Cryptosporidium parvum Induces an Endoplasmic Stress Response in the Intestinal Adenocarcinoma HCT-8 Cell Line*

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Background: Invasion of host cells by Cryptosporidium parvum results in an endoplasmic reticulum (ER) stress response. Results: Host cell calreticulin, GRP78/BiP, Nrf2, and spermidine/spermine N\textsuperscript{1}-acetyltransferase increase, whereas poly(ADP-ribose) polymerase decreases.

Conclusion: C. parvum causes an ER stress response culminating in the synthesis of N\textsuperscript{3}-acetyl spermine by infected cells.

Significance: Host ER stress provides a source of polyamines for C. parvum, which lacks ornithine decarboxylase.

Invasion of human intestinal epithelial cells (HCT-8) by Cryptosporidium parvum resulted in a rapid induction of host cell spermidine/spermine N\textsuperscript{1}-acetyltransferase 1 (hSSAT-1) mRNA, causing a 4-fold increase in SSAT-1 enzyme activity after 24 h of infection. In contrast, host cell SSAT-2, spermine oxidase, and acetylpolyamine oxidase (hAPAO) remained unchanged during this period. Intracellular polyamine levels of C. parvum-infected human epithelial cells were determined, and it was found that spermidine remained unchanged and putrescine increased by 2.5-fold after 15 h and then decreased after 24 h, whereas spermine decreased by 3.9-fold after 15 h. Concomitant with these changes, N\textsuperscript{1}-acetyl spermine and N\textsuperscript{3}-acetylspermidine both increased by 115- and 24-fold, respectively. Increased SSAT-1 has previously been shown to be involved in the endoplasmic reticulum (ER) stress response leading to apoptosis. Several stress response proteins were increased in HCT-8 cells infected with C. parvum, including calreticulin, a major calcium-binding chaperone in the ER; GRP78/BiP, a prosurvival ER chaperone; and Nrf2, a transcription factor that binds to antioxidant response elements, thus activating them. However, poly-(ADP-ribose) polymerase, a protein involved in DNA repair and programmed cell death, was decreased. Cumulatively, these results suggest that the invasion of HCT-8 cells by C. parvum results in an ER stress response by the host cell that culminates in overexpression of host cell SSAT-1 and elevated N\textsuperscript{3}-acetyl polyamines, which can be used by a parasite that lacks ornithine decarboxylase.

Cryptosporidium parvum belongs to a ubiquitous and diverse group of intracellular apicomplexan parasites of both human and veterinary importance. Ribosomal RNA data have shown that within the Apicomplexa, C. parvum forms an early branch point near the dinoflagellates and is more closely related to Plasmoidium sp. than other Coccidia (1–3). We have previously shown that C. parvum and Toxoplasma gondii have only a partial polyamine biosynthetic pathway and are dependent upon host-derived polyamines (4, 5). C. parvum lacks ornithine decarboxylase (ODC)\textsuperscript{3} and synthesizes putrescine via a low activity arginine decarboxylase (6). Based upon kinetic data, it is unlikely that the parasite arginine decarboxylase will provide sufficient polyamines for parasite growth and survival. Both C. parvum and T. gondii have an active retroconversion pathway that utilizes spermidine/spermine N\textsuperscript{3}-acetyltransferase (SSAT) and acetylpolyamine oxidase (APAO) to convert spermine to spermidine (4, 5). Although intracellular, C. parvum is extracytoplasmic, and a significant barrier composed of four membranes separates the parasite from the host cell cytoplasm. Polyamines are cationic compounds that require specific transport mechanisms for their uptake or removal from the cell (7). Polyamine acetylation is a mechanism for neutralizing the charge on the terminal amines, allowing movement of the molecule across cell membranes in the absence of energy transporters (8, 9). In this study, we examined the effect of C. parvum upon polyamine synthesis and export by the intestinal epithelial cell line HCT-8. The results indicate that invasion of host cells by C. parvum results in an ER stress response that causes increased expression of human SSAT-1 (hSSAT-1), resulting in overproduction and excretion of N\textsuperscript{3}-acetylspermine and N\textsuperscript{3}-acetyl spermidine, which can be taken up by the parasite.

