Erythrocyte Stages of *Plasmodium falciparum* Exhibit a High Nitric Oxide Synthase (NOS) Activity and Release an NOS-inducing Soluble Factor

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Summary

Nitric oxide (NO), a highly diffusible cellular mediator involved in a wide range of biological effects, has been indicated as one of the cytotoxic agents released by leukocytes to counteract malaria infection. On the other hand, NO has been implicated as a mediator of the neuropathological symptoms of cerebral malaria. In such circumstances NO production has been thought to be induced in host tissues by host-derived cytokines. Here we provide evidence for the first time that human red blood cells infected by *Plasmodium falciparum* (IRBC) synthesize NO. The synthesis of NO (measured as citrulline and nitrate production) appeared to be very high in comparison with human endothelial cells; no citrulline and nitrate production was detectable in noninfected red blood cells. The NO synthase (NOS) activity was very high in the lysate of IRBC (while not measurable in that of normal red blood cells) and was inhibited in a dose-dependent way by three different NOS inhibitors (L-canavanine, N^G-amino-L-arginine, and N^G-nitro-L-arginine). NOS activity in *P. falciparum* IRBC is Ca^{++} independent, and the enzyme shows an apparent molecular mass <100 kD, suggesting that the parasite expresses an isoform different from those found in mammalian cells. IRBC release a soluble factor able to induce NOS in human endothelial cells. Such NOS-inducing activity is not tissue specific, is time and dose dependent, requires de novo protein synthesis, and is probably associated with a thermolabile protein having a molecular mass >100 kD. Our data suggest that an increased NO synthesis in *P. falciparum* malaria can be directly elicited by soluble factor(s) released by the blood stages of the parasite, without necessarily requiring the intervention of host cytokines.

N itric oxide (NO)[1] is a short-lived free radical gas produced by a wide variety of cell types, the best studied of which are vascular endothelial cells (1, 2), macrophages (3–5), and neurons (6). NO is generated by a class of nicotinamide adenine dinucleotide phosphate (NADPH)-depen-

dent NO synthases (NOS), which catalyze the conversion of L-arginine to L-citrulline and NO with a 1:1 stoichiometry (7, 8). At least three different isoenzymes have been characterized (9, 10): Two of them are constitutive and $Ca^{++}/$ calmodulin activated (endothelial and neuronal cNOS), and one is an inducible, Ca^{++}-independent iNOS detected in macrophages. NO synthesis occurs in response to a large number of stimuli such as cytokines, endotoxin, and Ca^{++}-mobilizing agonists. NO displays manifold activities, such as smooth muscle relaxation, inhibition of platelet aggregation and adhesion, and decrease of smooth muscle cell proliferation (9, 11, 12). These events have been partly attributed to NO-mediated activation of a soluble guanylate cyclase (13), but NO interacts with other intracellular targets as an ADP-

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[1] *Abbreviations used in this paper:* cNOS, constitutive nitric oxide synthase; DTT, dithiothreitol; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cell; iNOS, inducible nitric oxide synthase; IRBC, infected red blood cell; L-NAA, N^G-amino-L-arginine; L-NNA, N^G-nitro-L-arginine; NADPH, nicotinamide adenine dinucleotide phosphate; NO, nitric oxide; NOS, nitric oxide synthase; PNIF, *Plasmodium*-derived NOS-inducing factor; RBC, red blood cell; SNP, sodium nitroprusside; Vmax, maximal rate.

ribosyltransferase (14), which has been identified in some tissues as glyceraldehyde-3-phosphate dehydrogenase (15). Glyceraldehyde-3-phosphate dehydrogenase impairment, as well as inhibition of ribonucleotide reductase (16) and iron- and sulfur-containing enzymes (17), may account for NO-elicited cytotoxicity.

