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The Antileishmanial Agent Licochalcone A Interferes with the Function of Parasite Mitochondria

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Our previous studies have shown that licochalcone A, an oxygenated chalcone, has antileishmanial (M. Chen, S. B. Christensen, J. Blom, E. Lemmich, L. Nadelmann, K. Fich, T. G. Theander, and A. Kharazmi, Antimicrob. Agents Chemother. 37:2550–2556, 1993; M. Chen, S. B. Christensen, T. G. Theander, and A. Kharazmi, Antimicrob. Agents Chemother. 38:1339–1344, 1994) and antimalarial (M. Chen, T. G. Theander, S. B. Christensen, L. Hviid, L. Zhai, and A. Kharazmi, Antimicrob. Agents Chemother. 38:1470–1475, 1994) activities. We have observed that licochalcone A alters the ultrastructure of the mitochondria of *Leishmania* promastigotes (Chen et al., Antimicrob. Agents Chemother. 37:2550–2556, 1993). The present study was designed to examine this observation further and investigate the mechanism of action of antileishmanial activity of licochalcone A. Electron microscopic studies showed that licochalcone A altered the ultrastructure of *Leishmania major* promastigote and amastigote mitochondria in a concentration-dependent manner without damaging the organelles of macrophages or the phagocytic function of these cells. Studies on the function of the parasite mitochondria showed that licochalcone A inhibited the respiration of the parasite in a concentration-dependent manner, as shown by inhibition of O_2 consumption and CO_2 production by the parasites. Moreover, licochalcone A inhibited the activity of the parasite mitochondrial dehydrogenase. The inhibition of the activity of the parasite mitochondrial enzyme correlated well with the changes in the ultrastructure of the mitochondria shown by electron microscopy. These findings demonstrate that licochalcone A alters the ultrastructure and function of the mitochondria of *Leishmania* parasites.

Leishmaniasis is a major and increasing public health problem, particularly in Africa, Asia, and Latin America (18, 20), and it consists of a broad spectrum of diseases caused by different species of the protozoan genus *Leishmania*. More than 350 million people in the world are at risk of infection with *Leishmania* parasites. Over 12 million people are infected with different species of the parasite, and there are over 400,000 new cases each year (1). Recently, a dramatic increase in the number of visceral cases has been observed in southern Europe. The drugs available for the treatment of leishmaniasis are, in general, toxic and expensive and require long-term treatment (18). Large-scale clinical resistance against the most commonly used antileishmanial drug, antimonial agents, has been reported (19). The spread of drug resistance, combined with other shortcomings of the available antileishmanial drugs, emphasizes the importance of the development of new, effective, and safe drugs against leishmaniasis.

Our previous studies have shown that licochalcone A, an oxygenated chalcone, inhibits the in vitro growth of both *Leishmania major* and *L. donovani* promastigotes, exhibits a remarkably strong ability to kill the intracellular parasites of *L. major* amastigotes (5), prevents lesion development in mice infected with *L. major*, and reduces the parasite load in the spleens and livers of hamsters infected with *L. donovani* (6). Licochalcone A also exhibits potent antimalarial activity both in vitro and in vivo (7). The mechanism by which licochalcone A kills the parasites and protects animals from infection is not known. We have observed that licochalcone A alters the ultrastructure of the mitochondria of *Leishmania* promastigotes (5). The present study was designed to examine this observation further and investigate the mechanism of action of the antileishmanial activity of licochalcone A. The ultrastructural changes in the promastigotes and amastigotes of *L. major* incubated with licochalcone A, the respiration of the parasites, and the activity of the parasite mitochondrial dehydrogenase were investigated. The data indicate that licochalcone A alters the ultrastructure and function of the mitochondria of *Leishmania* parasites.

MATERIALS AND METHODS

Licochalcone A. Licochalcone A was synthesized in our laboratory as previously described (6, 7), and it was dissolved in 2% dimethyl sulfoxide in medium 199 to prepare a working solution of 1 mg/ml.

Parasite cultures. A World Health Organization reference vaccine strain of *L. major* (MHOM/IL/67/LRC-L437) originally isolated from a patient in Iran was cultured in medium 199 containing 10% heat-inactivated fetal calf serum. Incubation and growth of the parasite were carried out at 26°C. Promastigotes were harvested on culture day 4 and used.

