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Action of chloroquine on nitric oxide production and parasite killing by macrophages

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Abstract

Chloroquine is known to inhibit several functions of macrophages, but its effect on the nitric oxide (NO)-dependent parasite killing capacity of macrophages has not been documented. NO synthesis by interferon-γ-induced mouse and casein-elicited rat macrophages was significantly and irreversibly inhibited by chloroquine. The activity of the inducible NO synthase was not directly altered, but previous incubation of macrophages with chloroquine decreased it. Chloroquine did not alter arginase activity or arginine uptake. NADPH diaphorase activity, an indicator of NO synthase was impaired. Western blotting showed that inducible NO synthase synthesis was blocked by chloroquine. The blocking of NO formation by chloroquine resulted in increased infection of mouse peritoneal macrophages by *Trypanosoma cruzi* (*T. cruzi*). This suggests that chloroquine decreases NO formation by macrophages by inhibiting the induction of NO synthase. The findings are further evidence that NO is involved in the anti-parasitic response of macrophages.

Keywords: Macrophage; Nitric oxide (NO); Chloroquine; Inhibition of NO production; *Trypanosoma cruzi* infection

1. Introduction

Chloroquine is best known as an antimalarial drug (Wellems, 1992; Slater, 1993), but it also affects several functions of macrophages. It inhibits phagocytosis and protein synthesis (Antoni et al., 1986), antigen processing and presentation (Unanue, 1984; Lang and Kaye, 1991); it acts as a lysosomotropic agent by increasing lysosomal pH (Ziegler and Unanue, 1982) and it inhibits the transcription of tumor necrosis factor-α (TNF-α) (Zhu et al., 1993).

The role of NO in the killing of various eukaryotic parasites by macrophages is well documented (Adams et al., 1990; Green et al., 1990; Roach et al., 1991; Kremsner et al., 1992; Munoz-Fernandez et al., 1992; Metz et al., 1993; Plasman et al., 1994; Vespa et al., 1994; James, 1995). NO is produced in macrophages from L-arginine (L-Arg) by inducible NO synthase (MacMicking et al., 1997), an enzyme whose synthesis is induced by lipopolysaccharide and cytokines (typically by interferon-γ) (Lowenstein et al., 1993). Peritoneal macrophages elicited in vivo by casein also produce nitrite without any in vitro cytokine treatment and rat macrophages produce more NO than do mouse cells (Hrabák et al., 1996).

The intracellular parasitic protozoan, *Trypanosoma cruzi* (*T. cruzi*), which causes Chagas' disease (Tanowitz et al., 1992) multiplies in macrophages. However, macrophages suitably activated with interferon-γ can also inhibit *T. cruzi* infection by producing NO (Munoz-Fernandez et al., 1992; Metz et al., 1993; Plasman et al., 1994; Vespa et al., 1994). Macrophages also contain an arginase that breaks down arginine into urea and ornithine. We have therefore examined the effect of chloroquine on NO and urea production and the capacity of macrophages to control the *T. cruzi* infection by producing NO. The synthesis of NO by mouse macrophages stimulated in vitro with interferon-γ (Metz et al., 1993) and rat cells stimulated in vivo with casein (Hrabák et al., 1996) was assessed. In our experi-

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ments, *T. cruzi* was used as a tool to evaluate NO production by measuring NO-mediated killing of the parasite.

2. Materials and methods

2.1. Macrophages

Mouse peritoneal macrophages were removed from BALB/c mice (Bantin and Kingman Universal, Hull, UK) (Olivares and Vray, 1995) by lavage with Ca^{2+}–Mg^{2+}-free Hanks' balanced salt solution (HBSS) (Gibco, Grand Island, NY). They were suspended in RPMI 1640 medium containing 10% foetal bovine serum, placed in 96-well plastic culture dishes and allowed to adhere for 2 h. The adhered cells washed twice with the same medium and cultured for 24 h in fresh RPMI 1640 medium supplemented with 25 mM HEPES and 10% foetal bovine serum (mycoplasma free, endotoxin concentration less than 27.5 pg/ml measured by Coatest endotoxin test, Chromogenix, Mölndal, Sweden) containing 100 U/ml interferon-γ, kindly provided by Prof. A. Billiau and Dr. D. Herremans, K.U.L., Leuven, Belgium; 100 IU/ml penicillin and 100 μg/ml streptomycin. Rat peritoneal macrophages were obtained from Wistar rats (LATI, Gödöllő, Hungary) stimulated by injection of 2% dephosphorylated casein. They were suspended in complete HBSS, placed in culture plates and allowed to adhere for 2 h (Hrabák et al., 1991, 1996). They were then cultured in Dulbecco Minimal Essential Medium (DMEM) containing 2 mM L-Arg, 4.5 g/l glucose and 1 mM pyruvate, 5% foetal bovine serum, 100 IU/ml penicillin and 100 μg/ml streptomycin. Chloroquine was purchased from Sigma, dissolved in the culture medium (stock solution 10 mM) before adding to the cultured cells.