NO has been shown to be involved in different aspects of *Plasmodium falciparum* malaria. Serum levels of cytokines known to induce NO synthesis, such as TNF and IL-1, are increased in malaria patients (18), and TNF or IL-1 injection into healthy mice considerably increases NO generation (19, 20). Killing of malaria parasites by the mouse immune system correlates with increased levels of nitrogen oxides in plasma (21, 22). NO or its derivatives inhibit the in vitro growth of asexual blood stages of *P. falciparum* (23) and of exoerythrocytic stages of *P. berghei* (24) and *P. yoelii* (25). During schizogony in blood, the infectivity of *P. vinckei petteri* gametocytes was markedly reduced but could be restored by prior injection of an NOS inhibitor (26). NOS inhibitors reverse the inhibition of development of the hepatic stage of *P. yoelii* induced by IL-6 and TNF (25). The injection of irradiated malaria sporozoites (27) or the exposure to mosquitoes infected with irradiated sporozoites (28) induces NO synthesis in mice hepatocytes, which is responsible for a liver refractory status against reinfection. *P. falciparum*-infected human hepatocytes develop the ability to inhibit the growth of parasites via induction of NO synthesis, which is potentiated by IFN-γ (29). Finally, a link between NO production and cerebral malaria has been suggested. During infection, NO released by TNF-stimulated vascular cells could diffuse to nearby neurons, thus interfering with neurotransmission and contributing to neurological symptoms (18, 19). All of the results mentioned above point to the following scenario: During malaria infection, the enhanced synthesis of cytokines (TNF, IL-1, IFN-γ, IL-6, etc.) induces host cells (leukocytes, endothelium, hepatocytes, etc.) to produce NO, and NO reduces the growth and invasiveness of parasites. Excessive NO production may lead to negative side effects for the host, thus contributing to malaria complications.

The present work was designed to study whether the *P. falciparum* is able per se to synthesize NO and/or directly evoke NO production in human cells. Our results demonstrate that the infected human red blood cells (IRBC) produce NO and release a soluble factor that induces NOS in human endothelial cells, thus suggesting a new pathogenetic mode of parasite-host interaction.

Materials and Methods

Reagents

Medium M-199 and trypsin/EDTA were from GIBCO-BRL (Paisley, Scotland); FCS was from Irvine Scientific (Santa Ana, CA); plastic for cell culture was from Costar Italia (Milan, Italy); collagenase and ionomycin were provided by Boehringer Mannheim (Mannheim, Germany); the cationic exchange resin Dowex AG50WX-8, *N*-(1-naphthyl) ethylenediamine dihydrochloride, and sulfanilamide were from Aldrich Italia (Milan, Italy); and L-[2,3,4,5-

[³H]arginine monohydrochloride (54–62 Ci/mmol), L-[1-¹⁴C]ornithine (58 mCi/mmol), and cGMP radioimmunoassay kit were from Amersham International (Little Chalfont, UK). RPMI 1640, Percoll, Giemsa, sodium nitroprusside (SNP), trifluoperazine, W5, dithiothreitol (DTT), EDTA, NADPH, flavin adenine dinucleotide, nitrate reductase (from Aspergillus), Hepes (sodium salt), Tris-HCl, urease (type VII), diacetylmonoxime (2,3-butanedione monoxime), thiosemicarbazide, L-arginine, DL-citrulline, N^G-nitro-L-arginine (L-NNA), N^G-amino-L-arginine (L-NAA), L-canavanine, 3-isobuthyl-1-methylxanthine (IBMX), *N*-benzoylarginine ethylester, and formamidine were from Sigma Chemical Co. (St. Louis, MO). Other reagents were from Merck (Bubendorf, Germany) and were of the highest purity available. The composition of Hepes buffer was as follows: 145 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 10 mM Hepes (sodium salt), 10 mM glucose, and 1 mM CaCl₂, pH 7.4, at 37°C. The composition of Hepes-Na/EDTA buffer was as follows: 20 mM Hepes-Na and 2 mM EDTA, pH 6. The composition of Hepes/EDTA/DTT buffer was as follows: 20 mM Hepes, 0.5 mM EDTA, and 1 mM DTT, pH 7.2. Ionomycin was dissolved at 1 mM in DMSO and stored at −20°C until use; SNP was freshly prepared by dissolving at 100 mM in buffered saline. The protein content of cell monolayers was assessed with the modified micro-Lowry method (kit from Sigma Chemical Co.). Mouse mAbs anti-human endothelial cNOS (eNOS) and anti-murine macrophage iNOS directed against protein fragments of 179 amino acids (eNOS, $W^{1030} \rightarrow L^{1209}$) and 183 amino acids (iNOS, $F^{961} \rightarrow L^{1144}$), respectively, were from Transduction Laboratories (Lexington, KY). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA).

