inhibits T. gondii Replication—The de novo biosynthesis of PtdCho and its dominance as a major lipid in T. gondii membranes offered an excellent metabolic target to disrupt the membrane biogenesis of the parasite. We tested the effect of a choline analog, Etn(Me)$_2$, on the intracellular replication of T. gondii. Previous studies with Etn(Me)$_2$ in mammalian cells indicated it was an excellent choline analog that was extraordinarily well tolerated at high concentrations (10, 11). Surprisingly the presence of Etn(Me)$_2$ in tissue culture medium caused a marked inhibition of parasite replication as shown in Fig. 6. In contrast, the host cells grew well and appeared morphologically normal when exposed to the choline analog (data not shown). We quantified parasite replication by measuring the number of parasites produced after 48 h of culture following infection of HFFs at a multiplicity of 4. Typically this infection produced 50–100 parasites per host cell upon lysis after 48 h. The parasite number decreased with the increasing concentration of Etn(Me)$_2$, and at 2 mM the yield was reduced by 99.5% (Fig. 6). Further decreases in parasite yield occurred at 4 and 8 mM Etn(Me)$_2$, but these concentrations also reduced host cell growth. Therefore, we used 2 mM Etn(Me)$_2$ or lower concentrations to perform further studies on parasite metabolism.

We next sought to determine the time frame for the efficacy of Etn(Me)$_2$ upon parasite replication. HFF cells were incubated in Etn(Me)$_2$ added at times ranging from 120 h before infection to 36 h after infection, and the consequences of these treatments were determined. The results shown in Fig. 7 reveal that the Etn(Me)$_2$ remained almost fully effective at inhibiting parasite replication for up to 12 h after infection of the HFFs. Even when added at 24 h after parasite infection of HFFs, the choline analog was a potent inhibitor of parasite replication. We also observed that the effect of Etn(Me)$_2$ was reversible such that removal of the analog allowed the parasites to begin to grow normally after a few days. From these observations we conclude that Etn(Me)$_2$ is parasitostatic but not parasitocidal. We conducted additional experiments in which we manipulated the timing of HFF exposure to Etn(Me)$_2$ either before or after infection and conclude that the analog has no discernible effect upon invasion or infection but only affects events occurring as the parasite begins to divide within the host cell (data not shown).

The RNA metabolism of the parasites was also examined by following the incorporation of $[^3H]$uracil into macromolecular nucleic acids as shown in Fig. 8. The HFF cells either in the absence or presence of Etn(Me)$_2$ incorporated negligible radioactivity into RNA. In contrast, the parasite-infected HFF cells incorporated relatively high levels of $[^3H]$uracil into RNA compared with the host cells. Treatment of infected host cells with 2 mM Etn(Me)$_2$ for 22 h reduced the amount of labeled RNA by 90% as shown in Fig. 8. This reduction in RNA synthesis corresponded to the level expected if the parasites remained viable but failed to double every 8 h as occurred in the relevant control cultures.

Metabolism of Etn(Me)$_2$ by Host Cells and Parasites—Previous studies have shown that Etn(Me)$_2$ is readily taken up by tissue culture cells and incorporated into PtdEtn(Me)$_2$ (10, 11). As shown in Fig. 9, the host cells used in these studies metabolized Etn(Me)$_2$ into PtdEtn(Me)$_2$ and gradually accumulated the lipid up to as much as 21% of total phospholipid after 5 days. The increase in PtdEtn(Me)$_2$ was paralleled by a decrease in PtdCho. There were no significant changes in other cellular phospholipids, and the morphology of the cells examined by light microscopy appeared normal.

