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The Effect of Febrile Temperature on Plasmodium falciparum

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The Effect of Febrile Temperature on *Plasmodium falciparum*

by

Heidi S. Porter

A dissertation submitted to the faculty of

Brigham Young University

in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Microbiology and Molecular Biology

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BRIGHAM YOUNG UNIVERSITY

GRADUATE COMMITTEE APPROVAL

of a dissertation submitted by

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As chair of the candidate's graduate committee, I have read the dissertation of Heidi S. Porter in its final form and have found that (1) its format, citations, and bibliographical style are consistent and acceptable and fulfill university and department style requirements; (2) its illustrative materials including figures, tables, and charts are in place; and (3) the final manuscript is satisfactory to the graduate committee and is ready for submission to the university library.

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ABSTRACT

The Effect of Febrile Temperature on *Plasmodium falciparum*

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Department of Microbiology and Molecular Biology

Doctor of Philosophy

Previously it has been shown that cultures of *Plasmodium falciparum* died following exposure to a febrile temperature of 40°C, as demonstrated by a decrease in parasitemia of the following generation. In the current study, the effect of 40°C treatment on culture media, erythrocytes, and parasite glucose consumption, were ruled out as possible influences on parasite death, demonstrating that 40°C impacted the parasites directly. Metabolic profiling of DNA synthesis, protein synthesis, and glucose utilization during exposure to 40°C clearly indicated that febrile temperatures had direct effect on major metabolic pathways and parasite development, beginning 20-24 hr after erythrocyte invasion. The ring stages were relatively refractory to heat and recovered completely if returned to 37°C. The mechanism of parasite death was investigated for evidence of an apoptosis-like pathway in cells treated with 40°C, chloroquine, and staurosporine. Lack of typical physiological hallmarks, namely, caspase activation, characteristic mitochondrial membrane potential changes, and DNA degradation as

indicated by DNA laddering, eliminated 'classical', apoptosis as a mechanism of parasite death. Parasites dying under the influence of 40°C, staurosporine, and chloroquine initially appeared pyknotic in light and electron microscopy, as in apoptosis, but eventual swelling and lysis of the food vacuole membrane led to secondary necrosis. Initially, chloroquine did induce DNA laddering, but it was later attributed to occult white blood cell contamination. While not apoptosis, the results do not rule out other forms of temperature-induced programmed cell death.

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Introduction

History

Malaria is a parasitic febrile illness caused by organisms of the genus *Plasmodium* specifically, *P. falciparum*, *P. ovale*, *P. vivax*, and *P. malariae* in humans. *Plasmodium* organisms have been associated with human populations since the dawn of civilization. Early Egyptians suffered from this disease as evidenced by a description of characteristic malarial fevers in the Eber's papyrus, the earliest known medical text, circa 1500 B.C. Furthermore, at the temple of Dendara, in central Egypt, inscriptions describe malarial fevers that occurred after the flooding of the Nile along with a prophylactic chant that would ward off the fevers (Halawani and Shawarby, 1957). In more direct measurements, malarial antigens have been detected in mummified human remains dating back to 3200 B.C. (Miller et al., 1994).

The Greeks were the first recorded civilization to make a serious study of the clinical disease. Hippocrates, a Greek physician born in 460 B.C., not only determined that the disease was linked to marsh land, but also was able to distinguish the three different forms of the illness by carefully documenting fever frequency. The three were, quartan fever caused by an infection of *P. malariae*, tertian fever (*P. vivax and P. ovale*), and malignant tertian fever (*P. falciparum*).

Though known by many names through the ages such as tertian fever, ague, or marsh fever, the current name is derived from the Italian, *mal-* 'bad' and *aria-* 'air', which comes from the observation that the disease was more common near swamp land or marshes and was attributed to the bad air that emanated from

stagnant water. It was not until 1897 that mosquitoes were determined to be the vector of malarial infections.

In 1850 malaria was endemic to most of the world, including the United States, Europe, Russia, Asia, and Africa. Throughout the 20th century, the use of anti-malarial drugs to control the parasite and insecticides to limit the vector, led to a decrease in the world wide distribution of the organism especially in moderate climates. Malaria was eradicated from the United States in 1953.

Although largely eliminated in many industrialized nations, malaria continues to exact a monumental toll on less developed countries. In recent years, much of the progress towards malaria eradication in third world countries has been eroded by wide spread drug resistance of the parasite and insecticide resistance in the mosquito vector (Gallup and Sachs, 2001). Currently, it is estimated that world wide, there are between 300-500 million cases of malaria causing 1 - 3 million deaths annually (Sachs and Malaney, 2002). Greater than 80% of fatalities occur in sub-saharan Africa where a rising number of infections led epidemiologists to predict that without drastic intervention, the current rates of infection will soon rival those found in the early 1900's (Breman, 2001).

Plasmodium falciparum causes the deadliest form of malaria, causing >90% of malarial deaths world wide (Despommier et al., 2000). The organism has a complex life cycle consisting of several morphological stages in both human and mosquito hosts. In brief, mosquitoes of the *Anopheles* genus ingest male and female gametocytes while taking a blood meal from an infected human. Sexual reproduction and differentiation produce infectious sporozoites within the

mosquito over the next several days to weeks. During a subsequent blood meal, the mosquito releases the sporozoites into the human blood stream. They immediately invade hepatocytes where they undergo asexual reproduction resulting in production of thousands of merozoites. Upon release from the liver, merozoites enter erythrocytes and begin a continuous cycle of replication and rupture, followed by invasion of new red blood cells. Occasionally during the erythrocytic cycle, a newly released merozoite enters into an erythrocyte and begins gametocytogenesis, a 10 day process resulting in stages that are infectious to the mosquito vector. The life cycle continues if a receptive mosquito ingests viable gametocytes (Sherman, 1998).

Much of malaria research focuses on halting the erythrocytic cycle of *P. falciparum* as only these stages cause pathology in humans. Erythrocytic development is divided into three segments based on changes in parasite morphology. Initially merozoites enter the erythrocyte and differentiate into a phase known as the ring stage, named for a signet-ring-like appearance after giemsa staining. This stage exhibits minimal metabolism and has been compared to the G1 phase of the cell cycle in higher eukaryotes. As the parasites mature, they begin to engulf hemoglobin from the cytoplasm of the host red blood cell. Hemoglobin digestion takes place in a specialized organelle called the food vacuole where free-radical inducing heme molecules are polymerized into hemozoin to limit oxidative damage within the parasite. The visualization of hemozoin crystals denotes the beginning of the 14-hr trophozoite stage. During this stage the parasite prepares for division by synthesizing DNA, proteins, and

other bio-molecules needed for replication, similar to the synthesis phase of the cell cycle in metazoan cells. Unlike higher eukaryotes, there is no G2 phase of the cell cycle in the parasite, rather the division into multiple nuclei begins before all the DNA has been synthesized, with the parasites' "S phase" and "M phase" partially overlapping. Visually, the schizont stage begins when two or more nuclei are distinguishable and lasts ~12 hrs. Each parasite will eventually produce between 16-24 new merozoites that will enter new red blood cells and continue the 44-48 hr erythrocytic cycle (Arnot and Gull, 1998).

In ultrastructure studies of the erythrocytic cycle of *P. falciparum*, one or more parasites can be observed in the erythrocyte. During the mid to later stages of the erythrocytic cycle parasitized red blood cells often have 'knobs' seen protruding at the host cell membrane. The knobs are of parasite origin and consist of proteins that have been linked to cytoadherence and sequestration (Aikawa, 1988). When observed under the electron microscope, the parasites appear to have a double cell membrane. The outer cell membrane is known as the parasitophorous vacuole and is of host cell origin, though it has been extensively modified by the parasite. The parasitophorous vacuole often has extensions that are seen as whorls or sacs within the cytoplasm of the red blood cell. These are known as Maurer's clefts and are thought to participate in protein trafficking between host and parasite. The parasite vacuole often is very close to the parasite plasma membrane during the mid-late stages of the erythrocytic cycle and can be hard to distinguish. At the parasite cell membrane there are small ringed orifices known as cytostomes. As the parasite matures it begins feeding on the host cell

cytoplasm via these cytostomes, often engulfing large amounts of hemoglobin along with some of the parasitophorous vacuole and cell membrane into a single membrane bound sac which pinches off forming a hemoglobin filled vacuole. These hemoglobin vesicles eventually fuse with the central digestive parasite food vacuole which becomes microscopically visible at 18-22 hrs into the lifecycle. This food vacuole is an acidic compartment that is very easy to detect because of the large hemozoin crystals produced during hemoglobin digestion. The hemozoin crystals are electron dense and have an angular shape. The parasite also contains a plastid, which is often seen as a double membrane vacuole filled with membranous whorls, often near the food vacuole and mitochondria (Bannister et al., 2000). The mitochondrion of the parasite is branched in the mid-late stages of maturation and is achristate in nature. As the parasite matures, the DNA replicates and divides into 16-24 new nuclei. During nuclei division the nuclear membrane remains intact, although the nucleus is often hard to distinguish as its consistency under electron microscopy is often similar to that of the cytoplasm (Langreth, 1978).

Often during infection, the erythrocytic cycle is reasonably synchronous leading to the classic fever pattern occurring in *P. falciparum* infections every 48 hrs. The fever episodes are linked to the erythrocytic cycle and are parasite-density dependent, occurring as the parasitemia reaches the pyrogenic threshold (10^4 parasites/ μ l blood), typically a few days after organisms are found in the blood (Miller, 1958). As newly released merozoites enter red blood cells, fragments of the merozoite cell membrane and surface proteins are sheered off

and released into the serum. Several merozoite surface proteins are anchored into the cell membrane by glycosylphosphatidylinositol (GPI). Free *P. falciparum* GPI anchors act in an LPS-like fashion to stimulate macrophage production of the fever inducing cytokine, TNF- α , by signaling through the toll-like receptor (TLR) pathway, specifically TLR2 and TLR4 (Naik et al., 2000; Krishnegowda et al., 2005). Hemozoin, which is released as the merozoites escape from a red blood cell, may also play a minor part in fever production by signaling through the TLR9 pathway, though the exact role of this signaling remains to be established (Skorokhod et al., 2004; Coban et al., 2005).

It is not known what role fevers play in the lifecycle of the organism during infection. Several mathematical models suggest that fevers have an important function in limiting parasite number during infection. This allows the patient to survive and the erythrocytic cycle to continue thereby lengthening the time of transmission of the gametocyte to the mosquito (Gravenor and Kwiatkowski, 1998; Molineaux et al., 2001). *In vivo* studies in humans have yielded mixed results on the effect of fever suppression on the clinical course of *P. falciparum*. The administration of antipyretics during infection caused a delay in parasite clearance under some circumstances and no change in the course of infection in other studies (Brandts et al., 1997; Lell et al., 2001). The conflicting data may be explained by the concurrent administration of anti-malarial drugs during the studies, thus making it difficult to determine the true effect febrile temperatures have on parasites *in vivo*.

Kwiatkowski et al., (1989) initiated the *in vitro* work on fever temperatures in *P. falciparum* by describing the direct effect of febrile temperatures on the erythrocytic cycle. He demonstrated that by alternating daily incubation temperatures between 37°C and 40°C, mimicking the fever periodicity seen in natural infections, he could produce a synchronization of the organism *in vitro* that was similar to that often seen during infection. Growth curves showed that parasitemia decreased after exposure to 40°C. Furthermore, parasites apparently showed greater sensitivity to 40°C during the second half of the erythrocytic cycle (Kwiatkowski, 1989). Later, others confirmed the work of Kwiatkowski and expanded the temperature range studied. It was determined that the parasites became susceptible to 40°C beginning at 24 hrs into the erythrocytic cycle (Long et al., 2001). Both Kwiatkowski et al. and Long et al. assumed death of the parasite indirectly after exposure to 40°C, by measuring the parasitemia of the following generation. Possible culture parameter changes due to heat were not examined. Furthermore no direct measurement of parasite death during exposure to 40°C was taken. We determined that several issues regarding fever and parasite biology remained to be addressed.

The effect of 40°C on culture parameters

We hypothesized that parasite death caused by incubation at 40°C may be precipitated by heat's effect on components of the *in vitro* culture system rather than on the parasite. In culture, febrile temperatures may induce death by:

- 1) Inducing culture medium instability.
- 2) Producing permanent damage to red blood cells thus decreasing invasion rates.
- 3) Initiating increased metabolism in

the parasite leading to death by glucose starvation or pH changes induced by the accumulation of lactic acid in the culture media.

Plasmodium falciparum is cultured in a complex medium of RPMI (Roswell Park Memorial Institute) 1640 that is supplemented with either human serum or a lipid-rich serum substitute and culture parameters are very narrow. Slight changes in the nutritional value of the culture medium can render it unable to support growth of the organism. It is feasible that extended exposure to 40°C denatures vital heat sensitive elements of the media, rendering it incapable of supporting parasite growth.

Another factor in determining successful cultivation of the erythrocytic stages of *P. falciparum* is the condition of the host red blood cells. Invasion of red blood cells by merozoites is an active process requiring receptor/ligand signaling between the parasite and host. *Plasmodium falciparum* is especially adept at invasion of red blood cells entering both mature and immature erythrocytes with equal proclivity. However, as erythrocytes are collected, stored in preservative, and placed into culture conditions, several physiological changes occur such as, ATP depletion, membrane digestion and shape modifications, which over time make the red blood cell less receptive to parasite invasion (Capps and Jensen, 1983; Gaczynska et al., 1989). Erythrocytes are viable to invasion for up to 30 days in refrigerated preservative. However, once in culture (37°C) they can only support invasion and growth of *P. falciparum* for about 4-5 days. It is for this reason that fresh erythrocytes must be given to the parasites *in vitro* every 3-4 days independent of parasitemia. We hypothesized that incubation of red

blood cells at 40°C for 24 hrs may hasten the deleterious changes seen in cultured red blood cells, making erythrocytes refractory to invasion earlier than expected during 37°C incubation. Furthermore, studies on the effect of high temperature on erythrocytes showed that incubation at 46°C for 30 min. led to weakening of erythrocyte cell membranes in cultured red blood cells (Prinsze et al., 1991).

Heat may also lead to parasite death by altering glucose consumption rates. During the erythrocytic cycle, *P. falciparum* is a homolactate fermentor, producing two molecules of both ATP and lactic acid for every molecule of glucose metabolized. The inefficient use of glucose leads to several considerations when growing the parasite in culture. First, the demand for glucose is considerable. Second, and more importantly, lactic acid build up rapidly decreases the pH of culture media which leads to death of the parasite (Jensen et al., 1983). For these two reasons, the media must be exchanged every 12-24 hrs depending on the number and stage of the parasites and special care must be taken in designing experiments to ensure adequate glucose supply and waste removal. Earlier work on glucose metabolism showed that lowering the incubation temperature during cultivation of *P. falciparum* led to a decrease in glucose metabolism rates (Rojas and Wasserman, 1993). We hypothesized that the opposite might also be true, that increasing incubation temperature may increase glucose metabolism of *P. falciparum*. The experiments of Long and Kwiatkowski were performed in culture media containing minimally adequate glucose concentrations for the organisms. It is therefore plausible that temperatures of 40°C could increase the metabolism of the parasite sufficiently to

exhaust glucose supply. Parasite death attributed to heat may have been, in reality, caused by glucose deprivation or acidosis of the medium precipitated by lactic acid accumulation. In this work, we sought to determine if febrile temperatures were having a detrimental effect upon the culture conditions discussed above. Moreover, we sought to extend the work of Long et al. (2001) by determining the timing and recoverability potential of *P. falciparum* after incubation at 40°C, and determine the impact of heat upon the major metabolic pathways of *P. falciparum*.

Characterization of cell death

Many cells have the ability to initiate an organized pathway or program of cell death in response to outside stimuli or intrinsic changes within the cell. Although many different types of programmed cell death have been described, they do have certain elements in common; 1) cell membrane remains intact during the majority of the pathway; 2) process requires energy; 3) presence of regulatory molecules; 4) morphological and physiological changes can be consistently reproduced (Kerr, 2002; Guimaraes and Linden, 2004). The idea of a program of cell death is in opposition to necrosis, which is an uncontrolled degradation of the cell often occurring after extreme cellular insult.

The most studied and recognizable form of programmed cell death is apoptosis. This form of cell death consists of specific morphological and physiological changes within the cell, a partial summary of which is shown in Table I. They are usually precipitated by activation of a family of cysteine-aspartate proteases called caspases. Though once thought of as a process specific

to multicellular organisms, recently apoptotic-like processes have been discovered in at least nine species of single celled eukaryotes. Organisms such as *Leishmania spp.*, *Trypanosoma spp.*, and other lower order eukaryotes have exhibited apoptosis-like hallmarks such as DNA laddering, membrane blebbing, phosphatidyl serine exposure, and maintenance of membrane integrity during the death process (Nguewa et al., 2004). The regulatory molecules were not ascertained, though several of the organisms showed signs of caspase-like protein activation. *Leishmania major* parasites undergoing differential cell death exhibited the ability to cleave synthetic caspase substrate, DEVD-AMC. Cleavage of the substrate was blocked by the pan-caspase inhibitor Z-VAD (Zangger et al., 2002). Similar results were found in *L. major* exposed to staurosporine as well, though a caspase gene homolog has not been discovered in the genus (Arnoult et al., 2002).