Ultrastructure studies. Electron microscopic studies were carried out to examine the effect of licochalcone A on the ultrastructure of both the promastigote and amastigote forms of the parasite as previously described (5). Briefly, for the promastigote study, a suspension of 3×10^6 *L. major* promastigotes per ml was incubated in the presence of either licochalcone A or medium alone for 24 h at 26°C. After incubation, the promastigotes were centrifuged, resuspended in 1 ml of medium, fixed with 3% glutaraldehyde, and embedded in Noble agar. After postfixation in OsO_4, blocks were stained in 2% uranyl acetate and embedded in Vestopal W. The sections were poststained with magnesium uranyl acetate and lead citrate and examined in a Philips 201C electron microscope at 60 kV. For the amastigote study, human peripheral blood monocyte-derived macrophages (MDM) were infected with *L. major* promastigotes as described previously (5) and then incubated with licochalcone A for 72 h at 37°C. An uninfected macrophage culture treated with licochalcone A was used as a control. The macrophages were removed from the cultures by treatment with trypsin, centrifuged at

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$500 \times g$, and then resuspended in 1 ml of medium. The rest of the steps were the same as in the promastigote study.

Monocyte chemiluminescence and viability. A luminol-enhanced chemiluminescence assay was used to determine the oxidative burst response of human peripheral blood neutrophils and monocytes (13). The monocytes were incubated with various concentrations of licochalcone A for 1 h at 37°C, and the cell response to opsonized zymosan was determined. Cell viability after overnight incubation with licochalcone A was determined by trypan blue dye exclusion.

Monocyte phagocytosis of *Candida albicans*. The effect of licochalcone A on human monocyte phagocytosis of *C. albicans* was assessed by a method previously described (17). Briefly, 1 ml of 2×10^6 human peripheral blood mononuclear cells per ml was incubated with 1 ml of licochalcone A in different concentrations in minimal essential medium or medium alone for 30 min at 37°C. After being washed twice, the cells were incubated with 2 ml of 5×10^6 *C. albicans* cells per ml for 30 min at 37°C. The cells were then washed twice, and one portion of the cells was cytocentrifuged and stained for nonspecific esterase. The number of yeast cells ingested by 200 monocytes was determined by direct microscopy, and the number of yeast cells per monocyte was calculated. Another portion of the cells was used for an ultrastructure study (the same procedure as described above).

Respiration of the parasite (oxygen consumption and changes in carbon dioxide and pH). A suspension of 4.95 ml of promastigotes (2×10^6/ml) was incubated at 26°C in the presence of 50 μl of licochalcone A at different concentrations or medium alone in sealed bottles. Oxygen consumption and changes in carbon dioxide and pH were measured at 2, 4, 8, 24, 48, and 72 h after incubation with an ABL4 acid-base laboratory (Radiometer, Copenhagen, Denmark).

Activity of mitochondrial dehydrogenases. The effect of licochalcone A on the activity of parasite mitochondrial dehydrogenases was determined by a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium method as described previously (2). This method was developed from the 3-(4,5-dimethylthiasolyl-2-yl)-2,5-diphenyltetrazolium bromide method commonly used to study mitochondrial dehydrogenase activity (8, 10). In this method, we used a new type of substrate, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium, which is converted into a soluble, formazan-like dye complex by the parasite mitochondrial dehydrogenase. Briefly, 5×10^4 promastigotes were seeded in 96-well flat-bottom microtrays and incubated with different concentrations of licochalcone A or medium alone at 26°C. After 3, 6, 12, 24, 48, and 72 h, 25 μl of 3-(4,5-dimethylthiazolyl-2-yl)-2,5-(diphenyltetrazolium bromide–phenazin metasulfate) was added to each well and further incubated for 3 h at 37°C, whereafter the optical densities 492 nm were measured directly with a Titre-Tech 96-well scanner.

Statistical analysis. A paired two-tailed *t* test was used for analysis of the data. *P* values of <0.05 were considered significant.

RESULTS

Electron microscopic studies were carried out to determine possible ultrastructural changes in parasites incubated with licochalcone A. Unaffected control promastigotes contained long, slender, dark mitochondria (Fig. 1A). After addition of only 1 μg of licochalcone A per ml, some ultrastructural changes were induced in the mitochondria because of enlargement and disruption of their structure (Fig. 1B). Increasing concentrations of licochalcone A of up to 5 μg/ml aggravated these changes, but some cristae were still found intact (Fig. 1C and D). After addition of 10 μg of licochalcone A per ml, the deformed and swollen mitochondria filled up a great part of the cytoplasm of the promastigotes (Fig. 1E and F). In all of these experiments, the other organelles appeared to be unaffected and no morphological changes were seen in the kinetoplast (Fig. 1F). The kinetoplast is a section of the mitochondrion located just below the basal body of the flagellum.