EXPERIMENTAL PROCEDURES

C. parvum—Oocysts were obtained from G. & S. Pritchard (Bunch Grass Farm, Deary, ID). Oocysts were passaged in 2–5-day-old calves, collected, and purified on CsCl gradients as

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3 The abbreviations used are: ODC, ornithine decarboxylase; SSAT, spermidine/spermine N\textsuperscript{1}-acetyltransferase; APAO, acetylpolyamine oxidase; hSSAT-1, human SSAT-1; MEM, minimal essential medium; hSSAT, human spermine oxidase; hSSAT, C. parvum SSAT; Binice, N/N-bis(2-hydroxyethyl)glycine; CAPS, 3-(cyclohexylaminopropane)sulfonic acid; ER, endoplasmic reticulum; CHOP, CCAAT/enhancer-binding protein homologous protein; PARP, poly(ADP-ribose) polymerase; UPR, unfolded protein response; PERK, PKR-like ER-associated protein kinase.
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Oocysts were then surface-sterilized using 10% (v/v) Clorox®, washed, and shipped in potassium dichromate. Oocysts were washed with distilled H2O to rinse them free of potassium dichromate and washed with 10% (v/v) Clorox before being suspended in minimal essential medium (MEM) containing 10% horse serum. Human adenocarcinoma cells (HCT-8, ATCC CCL-244) were grown to confluence on 75-cm² flasks in MEM containing 10% horse serum. One set was infected with 2 × 10⁷ freshly excysted sporozoites, and a second set was maintained as uninfected controls. The medium was removed from the plates at 3-h intervals from 0 to 9 h, and a 24-h time was included. HCT-8 cells were collected by trypsin treatment and washed with fresh MEM, and the cell pellets were stored at −70 °C until assayed for polyamine-metabolizing enzymes and RT-PCR studies as described below.

Polyamine Analysis—Polyamines were separated and identified by reverse-phase HPLC using a PerkinElmer LC 410 system coupled to a C18 10-μm column (4.5 × 250 mm) at a flow rate of 1 ml/min. The method employed a 40-min discontinuous gradient starting with 90% (v/v) buffer A (0.1M NaH2PO4, 8 mM octanesulfonic acid, and 0.05 mM EDTA) and 10% (v/v) buffer B (acetonitrile). The initial conditions were held for 5 min before changing to 20% buffer B for 15 min, 40% buffer B for a further 10 min, and then returning to 10% buffer B for 10 min. Samples and standards were mixed 1:1 with 1.5 mM phthalaldehyde (dissolved in 0.5 mM boric acid, 0.43 mM KOH, and 0.014 M β-mercaptoethanol) using a post-column pump and detected using a dual detection system employing a PerkinElmer fluorescence detector (excitation wavelength, 320 nm; and emission wavelength, 455 nm) and a β-RAM radiometric detector (IN/US Systems). Areas under the peaks were determined using β-RAM computer software (IN/US Systems).