2.2. Chemical determinations

The NO concentration in the culture medium was evaluated by measuring nitrite, its stable degradation product, by the Griess' reaction (James and Glaven, 1989) using sodium nitrite as standard. The urea concentration in the medium was measured colorimetrically using urea as standard (Coulombe and Favreau, 1963). The urea content of macrophage-free RPMI—foetal bovine serum was also measured and subtracted. Absorbances were measured with a microplate ELISA reader (Titertek Multiscan MCC/340, MKII EFLAB, Finland) at 540 nm.

2.3. Enzyme assays

Arginase activity was measured directly by determining the urea released in 15 min using 20 mM L-Arg as substrate (Coulombe and Favreau, 1963). One unit of enzyme activity releases 1 μmol urea/min. NO synthase activity was determined directly in lysed rat macrophages. Macrophages (3×10^6) were depleted of L-Arg for 24 h, lysed by adding 5 mM HEPES, 10 μM tetrahydrobiopterin, 1 mM NADPH and U-^{14}C-labeled L-Arg (diluted with unlabeled L-Arg to 20 μM and 5 kBq/nmol specific radioactivity). The ^{14}C-labeled L-citrulline (L-Cit) formed from U-^{14}C-labeled L-Arg was measured by incubating this mixture for 60 min at 37°C. The reaction was stopped by adding 5 mM unlabeled L-Arg and 5 mM L-Cit. An aliquot of 30 μl was spotted on a thin layer chromatography (DC-Alufolien, Kieselgel 60, Merck) and L-Arg and L-Cit were separated on the plate by using a solvent of chloroform–methanol–ammonia–water (1:9:6:2). Amino acids were detected by ninhydrine and L-Cit spots were cut out, placed in a toluene-based scintillation cocktail containing 1/3 Triton-X-100 their radioactivities were measured in an LS 300 Beckman liquid scintillation spectrometer. Enzyme activity is given in nmol L-Cit produced/mg protein/min. Protein concentration was determined according to Lowry et al. (1951).

NADPH diaphorase activity was estimated essentially according to Janssens et al. (1992), using a reagent containing 100 mM Tris–HCl (pH 7.4), 1 mM NADPH, 0.4 mM nitroblue tetrazolium and 0.5% Triton-X-100 (Sigma, St. Louis, MO). Macrophages (10^6/well) were cultured for 24 h, washed and incubated with 50 μl reagent for 2 h. The violet formazane colour was measured in an ELISA-reader at 540 nm.

2.4. Measurement of L-Arg uptake

Macrophages (10^6/well in 96-well plates) were cultured in 100 μl HBSS containing various doses of chloroquine in 96-well plates. L-Arg uptake was measured by incubating the cells with ^{14}C L-Arg (3 kBq, 0.2 mM final concentration for 5 min). The reaction was stopped by adding 1 M unlabeled L-Arg in HBSS. Cells were washed three times with cold HBSS and then dissolved in 0.5 N NaOH. The radioactivity of these samples was measured by a β-counter and the uptake was given in nmol/10^6 cells/min. A K_t of ≈0.1-mM for L-Arg uptake by casein-elicited rat macrophages was determined in preliminary studies and 5 min was found to be in the linear range of arginine uptake (Baydoun et al., 1993).

2.5. Check of cell viability

Trypan blue 0.04% in 0.9% NaCl (100 μl) was mixed with an equal volume of cell suspension for 10 min. Cells were then counted under a light microscope and the percentage of dead cells calculated.