Phagocytozed amastigotes showed a normal ultrastructure in human MDM grown in normal medium (Fig. 2A and B). Increased concentrations of licochalcone A changed the ultrastructure of the engulfed amastigotes, and 5 μg of licochalcone A per ml produced an enlarged mitochondrion (Fig. 2C and D) with no damaging effect on the mitochondria of the macrophage (Fig. 2D). When the concentration of licochalcone A was increased to 10 μg/ml, very marked morphological changes were induced in the amastigotes (Fig. 2E), and again no ultrastructural alteration was observed in the mitochondria of human MDM (Fig. 2E).

Human monocytes grown in medium with 10 μg of licochalcone A per ml showed no ultrastructural defect in the mitochondria or other cell organelles (Fig. 3A and B). The normal phagocytic effect of human monocytes on *C. albicans* induced no change in the ultrastructure of the mitochondria of the monocytes (Fig. 3C and D), and after addition of 10 μg of licochalcone A to the medium, no damaging effect on the mitochondria (Fig. 3E and F) or other cell organelles was seen. The average number of *C. albicans* yeast cells per monocyte incubated in the presence of 10 μg of licochalcone A per ml was 1.9, compared with 2.1 in the control monocytes, indicating that licochalcone A did not influence the phagocytosis of *C. albicans* by human monocytes.

Table 1 shows that in human monocytes about 45 and 40% of the mitochondria of monocytes were influenced by licochalcone A at concentrations of 80 and 160 μg/ml, respectively, and all of the mitochondria of monocytes were affected at a concentration of 320 μg/ml. At a concentration of 500 μg of licochalcone A per ml, the chemiluminescence response of human monocytes was inhibited by 70%, whereas 90% of the cells were viable.

Figure 4 shows that licochalcone A markedly inhibited the oxygen consumption of the parasites, the accumulation of CO_2, and the pH decline in the parasite culture. After 2 h of incubation, the concentration of oxygen in control promastigotes was 22.6 ± 0.66 kPa, and after 72 h it dropped sharply to 12.8 ± 0.79 kPa. After 72 h of incubation of the parasites with 5 or 10 μg of licochalcone A per ml, there were no or very slight changes in the oxygen contents (Fig. 4A). The CO_2 content of the parasite cultures was slightly increased in the presence of 5 μg of licochalcone A per ml and unchanged in the presence of 10 μg/ml (Fig. 4B). Similarly, there were no changes in the pH of the culture of parasites in the presence of 5 or 10 μg of licochalcone A per ml (Fig. 4C).

A time- and dose-dependent inhibitory effect of licochalcone A on the activity of parasite mitochondrial dehydrogenase was found (Fig. 5). When promastigotes were incubated with licochalcone A for a longer period, the inhibitory effect on the activity of parasite mitochondrial dehydrogenase was stronger and the dose of licochalcone A required was much less. The 50% inhibitory concentration of licochalcone A after 48 h of incubation was 1.7 μg/ml. The parasites died after 48 to 72 h of incubation. At higher concentrations (>20 μg/ml), the parasites died more rapidly. This indicates that the inhibition of mitochondrial activity occurs earlier and at concentrations lower than parasiticidal concentrations.

DISCUSSION

Previously we have demonstrated that licochalcone A exhibits strong antileishmanial and antimalarial activities (5–7). However, the mechanism by which licochalcone A kills the parasites and protects animals from infection is not known. It is important to understand the mechanism of action of the antiprotozoal activity of licochalcone A. Our preliminary studies showed that licochalcone A destroyed the mitochondria of *Leishmania* promastigotes (5). In this study, we further examined the effects of licochalcone A on the ultrastructure and function of the mitochondria of *L. major* parasites. The data indicate that licochalcone A alters the ultrastructure and function of the mitochondria of *Leishmania* parasites.