SSAT Expression—Real-time quantitative RT-PCR (TaqMan assay) with a PerkinElmer ABI PRISM 7700 sequence detection system was used to measure the expression of hSSAT-1, hSSAT-2, human spermine oxidase (hSMO), and hPAPO. The mRNA levels of the gene of interest and those of the internal standard (human actin) were measured concurrently from the same cDNA preparations. Total RNA was extracted using Qiagen RNeasy spin columns. cDNA was synthesized using SuperScript II reverse transcriptase, followed by PCR using the ABI PRISM 7700 system. The detection and quantitation of PCR products with the ABI PRISM 7700 system were performed by fluorescence using gene-specific primers and a fluorescent probe. The comparative C_{T} method of quantitation was used as described previously (12). All mRNA expression values are ratios to human actin, and all values are × 10⁻³. Data shown are the fold increase for treated samples relative to untreated controls. The primers and probes for hSSAT-1, hSSAT-2, and human actin were purchased from Applied Biosystems (Foster City, CA) as ready-to-use kits (hSSAT-1, Assays-on-Demand, Assay Hs00161511_m1; hSSAT-2, Assays-on-Demand, Assay Hs00374138_g1; and human actin, predeveloped assay reagent, catalog no. 4310881E). The hSSAT-1 probe lies on the exon 3/exon 4 junction (accession no. NM_002970). The hSSAT-2 probe lies on the exon 2/exon 3 junction (accession no. AF348524). The primers and probes for hSMO and hPAPO were designed through Applied Biosystems under the Assay-by-Design option and are as follows: hSMO (accession no. AK000753), 5'-GCGAGTGGCCGAGATCTG-3' (forward primer), 5'-CGCCGAGTTTTGGGAATGTGGT-3' (reverse primer), and 5'-FAM-TCACAGGGAACCCC-nonfluorescent quencher-3' (probe); hPAPO (accession no. XM_113593), 5'-GGTTCGGAAGGCTCATTTGG-3' (forward primer), 5'-GGCAATGAACCCACAGAACAC-3' (reverse primer), and 5'-FAM-TGGACAGCAGC-AAGG-nonfluorescent quencher-3' (probe) (12); and C. parvum SSAT (CpSSAT; accession no. EAK87438), 5'-GACGCAGCACAAGATCATCTCTCTTGTGAAGTTAGAAAGGC-3' (forward primer) and 5'-GAGGAGGAGGGCCTTTAATCTGTGATTGTTGTCAATC-3' (reverse primer) (4). Enzymes were assayed as described below. One unit activity is defined as 1 nmol of substrate consumed or product formed from the substrate in 1 min at 37 °C.

Enzyme Assays—SSAT activity was determined as described by Libby et al. (13) using 100 μM Bicine buffer (pH 8.0) containing 17 mM [1-14C]acetyl-CoA (60 μCi/mmole) and supplemented with 50 μM unlabeled acetyl-CoA, 500 μM spermine, and 25 μg of protein. The reaction was stopped after 30 min with ice-cold 50 mM hydroxylamine, placed in a boiling water bath for 3 min, cooled, and centrifuged at 9000 × g for 1 min to remove precipitated protein. The supernatant (50 μl) was spotted onto filter discs, dried, and washed with 6 × 200–ml changes of distilled water to remove unreacted [1-14C]acetyl-CoA, with a final wash with 200 ml of methanol. The dried discs were placed in 10 ml of OmniFluor, and the radioactivity present as [14C]acetylspermine was counted using a Beckman Tri-Carb 1600CA liquid scintillation counter (PerkinElmer Life Sciences). Blanks containing [14C]acetyl-CoA and protein without spermine or containing [14C]acetyl-CoA and spermine without protein were also analyzed and subtracted from the experimental results. APAO was determined spectrophotometrically at 420 nm by measuring the amount of peroxide formed in incubations containing 1 mM N⁵-acetylspermine, 10 mM glycine (pH 8.0), 5 units of HRP, 10 mM 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt, and 50 μg of sample protein in 1-ml cuvettes. ODC activity was measured by trapping the 14CO₂ released from L-[1-14C]ornithine (42.5 Ci/mmol) using a reaction mixture containing 0.2 mM acetate (pH 6.5), 60 μM pyridoxal 5'-phosphate, and 3 mg/ml BSA in a final volume of 0.5 ml (14). The reaction was started by the addition of 10–30 μg of protein and incubated for 30 min in a shaking water bath at 37 °C. Reactions were stopped by the addition of 1 ml of 40% (w/v) trichloroacetic acid and incubated for an additional 30 min.