2.6. Western blot analysis of inducible NO synthase production

Untreated macrophages and macrophages (2×10^6 cells) treated with interferon-γ (100 U/ml) and lipopolysaccha-

ride (10 ng/ml) were cultured for 24 h with or without 100 μM chloroquine. Cells were then disrupted in 5-mM HEPES at 0°C, mixed with an equal volume of sample buffer (4% sodium dodecyl sulfate (SDS)–10% β-mercaptoethanol–0.1% bromophenol blue) and heated at 100°C for 2–3 min. Samples (25 μl) were separated by SDS polyacrylamide gel electrophoresis in 7.5% gels at pH 8.3. Separated proteins were blotted onto a Hybond-C (Amersham) nitrocellulose membrane and the membranes were incubated with a rabbit anti-inducible NO synthase polyclonal antibody (BIOMOL Res., Plymouth, USA; 1:500 dilution) for 24 h. The membranes were washed with phosphate-buffered saline (PBS, pH 7.4) containing 0.1% Tween-20 and incubated with a goat anti-rabbit IgG polyclonal antibody conjugated to horseradish peroxidase (Sigma, dil. 1:1000) for 1 h. Finally, bound peroxidase was detected by incubation with 0.6 mg/ml 4-chloro-1-naphthol (Sigma) and 0.6 μl/ml 30% H_2O_2 for 30 min. Incubations with antibody and washings were done in PBS containing 0.1% Tween-20 and 3% fat-free milk powder.

2.7. The study of the parasitic parameters of macrophages

T. cruzi trypomastigotes (Tehuantepec strain) were obtained from blood (containing 10 U/ml heparin) taken from X-ray irradiated and infected F344 Fisher male rats (Iffa Credo, Brussels, Belgium). They were isolated by ion exchange chromatography on DEAE cellulose (Whatman DE 52) equilibrated with PBS containing glucose, washed and resuspended in endotoxin-free PBS. Murine peritoneal macrophages were activated by incubating them with mouse recombinant interferon-γ (100 U/ml), for 24 or 48 h. Induced cells were infected with *T. cruzi* by incubation (10:1 parasite-to-cell ratio) for 16 h, washed with prewarmed RPMI culture medium to remove free parasites, fixed with methanol, stained with Giemsa and at least 200 cells/well were examined under the light microscope. The percentage of infected macrophages and the mean number of amastigotes/infected cell were recorded. Culture medium with or without interferon-γ was renewed every 24 h for a total of 72 h. Endotoxin concentration was under 5 pg/ml. Murine peritoneal macrophages were treated with chloroquine according to three protocols: (1) preincubated for 2 h with chloroquine and then induced with interferon-γ for 24 or 48 h; (2) incubated with chloroquine plus interferon-γ for 24 or 48 h; and (3) incubated with chloroquine for 2 h, induced for 24 h with interferon-γ and then infected with *T. cruzi*. Traces of endotoxin in the added chloroquine (less than 30 pg/ml) were neutralized by adding 10 U/ml polymixine B (Verdot et al., 1996).

2.8. Statistical evaluation

Significance was tested by a two-sample unpaired *t*-test. Both *P* values and standard deviations were calculated by the Statistica 5.0 software.

3. Results

3.1. Effect of chloroquine on NO production by interferon-γ-nduced murine peritoneal macrophages

Chloroquine decreased the NO production by murine peritoneal macrophages treated with interferon-γ for 24 and 48 h (Fig. 1) at concentration as low as 1 μM. Incubation of murine peritoneal macrophages with chloroquine for 2 h before induction with interferon-γ also reduced nitrite formation, but higher concentrations of chloroquine (10 μM or over, Fig. 2A) were required. In both cases, chloroquine also decreased the additional NO synthesis caused by *T. cruzi* infection (Fig. 2A).

Murine peritoneal macrophages were cultured for 72 h in RPMI 1640 medium to deplete the cells of endogeneous arginase. Macrophages were then cultured in RPMI 1640 containing 10% foetal bovine serum and 10 μg/ml lipo-