In *P. falciparum*, gene expression studies have reported modifications of transcription levels specific genes during heat-induced death of the cell. Early work reported that exposure to 39°C led to an increased production of the heat shock protein, PfHSP70 (*Plasmodium falciparum* heat shock protein 70) (Biswas and Sharma, 1994). Recently, a more complete microarray analysis determined that exposure to febrile temperatures (41°C) for 2 hrs produced significant alterations in 336 out of 5300 mRNA expression profiles (Oakley et al., 2007). Many of the upregulated mRNA's were involved in protein trafficking with 47% of the gene products located in the cell membrane or cytoplasm of the host erythrocyte. As expected, the upregulated genes included PfHSP 70 as well as

several members of the DnaJ chaperone family. HSP 70 and cofactor DnaJ (human HSP 40 homologue) are the only upregulated genes known to be linked to forms of programmed cell death in other organisms. It is possible that there are others, however, as the function of some of the genes that exhibited fluctuation have yet to be determined.

Al-Olayan et al. (2002) investigated the mosquito mid-gut stages of *Plasmodium berghei* which causes malaria in mice. Upon ingestion by a mosquito, gametocytes, found in the blood of the vertebrate host, rapidly mature into gametes with fertilization taking place in the mosquito midgut. Resulting zygotes form ookinetes which burrow into the basil lamina of the mosquito and form oocysts. Oocysts go on to produce sporozoites which are infectious to the vertebrate host. Although several thousand ookinetes may be produced, only 10 - 200 oocysts ultimately end up within the basil lamina of the mosquito gut. Al-Olayan et al. (2002) sought to determine the fate of ookinetes that did not result in oocyst formation. It was determined during *in vivo* and *in vitro* experimentation was that more than 50% of ookinetes died within 24 hrs of formation. When assayed, the dying cells exhibited several classical hallmarks of apoptosis namely, caspase activation, DNA fragmentation (TUNEL+), phosphatidyl serine exposure, chromatin condensation, and membrane blebbing. Further testing indicated that the process was active, requiring protein synthesis and was blocked by addition of caspase inhibitors (Al-Olayan et al., 2002).

Evidence of an apoptotic-like process has been reported in *P. falciparum* as well. Using the classic anti-malarial, chloroquine, to promote cell death,

characteristic oligonucleosomal fragmentation or DNA laddering was reported (Picot et al., 1997). Furthermore, exposure to the antimalarial drugs, mefloquine and artemesinin, lead to a decrease in glutathione levels in *P. falciparum*, which has been associated with apoptosis in higher eukaryotes (Pankova-Kholmyansky et al., 2003).

Using these studies as a starting point we sought to determine if cell death initiated by exposure of *P. falciparum* to 40°C, exhibited hallmarks of apoptosis. We also extended the investigation to examine other initiators of cell death namely, staurosporine, a protein kinase inhibitor known to initiate apoptosis-like events in multicellular and unicellular eukaryotes (Scarlett et al., 2000), and chloroquine an antimalarial drug which was reported to induce a classic apoptosis characteristic in cultures of *P. falciparum* (Picot, et al., 1997).

Chloroquine is a 4-aminoquinilone, similar in structure to the first antimalarial drug, quinine. The drug is a weak base and its activity is dependent upon its concentration in the acidic food vacuole during exposure. Morphological studies of parasites exposed to chloroquine showed swelling of the food vacuole and decrease in trafficking and fusion of hemoglobin-laden vacuoles with the food vacuole (Jacobs et al., 1988). The exact mechanism of action of chloroquine within the food vacuole is currently unknown, as previously reviewed (Bray et al., 2005).

Both lower and higher eukaryotic cells have exhibited signs of apoptosis after exposure to chloroquine. Erythrocytic cultures of *P. falciparum* exhibited apparent DNA laddering after exposure to chloroquine (Picot et al., 1997) and

chloroquine initiated an apoptotic pathway in metazoan organisms in transformed epithelial cells (Turco et al., 2000), neuronal cells (Zaidi et al., 2001), and peripheral white blood cells (Meng et al. 1997). We sought to confirm and extend the work of Picot et al., (1997) by further study of chloroquine as an initiator of cell death in *P. falciparum*.

Staurosporine is an alkaloid that was initially discovered in the bacterium *Streptomyces staurosporeus*. It is a broad spectrum protein kinase inhibitor, inhibiting many serine-threonine kinases, previously reviewed in (Ruegg and Burgess, 1989; Hidaka and Kobayashi, 1992). Exposure to low levels of staurosporine leads to cell death, specifically apoptotic death, in many cell types (Canzoniero et al., 2004; Zhang et al., 2004). Because staurosporine inhibits a wide variety of proteins, it has been difficult to elucidate activation of a specific apoptotic pathway. Several pathways seem to be involved depending on cell type and environment (Deshmukh and Johnson, 2000; Feng and Kaplowitz, 2002). Staurosporine has also been used to induce an apoptosis-like pathway in single celled eukaryotes such as *Leishmania major*, leading to phosphatidyl serine exposure, characteristic visual changes and DNA degradation (Arnoult et al., 2002). Studies done in *P. falciparum* showed that the parasite is sensitive to staurosporine exposure leading to inhibition of the erythrocytic cycle, though the process was not characterized (Dluzewski and Garcia, 1996; Gazarini and Garcia, 2003). We sought to determine if staurosporine exposure initiated apoptosis in *P. falciparum*.

The indicative markers of apoptosis we chose to examine were: changes in metabolic profiles, DNA degradation as measured by TUNEL and oligonucleosomal fragmentation (DNA laddering), caspase activation, mitochondrial membrane potential changes and morphological changes observed by light and electron microscopy.

Materials and Methods

Materials

RPMI 1640 (25 mM HEPES buffer with L-glutamine without NaHCO₃), Albumax I, penicillin-streptomycin (1,000 units/ml-10,000 µg/ml) (*Invitrogen, Grand Island, NY*); sodium bicarbonate (*EMD Chemical Inc, Gibbstown, NJ*); sodium citrate (*J.T. Baker CO, Phillipsburg, NJ*); citric acid, potassium chloride (*Mallinckrodt Inc., Paris, KY*); dextrose (*La Pine Scientific, Norwood, NJ*); sodium monobasic phosphate (*Spectrum Chemicals, Gardena, CA*); adenine, sorbitol, gelatin (300 bloom), L-phenylalanine-[ring-2,6-³H(N)] (1 mCi/ml), glucose (HK) assay kit, hypoxanthine-[2,8-³H] (1 mCi/ml), giemsa modified stain (*Sigma, St. Louis, MO*); Phisioigel (*Roger Bellon Laboratories, Newilly, France*); modular incubator chamber (*Billups-Rothenberg, Del Mar, CA*) ; specialty gas (*Airgas specialty gasses, Radnor, PA*); Cell Harvestor (*Inotech Biosystems, Rockville, MD*) percoll- PVP coated silica, carbonylcyanide m-chlorophenylhydrazone (CCCP), eukaryotic protease inhibitor cocktail (AEBSF), aprotin, leupeptin, bestatin, pepstatin A, E-64), saponin, micrococcal nuclease (500units), DiOC₆(3), SYBR green I (*Molecular Probes, Eugene, OR*);

magnesium chloride, sucrose, calcium chloride (*Columbus Chemical Industries , Columbus WI*); EDTA (*EMD chemicals Inc., Rochester, NY*); Tris HCl , RNase A, phenol/chloroform/isoamyl alcohol (25:24:1), sodium acetate (*Fisher Scientific, Pittsburg, PA*); proteinase K, 100 bp DNA Ladder (*Invitrogen, Grand Island, NY*)

Culturing parasites

Plasmodium falciparum strain CSC-1 (Honduras) was cultured using the Trager and Jensen method (Trager and Jensen, 1976) with slight modification (Jensen, 2002). In brief, parasites were allowed to invade freshly washed erythrocytes that had been refrigerated for 1-12 days in the blood preservative CPDA-1 (26.3 g NaCitrate, 3.27 g citric acid, 2.2 g sodium monophobasic phosphate, 31.94 g dextrose, 0.27 g adenine, H₂O up to 1 L). Culture media consisted of RPMI 1640 (16 g/L) supplemented with NaHCO₃ (0.2% w/v), penicillin-streptomycin (18 units/ml-180 µg/ml), Albumax I (0.27% w/v), and hypoxanthine (198 µM). Parasites were incubated in modular incubation chambers with an atmosphere of 94% N₂, 5% CO₂, and 1% O₂ at 2% hematocrit.

Treatment and Controls

Growth temperature was maintained at 37°C except when subjected to febrile temperatures (40°C). In chloroquine experiments, stock solutions of chloroquine phosphate were diluted into culture media to a final concentration of 100 µM. Comparisons were made between cultures fed with chloroquine-supplemented media or control. In staurosporine investigations, comparisons were made between identical cultures treated with staurosporine (5 µM) or control

media, each containing equal concentrations of the solvent dimethyl sulfoxide (DMSO).

Synchronization of parasites

Parasites were synchronized to a 4-5 hr window using a combination of the gel-separation (Jensen, 1978) and sorbitol (Vernes et al., 1984) techniques. In short, organisms were suspended in a gelatin solution (0.25% gelatin, 50% Physiogel) for 30 min. Gelatin-containing solutions significantly enhanced rouleux formation in uninfected and ring infected erythrocytes leaving more mature stages of the parasite in suspension. Once the uninfected and ring-infected erythrocytes had settled, the mature-stage parasites were isolated from the supernatant by gentle centrifugation and allowed to invade fresh erythrocytes for 4 hrs after which all trophozoite and schizont infected cells were lysed by sorbitol treatment leaving highly synchronous ring-stage cultures having a 0-4 hr window (Jensen, 2002). Throughout the body of this text, this time point is referred to as 0 hrs post-invasion (PI).

Giemsa-Stained Thin Films and pH

All experiments were confirmed by visualization of giemsa-stained thin films. Thin films were stained for 10-12 minutes with a 10% solution of modified giemsa stain dissolved in RPMI 1640 (pH 7.4).

³H-Phenylalanine and ³H- Hypoxanthine incorporation assays

Microcultures were established in 96-well plates having between 2-4% hematocrit and always <5% parasitemia. After parasites were aliquoted, each well was filled with 100 µl of L-phenylalanine-[Ring-2,6-³H(N)] or hypoxanthine

–[2,8-³H] labeled media, final concentration 2 μCi/well. Incorporation times and temperatures varied according to the experiment. Unless otherwise specified, the incorporation time was >18 hrs. In DNA and protein synthesis profile experiments, the concentration of ³H- phenylalanine and ³H-Hypoxanthine was increased to 4 μCi/well. During hypoxanthine assays, culture media having a lower concentration of hypoxanthine (17μM) was used. An Inotech harvester collected cells onto a glass fiber filter and radioactivity was assessed after drying (≥18h) using an Inotech gas scintillation reader (Jensen, 2002). In some experiments parasites incubated at 37°C or 40°C were transferred from the 96-well plate in which they were incubated to a new 96-well plate for simultaneous harvest. To control for pipetting error, a well from each sample was counted by hemocytometer and cpm was expressed as cpm per 10⁶erythrocytes.

Glucose Assay

The glucose assay was performed using the Glucose Assay (HK) Kit according to manufacturer's instructions. In summary, media was decanted from cultures and a solution containing ATP, NAD, hexokinase, and glucose-6-phosphate dehydrogenase was added. Glucose was converted to 6-phosphogluconate along with a reduction of NAD to NADH in equal molar amounts. The resulting increase in absorbance at 340 nm is directly proportional to the amount of glucose in the sample.

Mitochondrial membrane potential assay

Ethanol stock solutions of DiOC₆(3) and carbonylcyanide m-chlorophenylhydrazone (CCCP) were prepared in advance and kept in the dark at

4°C. Fresh working solutions diluted in RPMI 1640 were prepared immediately before use. Parasites were isolated from infected erythrocytes by microcentrifugation on a percoll gradient (using a modification of McNally et al., (1992). In brief, 500 µl of 45% percoll in RPMI 1640 were added to a 1.5ml tube and 500 µl of 35% percoll in RPMI 1640 were carefully overlaid. One hundred twenty µl of concentrated parasites were added. The tube was centrifuged at 5500 g for 15 min. After centrifugation late stage parasites (trophozoites and shizonts) were found in the upper half of the tube. They were removed and washed twice in media. This led to a yield of 85-95% infected erythrocytes. For each experiment a sample was stained with giemsa to determine parasite concentration. Parasites were then incubated with CCCP (200µM) or ethanol control for 1 hr at 37°C or 40°C. The cells were then stained with DiOC₆(3) for 60 min, washed twice in media and immediately viewed by flow cytometry, 488 nm argon laser, FL1- 525 nm emission, 10,000 cell count per sample.

Caspase Activation Assays

Pan-caspase activation was assayed using the CaspaTag™ Fluorescein Caspase (VAD) Activity Kit. Activity of specific caspases 8, 9, and 10 were determined using CaspaTag™ Caspase-8 (LETD) Activity Kit, CaspaTag™ Caspase-9 (LEHD) Activity Kit, or CaspaTag™ Caspase 10 (AEVD) Activity kit, respectively (Millipore, Billerica, Massachusetts). All assays were conducted according to manufacturer's instructions. Stained cells were analyzed using flow-cytometry, 488nm argon laser, FL1-525nm emission, 10,000 cell counts per

sample (Coulter Epics XL, Beckman-Coulter, Fullerton, California) and confirmed visually by fluorescent microscopy.

Oligonucleosomal DNA Fragmentation (DNA laddering) Assay

Parasites were partially synchronized during ring stage by sorbitol lysis. For sufficient DNA, at least eight 100 mm plates with parasitemia >10% at a 2% hematocrit were needed per electrophoresis lane. Cells were allowed to progress to early trophozoite stage before being treated. DNA extraction was performed using the protocol of Cary et al., (1994) with modifications. A brief summary of the modified method follows.

Nuclei isolation and lysis

Each sample was suspended in three pellet volumes of 0.05% saponin at 4°C for 10 min. Subsequently, 25 pellet volumes of media were added, mixed well and then centrifuged at 600 g for 5 min. Two other washes using 30 pellet volumes of media were then carried out. Pellets were suspended in 1 ml of hypotonic buffer (10 mM Tris-HCl, 1.5 mM MgCl₂, 15 mM KCl, 5.2 mM AEBSF, 4 μM aprotinin, 0.1 mM leupeptin, 0.2mM bestatin, 0.075 mM pepstatin A, 0.07 mM E-64) at 4°C for 30 min. The parasites were lysed using a Thomas 153 tissue homogenizer (100 strokes). Ruptured cells were suspended in hypotonic buffer and sucrose (0.34M) on ice for 1 hr and then pelleted at 16,000 g for 4 min. Each pellet was suspended in hypotonic buffer.

Enzyme Digest

Cell extracts were incubated with CaCl₂ (0.05 M) for 30 min. Afterwards micrococcal nuclease (50units/ml) was added to some samples to serve as a

positive control. Micrococcal digests were performed at 30°C for 0-3 min before a stop solution of EDTA (10 mM) was added and extracts were chilled on ice. RNA digestion was completed at 50°C for 1.5 hrs by RNase A (100 µg/ml) followed by Proteinase K (800 µg/ml) digestion under similar conditions in all samples.

DNA extraction

To extract protein from each sample, one volume of phenol/chloroform/isoamylalcohol (25:24:1) was added and mixed gently for 10 min. The suspension was centrifuged at 6,000 g for 10 min and the supernatant decanted to a new tube. The previous series of steps was performed twice. Sodium acetate was added to the supernatant bringing the final concentration to 0.3 M and tubes were mixed well. We precipitated DNA by adding 2.5 volumes of ice cold 100% ethanol and incubating samples overnight at -20°C. Pellets were then washed in 70% ethanol, air dried and dissolved in TE (10 mM Tris-HCl, 1 mM EDTA).

Electrophoresis

Samples were electrophoresed on a 1.5% gel at 100 volts in TBE (10.8 g Tris-base 5.5 g boric acid 0.832 g EDTA in 1 L H₂O). DNA was then visualized by staining gel with SYBR green I (1:10,000) for 1 hr followed by UV illumination. Positive controls, showing DNA ladders, consisted of DNA extracted from the established cell line, HL-60, which had been exposed to staurosporine (5 µM) to induce apoptosis or parasite DNA digested with

micrococcal nuclease (50 units/ml). All lanes consisted of 5-10 μ g DNA per lane unless otherwise specified.

White Blood Cell Depletion

Erythrocytes used in some chloroquine experiments were exhaustively depleted of white blood cells using Dynabeads CD45 kit (Invitrogen, Carlsbad, California) following the manufacturer's instructions. Erythrocytes were further incubated with 1% (w/v) DNase I at 37°C for 30 min to remove extracellular DNA. Concentrated parasite cultures were then allowed to expand into the purified erythrocytes for 1-2 cycles before DNA ladder experiments were initiated.