Cell Cultures

Human Umbilical Vein Endothelial Cells (HUVEC). HUVEC were obtained by treating human umbilical cord veins with collagenase from *Clostridium histolyticum* (Boehringer Mannheim) (10 mg/ml medium 199) cultured in 75 cm² plastic flasks in medium 199 containing 20% FCS and characterized as previously described (30).

P. falciparum Parasites. *P. falciparum* parasites (strains FCR-3, D2, D6, W2, HBR3) were kept in culture in human red blood cells (RBC) (type 0⁺ or A⁺) suspended in growth medium (RPMI 1640 supplemented with 10% heat-inactivated human AB⁺ plasma, 32 mM NaHCO₃, 25 mM Hepes, and 10 mM glucose, pH 7.4) as described previously (31). To obtain packed RBC, 1 ml of suspensions (1% hematocrit) of IRBC (10–20% parasitemia) or RBC was centrifuged at 1,000 g for 10 min and washed twice with PBS. When it was necessary to eliminate the RBC cytosol, the RBC membrane was permeabilized by Sendai virus. This treatment results in the release of >95% of host cell cytosol and does not impair parasite viability (32). Red cell suspensions (1 ml, 1% hematocrit and 10–20% parasitemia) in wash medium (culture medium without human plasma and bicarbonate) were incubated with Sendai virions (20–50 μg protein/ml, equivalent to 400–600 hemagglutination U) for 10 min at 37°C and centrifuged for 5 min at 2,200 g (31). The supernatant, consisting of host cell cytosol, was removed, and the pellet containing the parasites was washed twice in wash medium, resuspended in 1 ml Hepes/EDTA/DTT, and sonicated and diluted (see below) for NOS measurement.

Rodent Plasmodia. *P. yoelii nigeriensis* (NIG strain), *P. yoelii killicki* (194 ZZK strain), *P. yoelii yoelii* (265 BY and 17 X strains), *P. vinckei petteri* (279 BY strain), *P. vinckei vinckei* (67 strain), *P. chabaudi chabaudi* (864 VD strain), *P. chabaudi adami* (887 KA strain), *P. berghei* (ANKA strain), and *P. berghei* (NK 65 strain) were maintained in the laboratory by cryopreservation. Male outbred Swiss, C57BL/6, and CBA/Ca mice (18–20 g) (Iffa Credo,

L'Arbresle, France) were infected by injecting intraperitoneally 0.2 ml of thawed parasitized blood diluted 1:2 with isotonic saline. When parasitemias (23.9 ± 5.6%; n = 8) were patent, 1 ml of whole blood was withdrawn from the retroorbital sinus with a heparinized Pasteur pipette. RBC from control mice were withdrawn by the same procedure explained before. After removing plasma, platelets, and leukocytes by centrifugation, packed erythrocytes were frozen in liquid nitrogen and checked for NOS activity in triplicate after 24 h.