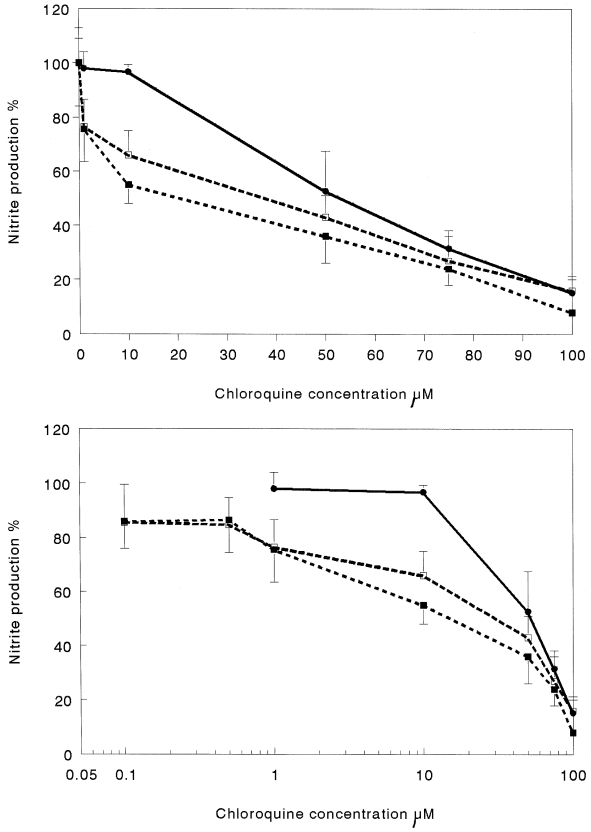

Fig. 1. Effect of chloroquine on nitrite production by mouse and rat macrophages. Macrophages (3×10^5 murine peritoneal macrophages and 10^6 rat cells/well in 200 μl) were cultured in DMEM—5% foetal bovine serum (rat) and in RPMI—10% foetal bovine serum (mouse) for 24 h. 100% production was 19.3 ± 2.9 and 31.2 ± 4.3 nmol nitrite/10^6 cells for interferon-γ-induced mice (in 24- and 48-h cultures, respectively) and 23.5 ± 5.4 nmol nitrite/10^6 cells for rats. Casein-induced rat macrophages (-●-); mouse peritoneal macrophages activated with interferon-γ for 24 h (-■-); mouse peritoneal macrophages activated with interferon-γ for 48 h (-□-); Data are means ± S.D. of six independent experiments.

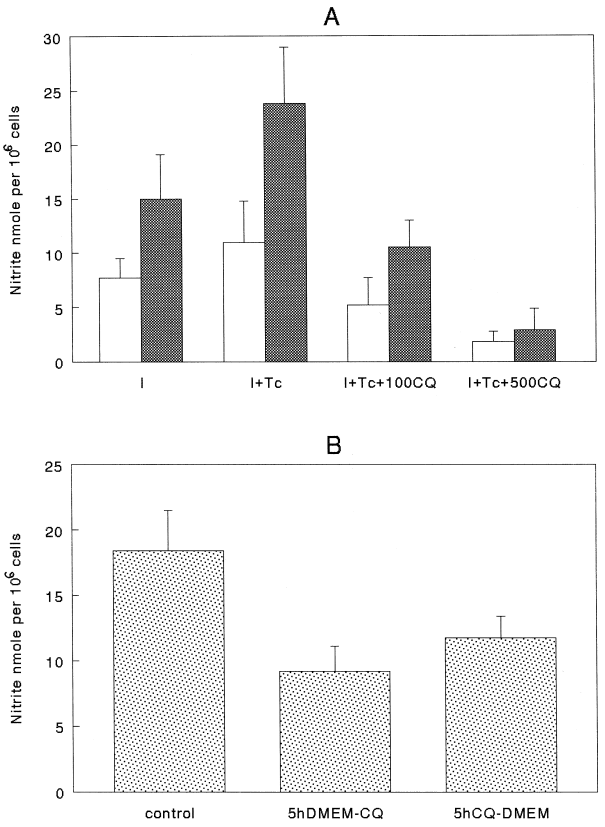

Fig. 2. The irreversible effect of chloroquine on rat macrophages. (A) The effect of *T. cruzi* and chloroquine on NO production by interferon-γ-induced murine peritoneal macrophages (100 U/ml interferon-γ was added to 2×10^5 cells for 24 h). Nitrite was measured in the medium of cell cultures after 24 h (open bars) and 48 h (dark bars) infection. I = murine peritoneal macrophages induced by interferon-γ followed by incubation with medium for 24 or 48 h; I + Tc = murine peritoneal macrophages induced by interferon-γ and then infected with *T. cruzi* for 24 or 48 h; I + Tc + numerals = murine peritoneal macrophages induced by interferon-γ and infected with *T. cruzi* as described above, but incubated with chloroquine for 2 h before induction (final chloroquine concentrations (μM) are indicated by numbers); *T. cruzi* causes a weakly (24 h, $0.01 < P < 0.05$) and a strongly (48 h: $P < 0.01$) significant enhancement of nitrite production. This nitrite production is significantly inhibited by 100 μM (24 h: $P < 0.01$; 48 h: $P < 0.001$) and by 500 μM ($p < 0.001$) chloroquine treatment. (B) Effect of chloroquine on NO production by casein-elicited macrophages. Macrophages (10^6 cells/well in 200 μl) were cultured for 24 h. Control bar represents an experiment without chloroquine by medium change at 5 h (control, 100%). Bar DMEM-CQ: DMEM—foetal bovine serum alone for the first 5 h and replaced by DMEM—foetal bovine serum plus 50 μM chloroquine for 19 h, the drug decreased significantly the nitrite production ($n = 6$, $p < 0.01$); Bar CQ-DMEM: DMEM—foetal bovine serum plus 50 μM chloroquine present during the first 5 h and replaced by DMEM—foetal bovine serum alone for 19 h. Drug treatment caused the significant decrease of nitrite production either added at the beginning and removed at 5 h ($P \leq 0.01$) or added at 5 h for the additional 19 h ($p < 0.01$). Difference between treated (DMEM-CQ and CQ-DMEM) samples was not significant ($P > 0.05$). Data are means ± S.D. ($n = 6$).