Transmission Electron Microscopy

Parasites were synchronized (4 hr window) and allowed to grow for 24 hrs. Cultures were partitioned into 6 identical plates and placed in experimental or control conditions. Every 3-5 hrs, a control and experimental plate were harvested and the parasites concentrated by gel-separation (Jensen, 1978). Fixation for transmission electron microscopy was performed using a modification of a method previously described in Yayon et al., (1984). Briefly, parasites were washed twice in phosphate buffered saline (pH 7.4) and suspended in fixative (1.6% glutaraldehyde, 0.1 M sucrose, 0.2 mM CaCl₂, 0.1 M sodium cacodylate) for 0.5 hrs. Samples were then washed 3 times in wash buffer (0.12 M sucrose, 0.1 M sodium cacodylate) and stained with osmium buffer (1% OsO₄, 0.12 M sucrose, 0.1 M sodium cacodylate) for 0.5 hrs. After two brief washings, the pellets were kept in distilled H₂O until the remaining samples in the time

series reached this point. All parasites were then stained over night in 0.5 M uranyl acetate followed by three H₂O washes. Water was expelled from the samples through a dehydration series of ethanol, (10%-100% in ten percent increments) and acetone (100%). The cells were embedded in SPURS by gradual increase in resin concentration (1 part resin: 2 parts acetone, 2 parts resin: 1 part acetone, 100% resin). Samples were then polymerized at 70°C. Thin sections were cut by a diamond knife, post stained with Reynold's lead citrate and visualized on a Tecnai T-12 transmission electron microscope.

Statistical analysis

Error bars are 95% confidence intervals for the means. Statistical significance was determined by unpaired t-test using a 95% confidence level, p-value ≤ 0.05 . All experiments consisted of 2-4 independent trials.

Results

The effect of 40°C on culture media, erythrocytes, and parasite glucose metabolism.

To determine if heat treatment of 40°C had an effect on the ability of culture medium to support growth of *P. falciparum*, RPMI-1640 with Albumax I, NaHCO₃ and antibiotic supplementation, was pre-treated at 37°C or 40°C for 24 hrs before being used to support parasite development. A temperature of 40°C was used throughout the study because it is common to see fevers of this magnitude clinically and Long et al., (2001) determined that 40°C but not 39°C

led to significant parasite death in a short amount of time. Subsequently, identical parasite cultures were fed with the pre-treated media and parasite growth was assessed by 24 hr ³H-phenylalanine incorporation at 37°C or 40°C. Pre-treatment of media yielded no significant differences in parasite growth (Fig. 1).

Because high temperatures have been shown to alter red blood cell physiology *in vitro* we sought to determine if 40°C treatment induced changes in the suitability of cultured erythrocytes to serve as host to *P. falciparum*.

Erythrocytes were pre-treated by incubation in culture conditions at 37°C or 40°C for 48 hrs. Next, late stage parasites were introduced and were allowed to invade the pre-treated erythrocytes at 37°C for 8 hrs. Cultures were treated with sorbitol to remove residual late stage parasites and newly-parasitized red blood cells were evaluated for their ability to sustain *P. falciparum* development by measuring growth of parasites by ³H-phenylalanine incorporation at 37°C. Erythrocytes exposed to 40°C for 48 hrs exhibited no permanent decrease in ability to support growth of *P. falciparum* (Fig. 2).

To eliminate the possibility that parasite death after exposure to febrile temperatures, as seen in earlier experiments, was a result of glucose starvation or lactic acid accumulation, synchronized parasites were grown at 37°C or 40°C for 40 hrs. Samples of culture media were removed and assayed for glucose concentration at 24 and 40 hrs. Our results indicated that metabolism of glucose does not markedly increase with exposure to febrile temperature; rather there was a significant decrease in glucose consumption (p= 0.03) that could be seen as early as 24 hrs (Fig. 3).

Determining the timing of parasite sensitivity to 40°C

Synchronized parasites were incubated at 40°C beginning at 0 hrs PI. Samples were returned to 37°C at various time points and allowed to recover for 36 hrs before a ³H-phenylalanine incorporation assay was performed. Ring-stage parasites (0-18hrs) were not damaged by exposure to 40°C, progressing through normal erythrocytic development when returned to 37°C. However, at 25 hrs PI, parasites became sensitive to 40°C treatment, exhibiting a low amount of phenylalanine incorporation and cell growth, as assessed by giemsa-stained thin films. There was no increase of cell growth if parasites were allowed to recover at 37°C for ≥ 36 hrs before assessment (Fig. 4). If 40°C treatment of the parasites was begun at 24 hrs PI, the cultures exhibited general decline beginning after 6 hrs of heat exposure (Fig. 5).

Metabolic profiling of P. falciparum during cell death

In an attempt to identify metabolic processes directly affected by 40°C, we assayed parasite metabolic profiles of protein synthesis, DNA synthesis, and glucose consumption during exposure to 37°C or 40°C and compared the results (Fig. 6). Furthermore, changes in the major metabolic pathways were assayed in the parasites during exposure to chloroquine or control (Fig. 7) and staurosporine or control (Fig. 8). In all cases, 40°C, chloroquine, and staurosporine treatments exerted a significant negative impact on protein synthesis, DNA synthesis, and glucose consumption. In general, staurosporine treated cells exhibited a

significant decrease in all three profiles earlier than parasites treated with chloroquine or 40°C.

Characterization of cell death

To determine whether parasite death, as seen under the influence of 40°C, chloroquine, or staurosporine might be due to apoptosis, we examined the dying parasites for markers often associated with this form of programmed cell death. Much of our work characterizing apoptosis was accomplished in trophozoite stage parasites (24+ hrs PI), because early experiments established that the parasites were sensitive to all three initiators of cell death at that time.

Mitochondrial membrane potential ($\Delta\Psi_m$)

To assay $\Delta\Psi_m$, a cationic lipophilic dye, DiOC₆(3) was used to stain the negatively charged interior of an active mitochondria and measured by flow cytometry. To ensure the changes that we observed in fluorescence were due to depolarization of the mitochondria and not artifact, several controls were used. The working concentration of DiOC₆(3) was calibrated to the cell type tested as overloading can lead to staining of other organelles that may exhibit a charge differential such as the cell membrane or endoplasmic reticulum (Bergeron et al., 1994). Others had determined that DiOC₆ concentrations in the nM range were efficient in detecting changes in mitochondrial membrane potential in *P. falciparum*. Furthermore, they demonstrated that fluorescence, as seen during incubation with low concentrations of DiOC₆(3), was abolished by classical electron transport chain inhibitors such as antimycin, and cyanide (Srivastava et al., 1997). We confirmed that low levels of DiOC₆(3) preferentially stained

mitochondria as observed by fluorescent microscopy. Concentrations of DiOC₆(3) in the range of 10 nM or less exhibited characteristic labeling of the branched mitochondrion within the parasite and low background staining of the *P. falciparum* cytoplasm and erythrocyte (data not shown) (Divo et al., 1985). As a further control, parasites were incubated with carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP). This molecule is a protonophore and specifically disrupts the proton gradient in the inner membrane space of active mitochondria by serving as a membrane-soluble proton carrier. Fluorescence seen in *P. falciparum* stained with DiOC₆(3) was dissipated by incubation with CCCP (Fig. 9) indicating that a change in fluorescence intensity correlated with a change in mitochondrial membrane potential.

Because white blood cells were shown to be a confounding variable in other experiments (discussed later), we sought to ascertain the possible contribution of active mitochondria in contaminating white blood cells to fluorescence in parasite cultures. Whole blood was collected, placed in preservative for 2-10 days at 4°C and divided into two samples. One sample was infected with *P. falciparum* and both blood samples, the infected and uninfected, were incubated at 37°C for 24 hrs. Mitochondrial membrane potential was assayed by DiOC₆(3) labeling of both cultures (Figure 10.) We concluded that cultured blood cells did not contribute significantly to fluorescence seen in $\Delta\Psi_m$ assays of parasite cultures.

We examined the effect of 40°C incubation temperatures as well as chloroquine and staurosporine treatments on the mitochondrial potential of *P.*

falciparum. Large numbers of parasites were grown and synchronized. The synchronized parasites were exposed to control or treatment conditions and then isolated from uninfected erythrocytes by percoll gradient centrifugation. Because of the large initial population of parasites needed (40 -100mm petri dish cultures at high parasitemia were required per time period sampled), each time period was assayed separately. Parasites exhibited loss of $\Delta\Psi_m$ coincident with sensitivity to 40°C treatment at 24 hrs PI and remained low throughout 44 hrs PI (Fig. 11). Parasites exposed to chloroquine (Fig. 12) or staurosporine (Fig. 13) beginning at 24 hrs PI exhibited permanent loss of ($\Delta\Psi_m$) within 8 hrs of exposure to treatment.

Most samples exhibited two clearly defined populations during DiOC₆(3) staining, one with high mean fluorescence intensity (HiMFI) and one with a low mean fluorescence intensity (LowMFI). These two populations likely correlate with parasites that have high mitochondrial membrane potential and those with low mitochondrial membrane potential, respectively. To further analyze results, HiMFI populations and LowMFI populations were examined closer. Both HiMFI and LowMFI peaks were tightly gated, the ratio of events in each population to total events in the sample and the mean fluorescence intensity of each population calculated (Table II). We determined that in most cases the HiMFI and LowMFI populations in treated and untreated cells exhibited similar mean fluorescent intensity, with one notable exception. Parasites grown at 40°C for 44 hrs exhibited a large LowMFI population (Fig. 11C), yet there also was a small HiMFI population. Upon further analysis of the small HiMFI population, it

was apparent that the mean fluorescent intensity of the HiMFI cells in 40°C treated cultures at 44 hrs was half of that seen in the HiMFI population of the 37°C controls at the same time point (Table II, A. lines 5 and 6). This is an indication that the small number of survivors seen in 40°C-treated cells at T44 exhibit lower mitochondrial membrane potential than the parasites kept at 37°C, and may be in the early stages of cell death.

Caspase Assay

Synchronized parasite cultures assessed for pan-caspase activity and for activation of specific caspases 8, 9, and 10 showed unexpected caspase activity in healthy cultures. Parasites treated at 40°C and 37°C for 12 hrs exhibited similar fluorescence profiles when caspase activity was assayed using a pan-caspase probe, even though 40°C treated cells were clearly dying as visualized by giemsa-stained thin films (Fig. 14). Interestingly, parasites treated with staurosporine or control for 12 hrs and then stained with a probe specific for caspase 8 activity exhibited a significant difference in staining. Parasites treated with staurosporine exhibited low fluorescence intensity compared to healthy controls (Fig. 15). In general, caspase 9, and 10 showed similar results to caspase 8 assays in staurosporine treated cells and controls (data not shown).

Oligonucleosomal fragmentation (DNA laddering)

To ensure we extracted an adequate amount of DNA from parasite cultures to visualize DNA laddering if present, human cells (HL-60) were incubated with staurosporine to induce apoptosis and serve as the positive control. Identical parasite cultures were incubated at 37°C or 40°C for 16 hrs. Ten µg of

DNA were extracted from parasites or HL-60 cells and DNA was examined after gel-electrophoresis. We were able to visualize DNA laddering in DNA extracted from HL-60 cells which had been treated with staurosporine (Fig. 16, lane 6) but not in equal amounts of DNA extracted from 37°C or 40°C treated parasites (Fig. 16, lane 2 and 3). Likewise, staurosporine treatment of parasites for 10 hrs did not induce parasite DNA fragmentation (Fig. 17A). It is probable that DNA fragmentation after staurosporine exposure would have been visualized if present, as we were able to visualize DNA laddering in parasite DNA that had been digested with micrococcal nuclease after extraction from cultures (Fig. 17B, lane 3) and not in DNA extracted from staurosporine treated parasites (Fig. 17B lane 5).

In early chloroquine experiments, DNA extracted from parasites exposed to chloroquine often exhibited DNA laddering (Fig. 18A lanes 5, 6). However, DNA laddering was also observed in preserved blood controls after exposure to chloroquine as well (Fig. 18B, lane 1). To determine if the observed ladders in parasites treated with chloroquine resulted from occult white blood cell (WBC) contamination, we immunodepleted WBC's from the erythrocytes by using CD45 coated magnetic beads before culturing with parasites. After cultures were depleted of WBC's, the blood used to culture parasites contained an extremely small amount of DNA, no longer significantly contributing to culture DNA content (Fig. 18 C, lane 1, 2). After elimination of WBC's DNA ladders could no longer be demonstrated in parasite cultures incubated with chloroquine (Fig. 18C, lane 6). Furthermore, DNA ladders could no longer be induced in

parasite DNA by digestion with micrococcal nuclease, instead a smeared band occurred in parasite DNA that had been digested with micrococcal nuclease at ~100 bp (Fig. 18C, lane 5 and 7). Figure 18C is displayed as a reverse image because it improved visualization of the diffuse banding at 100 bp produced by micrococcal nuclease digestion of parasite DNA.

Morphological changes as visualized by light microscopy and ultrastructure analysis

Morphological changes during death were observed by light microscopy and ultrastructure analysis. Ring-stage parasites appeared normal in giemsa-stained thin films during exposure to 40°C. However, after 24 hrs PI, parasites exposed to febrile temperatures began showing abnormalities such as obviously retarded development, most appearing pyknotic with a lesser proportion showing vacuolation (Fig. 19A). Chloroquine treated parasites appeared normal until late ring or early trophozoite stage after which they appeared markedly vacuolated with a few pyknotic forms (Fig. 19B). Every stage of the erythrocytic cycle appeared affected by staurosporine treatment exhibiting obvious retardation of development with a mixture of pyknotic and vacuolated phenotypes (Fig. 19C).

Ultrastructure analysis of trophozoites exposed to 40°C exhibited cytoplasmic condensation after 6 hrs of treatment in 5-6% of parasitized cells (Fig. 20, B), followed by food vacuole swelling and organelle lysis. About 50% of parasites became obviously necrotic after 9 hrs of exposure (Fig. 20, C & D). Parasites treated with chloroquine displayed notable cytoplasmic vacuolation in 10% of parasites after 5 hrs, followed by food vacuole swelling and lysis

beginning 8 hrs after exposure. At 12 hrs of chloroquine exposure >90% of parasites exhibited food vacuole swelling and digestion of the cytoplasm (Fig. 21). Staurosporine-treated cells were similar in appearance to parasites cultured at 40°C, although 50% of parasites exhibited obvious food vacuole swelling and lysis after only 5 hrs of treatment. After 12 hrs of treatment parasite numbers were lower with nearly all parasites exhibiting necrotic morphology (Fig. 22). More than 50 parasites were visualized at each time period.

DISCUSSION

Metabolic profiling during cell death

Initial studies by Kwiatkowski et al. (1989) and Long et al. (2001) determined that temperatures of 40°C caused a marked reduction in parasite number in cultured *P. falciparum*. Their conclusions were based on the notable decreases of parasites in the subsequent culture generation with the logical assumption that febrile temperatures were responsible for reduction of parasitemia. We have confirmed and extended earlier observations by determining that the reduction in the parasite population was indeed a parasite phenomenon and not an artifact of the *in vitro* system. Furthermore, we have confirmed that the parasites became sensitive to 40°C incubation at 20-24 hrs PI and determined that the ring stages exposed to febrile temperatures developed normally when returned to 37°C, unlike trophozoite and schizont stages which once committed, were unable to recover after 36 hrs at 37°C.

Direct measurement of metabolic pathways during exposure to 40°C, chloroquine, and staurosporine showed that each of these treatments had a profound effect on the metabolism of the parasite. Normally ring stages of the parasite are metabolically less active than the trophozoite or schizont stages, with protein synthesis steadily rising from 0 hrs PI to peak at 27 hrs PI during trophozoite stage and progressively declining throughout the remaining erythrocytic cycle (Graeser et al., 1996). In control cultures expected protein synthesis profiles were observed whereas in treated cultures the parasites exhibited a significant decrease in protein synthesis. DNA synthesis was also severely curtailed by all treatments, with parasites showing little to no increase from 0 hrs PI. Normally parasites begin synthesis of DNA in the latter half of the trophozoite cycle, peaking at 30 hrs PI which rapidly tapers off before schizogony occurs (Inselburg and Banyal, 1984). Glucose metabolism is the main source of energy for the parasite. Typically glucose consumption rates remain steady at a low rate during the ring stages of the parasite and exponentially increase as the organism reaches the schizont stage. In all courses of treatment, the parasites exhibited a significant decrease in glucose consumption.