Measurement of NOS Activity in Cell Lysates

Human or murine RBC and IRBC were resuspended in 1 ml Hepes/EDTA/DTT buffer and sonicated (two 12-s bursts on crushed ice); if necessary (falciparum IRBC), before the test, the hemolysate was further diluted 40-fold with the same buffer. HUVEC grown to confluence in 75-cm^2 flasks were detached by trypsin/EDTA (0.05:0.02% vol/vol). Cells from 8–12 flasks were pooled, washed with PBS, and then resuspended in 1 ml of Hepes/EDTA/DTT buffer and sonicated on ice with three 10-s bursts. In each test tube, the following reagents were added to 100 μl cell lysate up to a final volume of 120 μl and the indicated final concentrations: 2 mM NADPH, 1.5 mM CaCl$_2$, 1–100 μM L-arginine, and 2.5 μCi L-[^{3}H]arginine (0.4 μM) (33, 34). When indicated, NOS inhibitors were added to the other reagents. A blank was prepared by replacing the cell lysate with an equal volume of Hepes/EDTA/DTT buffer. After a 15-min incubation at 37°C, the reaction was stopped by adding 2 ml cold Hepes-Na/EDTA buffer. The whole reaction mixture was applied to 2-ml columns of Dowex AG50WX-8 (Na$^+$ form) and eluted with 4 ml of water. The radioactivity corresponding to [^{3}H]citrulline content in ~6.1 ml eluate was measured by liquid scintillation. In *P. falciparum* IRBC, NOS activity was expressed as picomoles citrulline produced/min per 10^7 lysed parasites; this calculation was based on the number of RBC (counted by a Bürker chamber), their parasitemia, and an average of 1 trophozoite or 1.2 ring per IRBC; in HUVEC, NOS activity was expressed as picomoles citrulline produced/min per milligram cell protein.

Western Blots

Human RBC, trophozoite IRBC, and HUVEC were directly solubilized in boiling Laemmli buffer, and proteins, separated by SDS-PAGE (8%), were transferred to nitrocellulose sheets and probed with an mAb anti-iNOS (1:500) or anti-eNOS (1:250) and detected by enhanced chemiluminescence (Amersham International).

Measurement of Citrulline Synthesis in Intact Human RBC and HUVEC

Citrulline synthesis was measured by a radiometric method (detection of extracellular concentration in short-term experiments) and by a colorimetric method (measurement of extracellular citrulline accumulation during long-term incubations). Radiometric citrulline detection in RBC was performed as a modification of previously described procedures (32–34). RBC (2.5 × 10^7) were resuspended in 0.1 ml Hepes buffer containing 10 μCi (1.6 μM) L-[^{3}H]arginine. After incubation at 37°C for 15 min in the presence of 2 μM ionomycin or DMSO (as a control), the reaction was stopped by washing cells with cold PBS containing 5 mM L-arginine and 4 mM EDTA. The samples were centrifuged at 12,000 g for 10 s, and 50 μl supernatant was mixed with 2 ml Hepes-Na/EDTA buffer. The mixture was applied to a Dowex column, and [^{3}H]citrulline-related radioactivity was counted in the eluate, as indicated before. [^{3}H]Citrulline synthesis in HUVEC was mea-

sured in a similar way, as previously described (35). Citrulline synthesis was expressed as picomoles citrulline produced/min per 10^7 parasites (IRBC) or picomoles citrulline produced/min per milligram cell protein (HUVEC). Colorimetric citrulline detection in the extracellular medium of RBC suspensions was performed according to Boyde and Rahmatullah (36). Briefly, after urease treatment (2 U/ml, 30 min, 37°C) and deproteinization with 5% TCA and centrifugation, 0.1 ml supernatant was mixed with 3 ml chromogenic solution and boiled. After cooling, absorbance at 530 nm was measured. Citrulline synthesis was expressed as nanomoles citrulline produced/24 h per 10^7 parasites; because of the different experimental conditions (1.26 mM extracellular L-arginine in the latter setting), these results cannot be superimposed on those obtained by the radiometric technique (where the extracellular L-arginine concentration was only 1.6 μM, and much arginine is produced intracellularly during the digestion of host cell cytosol by the parasite).

Measurement of Nitrite

Nitrite production was measured by adding 0.15 ml of medium from human RBC, IRBC, and HUVEC cultures to 0.15 ml of Griess reagent (37), and, after 30 min of incubation at room temperature in the dark, absorbance at 540 nm was measured using a microplate reader.