polysaccharide with or without 500 μM chloroquine for 48 h to induce new arginase production. Even this high concentration of chloroquine did not inhibit the de novo arginase synthesis, as shown by the urea measured in the medium. Thus, chloroquine did not affect the induction of arginase (Table 2).

3.2. Effect of chloroquine on NO production by macrophages from casein-elicited rats

Chloroquine also inhibited NO formation by casein-elicited rat peritoneal macrophages in a concentration-dependent manner (Fig. 1), however, the effect was observed over 10 μM chloroquine. The reversibility of the effect of 50 μM chloroquine was investigated further by two methods. First, chloroquine was added to the cell culture for 5 h and the medium was changed to medium without chloroquine. Second, cells were cultured in DMEM without chloroquine for 5 h and placed in medium containing 50 μM chloroquine. Chloroquine present during the first 5 h of incubation irreversibly inhibited NO production; chloroquine added after 5 h also caused a marked inhibition (Fig. 2B).

3.3. Effect of chloroquine on inducible NO synthase and arginase activity, L-Arg uptake and cytotoxicity

Rat macrophages (3×10^6/well) were incubated with 50 or 100 μM chloroquine for 5 h and then cultured in L-Arg-free DMEM—10% foetal bovine serum without chloroquine for 24 h. ^{14}C L-Arg was added and NO synthase was measured by the formation of ^{14}C L-Cit. Chloroquine caused a significant decrease (56% of control) in L-Cit formation at a concentration as low as 50 μM (Table 1).

Chloroquine (50–100 μM) caused no significant decrease in NO synthase when it was measured in the presence of the drug, but without a previous chloroquine treatment. The drug also did not affect arginase activity (measured directly on arginase in the medium of adhered murine macrophages, Table 1).

The chloroquine concentrations which inhibited nitrite production did not alter arginine uptake into the cells (data not shown). Chloroquine was also not directly toxic at the concentrations used, at least in the 2- and 5-h experiments by trypan blue exclusion.

3.4. Effect of chloroquine on NADPH diaphorase activity

Inducible NO synthase requires NADPH as a cofactor to function as an oxygenase. Thus, inducible NO synthase can also be detected by its NADPH diaphorase activity. The diaphorase activity of cells incubated with or without chloroquine was measured. Macrophages incubated with chloroquine for 5 h reduced less nitroblue tetrazolium to formazane than controls (Fig. 3), indicating that NADPH diaphorase activity was impaired. Chloroquine also inhibited the diaphorase activity of mouse and rat macrophages cultured for 5 h in chloroquine-free medium and then for 5

Table 1
The effect of chloroquine on macrophage activities

Chloroquine concentration	Arginase activity (%)	NO synthase		L-Arg uptake (%)
		Activity (%)	Induction (%)	
Control	100	100	100	100
10 μM	117	n.d.	94 ± 8	89 ± 12
50 μM	111	88 ± 6	56 ± 9	93 ± 6
75 μM	104	n.d.	40 ± 6	n.d.
100 μM	106	87 ± 6	23 ± 12	94 ± 16

Arginase activity was measured spectrophotometrically by the release of urea and the mean value of two experiments are given. Inducible NO synthase activity was measured by labeled Cit formation after a 24-h Arg-depletion. Drug was present only during the measurement. Decrease of activity was not significant ($n = 4$, $p \geq 0.05$). The effect of chloroquine on the induction of NO synthase was also measured by Cit formation, however, drug was present in Arg-free DMEM for 24 h, then cells were washed and the activity was measured in the absence of the drug. The inhibitory effect of chloroquine was significant from 50 μM ($n = 4$, $p < 0.01$).
L-Arg uptake was measured by using [^{14}C]L-Arg as described in Section 2. Arginine transport was not affected significantly by the drug ($n = 4$, $p > 0.05$). Data are means ± S.D., except arginase where statistical evaluation was not performed ($n = 2$). n.d. = not determined. Control (100%) values: 5.2 U/mg protein for arginase, 94.54 ± 12.66 pmol/mg protein/min for NO synthase activity and induction, 51.98 ± 2.72 pmol Arg/10^6 macrophages for uptake.

to 24 h in medium containing chloroquine. Thus, two activities of NO synthase (NO production and diaphorase) were inhibited by the same treatment. NADPH-diaphorase activity (measured by L-Cit formation) was not directly inhibited by 100-μM chloroquine (data not shown).