SMO was assayed by determining the H2O2 formed due to the oxidation of spermine by converting homovanillic acid into a highly fluorescent compound in the presence of HRP (15). The samples were prepared in a 1-ml reaction containing 8.3 mM sodium borate buffer (pH 9.0), 0.04 mg of HRP, 0.5–1.0 mg of homogenate protein, 0.1 mg of homovanillic acid, and 2.5 mM spermine. Before the addition of homovanillic acid and spermine, the tubes were preincubated for 20 min with shaking at 37 °C to remove endogenous substrates of H2O2-producing enzymes. After preincubation, homovanillic acid and spermine were added, and the reactions were incubated for 1 h at 37 °C.
The enzyme activity was stopped by the addition of 2.0 ml of 0.1 M NaOH solution. The fluorescence intensity was measured with excitation at 323 nm and emission at 426 nm. Background fluorescence was determined by the addition of spermine to the reaction mixture after inactivation of the enzyme by 5 M NaOH at 37 °C.

*Interconversion of Radiolabeled Amines and Polyamines—* HCT-8 cells were grown to confluence in 6-well plates containing 5 ml of MEM supplemented with 10% horse serum at 37 °C in a 5% CO₂ incubator. Either 0.06 μmol of [U-14C]arginine (320 mCi/mmol) mixed with 1 mM 1-arginine or 0.010 μmol of [14C]spermine (N,N'-bis[3-aminopropyl]-1,4-14C]tetramethyl-ylene-1,4-diamine; 104 mCi/mmol) mixed with 1 mM spermine was aseptically added to the wells and incubated for 2 h. The medium was aseptically removed, and the HCT-8 cells were washed three times with 5 ml of sterile 10 mM potassium phosphate (pH 7.4) containing 150 mM sodium chloride (PBS) to remove any extracellular labeled substrate. Finally, 5 ml of fresh MEM containing 10% horse serum was aseptically added to the HCT-8 cells along with 5 × 10⁴ *C. parvum* oocysts and incubated for 24 h at 37 °C in a 5% CO₂ incubator. After 24 h, the medium was removed, and *C. parvum* was separated by centrifugation at 3330 rpm for 10 min using an Eppendorf 5810R bench top centrifuge (Brinkmann Instruments). The spent medium and parasites were analyzed for polyamines by HPLC as described above. Protein was determined by the method of Lowry et al. (16).

**Analysis of Endoplasmic Reticulum (ER) Stress Proteins—** For Western blots, proteins (50 μg) were separated by 10–14% gradient SDS-PAGE. Briefly, the separated proteins were transferred to a nitrocellulose membrane in a semidy blotting chamber (Bio-Rad) according to the manufacturer’s protocol or in a transfer apparatus in 10 mM CAPS in 15% methanol (pH 10.6).

Blots were blocked with 5% milk in Tris-buffered saline (pH 7.6) containing 0.05% Tween 20 and probed with the following rabbit anti-human antibodies from Santa Cruz Biotechnology at a concentration of 0.4 μg/ml: GRP78 (glucose response protein 78), calreticulin, Nrf2 (NF-E2-related factor 2), and actin. In addition, rabbit anti-human anti-phospho-eIF2α from Cell Signaling Technology was used at a dilution of 1:1000.

**ER Stress Induces Activation of CCAAT/Enhancer-binding Protein Homologous Protein (CHOP)—** To assess the activity of ER stress-regulated apoptosis pathways, we examined the expression of CHOP. Anti-human CHOP was obtained from MBL International Corp. (Woburn, MA). Blots were incubated with primary antibody overnight at 4 °C with gentle shaking and then incubated with HRP-conjugated mouse anti-rabbit secondary antibody (1:10,000; Biomeda Corp., Foster City, CA) for 1 h at room temperature. Blots were exposed using a chemiluminescence detection method (ECL detection system, Amer sham Biosciences).

Poly(ADP-ribose) polymerase (PARP) was assayed using a universal PARP colorimetric assay kit with histoate-coated strip wells. Detection based on an ELISA employing mouse anti-PARP monoclonal antibody and HRP-conjugated goat anti-mouse IgG was performed as described by the manufacturer (Trevigen, Gaithersburg, MD). Etoposide (20 μg/ml) was used as a positive control.