Programmed cell death

Many cells have the ability to initiate an organized pathway of programmed cell death (PCD) in response to outside stimuli or intrinsic messages originating within the cell. The most studied and recognizable form of PCD is apoptosis. This form of PCD consists of specific morphological and physiological changes that are usually precipitated by activation of a family of

cysteine-aspartate proteases called caspases, as previously reviewed in (Kerr, 2002; Guimaraes and Linden, 2004). The phenomenon of programmed cell death is in contrast to another type of cell death known as necrosis, an uncontrolled degradation of the cell occurring after extreme physical or chemical insult (Cohen, 1993). Because parasite death at febrile temperatures was reasonably stage-specific, and since 40°C is probably insufficient in itself to induce necrosis, we initially postulated that febrile temperatures might be inducing an apoptotic-like pathway in the parasites. The evolutionary rationale for such a pathway was that of self-limitation, restricting the erythrocytic cycle and sparing the host long enough to allow for sufficient development and transmission of gametocytes to the mosquito. Gametocyte development begins as merozoites enter red blood cells and a divergent developmental pathway is initiated instead of the standard erythrocytic cycle. Maturation of the gametocyte occurs during sequestration in the bone marrow. After 10 days, the mature gametocytes, infectious to the mosquito, are released into the circulation. Mathematical modeling suggests that given the lengthy maturation cycle of gametocytes, the host would succumb to the erythrocytic stage parasites before adequate transmission of the gametocytes unless there was a limitation to the growth of the erythrocytic cycle (Gravenor and Kwiatkowski, 1998). Furthermore, lower parasitemia is beneficial to gametocyte transmission because high numbers of parasites lead to acidosis in the host. As the pH of the blood reaches 7.2 there is a sharp decrease in gametocyte viability after transmission (Butcher et al., 1996). Mathematical modeling based on untreated infections and recorded fluctuation in host temperature indicates that

fever plays a limiting role on the erythrocytic cycle (Molineaux et al., 2001). The limitation of one stage of a parasite in deference to another has been seen in other unicellular eukaryotes as well. Members of the genera *Trypanasoma* (Ameisen et al., 1995) and *Leishmania* (Zangger et al., 2002) exhibited widespread death in an early stage of the life cycle as a second infectious stage matured. Upon examination of the dying leishmanial parasites, several hallmarks of an apoptotic-like process were discovered. We hypothesized that self-limitation of *P. falciparum* by febrile temperatures could be inducing a similar apoptotic-like program of cell death. Determination of the types of cell death employed in malarial parasites is of particular interest because of their potential role as targets for anti-malarial therapy.

Mitochondrial membrane potential

Changes in mitochondrial membrane potential are frequently used as an index of programmed cell death. Often in the process of apoptosis in higher eukaryotes, $\Delta\Psi_m$ is lost early as molecules used in initiation and regulation of the apoptotic pathway are released from the mitochondria. However, a recent survey of the *P. falciparum* genome has revealed that other than cytochrome c, the parasite contains no homologues of pro-apoptotic proteins often found in the mitochondria such as AIF, or endonuclease G (Deponate and Becker, 2004). We observed loss of $\Delta\Psi_m$ early in the cell death process initiated by all three initiators of cell death but no return of membrane potential. Apoptosis is an active process, and the early initial decrease in $\Delta\Psi_m$ is often, though not always, recovered to allow for energy generation throughout the rest of the pathway

(Kroemer et al., 1998; Skulachev, 2006). Energy generation plays a very important role in the completion of apoptosis. It has been shown that insufficient ATP levels during initiation or progression of apoptosis will quickly lead to a necrotic event in higher eukaryotes (Eguchi et al., 1997). The single mitochondrion in the erythrocytic cycle of *P. falciparum* is achristate in nature, growing from a small disk in the ring stage to a branched web-like organelle in the schizont stages. The tips of the mitochondrial ‘branches’ are broken off during schizogony and each new merozoite receives a small mitochondrion (Divo et al., 1985). Mitochondrial membrane potential is required for parasite survival but has not been linked to ATP synthesis. It has recently been shown that the electron transport chain is used for the sole purpose of dealing with excess electrons that are generated in pyrimidine synthesis (Painter et al., 2007). *Plasmodium falciparum* must synthesize pyrimidines directly as they have no salvage pathway. As *P. falciparum* was transfected with gene constructs coding for *Saccharomyces cerevisiae* pyrimidine synthesis enzymes that utilize alternative electron carriers, the parasite became insensitive to electron chain poisons. Surprisingly, stopping flow of electrons down the electron transport chain did not completely dissipate mitochondrial membrane potential, which was still shown to be necessary for parasite survival. The electron transport chain is only partially responsible for maintenance of the membrane potential in mitochondria. It is hypothesized that reversal of ATP synthase may generate membrane potential as well (Lill and Muhlenhoff, 2005). In cells that do not rely on the mitochondria for energy or release of pro-death molecules, it is unclear

what meaning, dissipation of mitochondrial membrane potential has. In other organisms that do not rely on energy generation from mitochondrial activity, such as yeast grown in excess glucose or eosinophils, mitochondrial activity was shown to still be necessary for the initiation of the early stages of apoptosis, possibly as a generator of reactive oxygen species (Peachman et al., 2001; Pozniakovsky et al., 2005). The role of the mitochondria during apoptotic-like events in other lower eukaryotes is unclear. Both *Leishmania spp.* (Lee et al., 2002) and *Trypanosoma spp.* (Ridgley et al., 1999) have exhibited loss of $\Delta\Psi_m$ relatively early in apoptotic-like death in the parasites. Further experimentation will need to be done to determine the relative importance of early and permanent loss of $\Delta\Psi_m$ during parasite death, and whether it is a cause or effect of the cell death process.

Caspase activity

Caspases are proteases that function as signal transduction molecules and molecular promoters of apoptotic pathways in higher eukaryotes (Degterev et al., 2003). Caspase-like activity has been detected in apoptotic events in protozoans such as *Leishmania spp.* (Arnoult et al., 2002) and the malarial parasite *P. berghei* (Al-Olayan et al., 2002) despite the fact that no caspase homologues have been discovered in these organisms or in any other unicellular eukaryotes (Mottram et al., 2003). However, another related class of cysteine proteases, the metacaspases, has been sequenced in many protozoans (Uren et al., 2000), although their function and possible participation in cell death have yet to be elucidated. We determined that there was no increase of caspase activation

during *P. falciparum* death. In fact, caspase activity was notable in healthy parasites and remained unchanged or decreased as parasite death progressed. This data is in direct contrast to Al-Olayan et al., (2002) and their work in the malarial parasite *P. berghei*. There may be several reasons why this is so. The activity that we observed could have been caspase activity in the erythrocyte host and not the parasite itself. Erythrocytes do have caspases that are active early in red blood cell differentiation (Zermati et al., 2001), however it is unknown what effect *P. falciparum* has on the status of the host caspases. Furthermore, the *P. berghei* experiments were conducted in the extracellular ookinete stage found in the mosquito, which exhibits vastly different gene expression than erythrocytic stages, even within species (Florens et al., 2002). It is unlikely that the caspase-like activity we observed was due to parasite caspase activation because the *P. falciparum* genome contains no caspase sequences (Wu et al., 2003). In Fig 13A, there are four distinct populations of cells, each with increasing fluorescence. This particular pattern of ‘caspase activity’ does not resemble those seen in the apoptotic cells of other organisms, however the distribution is similar that seen in unsynchronized *P. falciparum* cultures that have been stained with the DNA dye Hoechst 33342 or the membrane potential indicator DiOC₁(3) (Jacobberger et al., 1983). In these published assays the populations represented stages of the erythrocytic cycle, such as rings, trophozoites (early and late), and schizonts. During the caspase assays we performed, parasites were not highly synchronized. It is possible that the caspase probe used could be cross-reacting with other parasite proteases. Research has shown that DEVD-FMK type probes, such as the

one used in the study, are able to cross react with papain-like cysteine proteases (Rozman-Pungercar et al., 2003). In viable parasites these probes may have been reacting to cysteine proteases such as falcipain, found in the digestive food vacuole (Rosenthal et al., 1987). The distribution of the fluorescence would then represent the level of protease activity that varies with stages of the erythrocytic cycle, rather than true caspase activity.

DNA degradation

DNA degradation, specifically oligonucleosomal fragmentation leading to production of 180 bp multimers (appearance of DNA ladders on agarose gels) is another common marker of apoptosis (Cohen, 1993). Earlier work reported by Picot et al. (1997) showed DNA laddering in *P. falciparum* after exposure to chloroquine. Initially, we also visualized DNA ladders in parasites exposed to chloroquine, but upon further experimentation determined that the DNA ladders were due to white blood cell DNA contamination in the parasite cultures. It has previously been demonstrated that chloroquine is able to induce apoptosis in peripheral blood lymphocytes (Meng et al., 1997), which explains our findings that chloroquine could induce DNA laddering in blood used to culture the parasites. After measures were taken to deplete the erythrocytes of all white blood cells, chloroquine-induced ladders disappeared. It is probable that the DNA ladders reported by Picot et al. (1997) were due to chloroquine-induced apoptosis in white blood cell contaminants in cultures.

Micrococcal nuclease is an enzyme that has a preference for the 'linker' region DNA which is between nucleosomes (Felsenfeld, 1978). Often during

apoptosis, nucleases are activated which are specific for the internucleosomal or 'linker' regions of DNA such as CAD (caspase-activated Dnase) (Samejima and Earnshaw, 2005). Digestion of intact chromatin by linker-specific nucleases results in DNA fragments in multimers based on nucleosomal spacing, and DNA laddering when visualized through gel-electrophoresis. We used micrococcal nuclease as a positive control in this work. DNA was extracted from parasite cultures and treated with protease inhibitors to keep nucleosomes intact during the extraction process. Extracted DNA was digested with micrococcal nuclease to induce laddering. Ladders were induced in our control cultures, they appeared to have 'rungs' ~180bp apart and showed a lighter smear between the bands (Fig. 17B, and 18A). There are a few possible reasons for the variation in fragment size. During the DNA extraction process it is probable that some nucleosomes were displaced leaving stretches of DNA that were open to random nuclease digestion. Micrococcal nuclease will not produce a ladder if nucleosomes are not intact but instead produces a continuous smear of various sized fragments. Apoptotic nuclease CAD, though specific for linker DNA, does not need intact nucleosomes to produce DNA ladders (Widlak et al., 2000).

Although nucleosome displacement in the parasites may play a role in the variable fragmentation, it is more likely that another scenario is occurring. Once white blood cell DNA was removed from the cultures, we were unable to generate DNA ladders by digestion of parasite DNA with micrococcal nuclease. It is likely that the DNA ladders observed in control parasite cultures in early experiments originated from dead and dying contaminating white blood cells

whose chromatin, in some measure, was no longer intact, thus giving rise to some smearing. After white blood cell removal by magnetic beads coated with CD45, digestion of intact parasite DNA by micrococcal nuclease produced a thick band at about 100 bp (Fig. 18C lanes 5, 7). The origin of this thick band, though parasitic in nature, is not known. Little is known about the nucleosomal spacing of *P. falciparum* or the effect of micrococcal nuclease on parasite DNA. One study used micrococcal nuclease to determine that the space between nucleosomes was 180 bp (Cary et al., 1994). Unfortunately, they used standard blood preservation techniques and did nothing to control for white blood cell contamination. It is likely that the ladders observed and interpreted to determine nucleosomal spacing in that report were actually from white blood cell DNA contamination. The parasite does have 4 core histones which are homologous to higher eukaryotic histones, H2A, H2B, H3, and H4 which form an octamer around which it is presumed parasite DNA is wound (Longhurst and Holder, 1997; Miao et al., 2006). It is possible that the spacing of *P. falciparum* histones varies from higher eukaryotes. Nucleosomal spacing of 100 bp would lead to a DNA ladder with bands at multiples of 100 bp. If digestion were allowed to progress to its endpoint, all DNA would be digested into 100 bp fragment leaving a thick band at 100, as we observed. Further research is needed to determine if this hypothesis is correct.

In other attempts to demonstrate parasite DNA degradation during temperature-induced parasite death, we used the terminal deoxynucleotidyl transferase biotin-dUTP-nick-end-labeling assay (TUNEL) to determine overall

DNA damage by measuring breaks and nicks in the DNA strand. Although we tried several modifications of this assay we were unable to produce consistent results. Caution must be taken when using this assay as it can give false positive results in rapidly dividing cells (manufactures observations). During the erythrocytic cycle, *P. falciparum* rapidly divides into 16-24 new cells, going from 1N to 24N in ~14hrs. Furthermore the exact mechanism of DNA replication and karyokinesis is not a well understood process in *P. falciparum*. Extensive research will have to be conducted to determine the usefulness of the TUNEL assay as a viable marker of DNA damage in *P. falciparum*.

Morphological changes

In the early studies, apoptosis was characterized by morphological changes during cell demise (Kerr et al., 1972). Apoptotic cells were described as appearing shrunken or pyknotic, having condensed cytoplasm and chromatin, intact organelles, and blebbing from the cell membrane. Necrotic cells, on the other hand, exhibited cytoplasmic and organelle swelling followed by rupture of the cell membrane. Previous studies examining the morphology of *P. falciparum* after death induced by exposure to hyper-immune serum showed developmentally retarded and pyknotic parasites, known as “crisis forms” (Jensen et al., 1982). These changes appeared morphologically similar to cells undergoing apoptosis. In the current study, giemsa-stained thin films of parasites undergoing temperature-induced death showed many pyknotic cells reminiscent of apoptosis, while other parasites appeared swollen and heavily vacuolated, resembling

necrotic cells. Parasites killed by staurosporine and chloroquine appeared similar under light microscopy.

Ultrastructural examination of the dying parasites showed that many cells exhibited condensation of the cytoplasm with food vacuole swelling and lysis (Figs. 20-22). We postulate that the parasites appearing vacuolated under light microscopy may represent the parasites with necrotic cytoplasm seen by electron microscopy. Thus early visual changes during death were often reminiscent of cells undergoing apoptosis but subsequent food vacuole swelling and lysis may have induced a 'self digestion' or secondary necrosis of the parasite. The malarial parasite food vacuole is an acidic digestive organelle containing cysteine and aspartic proteases used to digest hemoglobin and polymerize heme (Olliaro and Goldberg, 1995). The homologue to the parasite food vacuole in higher eukaryotic cells is the secondary lysosome. Research on the role of these lysosomes during programmed cell death in higher eukaryotes has shown that large scale leakage or breakdown of the lysosome membrane leads to a necrotic type of death in dying cells, having similar morphology as *P. falciparum* after exposure to heat, chloroquine or staurosporine (Bursch, 2001; Artal-Sanz et al., 2006). Because the current work has focused on the changes seen in parasite death during the later stages of the erythrocytic cycle, when a large food vacuole is active, it would be of interest to characterize parasite death when no food vacuole was present or to attempt to ameliorate the action of food vacuole enzymes.

Heat shock protein 70 (HSP70) is a member of family of chaperone proteins whose homologues are widely distributed from bacteria to higher eukaryotes. Initially discovered after heat exposure, the chaperones promote cell survival by stabilization and refolding of damaged proteins in higher eukaryotes (Hartl, 1996). Later direct interaction of HSP70 with specific molecules involved in death or survival pathways highlighted HSP70's role in regulation of 'life or death' decisions. In human cells, HSP70 can associate with BAG-1, preventing its interaction with the anti-apoptotic factor Bcl-2 (Townsend et al., 2003). Furthermore, apoptosis inducing factor (AIF) is directly inhibited by HSP70, preventing activation of caspase 9 and halting apoptosis (Beere et al., 2000). Of interest to this work, was the recent discovery that HSP70 can be located to the lysosome and serves to stabilize the lysosomal membrane. Upregulation of HSP70 led to a decrease in permeability and leakage of lysosomal cysteine proteases (Nylandsted et al., 2004). Such leakage has been shown to initiate cell death (Bursch, 2001). It would be of interest to determine if PfHSP70 plays a similar role in the parasite food vacuole. If so, it is possible that one purpose of upregulating HSP70 during heat-induced death is to strengthen the food vacuole (lysosome homologue) membrane and prevent cell demise.

Evolutionary rationale

Our results are similar to those recently reported on drug-induced death of the erythrocytic cycle of *P. falciparum* exhibiting no detectable hallmarks of apoptosis (Nyakeriga et al., 2006). However, ours and others' results do not rule out the possibility that *P. falciparum* may exhibit some form of programmed cell

death, though we were unable to find biochemical or physiological evidence of classical apoptosis per se. In many cells undergoing apoptosis or another form of programmed cell death, necrosis is often the eventual end-game of cell death (Unal-Cevik et al., 2004). The possibility remains that febrile temperatures may induce some form of programmed cell death that eventually results in the loss of membrane integrity of the parasite's food vacuole. Lysis of the food vacuole membrane would then lead to an uncontrolled digestion of the parasite, resulting in secondary necrosis in *P. falciparum* as appears to be the case in our study.