3.5. Inhibition of inducible NO synthase induction detected by Western blotting

The above results suggested that chloroquine did not directly inhibit NO synthase, but inhibited the enzyme's induction. Western blots of extracts of uninduced, interferon-γ-induced and interferon-γ-induced, chloroquine-treated macrophages were compared. Only the induced, untreated samples reacted with anti-inducible NO synthase antibody, indicating that inducible NO synthase was produced only in response to interferon-γ, in the absence of chloroquine. Uninduced samples and induced samples treated with 100 μM chloroquine were negative (Fig. 4).

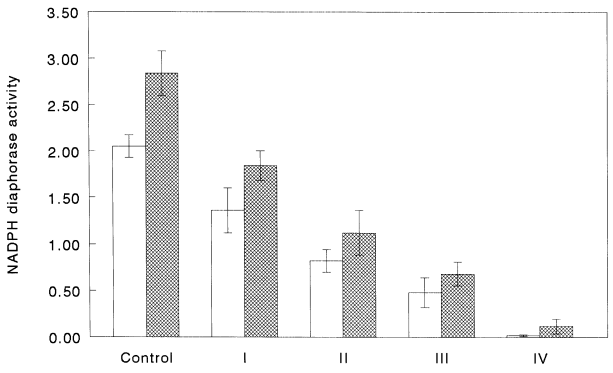

Fig. 3. The effect of chloroquine on NADPH diaphorase activity. Activity is in arbitrary units (means ± S.D. of six independent experiments performed in duplicate). Open bars: murine peritoneal macrophages induced by interferon-γ, dark bars: casein-elicited rat macrophages. Macrophages (3×10^5 murine peritoneal macrophages and 10^6 rat cells/well in 200 μl) were cultured for 24 h, chloroquine was added for the initial 5 h, and then replaced by chloroquine-free medium (Bar I for 50 μM, bar II for 100 μM chloroquine). In other experiments, macrophages were cultured first in chloroquine-free medium for 5 h and then in chloroquine-medium until 24 h (Bar III for 50 μM, bar IV for 100 μM chloroquine). Control samples were cultured in chloroquine-free medium. Medium was RPMI —10% foetal bovine serum for murine and DMEM—5% foetal bovine serum for rat macrophages.

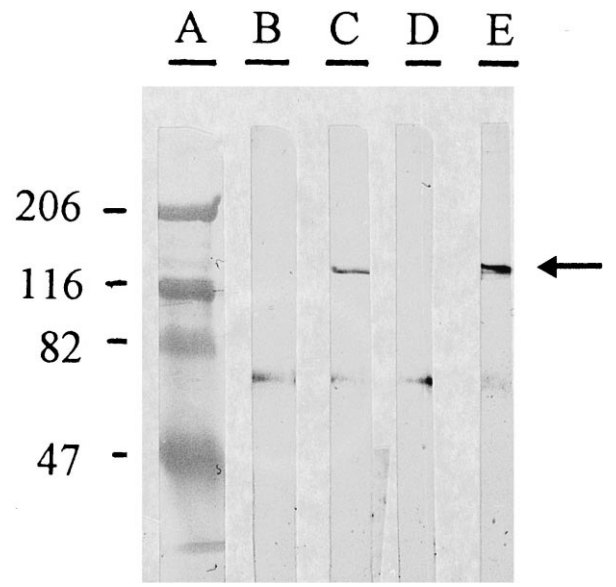

Fig. 4. Western blot analysis of inducible NO synthase in macrophage treated with chloroquine. Murine peritoneal macrophages (3×10^6 cells/well in 0.5 ml) were cultured for 24 h in RPMI—10% foetal bovine serum plus 100 μM chloroquine. The cells were solubilized, electrophoresed (SDS-PAGE) and the resulting proteins blotted onto nitrocellulose. Lane A: molecular weight standard (pre-stained by coomassie blue; BioRad Laboratories, Hercules, USA), molecular masses indicated and expressed in kilodaltons on the left side; lane B: macrophages without inducer; lane C: macrophages induced by interferon-γ (100 U/ml); lane D: macrophages induced by lipopolysaccharide (1 ng/ml) plus interferon-γ (100 U/ml) in the presence of 100-μM chloroquine, lane E: macrophages induced by lipopolysaccharide (1 ng/ml) plus interferon-γ (100 U/ml). An arrow on the right side indicates the position of inducible NO synthase. Width of gel pockets are indicated on the top. Five blot experiments were performed with the same result.