We next examined the influence of Etn(Me)$_2$ upon phospholipid metabolism of T. gondii within the host cell and after release from the host cell. To study the metabolism within the host cell, we grew infected HFFs for 48 h in the presence of 0.5 mM Etn(Me)$_2$, which resulted in a reduced but adequate number of parasites for phospholipid analysis (see Fig. 6). The results of these experiments are shown in Fig. 10 and demonstrate that T. gondii accumulated up to 44% of its phospholipids as PtdEtn(Me)$_2$ and concomitantly decreased the PtdCho content from 75 to 33% of total phospholipid. From these results we infer that, at 2 mM Etn(Me)$_2$, the level of the PtdEtn(Me)$_2$ in the parasite is likely to be substantially higher. Comparison of the PtdEtn(Me)$_2$ levels of HFFs incubated with 2 mM Etn(Me)$_2$ (see Fig. 9) reveals that after 48 h of incubation the lipid only comprised about 6% of the phospholipid pool of...
infection 0.5 mM Etn(Me)$_2$ was added. After 48 h the parasites were readily synthesize PtdEtn(Me)$_2$. Repeatedly we observed that the parasite possesses the transport and enzymatic machinery to pholipid pool. From these results we conclude that the free T. gondii infected with T. gondii in intracellular type medium containing H$_3$PO$_4$ and lipid could actively synthesize PtdEtn(Me)$_2$ up to levels as high as 26% of the nascent pholipid. The effects of Etn(Me)$_2$ upon the incorporation of $[^3H]$Cho into phos-

The accumulation of PtdEtn(Me)$_2$ by the parasite could be a consequence of de novo synthesis of the lipid by the organism or transfer of the preformed lipid from the host cell. Although the large discrepancy in PtdEtn(Me)$_2$ content between the host cell and the parasite makes it likely that T. gondii synthesizes the lipid autonomously, we performed additional experiments to directly test this hypothesis. Isolated parasites were incubated in intracellular type medium containing H$_3$PO$_4$ and lipid precursors in addition to 2 mM Etn(Me)$_2$. After 4 h the lipids were extracted from the parasites and analyzed by thin layer chromatography and autoradiography. The results shown in Fig. 11 demonstrate that the parasite could actively synthesize $^{32}$P-labeled PtdCho, PtdIns, PtdOH, and PtdEtn. Incorporation of 2 mM Etn(Me)$_2$ in the medium led to the synthesis of PtdEtn(Me)$_2$, up to levels as high as 26% of the nascent pholipid pool. From these results we conclude that the free parasite possesses the transport and enzymatic machinery to readily synthesize PtdEtn(Me)$_2$. Repeatedly we observed that the PtdEtn(Me)$_2$ and PtdEtn spots of the newly synthesized lipids were visualized by iodine staining and scraped for quantification of their phosphorus content.

In addition to these experiments we also examined the direct effects of Etn(Me)$_2$ upon the incorporation of $[^3H]$Cho into phos-

FIG. 9. Human fibroblasts synthesize PtdEtn(Me)$_2$ from Etn(Me)$_2$. Human foreskin fibroblasts were grown to confluence and exposed to 2 mM Etn(Me)$_2$ for the times indicated. The cells were harvested, and lipids were extracted and subjected to two-dimensional thin layer chromatography on a silica gel 60 plate (first dimension, chloroform:methanol:NH$_4$OH (65:35:5, v/v); second dimension, chloroform:acetic acid:methanol:water (75:25:5:2.2, v/v)). The lipid bands were scraped from the plates and quantified by liquid scintillation spectrometry. Values are means ± S.E. for three experiments.

The accumulation of PtdEtn(Me)$_2$ by the parasite could be a consequence of de novo synthesis of the lipid by the organism or transfer of the preformed lipid from the host cell. Although the large discrepancy in PtdEtn(Me)$_2$ content between the host cell and the parasite makes it likely that T. gondii synthesizes the lipid autonomously, we performed additional experiments to directly test this hypothesis. Isolated parasites were incubated in intracellular type medium containing H$_3$PO$_4$ and lipid precursors in addition to 2 mM Etn(Me)$_2$. After 4 h the lipids were extracted from the parasites and analyzed by thin layer chromatography and autoradiography. The results shown in Fig. 11 demonstrate that the parasite could actively synthesize $^{32}$P-labeled PtdCho, PtdIns, PtdOH, and PtdEtn. Incorporation of 2 mM Etn(Me)$_2$ in the medium led to the synthesis of PtdEtn(Me)$_2$, up to levels as high as 26% of the nascent pholipid. From these results we conclude that the free parasite possesses the transport and enzymatic machinery to readily synthesize PtdEtn(Me)$_2$. Repeatedly we observed that the PtdEtn(Me)$_2$ and PtdEtn spots of the newly synthesized lipids were visualized by iodine staining and scraped for quantification of their phosphorus content.