Although we were unable to find specific evidence of programmed cell death in the erythrocytic cycle of *P. falciparum*, the evolutionary argument for a type of PCD in malaria still stands. Apoptosis or other forms of programmed cell death were first discovered in multicellular organisms exhibiting several intrinsic and extrinsic pathways. The cell suicide pathways in multicellular organisms seem to have evolved to regulate cell type and number, culling malfunctioning or obsolete cells to protect the organism as a whole. Specifically, PCD has been demonstrated as necessary during embryogenesis and organ sculpting during differentiation, with the goal being the destruction of unnecessary cells (Prindull, 1995; Meier et al., 2000). This pattern is followed throughout the lifecycle of the organism as environments change. One of the most studied examples is that of apoptosis in the human immune system. Apoptosis is used to deplete the immune cell population of non-useful cells before and after a response to a specific pathogen (Williams, 1994). In addition to deleting unwanted cells or cells that have outlived their usefulness, PCD is also a means for multicellular organisms to

stop cells that are malfunctioning or damaged in some way. For instance, cells that have lost control of the cell cycle often initiate a PCD pathway (Alenzi, 2004). Furthermore, many cells that are damaged, for instance by heat shock, will remove themselves from the population using an apoptotic pathway (Punyiczki and Fesus, 1998). Besides elimination of unwanted or damaged cells, the use of PCD to kill cells gives the advantage of having the contents of the cells contained as the cells die, thus not alarming the host immune system and causing an unnecessary inflammatory reaction.

In recent years it has been shown that PCD pathways are not relegated to multicellular organisms. What would be the evolutionary purpose for a single cell to have the ability to select itself from a population? We propose that the evolutionary reasoning might not be much different from those in multicellular organisms. Unicellular parasites have exhibited PCD after exposure to cellular stresses such as heat shock or oxidative damage. *Leishmania donovoni* parasites showed evidence of an apoptosis-like pathway during death initiated by high temperatures (Raina and Kaur, 2006). Another circumstance where PCD may be useful to single celled organisms is during development and differentiation.

Many of the single celled eukaryotes that have displayed evidence of PCD have complex lifecycles with several different stages and host organisms or cells. For example *P. falciparum* has both a warm and cold-blooded host, and differentiates into five major stages each adapted to a separate environment. One stage gives rise to the second. The second stage is destined to infect a different cell or organism than the first. However, for a time they share the same environment,

with the first stage in its preferred host and the second waiting for transmission. At this point they are in direct competition with each other for limited nutrients within the host. It is clear however, that for the lifecycle to continue, the second stage must progress to a new host and is thus, in an evolutionary sense, more important than the first stage. The situation is reminiscent of the rise of different tissues during embryogenesis, when at different times, more of one type of cell is needed and less of another. It would be an advantage to the parasite to somehow limit the first stage to ensure transmission of the second; in a sense, one generation sacrificing itself for the next. This self-imposed limitation could be accomplished by initiation of programmed cell death (Knight, 2002; Nguewa et al., 2004). Such a mechanism has been demonstrated in two species of *Trypanosoma*, described in Welburn et al., 1997. *Trypanosoma cruzi* spends part of its life cycle in a triatomid bug. The infection begins when the bug ingests a trypomastigote during a blood meal. Upon reaching the midgut the trypomastigote differentiates into the (procyclic) epimastigote stage. Epimastigotes are adapted to the mid gut environment and divide rapidly. Eventually some of them differentiate into metacyclic trypomastigotes, the stage that is infectious to the animal host. When grown *in vitro*, the first stage (procyclic) divides rapidly and then begins to differentiate into the second stage (metacyclic trypomastigotes). At this point there is mass cell death in the procyclic stage. When assayed, the dying procyclics exhibited many signs of PCD, specifically apoptosis, such as self-containment, chromatin condensation, DNA laddering, and membrane blebbing (Ameisen et al., 1995). Similar results

were discovered in *Trypanosoma brucei rhodesiense* as well, with the addition that the apoptosis-like process required protein synthesis (Welburn et al., 1996). The trypanosomatids are one of the earliest diverging eukaryotes, and it is likely that later-diverging eukaryotes such as *P. falciparum* would have a similar mechanism in place.

As previously discussed, an apoptosis-like process was discovered in the mosquito mid-gut stages of *P. berghei*, during the differentiation of ookinetes to the oocyst stage *in vivo*. The dying ookinetes showed several hallmarks of apoptosis, such as self-containment, phosphatidyl serine flip, chromatin condensation and DNA degradation (Al-Olayan et al., 2002). From an evolutionary stand point, a form of programmed cell death may yet be discovered in the erythrocytic cycle of the parasite while it is in competition with the next stage (gametocytes) as a means of limitation of the erythrocytic cycle. It would be of interest to characterize the effect of febrile temperatures on parasites *in vivo* or in newly adapted cultures. It is possible that cells kept in long term culture, such as the ones used in this study, may have lost the ability to self-initiate and control their demise, whereas natural selection maintains such a program of cell death in wild populations.

Summary

Others have observed that the erythrocytic cycle of *P. falciparum* exhibits decreasing parasitemia following exposure to 40°C in culture. We determined that this decrease is a direct result of 40°C temperatures on the parasite and not an artifact of the *in vitro* culture system. Furthermore, we

determined that 40°C has little effect on early stages of the parasite, which can recover completely if returned to 37°C. At 24 hrs into the lifecycle, as the parasite transitions into a metabolically active stage, it begins to exhibit sensitivity to 40°C treatment. Heat has a profound effect upon the parasite's major metabolic pathways at this point, causing inhibition of protein synthesis, DNA synthesis and glucose metabolism.

We hypothesized that death induced by 40°C incubation could be an apoptosis-like process as others had observed morphological and physiological characteristics in *P. falciparum* during death (Picot et al., 1997; Al-Olayan; 2002). Parasites may limit the erythrocytic cycle to ensure transmission of the gametocytes. During characterization of cell death we expanded the mechanisms of cell death induction to include chloroquine and staurosporine. We determined that 40°C, chloroquine, and staurosporine induced death in *P. falciparum* that was lacking many of the morphological and physiological characteristics of apoptosis.

All three methods induced early, long-term loss of mitochondrial membrane potential. In higher eukaryotes this can be a sign of a necrotic event, however little is known about the significance of dissipation of mitochondrial membrane potential during cell death in lower eukaryotes. More study will have to determine what role if any the mitochondrion plays in the parasite during death.

Caspase activation is another classical hallmark of apoptosis in higher eukaryotes and similar activity has been shown in the malarial parasite *P. berghei* (Al-Olayan, 2002). We observed that the caspase assay used by Al-Olayan (2002) and others is ineffective in the erythrocytic cycle of *P. falciparum*.

Healthy parasites often stained positive for caspase activity whereas dead and dying parasites exhibited equal or less staining. It is likely that the probe used is cross reacting with other proteases found within the parasite.

DNA degradation, specifically the formation of DNA ladders is a classical hallmark of apoptosis seen in both higher and lower eukaryotes. We observed no DNA laddering during cell death in any form. Furthermore, we showed that previous DNA laddering seen in *P. falciparum* was an artifact, likely due to white blood cell contamination in parasite cultures (Picot et al., 1997).

Originally, characteristic changes in morphology were cardinal in differentiation between apoptosis and necrosis. Morphologically, the parasites exhibited early visual signs of apoptosis, being condensed and pyknotic. However, all three initiators of cell death led to food vacuole swelling and a complete digestion of the cell, presumably from leakage of food vacuole enzymes into the cytoplasm causing a secondary necrosis. Further study is necessary to determine the exact mechanism of food vacuole lysis and the level of control that the cell has over the process.

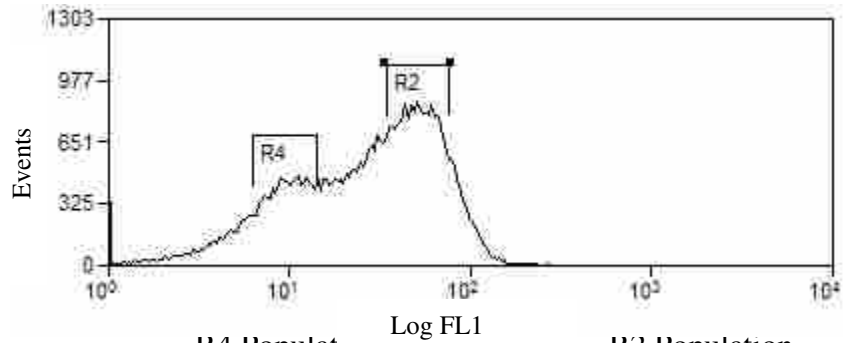
Continuing study of cell death in *P. falciparum* is of importance to further decipher cell death pathways in the parasite. If the cell death pathways can be elucidated they may open up a novel avenue of malarial treatment. Cell death molecules may serve as targets for anti-malarial drugs, giving a much needed addition to our side in the war with malaria.

**A Comparison of Typical Morphological and Physiological Characteristics
Found During Apoptosis or Necrosis in Metazoan Cells**

	Apoptosis	Necrosis	
Pyknotic in appearance	+	-	(Kerr et al., 1972)
Cell membrane intact	+	-	(Kerr et al., 1972)
Cytoplasmic vacuolization	-	+	(Kerr et al., 1972)
Chromatin condensation/marginalization	+	-	(Wyllie et al., 1984)
Organelle morphology	Intact	Early Swelling Lysis	(Wyllie, 1980)
Nuclear fragmentation	+	-	(Cohen, 1993)
Phosphatidyl serine exposure	+	-	(Fadok et al., 1992)
Decrease in $\Delta\Psi_m$	Transient/Late	Early Permanent	(Skulachev, 2006)
Protein synthesis required	+/-	-	(Cohen, 1991) (Martin et al., 1990)
Active process	+	-	(Skulachev, 2006)
TUNEL	+	-/+	(Sellins and Cohen, 1991)
Internucleosomal DNA fragmentation (200bp ladder)	+	-	(Wyllie, 1980)
Modified by Caspase inhibitors	+	-	(Ekert et al., 1999)

Table I.

Mitochondrial Membrane Potential in *Plasmodium falciparum* during exposure to 40°C , Chloroquine, and Staurosporine



	<u>R4 Population</u>		<u>R2 Population</u>	
	% of total population	MFI±S.D.	% of total population	MFI±S.D.
A.				
37°C T24	19.1	9.8 ±2.2	36.2	51.6 ± 11.8
40°C T24	42.2	13.24 ± 3.1	7.9	59.6 ± 12.25
37°C T32	8.2	24.11 ± 5.5	40.62	341.6 ± 59.3
40°C T32	47.8	14.92 ± 3.31	≤1	-----
37°C T44	7.6	22.5 ± 7.0	46.3	624.8 ±104.3
40°C T44	39.7	21.8 ± 4.11	3.2	335.4 ± 58.8
B.				
Control T2	3.7	52.2 ± 9.5	57.8	395.0 ± 67.5
CQ T2	10.1	20.1 ± 6.0	34.8	339.0 ± 59.0
Control T6	2.0	11.81 ± 3.4	49.3	334.0 ± 54.7
CQ T6	28.8	17.8 ± 4.3	15.8	247.5 ± 69.9
Control T9	7.4	14.24 ± 3.56	49.2	191.75 ± 47.2
CQ T9	42.9	7.8 ± 1.9	5.3	114.0 ± 30.8
Control T12	5.5	14.68 ±3.64	52.0	229.9 ± 92.6
CQ T12	39.8	18.0 ± 4.4	6.8	310.0 ± 64.7
C.				
DMSO T8	27.1	12.4 ± 3.0	26.6	127.7 ± 34.2
Staur T8	52.4	12.53 ± 3.0	≤1	-----
DMSO T16	35.4	12.5 ± 3.0	15.51	531.8 ± 145.8
Staur T16	47.5	14.7 ± 3.8	≤1	-----

Mitochondrial membrane potential of *P. falciparum* was assessed by DiOC₆(3) staining. Generally there were two distinct cell populations exhibiting more (R2) or less (R4) fluorescence intensity. Each population was narrowly gated (as shown in diagram above-37°C T24) to contain the population peak. Mean fluorescence intensity (MFI) and percentage of total population was calculated for each population. **A)** Synchronized parasites kept at 37°C or 40 °C from 0 hrs post invasion(PI) at T24, T32, or T44 hrs. **B)** Synchronized parasites incubated with 100 μM chloroquine (CQ) or control from 24 hrs PI at T2, T6, T9, or T12 hrs. **C)** Synchronized parasites incubated with 5 μM staurosporine (Staur) or control from 24 hrs PI at T8 or T16 hrs.

Table II.

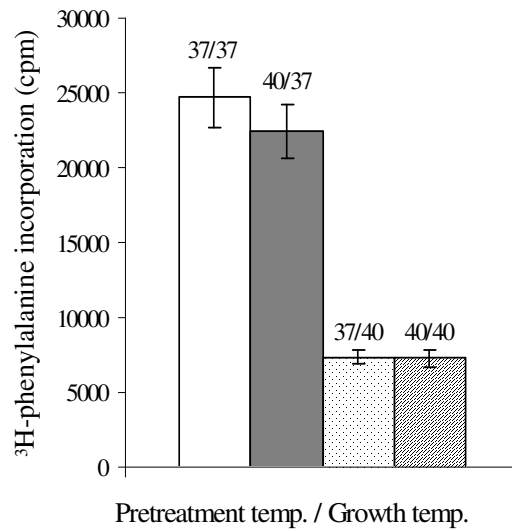


Figure 1. Febrile temperature (40°C) did not alter culture medium’s ability to support growth of *Plasmodium falciparum*. Culture medium was incubated at 37°C or 40°C for 24 hr. The pre-treated media was labeled with ³H-phenylalanine and incubated at 37°C and 40°C with healthy parasites for 24 hr. Error bars demonstrate a 95% confidence interval.

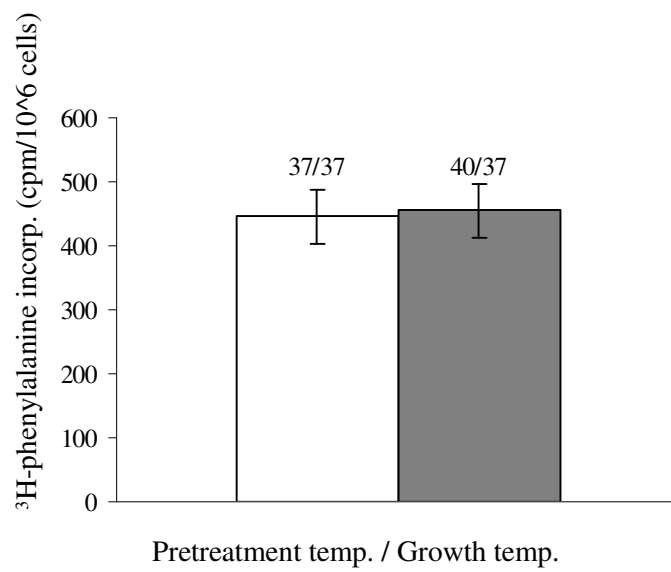


Figure 2. Erythrocytes incubated at 40°C show no long term decrease in ability to support *P. falciparum*. Red blood cells were incubated at 37 °C or 40°C for 48 hr. Late stage parasites were then allowed to invade the pre-treated erythrocytes for 8 hr at 37 °C. ³H-phenylalanine incorporations were performed on both cultures of newly-invaded erythrocytes.

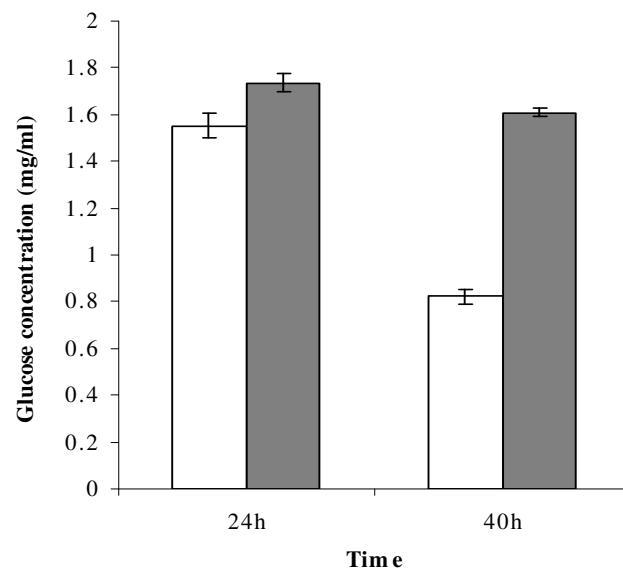


Figure 3. Exposure to febrile temperature decreases glucose metabolism in *Plasmodium falciparum*. Synchronized cultures were incubated at 37 °C or 40 °C beginning at 0 hr post-invasion (PI). Culture media was assayed for glucose concentration at 24 and 40 hrs. Error bars represent 95% confidence intervals.