Measurement of Nitrate

Since RPMI 1640–derived growth medium contains very high amounts of nitrate, we incubated RBC and IRBC (FCR-3 strain, ring stage) in the low-nitrate medium M-199 for 24 h. After this time, during which parasitemia (13 ± 2.7%; n = 3) did not significantly change, nitrate production was measured by a kinetic assay using *Aspergillus* nitrate reductase (38). 0.1 ml of extracellular medium was mixed with 0.2 ml of reaction mixture (final concentrations: 2.5 μM flavin adenine dinucleotide, 100 μM NADPH, and 56 mM K$_2$HPO$_4$, pH 7.5), and 18 mU of nitrate reductase was added. The oxidation of NADPH was monitored in a microplate reader by following the change in absorbance at 340 nm for 20 min (during this time the oxidation of NADPH was linear). The change in absorbance of fresh medium M-199 was taken as a blank.

cGMP Determination

Packed human RBC and IRBC (20 μl) were resuspended in 200 μl Hepes buffer containing 0.1 mM L-arginine and 0.2 mM IBMX; after 30 min incubation at 37°C, in the presence or absence of 2 μM ionomycin and 1 mM SNP, samples were centrifuged at 12,000 g for 10 s, supernatant was discarded, and the pellet was resuspended in 50 μl absolute ethanol. Solvent was evaporated by vacuum centrifugation, and the pellet was resuspended in 300 μl Tris/EDTA buffer (50 mM Tris-HCl, 4 mM EDTA, pH 7.5); after 10 min of shaking, samples were centrifuged and 100 μl supernatant was used for cGMP measurement. RBC cGMP content was measured by radioimmunoassay (39) and expressed as picomoles/10^7 RBC.

Effects of Drugs on Parasite Growth

Synchronous parasite cultures at the ring stage were cultivated in the presence of increasing drug concentrations for 24 h, and then [^{3}H]hypoxanthine was added to a final concentration of 10 μCi/ml. After an additional 24 h in culture, cells were harvested onto glass fiber filters, and the radioactivity was determined by liquid scintillation. The extent of [^{3}H]hypoxanthine incorporation was

taken as a measure of parasite viability, and this was compared with untreated control cultures. The IC$_{50}$ value ± SEM was calculated for each drug according to Desjardins et al. (40).

Statistical Analysis

Each experimental point was performed in duplicate or triplicate (per experiment), and all data are given as mean values ± SEM. Statistical analysis was carried out using the Student's t test for unpaired data.

Results

Characterization of NOS Activity in IRBC

Enzyme Activity and Kinetics. NOS activity was measured in the cell lysates of both normal human RBC and ring or trophozoite IRBC (FCR-3 strain). In RBC (n = 7), NOS activity was undetectable, whereas, in both ring and trophozoite IRBC, NOS activity was remarkable. IRBC NOS activity exhibited a substrate dependence that followed a Michaelis-Menten pattern (Fig. 1). In ring IRBC (n = 5), maximal rate (Vmax) was 444.4 ± 120 pmol citrulline/min per 10^7 lysed parasites, and half-maximal activity was detectable at 1.37 ± 0.3 μM L-arginine. In trophozoite IRBC (n = 6), the Vmax was 190.9 ± 49.5 pmol citrulline/min per 10^7 lysed parasites, and K_m = 3.8 ± 0.8 μM L-arginine. Vmax values were not significantly different in the two IRBC populations, whereas K_m for L-arginine was significantly higher in trophozoite IRBC (P = 0.03). NOS activity in IRBC
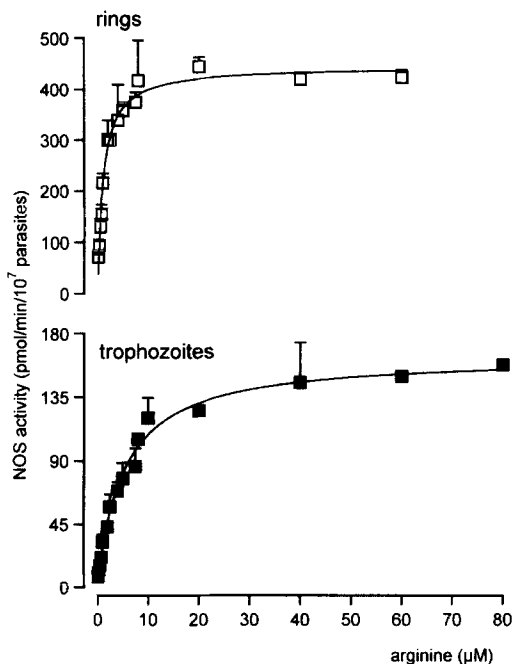