In addition to these experiments we also examined the direct effects of Etn(Me)$_2$ upon the incorporation of $[^3H]$Cho into phos-

Refer to the original source for the complete text and figures.
DISCUSSION

*T. gondii* is an important human pathogen capable of causing catastrophic disease in fetuses, neonates, transplant recipients, and immunocompromised individuals (1–3). Currently there is a paucity of information about the polar lipid metabolism of the parasite. In this report we characterized some of the fundamental features of aminoglycerophospholipid metabolism in the organism and identified a novel vulnerability of the parasite to manipulation of PtdCho metabolism.

Our initial studies identified the autonomous ability of the parasite to synthesize PtdSer, PtdEtn, and PtdCho. Under optimal conditions of soluble lipid precursor utilization we determined that free lipids have adequate synthetic capacity to produce all of the PtdEtn and 50% of the PtdSer required for a cell doubling. The free parasites could only synthesize PtdCho at ~5–10% of the rate required for a cell doubling. At present it is not clear whether PtdCho synthesis is sharply up-regulated in response to host cell invasion or whether significant quantities of PtdCho are actually acquired primarily from the host cell. Charron and Sibley (8) have suggested that the parasite can readily acquire PtdCho from the host cell. Our studies with extracellular and intracellular ionic conditions suggest that PtdCho synthesis is up-regulated in the high K+/Na+ environment that the parasite encounters upon invasion of the host cell. It is plausible that additional host cell factors may further increase PtdCho synthesis.

Both the precursor utilization experiments and the *in vitro* measurements of enzyme activity demonstrate that the parasite is capable of autonomous aminoglycerophospholipid synthesis outside the host cell. These studies clearly demonstrate the presence of the Kennedy pathways for PtdEtn and PtdCho synthesis, the base exchange pathway for PtdSer synthesis, and the PtdSer decarboxylase pathway as an additional route for PtdEtn synthesis. However, the quantification of the pathway rates either in intact parasites with precursors or by enzyme activity measurements reveals large discrepancies between the parasite’s quantitative requirements for new lipids for replication and the synthetic output. These discrepancies may be indicative of significant down-regulation of enzyme activities when the parasites undergo the transition from the intracellular to the extracellular phase of the life cycle. The mechanisms of down-regulation could occur at any of several levels ranging from gene expression to post-translational modification of enzymes. Among the catalytic activities measured, PtdSer decarboxylase was the highest. In comparison with other organisms the decarboxylase activity in *T. gondii* tachyzoites of the RH strain (10^8) was labeled with [3H]Cho (10 μCi, 25 μM) in the presence or absence of Etn(Me)_2 in extracellular type medium for 4 h at 37 °C. Lipids were extracted and quantified by liquid scintillation spectrometry.

**Fig. 12.** Etn(Me)_2 inhibits the synthesis of PtdCho by *T. gondii*. Purified *T. gondii* tachyzoites of the RH strain (10^8) were labeled with [3H]Cho (10 μCi, 25 μM) in the presence or absence of Etn(Me)_2 in extracellular type medium for 4 h at 37 °C. Lipids were extracted and quantified by liquid scintillation spectrometry.

**Fig. 13.** Etn(Me)_2 alters parasite membrane morphology and disrupts the development of new progeny. Human fibroblasts were grown to confluence and infected with *T. gondii* at a multiplicity of 3. The dishes contained either normal medium without additions (A) or medium supplemented to 0.5 mM Etn(Me)_2 (B) or 2 mM Etn(Me)_2 (C). Following an additional incubation period of 24 h the cells were processed for electron microscopy. Host mt, host mitochondrion; ER, endoplasmic reticulum. Inset shows parasite with abnormal lipid accumulation. Bars are 0.1 μm.
rates reported for yeast and mammalian cells. These high levels of catalytic activity suggest that PtdSer decarboxylase may play a previously unanticipated role in the biology of the parasite.