Electron microscopic studies showed that the ultrastructure of the mitochondria of promastigotes and amastigotes was changed in a concentration-dependent manner. In promastigotes, the mitochondria showed moderate edema with a low concentration of licochalcone A and greatly swollen organelles developed with 10 μg of licochalcone A. The same course of

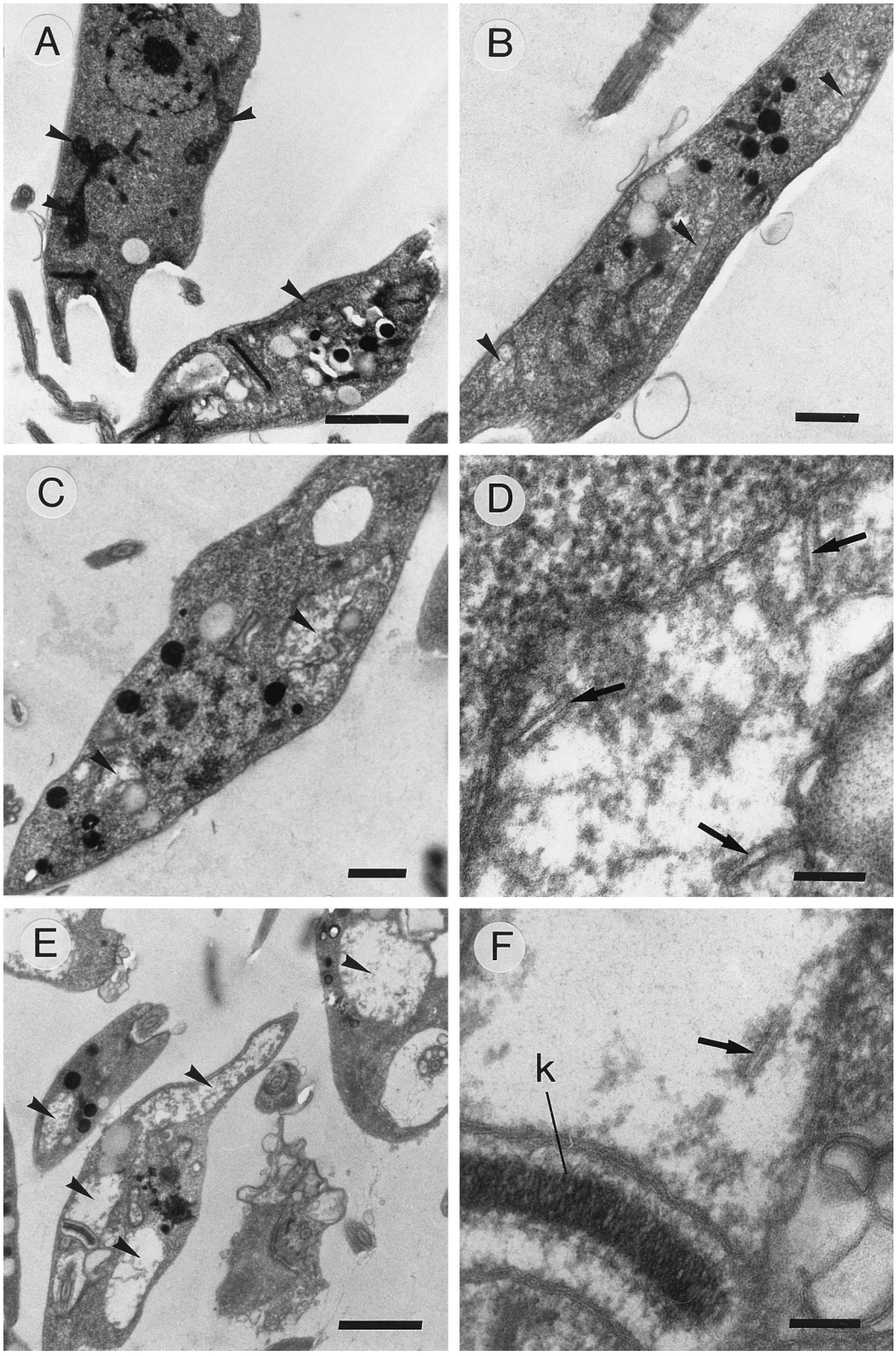

FIG. 1. Representative electron micrographs of promastigotes incubated in medium alone (A) or in the presence of 1 (B), 5 (C and D), or 10 (E and F) μg of licochalcone A per ml. (A) Sections of two promastigotes showing dense, slender mitochondria (arrowheads). Magnification, ×13,400. Bar, 1 μm. (B) Section of a promastigote with slightly enlarged mitochondria (arrowheads). Magnification, ×20,100. Bar, 0.5 μm. (C) A promastigote in which only the mitochondria seem to be enlarged (arrowheads). Magnification, ×17,600. Bar, 0.5 μm. (D) Higher magnification of one of the affected mitochondria with preserved cristae (arrows). Magnification, ×107,300. Bar, 0.1 μm. (E) Promastigotes with enlarged and disrupted mitochondria (arrowheads). Magnification, ×13,400. Bar, 1 μm. (F) Higher magnification showing part of an unaffected kinetoplast (k) and a mitochondrial crista (arrow). Magnification, ×107,300. Bar, 0.1 μm.