H₂O₂ was detected in *C. parvum*-infected and control cells using Fluoro H₂O₂™ a fluorescent hydrogen peroxide/peroxidase detection kit (Cell Technology, Inc., Mountain View, CA), and measured on a SpectraMax M2 microplate reader at an excitation wavelength of 540 nm and an emission wavelength of 600 nm. Tetracycline (10 μg/ml) was used as a positive control.

**RESULTS**

| Enzyme | Control | 9-h infected | 15-h infected | 24-h infected |
|--------|---------|--------------|---------------|---------------|
| SSAT-1 | 12.1 ± 2.3 | 117.2 ± 18.3 | 68.8 ± 9.8 | 58.5 ± 10.1 |
| APAO   | 0.15 ± 0.06 | 0.20 ± 0.07 | 0.19 ± 0.08 | 0.21 ± 0.14 |
| ODC    | 0.02 ± 0.01 | 0.05 ± 0.01 | 0.19 ± 0.09 | 0.21 ± 0.13 |

**TABLE 1**

Activity of polyamine-metabolizing enzymes in control and *C. parvum*-infected HCT-8 cells with increasing time of infection

Enzyme activity was determined as described under “Experimental Procedures.” Results are presented as the means ± S.D. of triplicate experiments.
infected with *C. parvum* compared with uninfected controls (Table 3). The mRNA levels of hSSAT-2, hAPAO, and hSMO were unchanged compared with control cells (Table 3). The increase in hSSAT-1 mRNA levels in *C. parvum*-infected HCT-8 cells was found to coincide with increased time of exposure to the parasites (Table 4). Collectively, these results indicate that *C. parvum* infection of HCT-8 cells results in a dramatic increase in host cell hSSAT-1, a key enzyme in polyamine retroconversion. As hAPAO and other host cell enzymes do not appear to increase, the net result is an increase in acetylated polyamines. It is well documented that acetylation of polyamines targets them for excretion; we therefore propose that acetylated polyamines pass through the host cell membrane and are available for the developing parasite, which is positioned in an extracytoplasmic vacuole. The acetylated polyamine is then further retroconverted to spermidine and putrescine by parasite enzymes (Fig. 2).

### Interconversion of Radiolabeled Polyamines—To demonstrate directly that *C. parvum* took up host-derived polyamines, HCT-8 cells were grown to confluence in 6-well plates incubated with [14C]radiolabeled arginine or spermine. Tracer studies demonstrated that significant [U-14C]arginine and [14C]spermine were taken up in 2 h and that [U-14C]arginine was converted to [14C]spermine (Table 5). Exogenous [14C]-labeled precursor amines were removed, and HCT-8 cells were infected with 10^3 *C. parvum* oocysts. During the 24-h incubation phase, the parasite completed its infection cycle, and the progeny were separated from the medium and analyzed for [14C]-radiolabeled spermine, which originated from the HCT-8 cells (Table 5). The results indicate that *C. parvum* recovered from [U-14C]arginine-incubated HCT-8 cells contained [14C]spermine as the sole radiolabeled product. The percentage of [14C]spermine in the parasite compared with that released into the medium was 25–33% in three separate experiments (Table 5). *C. parvum* recovered from [14C]spermine-incubated HCT-8 cells contained [14C]spermine as the sole radiolabeled product. The percentage of [14C]spermine recovered in the parasite compared with that in the medium was 17–36%. These results confirm that *C. parvum* is capable of obtaining significant amounts of spermine from the host.