Comparison of the precursor and enzymology studies with the genomic and dbEST information from T. gondii provides additional information about the ability of the parasite to synthesize lipids autonomously. The T. gondii dbEST of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) contains entries (accession numbers in parentheses) with homology to key enzymes of the Kennedy pathway for the synthesis of PtdEtn and PtdCho including ethanolamine kinase (CF246471), choline kinase (CD217337 and CV654538), phosphoethanolamine cytidylyltransferase (CB369604 and CB372649), phosphocholine cytidylyltransferase (CN18346 and CF246047), and CDP-choline/CDP-ethanolamine phosphotransferase (AW702647). ESTs for base exchange-type PtdSer synthase (CO055801 and CB411622) and PtdSer decarboxylase (CV549797, CO720987, and CN199397) are also present. The genomic data base for T. gondii also contains sequences annotated to be putative CDP-diaclyglycerol-dependent PtdSer synthase (CB025479 and CB186041), but we were unable to definitively detect this catalytic activity. Additional analysis will be required to elucidate whether this enzyme is present during other phases of the parasite life cycle.

Our results provide some important insights into phospholipid metabolism that were not predictable for this apicomplexan parasite. T. gondii readily synthesizes PtdSer and converts a substantial quantity of the nascent phospholipid to PtdEtn. This level of PtdSer decarboxylation is high compared with that occurring in host cells but is similar to that of the related parasite P. falciparum. However, unlike Plasmodium, T. gondii shows no significant formation of PtdCho from Ser and Etn precursors (7, 23, 24). This is true for PtdEtn derived from free Etn as well as that derived from PtdSer. These findings demonstrate that T. gondii is a choline or PtdCho auxotroph.

The reliance of T. gondii on an exogenous source of choline for PtdCho synthesis raises the possibility that this pathway may provide new opportunities for pharmacological attack upon its replication within host cells. We tested this idea by supplementing cultured cells with Etn(Me)2 and measuring the effects upon parasite growth and replication. Despite the findings that Etn(Me)2 acted as a choline analog and was innocuous to cultured mammalian cells (10), it profoundly altered the growth and lytic cycle of T. gondii tachyzoites. At a 2 mM concentration, the Etn(Me)2 greatly reduced the number of parasitophorous vacuoles visible in host cells and it also dramatically reduced the number of parasites visible per vacuole. Consequently the parasite burden per cell was greatly reduced over the course of infection, and the number of parasites lysing out of host cells at 48 h was reduced by almost 3 orders of magnitude. The degree of attenuation of RNA metabolism in the parasite was also completely consistent with the conclusion that parasite growth is only arrested by 2 mM Etn(Me)2. In addition, this effect upon parasite growth continued for several days past the normal 48-h period of host cell lysis. We know that the effects of Etn(Me)2 are parasitostatic because removal of this precursor from cultures led to recovery of the parasite over a period of 48–96 h. Yet another interesting effect of Etn(Me)2 is the onset of its action. The Etn(Me)2 was highly effective when added at the time of infection or up to 12 h after infection. Even when added 24 h after infection, the Etn(Me)2 inhibited parasite production at the normal 48-h period by 88%. These findings are consistent with a rapid alteration in lipid metabolism that markedly attenuates parasite growth and division.

A large number of choline analogs have been produced and evaluated for their ability to alter the growth of apicomplexan parasites, especially P. falciparum (25, 26). The major effect of these compounds appears to be alteration of PtdCho synthesis, but the exact mechanisms and sites of action of the most effective drugs are unclear. Etn(Me)2 has also been identified in these screens, but the details of perturbation of phospholipid metabolism have not been elucidated (25, 26). In the present study we found clear evidence that the accumulation of PtdEtn(Me)2 in conjunction with the reduction in PtdCho synthesis by T. gondii is particularly deleterious to the growth of the parasite within the host cell.