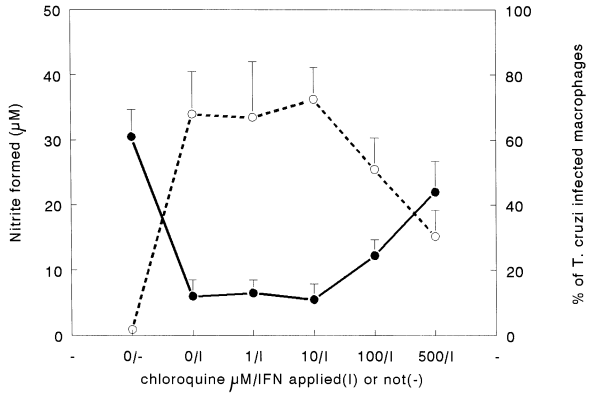

Fig. 5. Relationship between NO production and *T. cruzi* infection in murine peritoneal macrophages. The NO concentration (-○-, dotted line) was measured in the medium of cultured mouse murine peritoneal macrophages (2×10^5/well in 200 μl) treated with chloroquine at the indicated concentrations (μM) for 2 h, induced with 100 U/ml interferon-γ for 24 h, and then infected with *T. cruzi* (10:1 parasite to cell ratio). The percentage of infected macrophages (-●-, solid line) was measured 24 h after adding parasites to the induced murine peritoneal macrophages. Numbers indicate the chloroquine concentration during pre-treatment, I indicates the use of interferon-γ. Data are means ± S.D. of three independent experiments.

3.6. Effect of chloroquine on parasite killing by interferon-γ-nduced macrophages

Chloroquine decreased NO production, and consequently the anti-parasite response of interferon-γ-activated mouse macrophages. This effect depended on the drug concentration; there was a reciprocal relationship between NO production and the rate of infection, as indicated by the increase in the percentage of infected cells (Fig. 5) and by the increased number of amastigotes/infected cell (data not shown).

4. Discussion

Chloroquine markedly inhibits NO production by macrophages when the cells are incubated with concentrations of 50–100 μM (Fig. 1). This effect is due to blocked induction of inducible NO synthase rather than a direct effect on enzyme activity. 50–100 μM chloroquine does not significantly block the formation of L-Cit directly (Table 1), but the amounts of NO and L-Cit are decreased when cells are incubated with the drug for 2 or 5 h (Fig. 2), indicating that the effect of chloroquine is not reversible. The drug acts on interferon-γ-activated cells and on in vivo elicited macrophages that can synthesize NO. The exact target of this effect (i.e., transcription or translation) is not yet known. The finding that chloroquine inhibits NO formation when added 5 h after starting the culture suggests an effect after the translational period, as inducible NO synthase mRNA is usually synthesized during the early period (Morris and Billiar, 1994). Other systems using L-Arg (e.g., arginase, the arginine transport system) are not influenced during this period (Fig. 1, Table 1). NADPH diaphorase activity, which is an arginine-independent function of inducible NO synthase, is also decreased by chloroquine (Fig. 3). Western blot results (Fig. 4) has given an evidence that chloroquine inhibits the de novo synthesis, i.e., the induction of inducible NO synthase.

Chloroquine probably does not selectively inhibit the induction of inducible NO synthase, but has a broad effect on protein synthesis. Nevertheless, this inhibition is not analogous to the effects of general protein synthesis inhibitors, as shown by its lack of effect on the induction of arginase. Macrophages depleted of arginase can be induced to synthesize the enzyme by adding lipopolysaccharide. Cells incubated with lipopolysaccharide and chloroquine produce the urea and the amount is proportional to the arginase content of the macrophages. Both chloroquine-treated and untreated cells produced the same amount of urea (Table 2).