Figure 1. L-Arginine dependence of NOS activity in lysates of ring IRBC (n = 5; *top*) and trophozoite IRBC (n = 6; *bottom*). Each point represents mean values ± SEM; the curves are ideal plots obtained with the Michaelis-Menten equation: Y = (X × Vmax)/(X + K_m), where X = L-arginine concentration (0.4 μM L-[^{3}H]arginine + 1–100 μM L-arginine), and Vmax and K_m are the values obtained for each cell type by Lineweaver-Burk transformation.

was much higher than in HUVEC: Vmax in HUVEC was 18 ± 3.4 pmol citrulline produced/min per 10^7 lysed cells (n = 4). To exclude the presence of arginase activity, 50 mM L-valine (41) was added to the reaction mixture, and no change of the radioactivity in the column eluate was detected, suggesting that arginase activity was virtually absent in IRBC. The presence of other urea cycle–related enzymes was ruled out, since, after addition of 2 μCi [^{14}C]ornithine to the IRBC lysates, no ^{14}C-labeled substance eluted in the fraction corresponding to citrulline. An alternative source of citrulline in living systems is arginine deiminase, an enzyme converting L-arginine into L-citrulline and ammonia. Its activity is detectable in bacteria but not in mammalian cells (9). The existence of such an enzyme in IRBC was ruled out, as formamidine, a known inhibitor of arginine deiminase (34), did not influence arginine conversion even at 1-mM concentration. Moreover N-benzoylarginine ethylester (1 mM), a substrate for arginine deiminase (34), did not compete with [^{3}H]arginine. Since malaria cultures were run with whole human blood, NOS activity could possibly stem from activated leukocytes present in malaria cultures. This possibility was ruled out, since cultures prepared with washed RBC or with buffy coat–enriched RBC gave the same results (data not shown). Trophozoite IRBC suspensions (20–30% parasitemia, FCR-3 strain) were permeabilized by Sendai virus treatment as described in Materials and Methods and then centrifuged at 2,200 g for 5 min. NOS activity was measured (n = 3) both in the pellet fraction (134.08 ± 6.98 pmol/min per 10^7 parasites) and in the supernatant (5.6 ± 0.46 pmol/min per 10^7 parasites). As total activity in the whole lysate was 139.96 ± 6.52 pmol/min per 10^7 parasites, the erythrocyte cytosolic compartment contained only ~4% of IRBC NOS activity, and such activity is likely to be released from the small amount of parasites that are damaged during Sendai virus treatment. NOS activity did not differ significantly when measured in RBC lysates freshly prepared or stored for 24–72 h at −80°C or in liquid nitrogen. To clarify whether such a high NOS activity was a feature of FCR-3 strain only, we checked enzyme activity in RBC infected with other strains of *P. falciparum* (Table 1). NOS activity was clearly detectable in all strains of *P. falciparum* investigated. To ascertain whether NOS activity could be a common feature of *Plasmodium* parasites, we looked for its presence in Swiss murine RBC infected with 10 different strains of plasmodia: *P. yoelii nigeriensis* (NIG strain), *P. yoelii killicki* (194 ZZK strain), *P. yoelii yoelii* (265 BY and 17 X strains), *P. vinckei petteri* (279 BY strain), *P. vinckei vinckei* (67 strain), *P. chabaudi chabaudi* (864 VD strain), *P. chabaudi adami* (887 KA strain), *P. berghei* (ANKA strain), and *P. berghei* (NK 65 strain). NOS activity was not detectable in any of the RBC lysates obtained by infected or control mice (n = 3 for each condition). On infection with *P. berghei* ANKA, mice of susceptible strains such as CBA/Ca (42) and C57BL/6 (43) are known to develop a neurological syndrome, including seizures and coma, that is lethal in all untreated cases. IRBC from CBA/Ca (n = 3) and C57BL/6 (n = 3) strains infected with *P. berghei* ANKA did not show any NOS activity.