The evidence that Etn(Me)2 markedly alters phospholipid metabolism comes from two experiments. In one experiment we used levels of Etn(Me)2 near the IC50 (0.5 mM) for parasite production, so we could evaluate the effect of the molecule upon the parasite phospholipid composition within the host cell. This experiment demonstrated that the parasites accumulated high levels of PtdEtn(Me)2, making it the major phospholipid within the cell. The levels of PtdEtn(Me)2 in the parasite greatly exceeded the amounts made within the host cell over the same time period (48 h) strongly implicating autonomous synthesis of PtdEtn(Me)2 by the parasite. In a second experiment we demonstrated the direct synthesis of PtdEtn(Me)2 by free parasites using Etn(Me)2 and H32PO4. The results clearly demonstrated that the parasite can synthesize PtdEtn(Me)2 independently of the host cell. In additional studies we also found evidence that Etn(Me)2 competes for the incorporation of [3H]Chol into PtdCho. Thus the synthesis of PtdEtn(Me)2 within the parasite occurs with a concomitant decline in PtdCho synthesis. As described above, the rate of synthesis of PtdCho observed for the free parasite was less than that necessary for a cell doubling. This observation raises the possibility that PtdCho could be acquired from the host cell after infection. However, the parasite appeared unable to obtain sufficient PtdCho from host cells to bypass the effects of either Etn(Me)2 treatment or PtdEtn(Me)2 accumulation. These findings suggest that the recovery of PtdCho from the host cell by T. gondii is likely to be a relatively inefficient process when PtdCho synthesis is compromised. The accumulation of PtdEtn(Me)2 also makes it unlikely that the parasite is capable of significant phospholipid methylation. PtdEtn(Me)2 is normally produced as an intermediate in the conversion of PtdEtn to PtdCho. Organisms capable of phospholipid methylation typically will convert PtdEtn(Me)2 to PtdCho very rapidly (9).

Visualization of the parasites by electron microscopy revealed that Etn(Me)2 dramatically altered their membrane organization. Abnormal membrane structures and vacuolization were observed for cultures exposed to the choline analog. The micrographs suggest that the newly forming progeny are particularly affected in their membrane biogenesis. It remains unclear whether these alterations occurred as a consequence of depletion of PtdCho or the preponderance of PtdEtn(Me)2 in nascent membranes or both factors acting in concert.

In summary, our data provide an outline of aminoglycerophospholipid synthesis in T. gondii demonstrative of autonomous synthesis of PtdSer, PtdEtn, and PtdCho. The PtdEtn can be formed from PtdSer as well as Etn. The autonomous synthesis of PtdEtn occurs at rates sufficient for cell division, whereas that of PtdCho occurs at ~5–10% of that required for cell division. The rate of PtdCho synthesis increases under ionizing conditions that mimic the intracellular environment and may be subject to further modulation by factors within the host cell. The choline analog Etn(Me)2 is readily incorporated into PtdEtn(Me)2 by the parasite, and during replication within the...
host cell it becomes the major phospholipid of the parasite. The selective disruption of PtdCho synthesis concomitant with PtdEtn(Me)₂ formation dramatically arrests parasite replication and alters membrane structure and function. This sensitivity of PtdCho synthesis in *T. gondii* identifies a novel route for disrupting the parasite growth cycle in human cells and holds significant potential for new chemotherapeutic avenues of attack on the organism.

REFERENCES

Selective Disruption of Phosphatidylcholine Metabolism of the Intracellular Parasite *Toxoplasma gondii* Arrests Its Growth

Nishith Gupta, Matthew M. Zahn, Isabelle Coppens, Keith A. Joiner and Dennis R. Voelker

doi: 10.1074/jbc.M501523200 originally published online February 11, 2005

Access the most updated version of this article at doi: 10.1074/jbc.M501523200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 25 references, 14 of which can be accessed free at http://www.jbc.org/content/280/16/16345.full.html#ref-list-1