### Analysis of ER Stress Proteins—The involvement of N^-acetylssp-5|terminal|polyamines in the ER stress response of HCT-8 cells was examined by Western blot analysis of rabbit anti-human antibodies to known ER stress proteins. Protein expression of calreticulin, GRP78, NRF2, and phospho-eIF2α was increased in cell lysates from HCT-8 cells after a 9-h incubation with *C. parvum* compared with that in lysates prepared from control HCT-8 cells (Fig. 3). The means ± S.E. (n = 3 in each group) of results of bands obtained from triplicate analysis of GRP78 and phospho-eIF2α assessed by densitometry quantitate up-regulation of these proteins in infected cells compared with uninfected controls (Fig. 4). CHOP was also increased in lysates from HCT-8 cells after a 9-h incubation with *C. parvum* (Fig. 5). PARP, a protein involved in a number of cellular processes involving mainly DNA repair, was found to decrease in lysates of infected HCT-8 cells using mouse anti-human PARP monoclonal antibody and HRP-conjugated goat anti-mouse IgG in an ELISA/sandwich enzyme immunoassay (Fig. 6). It has previously been shown...
that a short exposure (15 min) of HeLa cells to H$_2$O$_2$ (15–50 $\mu$M) activates an unfolded protein response (UPR), resulting in increased expression of PRK-like ER-associated protein kinase (PERK), eIF2$\alpha$, and IRE1$\alpha$ (inositol-requiring enzyme-1$\alpha$) and ATF6 (activating transcription factor 6) cleavage, and that a longer exposure (1–3 h) to H$_2$O$_2$ induces ER-mediated apoptosis (47). Hydrogen peroxide was 50% higher in HCT-8 cells after 60 min of infection with C. parvum compared with that in uninfected control cells (Fig. 7).

**DISCUSSION**

C. parvum is an intracellular but extracytoplasmic parasite of the intestinal epithelial cells of vertebrates and is potentially lethal to immunocompromised individuals. Oocysts are ingested by drinking contaminated water or food, and they excyst in the gut to release sporozoites, which are responsible for invasion and infection of host epithelial cells (17). The intracellular development of sporozoites occurs in an intracellular vacuole that is separated from the host cell cytoplasm (17). Development of the parasite inside the vacuole results in the merozoite stage, which bursts from the intestinal epithelial cell and propagates the disease (17). The events that result in the transformation and development of the stages of parasitemia are complex and ultimately controlled by gene expression.
Polyamines are cationic molecules that interact with DNA and are involved in gene expression controlling the growth and development of all cells. Putrescine is the first polyamine in the biosynthetic pathway of the majority of cells, which, in eukaryotic cells, is synthesized from ornithine by ODC. *C. parvum* lacks ODC and has a low activity arginine decarboxylase, which is responsible for production of putrescine (6). The parasite also possesses a retroconversion pathway for polyamine salvage from spermine, utilizing an SSAT- and APAO-coupled pathway (4). The relative activities of polyamine biosynthetic enzymes and intracellular concentration of polyamines in *C. parvum* point to the uptake and retroconversion of host-derived polyamines to support growth and development of the parasite. For the parasite to adequately exploit the host cell polyamines, it would need to access polyamines separated by two sets of membrane bilayers or induce the host cell to provide polyamines by acetylation, a process in eukaryotic cells that has previously been demonstrated to neutralize the charge on polyamines and target them for export out of the cell (8, 9). We have demonstrated for the first time that infection of human epithelial cells by *C. parvum* results in elevated activity of SSAT-1 in the infected cells that coincides with the synthesis of large amounts N-acetylspermine, spermidine, and putrescine. To distinguish if this was due to parasite or host SSAT or a mixture of both, we developed an RT-PCR method to distinguish hSSAT from *Cp*SSAT. hSSAT-1 is a dimer or tetramer of ~65–80 kDa, with a subunit size of 20 kDa; contains 171 amino acids; and expresses a single 1.3-kb transcript (18). SSAT genes from mouse, rat, and hamster share regions of extensive similarity, including extensive 5′- and 3′-untranslated regions and a 200–300-nucleotide poly(A) tail (19). The catalytic active site contains eight arginine residues, five of which have been implicated in acetyl-CoA binding (18). The human enzyme has a short half-life (15 min) and is one of only two proteins lacking a PEST (proline-, glutamine-, serine-, and threonine-rich) sequence, which is common to rapidly degraded proteins (20–22). Amino acid residues 31–48 are rich in acidic amino acids that could react with proteases (21). Polyamine binding at this site may prevent protease attack and stabilize the protein (21), making it a good candidate for the amine substrate binding of the active site. Evidence from both human and mouse SSATs indicates a TATA-less promoter, a consensus sequences for a cAMP-responsive element at −487 bp, and several putative cis-acting transcriptional elements (19). *Cp*SSAT exhibits different kinetic characteristics compared with the host enzyme but is also found to be a homotetramer of 18 kDa, with arginine-rich motifs that are responsible for acetyl-CoA binding (4). *Cp*SSAT