Enzyme Features. NOS activity in *P. falciparum* IRBC

Table 1. *Nos Activity in Different Strains of P. falcipaum*

Strain	NOS*activity*	*n*
	pmol/min per 10⁷ parasites‡	
FCR-3	154.3 ± 12.05ⁱⁱ	4
D2	117.47 ± 26.19	4
D6	124.74 ± 29.9	4
W2	99.1 ± 13.3	4
HB3	106.4 ± 2.4	4

*Enzyme activity was measured in cell lysates as described in Materials and Methods, in the presence of 0.4 μM [³H]arginine alone.
‡ Parasitemias were, in each experiment, 15–20% trophozoites.
ⁱⁱ Means ± SEM.

(FCR-3 strain) appeared to be Ca^{++}/calmodulin independent, as it was not modified in the presence of EDTA (1 mM) and the calmodulin inhibitors trifluoperazine (0.1 mM) and W5 (0.1 mM). D-arginine, up to 1 mM, did not compete with L-[³H]arginine, indicating that IRBC NOS was stereospecific. The NOS activity was maintained after having filtered the lysate through a filter (Ultraspin; Cambio, Milano, Italy) with a cut-off at 100,000 daltons, whereas it was completely abolished when the lysate passed through an Ultraspin filter with a cut-off at 50,000 daltons; in the latter case the activity was completely recovered in the filter. To determine whether IRBC expressed NOS protein(s) antigenically similar to those
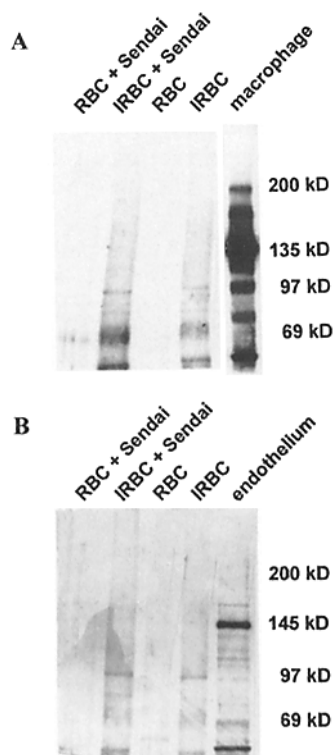

= L-NAA   □ = L-NNA   O = L-canavanine

Figure 3. Dose–response curve of NOS activity in the lysate of ring IRBC (*top*) and trophozoite IRBC (*bottom*) in the presence of three different NOS inhibitors. Measurement of enzymatic activity was performed as indicated in Materials and Methods in the presence of 2.5 μCi (0.4 μM) L-[³H]arginine. Enzyme activity is expressed as percentage of NOS activity (assumed to be 100% in the absence of inhibitors). Each point represents the mean value ± SEM of four different experiments performed in duplicate.

found in mammalian cells, as macrophages (iNOS) or endothelial cells (eNOS), we carried out Western blot analysis of total cellular proteins by using mAbs anti–murine macrophage iNOS and anti–human eNOS. Fig. 2 shows that IRBC, but not RBC, express a 97-kD protein recognized by both