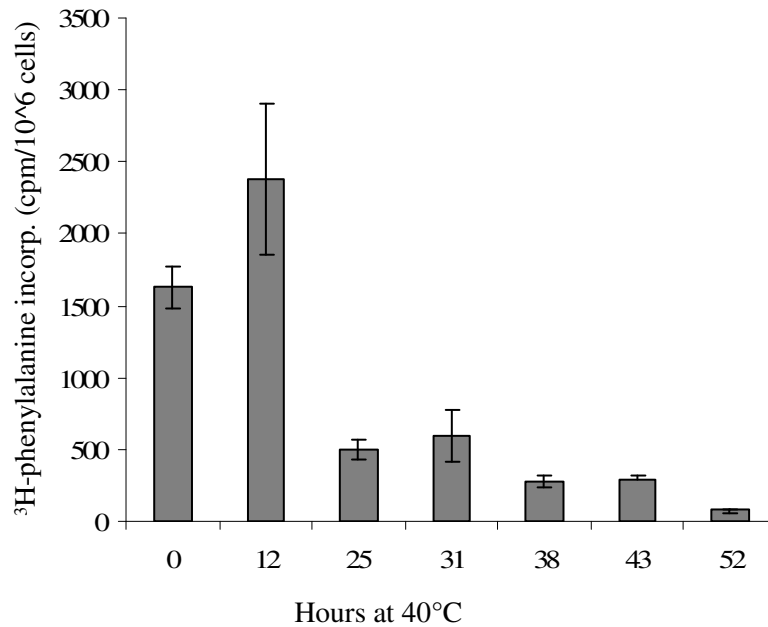


Figure 4. *Plasmodium falciparum* becomes sensitive to 40°C after 24 hrs. Synchronized parasites were incubated at 40°C at 0 hr post invasion (PI) for various time periods. Afterwards, cultures were returned to 37 °C for ≥36hours. Growth was assessed by ³H-Phenylalanine incorporation and visual counts. (Gamette, 2003)

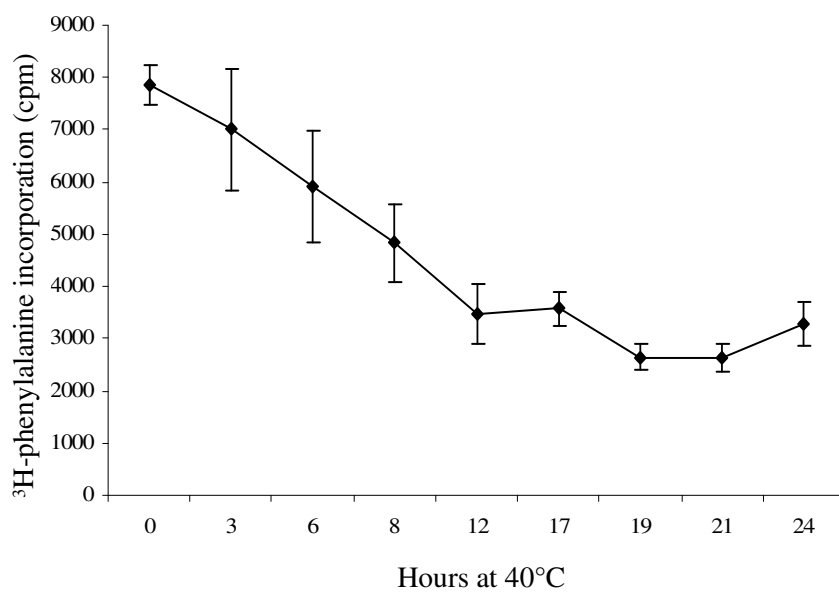


Figure 5. Trophozoites (24 hr PI) exhibited detectable sensitivity to 40°C after 6 hrs. Synchronized parasites incubated at 40°C beginning at 24 hr post-invasion (PI) for various time periods then transferred to 37°C for ≥24 hrs for recovery. Afterward, growth was assessed by ³H-phenylalanine incorporation and confirmed by visualization of geimsa-stained thin films. (Gamette, 2003)

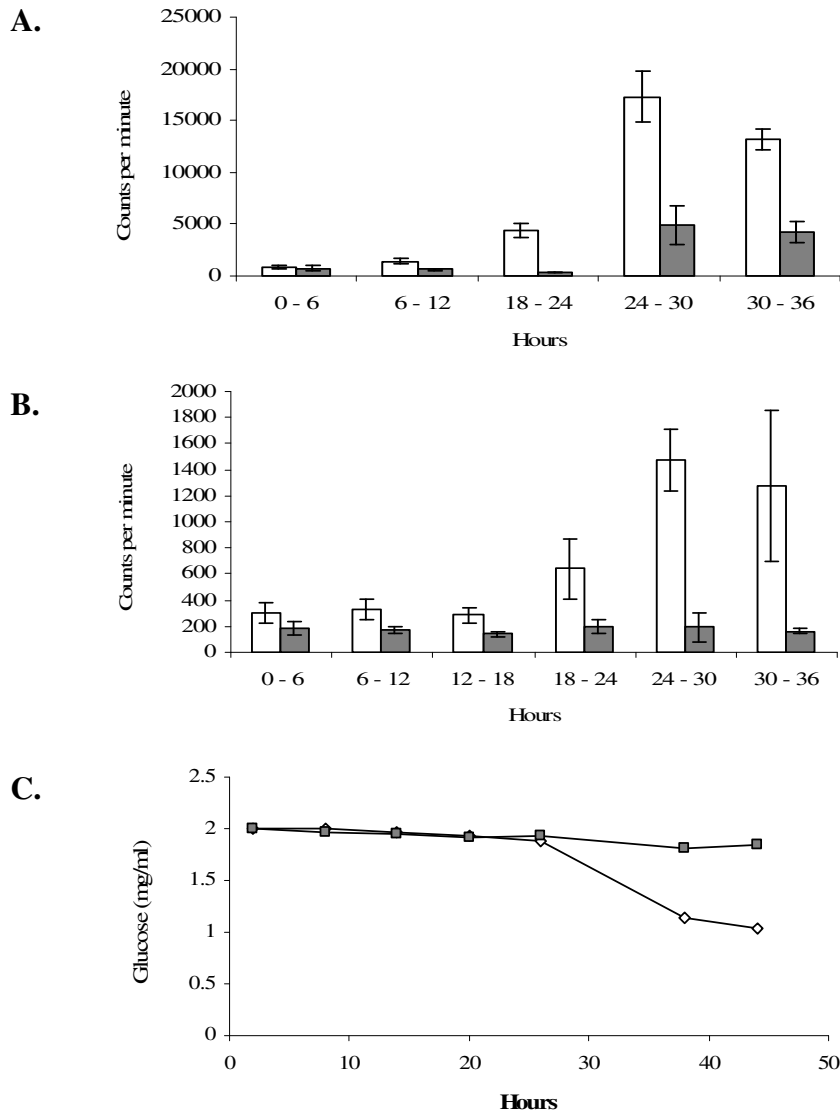


Figure 6. Exposure of *Plasmodium falciparum* to 40°C leads to inhibition of protein synthesis, DNA synthesis, and a reduction in glucose consumption. Synchronized parasites were incubated at 37°C □ or 40°C ■ beginning at 0 hrs post invasion (PI). A) Protein synthesis measured by [³H]-phenylalanine incorporation in 6 hr increments. B) DNA synthesis as measured by [³H]-hypoxanthine incorporation in 6 hrs increments. C) Glucose metabolism measurement. Y-axis represents concentration of glucose in culture media after parasite growth.

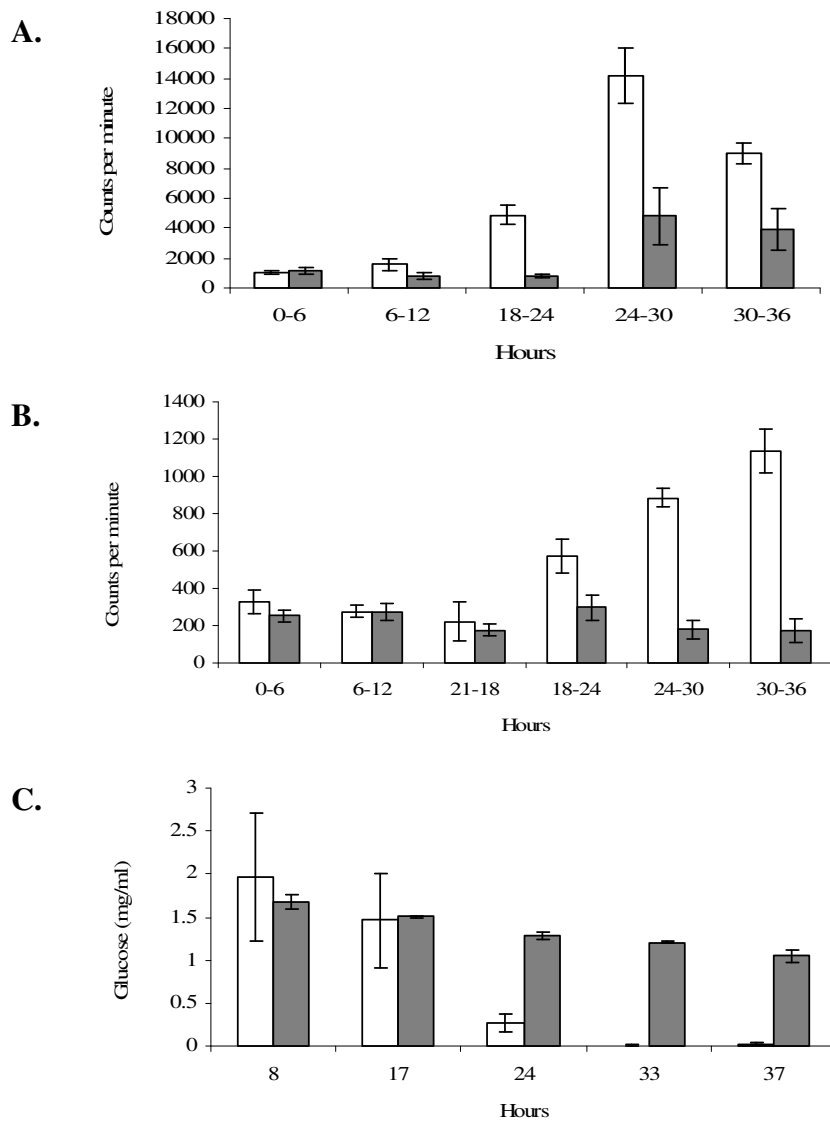


Figure 7. Exposure of *Plasmodium falciparum* to chloroquine (100 μ m) leads to inhibition of protein synthesis, DNA synthesis, and a reduction in glucose consumption. Synchronized parasites were incubated with control \square or chloroquine \blacksquare beginning at 0 hrs post invasion (PI). A) Protein synthesis measured by [3 H]-phenylalanine incorporation in 6 hr increments. B) DNA synthesis as measured by [3 H]-hypoxanthine incorporation in 6 hr increments. C) Glucose metabolism measurement. Y-axis represents concentration of glucose in culture media after parasite growth.

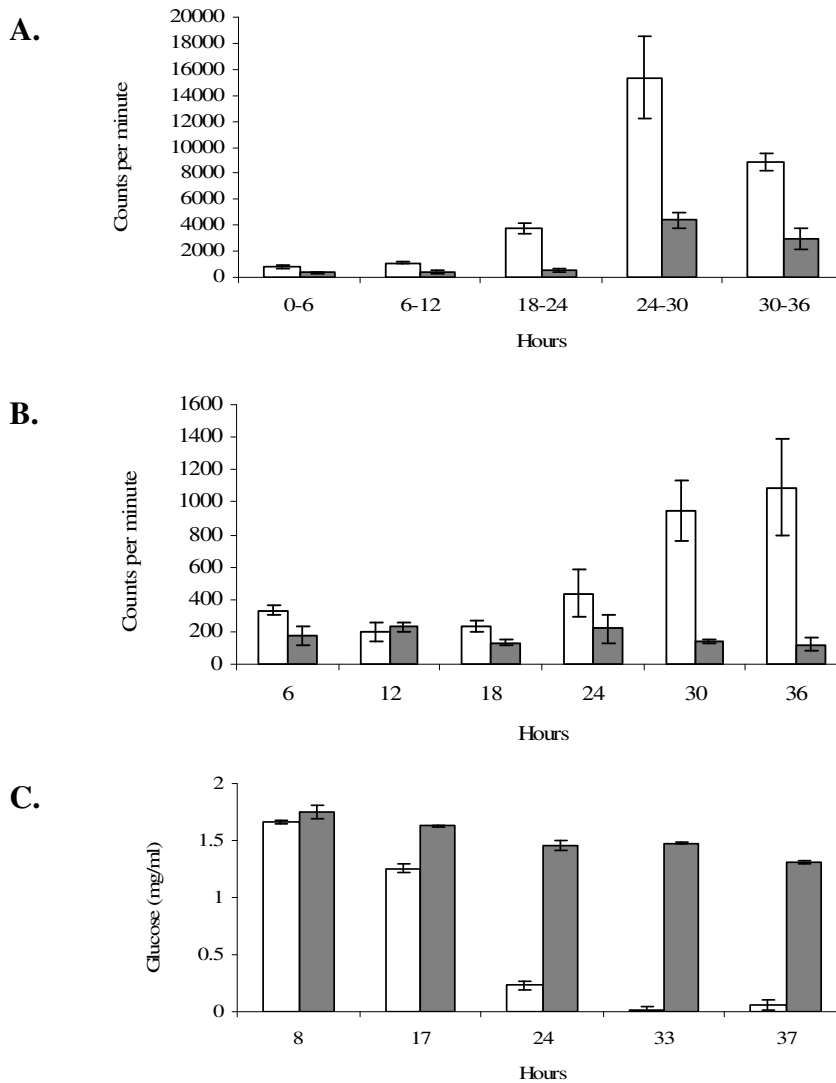
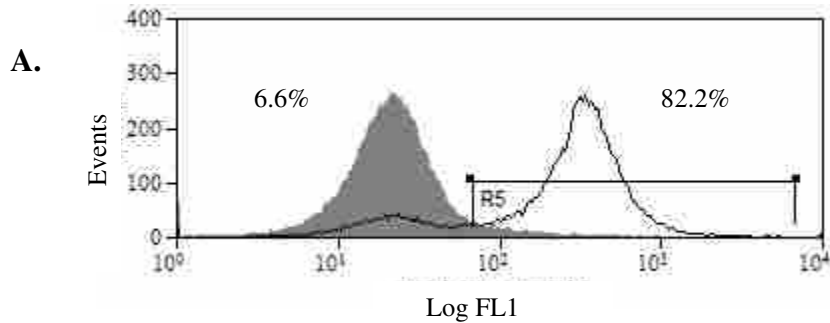
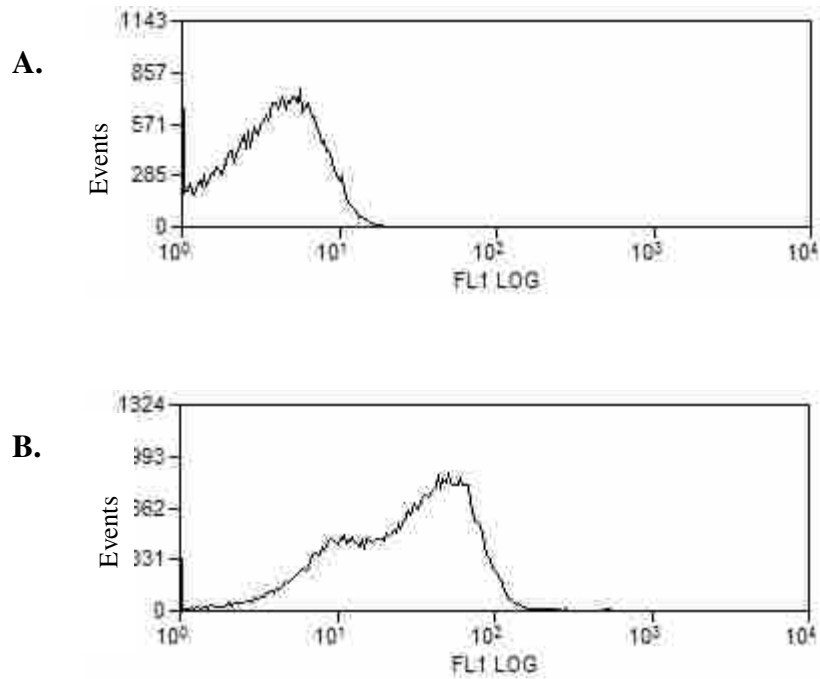


Figure 8. Exposure of *Plasmodium falciparum* to staurosporine (5 μ M) leads to inhibition of protein synthesis, DNA synthesis, and a reduction in glucose consumption. Synchronized parasites were incubated with DMSO (□) or staurosporine (■) beginning at 0 hrs post invasion (PI). A) Protein synthesis measured by [3 H]-phenylalanine incorporation in 6 hr increments. B) DNA synthesis as measured by [3 H]-hypoxanthine incorporation in 6 hr increments. C) Glucose metabolism measurement. Y-axis represents concentration of glucose in culture media after parasite growth.



DiOC₆(3)

Figure 9. Mitochondrial membrane potential dissipated in *P. falciparum* by addition of the protonophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP). Determination of mitochondrial membrane potential ($\Delta\Psi_m$) in *P. falciparum* as assessed by DiOC₆(3) staining. A) Synchronized parasites kept at 37°C for 32 hrs □. B) Identical culture incubated with the protonophore, CCCP, prior to DiOC₆(3) staining ■. Positioning of the R5 gate was determined using a consistent difference in fluorescence intensity exhibited between parasite populations incubated with and without CCCP. Number labels are the percentage of events in each sample that exhibited fluorescence intensity within the boundaries established by R5 with CCCP treated sample numbers on the left and ethanol control treated sample numbers on the right.