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**FIGURE 5.** Upper, expression of CHOP in uninfected (lanes 1–3) and *C. parvum*-infected (lanes 4–6) HCT-8 cells. GAPDH was used as a loading control. B, results are shown as the means ± S.D. of triplicate experiments.

**FIGURE 6.** PARP activity in control (uninfected) and *C. parvum*-infected HCT-8 cells compared with etoposide (20 μg/ml)-induced cells. PARP was determined by ELISA using mouse anti-PARP monoclonal antibody and HRP-conjugated goat anti-mouse IgG. Results are expressed as the means ± S.D. of triplicate experiments.

**FIGURE 7.** The production of H₂O₂ was examined by measuring the levels of H₂O₂ in control (uninfected) and *C. parvum*-infected HCT-8 cells and compared with HCT-8 cells containing 10 μg/ml tetracycline (positive control). Results are expressed in relative fluorescent units (RFU) using an excitation wavelength of 540 nm and an emission wavelength of 600 nm. Data are the means ± S.D. of triplicate experiments.
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was found to have 31% sequence similarity to the human enzyme (4) a feature exploited in this study to design primers that would distinguish CpSSAT from hSSAT-1. In this study, we have demonstrated that the parasite is capable of up-regulating hSSAT-1 synthesis by intestinal epithelial cells, resulting in increased host SSAT-1 enzyme levels. Using primers specific for the host enzyme and primers specific for the parasite, it was possible to show that hSSAT-1 has a 4-fold increased expression after 24 h of infection by C. parvum. These results were corroborated by the observed increase in SSAT-1 activity and the concomitant increase in intracellular acetylserpermine, which can be scavenged by the parasite (Fig. 1). The down-regulation of host cell proteins, particularly immunologic proteins, by parasites has been reported in Leishmania mexicana, Leishmania major, and Plasmodium falciparum (23, 24). Many of these effects are the result of changes in phosphorylation of cell signaling pathways. The obligate intracellular parasite Theileria parva causes a 12-fold induction of casein kinase II in bovine lymphocytes (25). C. parvum has previously been reported to have an immunomodulatory effect on host cells by selectively up-regulating human β-defensin-2, resulting in inhibition of TLR2 and TLR4 signals and activation of NF-κB (26). However, parasite-induced up-regulation of enzymes that provide essential growth components has not previously been reported in the literature but may be common in other parasites, particularly in the apicomplexan family, such as Toxoplasma gondii, which is also deficient in polyamine biosynthetic enzymes and has been reported to be a polyamine auxotroph (5, 27).

Early studies have shown that the specific ODC inhibitor DL-α-difluoromethylornithine not only failed to cure crypto-sporidiosis in patients but induced increased diarrhea and parasite shedding (28). Blockade of spermidine synthesis by inhibition of ODC results in elevation of the retroconversion pathway, in particular SSAT-1, which provides a source of spermidine (29, 30); the lack of parasite ODC and acquisition of host cell N-acetylserpermine explain in part these earlier observations with DL-α-difluoromethylornithine. The dependence of C. parvum upon host cell polyamine biosynthesis may be an important avenue to control infection by C. parvum via modulating host cell polyamine biosynthesis.