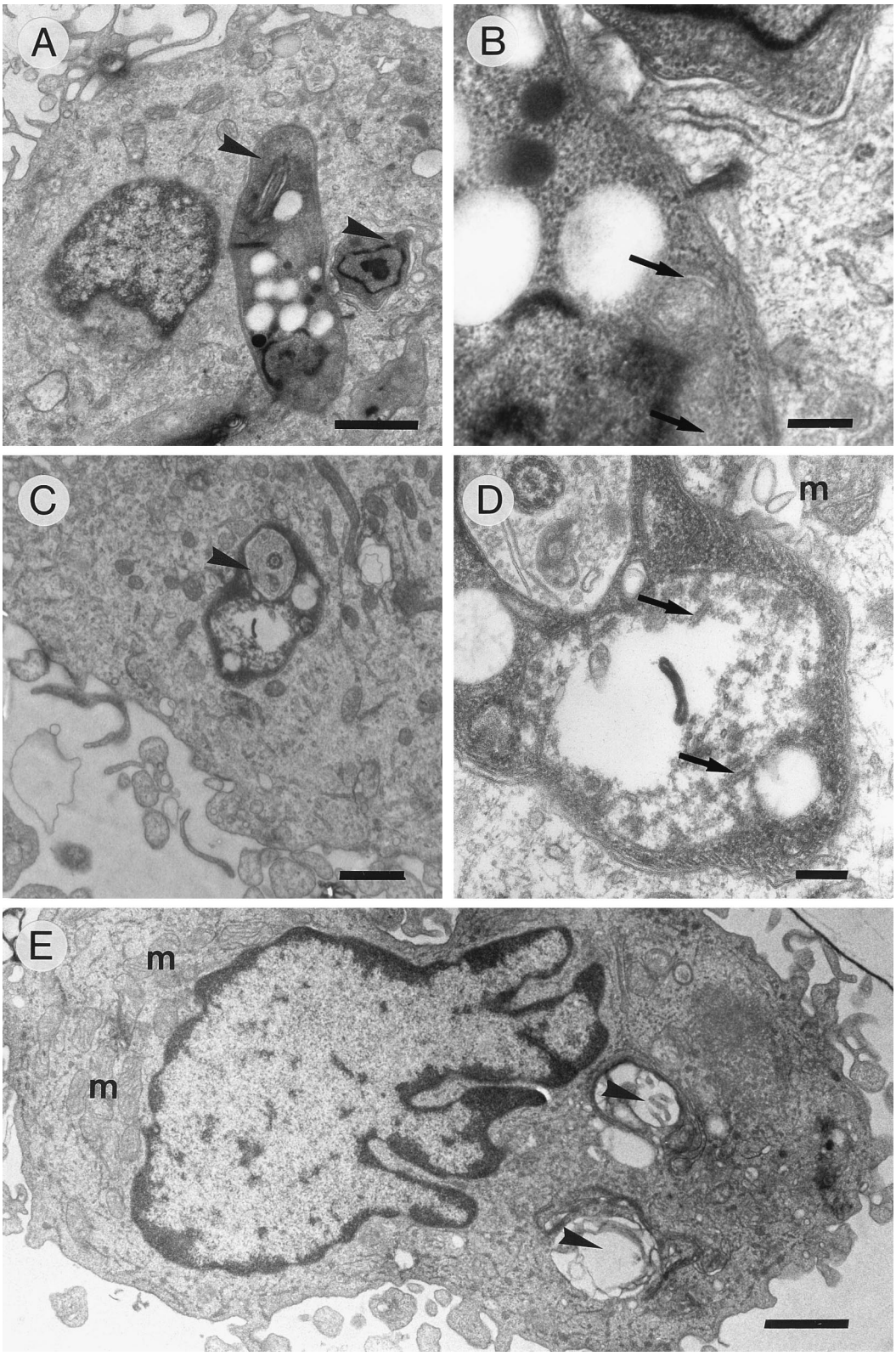

FIG. 2. Representative electron micrographs of human MDM infected with amastigotes and incubated in medium alone (A and B) or in the presence of 5 (C and D) or 10 (E) μg of licochalcone A per ml. (A) Part of an MDM infected with two normal-looking amastigotes (arrowheads). Magnification, ×13,400. Bar, 1 μm. (B) Higher magnification of part of one of the amastigotes showing a normal-looking mitochondrion with cristae (arrows). Magnification, ×54,700. Bar, 0.2 μm. (C) Part of an MDM with an affected amastigote (arrowhead). Magnification, ×9,700. Bar, 1 μm. (D) At a higher magnification, the affected amastigote shows an enlarged and disrupted mitochondrion with some intact cristae (arrows). Outside a normal-looking mitochondrion (m) from the macrophage is visible. Magnification, ×44,300. Bar, 0.2 μm. (E) MDM with two strongly degenerated amastigotes (arrowheads). Note the normal structure of the macrophage mitochondria (m). Magnification, ×13,400. Bar, 1 μm.