DiOC₆(3)

Figure 10. No significant mitochondrial membrane activity in cultured erythrocytes. Determination of mitochondrial membrane potential ($\Delta\Psi_m$) as assessed by DiOC₆(3) staining in A) Uninfected erythrocytes kept at 37°C for 24 hrs B) *Plasmodium falciparum* synchronized into erythrocytes and incubated at 37°C for 24 hrs. Parasites were isolated by percoll gradient before staining.

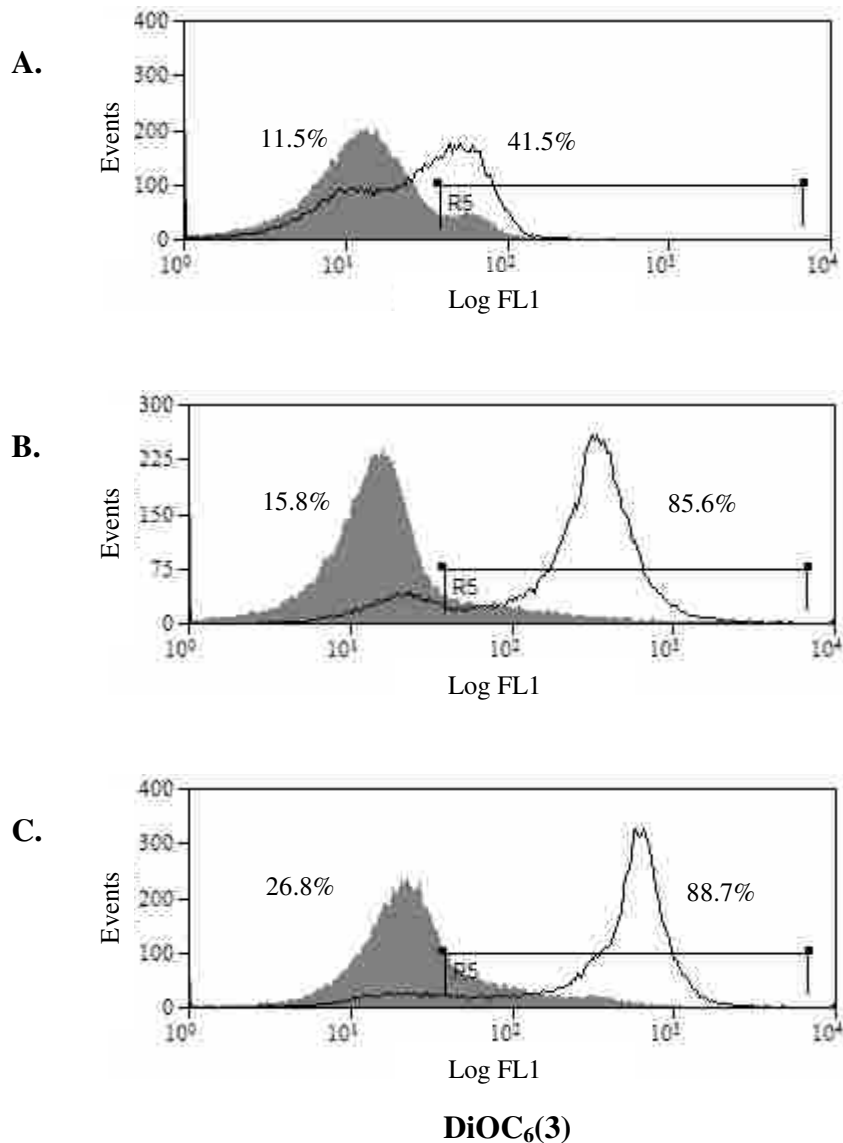


Figure 11. Exposure of *Plasmodium falciparum* to 40°C leads to a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). In separate experiments, synchronized parasites were incubated at 37°C □ or 40°C ■ beginning at 0 hrs Post Invasion (PI). Measurement of $\Delta\Psi_m$ was determined by flow cytometry using DiOC₆(3) staining at A) 24 hrs PI B) 34 hrs PI and C) 44 hrs PI. Y-axis represents number of events. X-axis corresponds to the intensity of fluorescence (mitochondrial activity) on a log scale. Positioning of the R5 gate was determined using a consistent difference in fluorescence intensity exhibited between parasite populations incubated with and without CCCP. Number labels are the percentage of events in each sample that exhibited fluorescence intensity within the boundaries established by R5 with 40°C treated sample numbers on the left and 37°C treated sample numbers on the right.

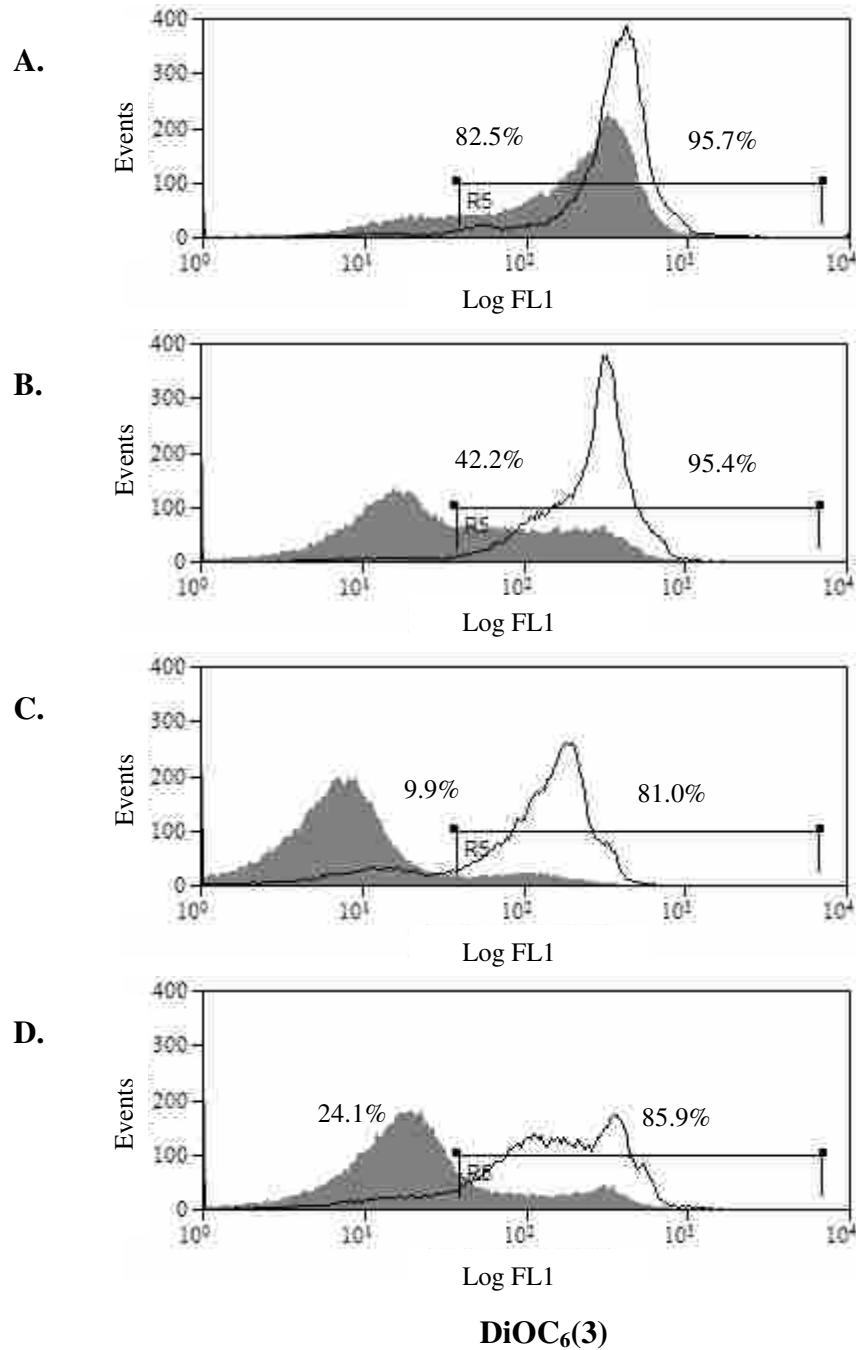
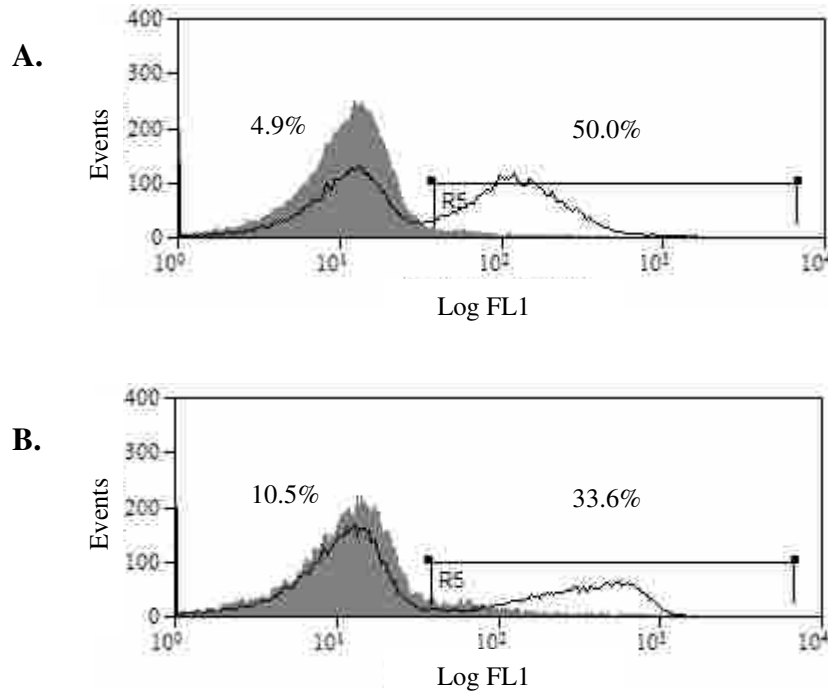


Figure 12. Exposure of *Plasmodium falciparum* to chloroquine leads to a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). In separate experiments synchronized parasites were incubated with control □ or chloroquine ■ beginning at 24 hrs post invasion (PI). Measurement of $\Delta\Psi_m$ was determined by flow cytometry using DiOC₆(3) staining at A) 2 hrs B) 6 hrs C) 9 hrs D) 12 hrs. Y-axis represents number of events. X-axis corresponds to the intensity of fluorescence (mitochondrial activity) on a log scale. Positioning of the R5 gate was determined using a consistent difference in fluorescence intensity exhibited between parasite populations incubated with and without CCCP. Number labels are the percentage of events in each sample that exhibited fluorescence intensity within the boundaries established by R5 with chloroquine treated sample numbers on the left and control treated sample numbers on the right. (Gamette, 2003)



DiOC₆(3)

Figure 13. Exposure of *Plasmodium falciparum* to staurosporine leads to a decrease in mitochondrial membrane potential ($\Delta\Psi_m$). In separate experiments, synchronized parasites were incubated with control or staurosporine at 24 hrs PI. Measurement of $\Delta\Psi_m$ was determined by flow cytometry using DiOC₆(3) staining at A) 8 hrs B) 16 hrs. Y-axis represents number of events. X-axis corresponds to fluorescence intensity (mitochondrial activity) on a log scale. Positioning of the R5 gate was determined using a consistent difference in fluorescence intensity exhibited between parasite populations incubated with and without CCCP. Number labels are the percentage of events in each sample that exhibited fluorescence intensity within the boundaries established by R5 with staurosporine treated sample numbers on the left and control treated sample numbers on the right. (Gamette, 2003)

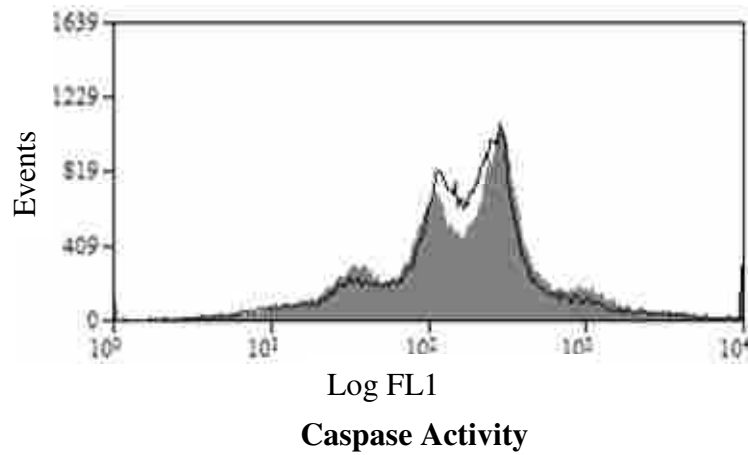
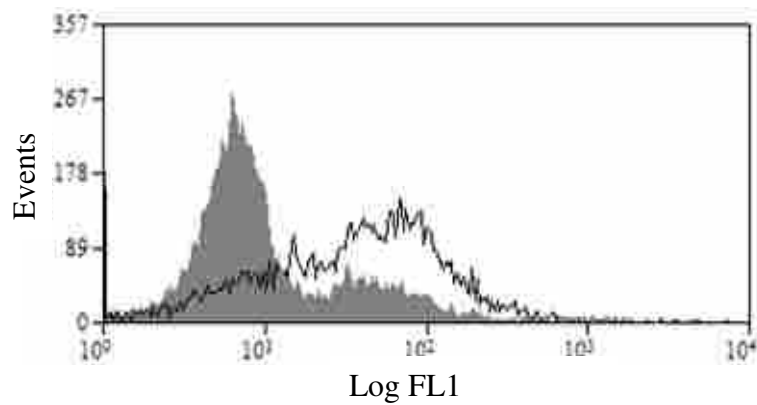


Figure 14. *Plasmodium falciparum* exhibits no increase in caspase-like activity during death induced by exposure to 40°C. Unsynchronized parasites were incubated at 37°C □ or 40°C ■ for 12 hrs. Caspase activity was measured using a pan-caspase detector.



Caspase Activity

Figure 15. *Plasmodium falciparum* exhibits a decrease in caspase 8 probe binding after staurosporine (5 μ M) treatment. Unsynchronized parasites were incubated at 37°C or 40°C for 12 hrs during the second half of the erythrocytic cycle. Caspase activity was measured using a probe specific for caspase 8. (Gamette, 2003)

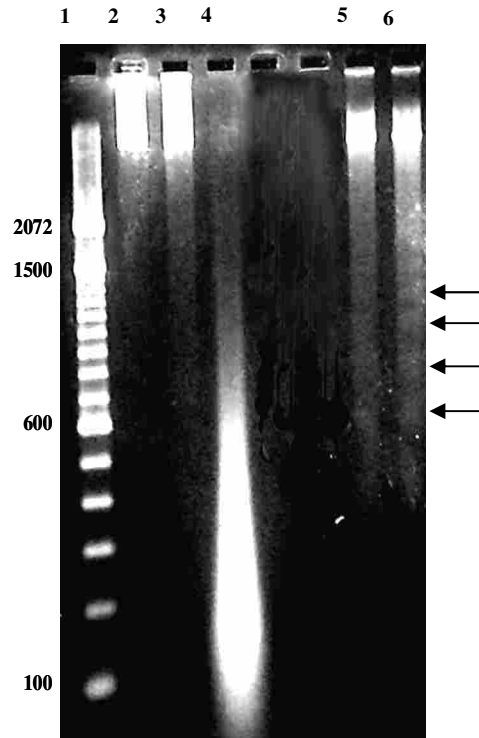
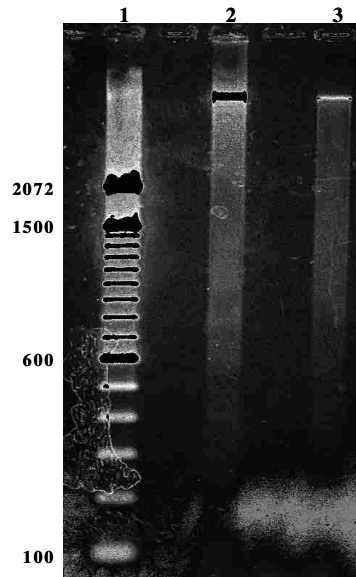


Figure 16. *Plasmodium falciparum* did not exhibit DNA laddering after exposure to 40°C. Parasites were exposed to 37°C or 40°C for 16 hrs. **1)**100bp size ladder; **2)**37°C; **3)** 40°C; **4)**37°C DNA digested with DNase I; **5)** HL-60 cells; **6)** HL-60 cells treated with staurosporine (5µM) for 12 hrs to induce apoptosis with typical DNA laddering. Arrows indicated DNA bands. All lanes contained 10 µg DNA. Unless otherwise indicated, all DNA comes from *P. falciparum* cultures.

A.



B.