The signal initiating the observed increase SSAT-1 expression was explored, and it was found that several proteins characteristic of the UPR or ER stress factors were increased. ER stress response refers to a combination of signaling and effector pathways used by cells to detect accumulation of misfolded proteins in the ER and to respond by implementing corrective measures or, if those fail, by initiating apoptosis. ER stress affects the expression of a wide array of genes regulating vital cell survival functions such as translation, post-translational modification, chaperones, and proteasome processing. It can cause the ER to expand, thereby increasing the capacity of response processes (31). ER stress has recently been shown to play important roles in normal physiology and in disease states (32). Because ER protein processing is dependent on calcium and energy supplies, it can be activated indirectly by perturbations in calcium stores, hypoxia, hypoglycemia, and viral infection. It can also be triggered by accumulation of oxidized fatty acids or by pharmacological agents that interfere with protein glycosylation, thus causing accumulation of unprocessed proteins. A triad of sensors located in the ER membrane monitors protein folding within the ER lumen and initiates a UPR upon detection of unfolded proteins (33, 34). ATF6 is cleaved in the Golgi, resulting in conversion into a transcription factor that traffics to the nucleus. ATF6 causes increased transcription of genes coding for UPR effector proteins, including chaperone/calcium proteins, GRP78, calreticulin, calnexin, protein folding, and protein-disulfide isomerase (35, 36). PERK is a transmembrane kinase that phosphorylates signaling molecules. PERK phosphorylation of eIF2α down-regulates general protein translation but up-regulates ATF4, a basic zipper transcription factor. ATF4 enhances expression of antioxidant genes such as glutathione synthase (35, 37, 38). PERK also phosphorylates Nrf2, which up-regulates the antioxidant relief element (39–41), resulting in increased expression of enzymes such as glutathione reductase, catalase, and thioredoxin, all involved in defense against reactive oxygen species and other electrophilic metabolites (39, 42). Recently, Nrf2 has also been shown to bind to PMF1 (polyamine-modulated factor 1), forming a complex that increases expression of SSAT (43). Finally, IRE1 (a transmembrane kinase with RNase activity) splices XBP1 mRNA, creating a transcription factor that up-regulates UPR effector proteins involved in ubiquitination and protein degradation (44). UPR activation is commonly detected by noting increases in the chaperones GRP78/BiP and calreticulin, the protein translation regulator eIF2α, and transcription factors ATF4 and XBP1 (35, 37, 38). Our data obtained with HCT-8 cells infected with C. parvum showed that induction of the UPR through PERK/Nrf2 signaling is confirmed by up-regulation of the key effector proteins GRP78, calreticulin, Nrf2, and eIF2α. C. parvum also induces NF-κB, which is known to shift UPR accumulation of unprocessed proteins (45). UPR and polyamines are connected via UPR activation of Nrf2, which in turn is involved in induction of SSAT; because SSAT induction has been associated with inhibition of cell cycling (46), it may be useful under conditions that elicit a UPR.

Our data show that Nrf2 becomes activated (phosphorylated), and because the SSAT transcription factor is formed by combining activated Nrf2 with PMF1, this is a critical step. Our data also show increases in the UPR-related chaperones GRP78, calreticulin, and protein-disulfide isomerase. One hypothesis that fits these data is induction of the UPR by C. parvum, a process known to lead to Nrf2 phosphorylation, resulting in SSAT activation. Alternatively, the UPR could be a consequence of increased SSAT triggered by another mechanism. PERK, one of the three ER sensors that independently initiate the UPR, phosphorylates Nrf2 upon activation, but the other two, ATF6 and IRE1, do not (data not shown). Increased SSAT activity resulting in increased polyamine turnover brings about a reduction in both arginine and oxidant stress, conditions known to initiate a UPR. Reduced arginine is the result of increased putrescine consumption required to maintain polyamine concentrations when cycling increases due to increased SSAT (putrescine is produced from ornithine, which is derived from arginine by the action of arginase). Oxidant stress results from the acetylated polyamines produced by SSAT being sub-
strates for APAO, an enzyme that removes the acetylated aminopropyl group from spermidine and spermine, producing H₂O₂ (a direct oxidant), and 3-acetylaminopropanal, which is also excreted by the host cell. Although the UPR can result in a successful response to cell stress, it can also lead to apoptosis if the stress cannot be managed. Our data showing induction of CHOP support the hypothesis that the UPR might play a role in the observed pathology of C. parvum in intestinal epithelia.

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