Chloroquine may also influence the interactions between TNF-α and NO formation. Although TNF-α is not an inducer of inducible NO synthase, there is considerable evidence that this cytokine potentiates NO synthesis if inducible NO synthase has been induced by lipopolysaccharide and/or interferon-γ (Munoz-Fernandez et al., 1992; Olivares and Vray, 1995). Chloroquine inhibits the transcription of TNF-α mRNA (Zhu et al., 1993). Chloroquine has also been shown to inhibit the conversion of pro-TNF-α protein molecules to the active cytokine in a macrophage cell line, without blocking the synthesis of its mRNA or of NF-κB (Jae-Yeon and Dae-Myung, 1997). The effective drug concentrations (100–250 μM) were similar to the effective concentrations in this study. Hence, chloroquine may have a similar effect on the processing of inducible NO synthase to its active form.

The induction of NO synthase is particularly important for protection against parasitic infections (Adams et al., 1990; James, 1991; Roach et al., 1991; Munoz-Fernandez et al., 1992; Metz et al., 1993; Pakianathan and Kuhn, 1994; Plasman et al., 1994; Vespa et al., 1994; James,

Table 2
The effect of chloroquine on arginase induction

Sample	Urea formation
Control	9.84 ± 1.68
LPS	36.27 ± 5.30
LPS + chloroquine	34.48 ± 6.12

5×10^5 murine peritoneal macrophages were depleted of arginase for 3 days.
A total of 10 μg/ml lipopolysaccharide was added to the cultures and the urea in the medium after 48 h was measured.
Urea production is given in nmol/10^5 cells.
Data are means ± S.D. of three independent experiments (with duplicates).
Lipopolysaccharide treatment causes a significant increase of urea ($p < 0.01$) and this was not decreased by chloroquine treatment ($p \gg 0.05$).

1995). Thus, blocking NO production could help the parasites survive. Macrophages treated with chloroquine had significantly more infected macrophages with more parasites/cell than untreated cells (Fig. 5). Another mechanism of parasite killing based on the lysosomotropic effect of chloroquine (Ziegler and Unanue, 1982; Ley et al., 1990) is not likely, because it should result in the increase of lysosomal pH, which would cause the release of NO from *S*-nitroso-compounds stable at low pH (Rubanyi, 1991), thus increasing the amount of measured NO. Ammonium chloride, another lysosomotropic agent does not have the same effects as chloroquine on NO formation (our unpublished results), or on TNF-α processing (Jae-Yeon and Dae-Myung, 1997).

Lastly, chloroquine also influences the production of several cytokines involved in the activation of macrophages, including their NO formation. The effect of TNF-α on macrophage inducible NO synthase has been mentioned. Interleukin-1 and TNF-α have been found to act synergistically on NO production (Rockett et al., 1994). Chloroquine inhibits both the transcription of TNF-α mRNA (Zhu et al., 1993) and cytokine processing (Jae-Yeon and Dae-Myung, 1997) and may decrease NO synthesis in this way. Blocking of TNF-α by a specific antibody indirectly decreases NO synthesis, impairing cell's defense against parasites (Munoz-Fernandez et al., 1992).

It should be noted that in vivo chloroquine treatment resulted in the increase of NO production in spleen (Prada et al., 1996). This difference may be due: (1) to the different cell types; (2) to the differences frequently observed between in vivo and in vitro results, especially in the case where malaria-infected mice were studied; (3) to a possible contamination of chloroquine with lipopolysaccharide which is an inducer of inducible NO synthase; in the absence of polymyxin B 1–50 μM chloroquine caused an elevation of NO production of macrophages induced by interferon-γ in vitro, due to the lipopolysaccharide contamination of the drug (our unpublished results); (4) to a possible lysosomotropic effect of chloroquine leading to the increase of pH and the release of NO (see above).

As the effect of chloroquine is mediated, at least partly, via inhibition of inducible NO synthase, the drug could perhaps be used in pathological processes in which induction of NO is involved. Septic shock seems to be a promising target, as the production of TNF-α is also inhibited by chloroquine (Zhu et al., 1993, Jae-Yeon and Dae-Myung, 1997). However, chloroquine also induces venodilation in hand veins (Abiose et al., 1997) causing a possible decrease in blood pressure which is unfavorable for septic shock. An inhibitor of induction has the advantage of have no effect on constitutive NOS activity. This is important, because even the most recent inducible NO synthase inhibitors are only of limited specificity. Another advantage of chloroquine is that its pharmacological properties and doses are known. While the concentrations of drug used in these in vitro experiments are somewhat higher than the clinical doses, in vivo studies have also demonstrated that pharmacological concentrations of chloroquine inhibit several monocyte/macrophage functions (Osorio et al., 1992).

In conclusion, chloroquine irreversibly inhibits NO production by mouse and rat macrophages and their NO-dependent functions. This should be taken into account when giving chloroquine to mice experimentally infected with parasites for drug studies.

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