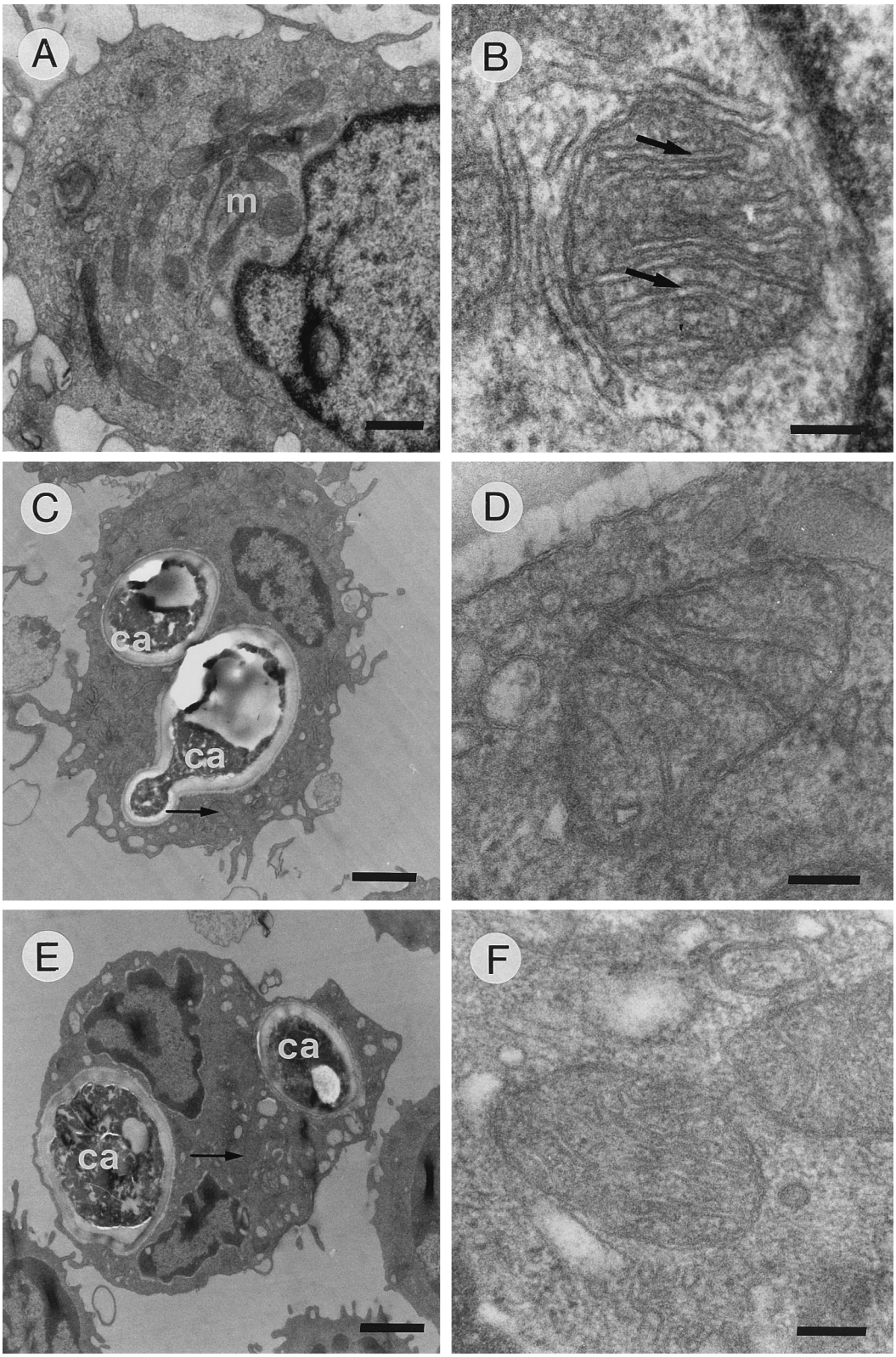

FIG. 3. Representative electron micrographs of human monocytes incubated with 10 μg of licochalcone A per ml (A and B), infected with *C. albicans* without licochalcone A (C and D), and infected with *C. albicans* after incubation with 10 μg of licochalcone A per ml (E and F). (A) Part of a monocyte with a high number of normal-looking mitochondria (m). Magnification, ×17,600. Bar, 0.5 μm. (B) Higher magnification of one of the mitochondria with normal-looking cristae (arrows) and matrix. Magnification, ×107,400. Bar, 0.1 μm. (C) Monocytes with phagocytized *C. albicans* (ca). Magnification, ×10,000. Bar, 1 μm. (D) High magnification of the mitochondrion indicated by the arrow in panel C, showing normal ultrastructure. Magnification, ×107,400. Bar, 0.1 μm. (E) MDM with phagocytized *C. albicans* (ca). Magnification, ×10,000. Bar, 1 μm. (F) High magnification of two mitochondria indicated by the arrow in panel E, showing normal ultrastructure. Magnification, ×107,400. Bar, 0.1 μm.