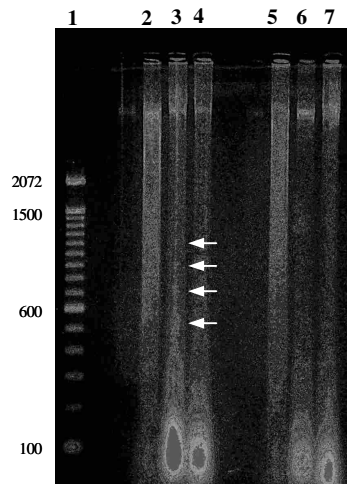


Figure 17. *Plasmodium falciparum* did not exhibit DNA laddering after exposure to staurosporine (5 μ M). **A)** Parasites treated with staurosporine or DMSO control for 10 hrs. **1)** 100bp size marker; **2)** staurosporine; **3)**DMSO. 9.5 μ g DNA in each lane. **B)** Parasites treated with staurosporine (5 μ M) or DMSO control for 10 hrs. **1)** size marker; **2)** DMSO; **3)** DMSO, DNA digested with micrococcal nuclease to induce DNA laddering for 1 min. **4)**DMSO, DNA digested with micrococcal nuclease to induce DNA laddering for 3min; **5)** staurosporine; **6)** staurosporine, DNA digestion with micrococcal nuclease for 1 min. **7)** staurosporine, DNA digestion with micrococcal nuclease for 3 min. Arrows indicated DNA bands.

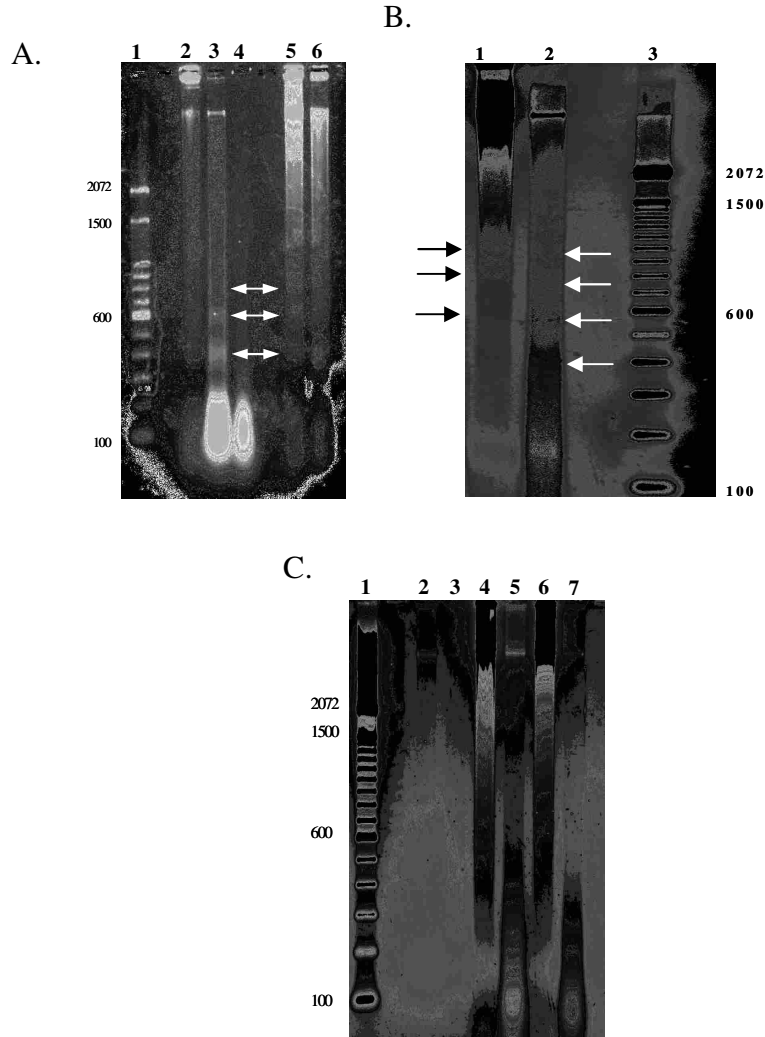


Figure 18. *Plasmodium falciparum* exhibited no detectible DNA laddering after exposure to chloroquine. **A)** Parasites exposed to chloroquine (100 μ M) or solvent control for 8 hrs. **1)** size marker; **2)** control; **3)** control digested with micrococcal nuclease for 1 min; **4)** control digested with micrococcal nuclease for 3 min. **5)** and **6)** chloroquine. **B)** Preserved blood kept at 4°C for 3 days. **1)** preserved blood cells treated with chloroquine (100 μ M) for 8 hrs; **2)** preserved blood cells digested with micrococcal nuclease; **3)** size ladder. **C)** Parasites were grown in white blood cell (WBC) depleted erythrocytes exposed to chloroquine (100 μ M) or solvent control for 8 hrs. **1)** size marker; **2)** WBC-depleted erythrocytes; **3)** WBC-depleted erythrocytes, digested with micrococcal nuclease; **4)** control parasites; **5)** control parasites digested with micrococcal nuclease; **6)** chloroquine treated parasites; **7)** chloroquine treated parasites, digested with micrococcal nuclease. Lanes 2 and 3 contained <1 μ g DNA. Panel C is displayed in reverse image to enhance contrast of 100 bp banding in lanes 5 and 7.

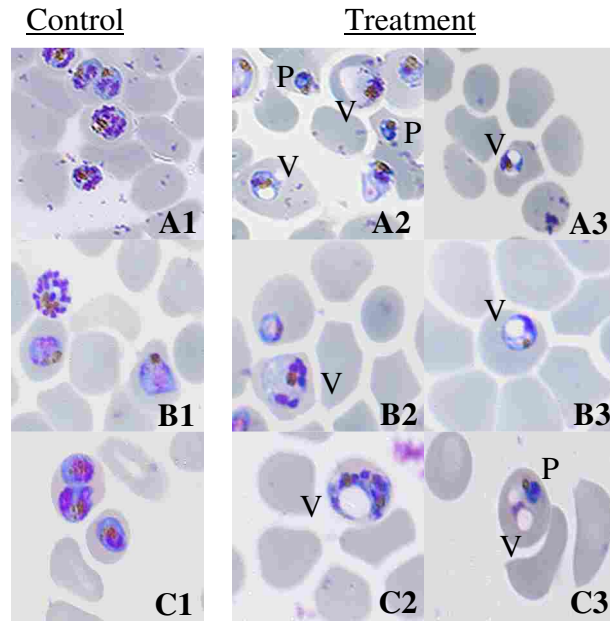


Figure 19. Visual assessment of the trophozoite stages of *Plasmodium falciparum* exposed to 40°C, staurosporine (5 µM), chloroquine (100 µM), and solvent or temperature controls after 9 hrs exhibited a mixture of pyknotic (P) and vacuolated (V) cells. **A1)** 37°C; **A2)** and **A3)** 40°C. **B1)** chloroquine control; **B2)** and **B3)** chloroquine. **C1)** staurosporine control; **C2)** and **C3)** staurosporine. Giemsa-stained thin films displayed at 1000x magnification.

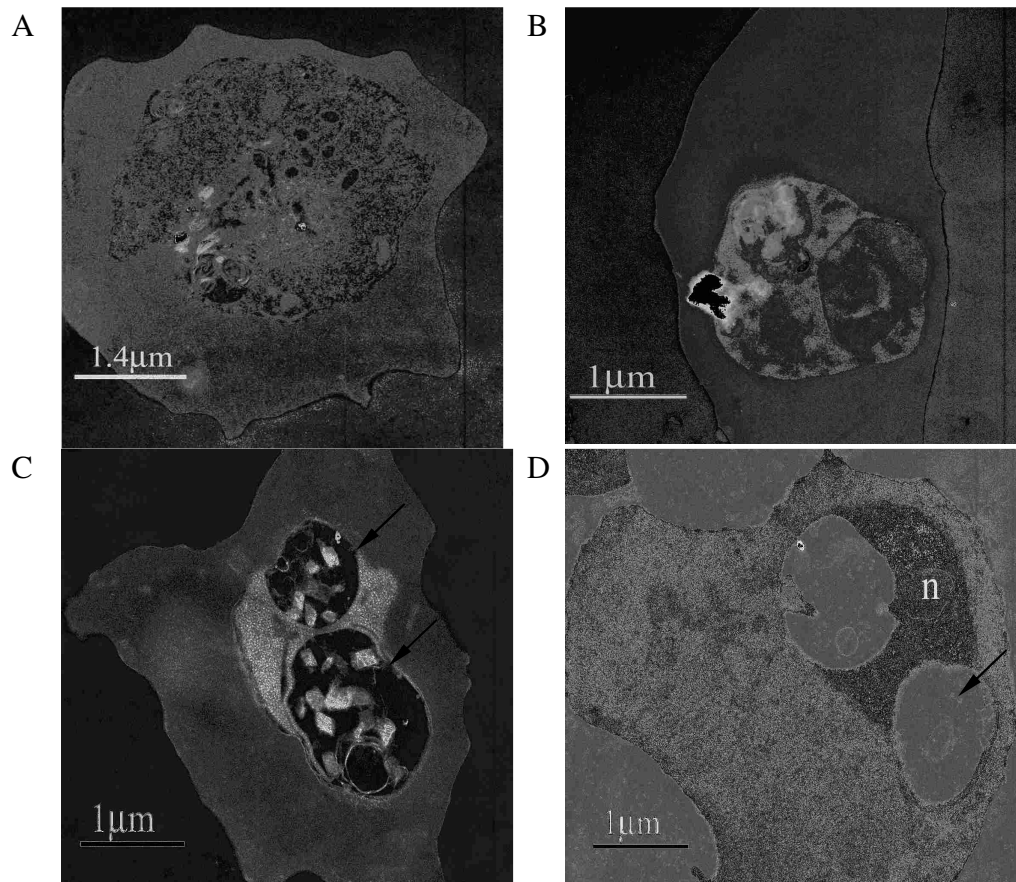


Figure 20. Ultrastructure examination of the erythrocytic cycle of *Plasmodium falciparum* shows condensation of cytoplasm with food vacuole swelling followed by digestion of the cell after exposure to 40°C. **A)** 37°C, 6 hrs 11,000x; **B)** 40°C, 6 hrs 15,000x; **C)** 40°C, 9 hrs 15,000x; **D)** 40°C, 9hrs 15,000x. Arrows indicate swollen food vacuoles. n represents nucleus

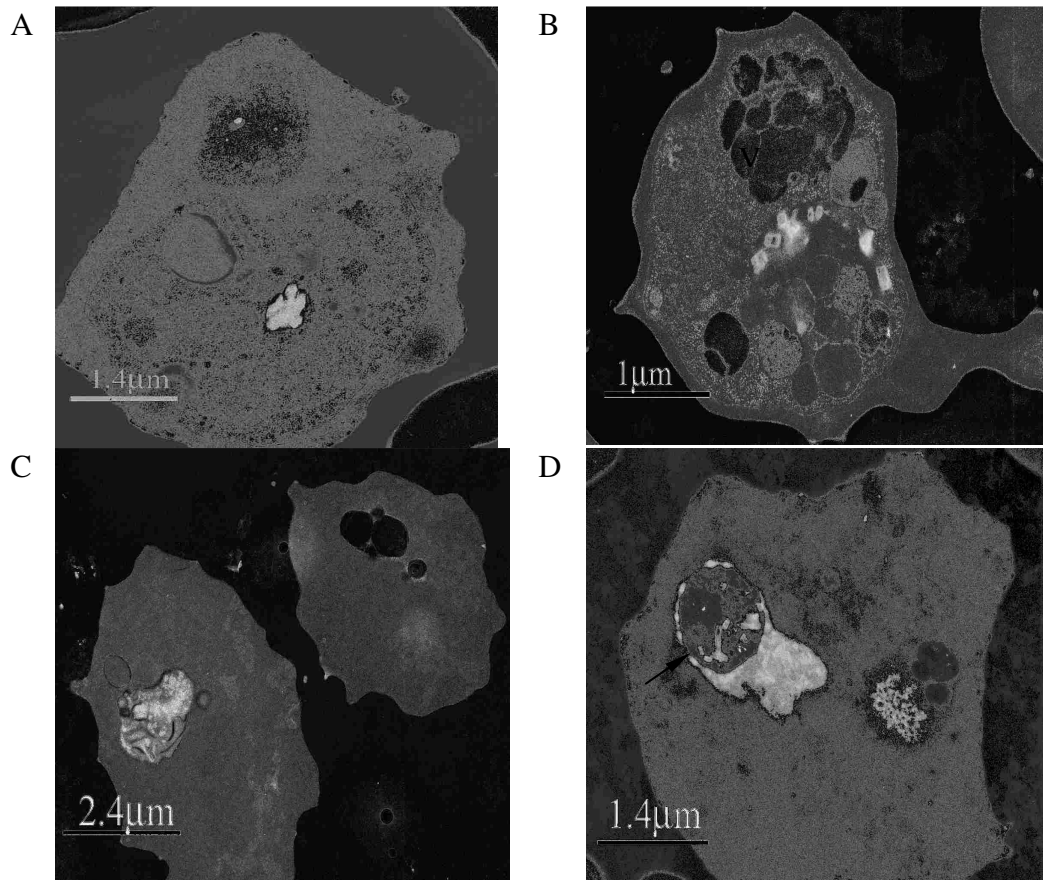


Figure 21. Ultrastructure examination of the erythrocytic cycle of *Plasmodium falciparum* shows extensive vacuolation of the cytoplasm with food vacuole swelling followed by digestion of the cell after exposure to chloroquine (100 μ M). **A)** solvent control, 12 hrs 11,000x; **B)** chloroquine, 4 hrs 11,000x; **C)** chloroquine, 12 hrs 6,500x; **D)** chloroquine, 12hrs 11,000x. Arrow indicate food vacuole swelling. V indicates vacuolization.

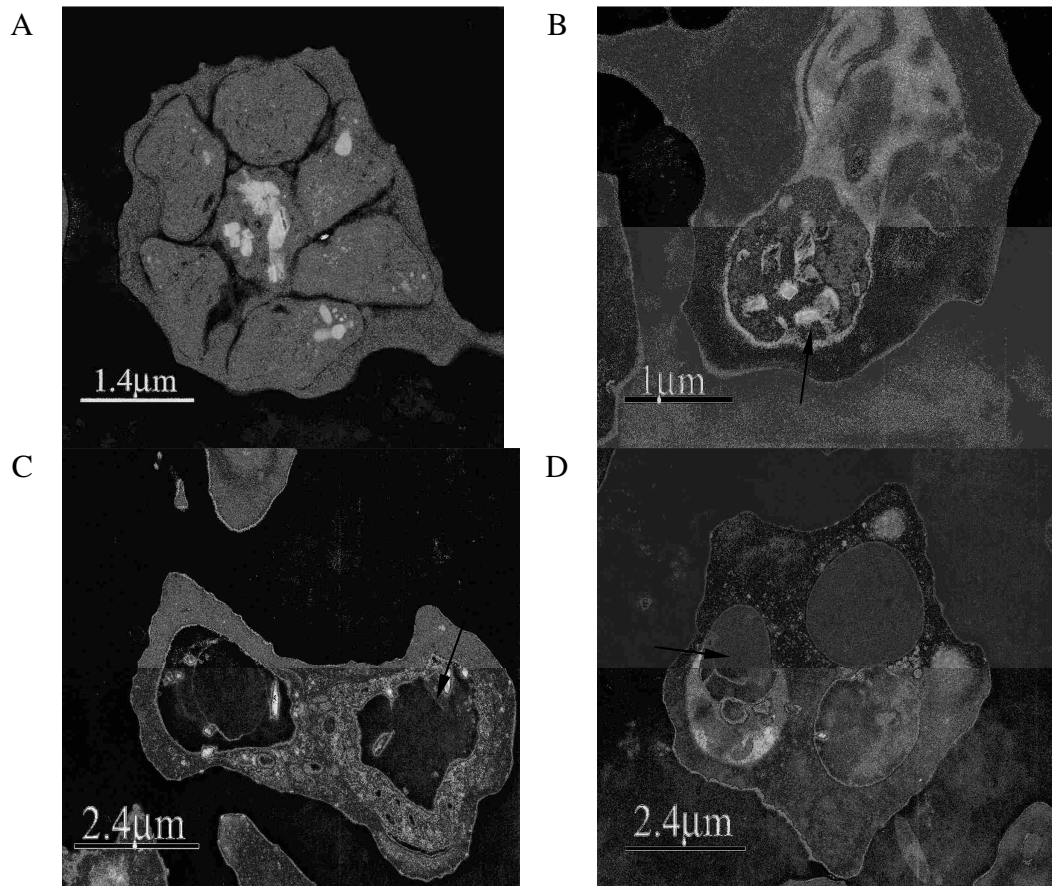


Figure 22. Ultrastructure examination of the erythrocytic cycle of *Plasmodium falciparum* shows extensive condensation of cytoplasm with food vacuole swelling and digestion of cell following exposure to staurosporine (5 μ M). **A)** solvent control, 12 hrs 11,000x; **B)** staurosporine, 5 hrs 15,00x; **C)** staurosporine, 9 hrs 6500x; **D)** staurosporine, 12hrs 6,500x. Arrows indicate food vacuole swelling.

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