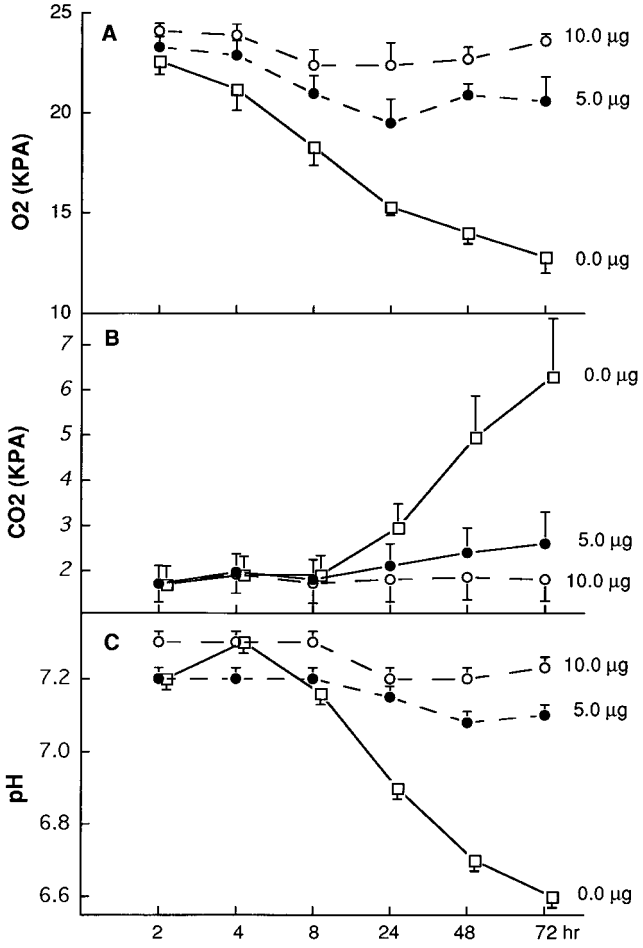

FIG. 4. Time study of the effect of licochalcone A on the oxygen (O_2) content (A) and changes in carbon dioxide (CO_2) (B) and pH (C) of *L. major* promastigotes. The data are from six experiments and are given as means ± 95% confidence intervals. KPA, kilopascals.

events was seen in amastigotes, except that in amastigotes other cell organelles were also heavily damaged. Cell organelles of macrophages, however, disclosed no ultrastructural changes at concentrations below 80 μg/ml. Thus, it seems that the effect of licochalcone A on amastigotes is followed by killing of the parasites by macrophages. Furthermore, the oxidative burst response, as measured by the chemiluminescence and phagocytic function of macrophages, was not influenced by fairly high concentrations of licochalcone A. As we have shown previously, oral administration of licochalcone A to rats at a dose of 1,000 mg/kg of body weight once a day for 2 weeks caused no observable toxicity (6).

Several methods were employed to examine the effect of licochalcone A on the function of parasite mitochondria. It was shown that licochalcone A inhibited the respiration of the parasites in a concentration-dependent manner, as shown by inhibition of O_2 consumption and CO_2 production by the parasites (Fig. 4). Licochalcone A also exhibited a dose-dependent inhibitory effect on the activity of the mitochondrial dehydrogenase of *L. major* promastigotes. The mitochondrial dehydrogenase activity measured in this assay includes the activities of various dehydrogenases in the mitochondrion, such as lactate and malate dehydrogenases (12) and succinate dehydrogenase (14), and has been shown to be mitochondrion specific (10, 16).

TABLE 1. Effect of licochalcone A on mitochondrial ultrastructure and chemiluminescence response of monocytes presented as a percentage of the cell response in medium alone and cell viability[a]

Licochalcone A concn (μg/ml)	% Mitochondria with normal morphology	Chemiluminescence (% of control)	Viability (%)
0	96	100	100
20	98	ND[b]	ND
40	94	101	100
80	55	94	100
160	60	114	100
320	0	91	100
500	ND	32	90

[a] The chemiluminescence and viability experiments were repeated three times, and data from one representative experiment are given. Mitochondrial ultrastructure of monocytes incubated in the presence of various concentrations of licochalcone A was examined under an electron microscope. Mitochondria with normal morphology or dilated were counted in 15 cells.
[b] ND, not done.

The inhibition of the mitochondrial function of the parasite correlated well with changes in the ultrastructure of the mitochondria shown by electron microscopy. These findings indicate that licochalcone A targets the parasite respiratory chain and mitochondria directly.

It was reported that some well-known antiprotozoal drugs and some potential antiprotozoal compounds influence the energy metabolism of the parasite. For example, sodium stibogluconate inhibits phosphorylation of ADP to ATP and the citric acid cycle of *L. mexicana* amastigotes (3, 4), and pentamidine isethionate damages the mitochondria of both promastigotes and amastigotes and the kinetoplast DNA core (9). Mycotoxin MT 81, an antibacterial, antifungal, and antileishmanial compound isolated from *Penicillium nigricans*, inhibits the respiration of *L. donovani* promastigotes (15). Atovaquone, a new antimalarial drug developed by Wellcome, inhibits *Plasmodium falciparum* dihydroorotate dehydrogenase, which is the major component of the electron transport chain of intraerythrocytic malaria parasites (11). Studies on several enzymes of the electron transport chain in mitochondria are

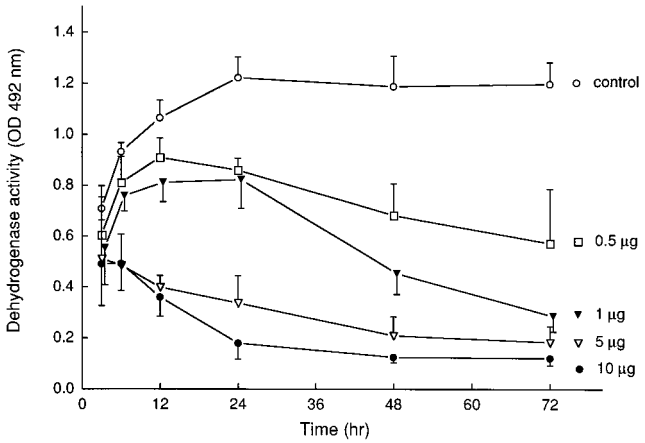

FIG. 5. Time study of the effect of licochalcone A on the activity of the mitochondrial dehydrogenase of *L. major* promastigotes. Promastigotes were incubated with licochalcone A for 3, 6, 12, 24, 48, and 72 h. The data are means ± 95% of confidence intervals of three experiments and are presented as optical densities (OD) at 492 nm indicating dehydrogenase activity.

warranted to document further the exact mechanism of action of licochalcone A on parasite mitochondria. Elucidation of the mechanism of action of this class of compounds is important for the development of these compounds into an antileishmanial and antimalarial drug.

In conclusion, the main discovery presented in this report is that licochalcone A alters the ultrastructure and function of the mitochondria of *Leishmania* parasites but not the mitochondria of host cells.

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