Figure 2. Western blot analysis of: whole-cell lysates from control (RBC) and *P. falciparum* IRBC, and pellets obtained by treating RBC and IRBC with Sendai virus. Experiments were performed with mAbs anti–murine macrophage iNOS (*A*) and anti–human endothelial cNOS (*B*). Standard lysates from murine macrophage and human endothelium, respectively, were used as positive controls. Representative blots of three different experiments giving similar results are shown.
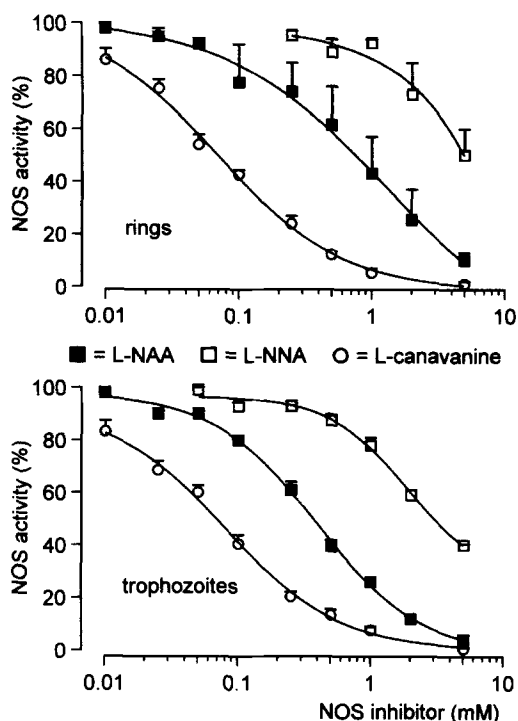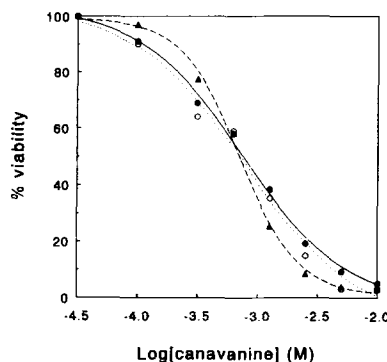

Figure 4. Effect of L-canavanine on the growth of *P. falciparum* strains in culture. Cultures at the ring stage (1% hematocrit, 2% parasitemia) were exposed to increasing concentrations of canavanine for 24 h. Then [³H]hypoxanthine was added, and cells were harvested 24 h later. The cell-associated radioactivity was determined and assumed as 100% viability. FCR-3 (*closed circles*), IC_{50} = 0.74 ± 0.06 mM; W2 (*open circles*), IC_{50} = 0.76 ± 0.11 mM; D6 (*closed triangles*) IC_{50} = 0.7 ± 0.029 mM. Results are from three different experiments.

anti-iNOS and anti-eNOS antibodies; other bands, corresponding to molecular masses of 85–70 kD, were less evident. Such results agree with ultrafiltration results, indicating that NOS activity is associated with protein(s) having an apparent molecular mass between 50 and 100 kD.

Enzyme Inhibition. *P. falciparum* IRBC (FCR-3 strain) NOS activity was differently inhibited by three NOS inhibitors, L-canavanine, L-NAA, and L-NNA (Fig. 3). IC_{50} values in ring IRBC ($n = 4$) and trophozoite IRBC ($n = 4$) were 0.07 and 0.08 mM (L-canavanine), 1.29 and 0.39 mM (L-NAA), ∿5 and 3.5 mM (L-NNA), respectively.

Effect of NOS Inhibitors on the Growth of Parasites. RBC infected with different strains of *P. falciparum* at the ring stage (1% hematocrit, 2% parasitemia) were incubated for 24 h with increasing concentrations of L-canavanine, the most effective NOS inhibitor in vitro. [³H]Hypoxanthine incorporation by IRBC, measured as a viability index, was inhibited in a dose-dependent way (Fig. 4), suggesting that NO production is relevant for the parasite. When L-NNA was used as inhibitor, parasite growth inhibition was lower than with L-canavanine, thus paralleling the different relative effects of the two compounds on NOS activity. IC_{50} values for L-NNA were as follows (means ± SEM): FCR-3, 5.25 ± 0.15 mM ($n = 3$); W2, 4.07 ± 0.13 mM ($n = 3$); and D6, 1.91 ± 0.13 mM ($n = 3$). On the other hand, the addition of exogenous NO, as SNP, was also toxic for IRBC cultures. The actual IC_{50} value for SNP was $9.33 ± 0.45 × 10^{-7}$ M ($n = 3$; FCR-3 strain), thus confirming previous results concerning inhibitory effect of SNP and other NO donors on intraerythrocytic stages of *P. falciparum* (44). It has already been demonstrated that the toxic effect of SNP is not attributable to ferricyanide moiety but rather to its NO-releasing property (44).

Evidences of NO Production in Intact Cells

Nitrite and Nitrate Production. Nitrite measurement performed with the Griess reagent did not show NO_2^- accumulation in the culture medium of both human RBC ($n = 6$) and IRBC suspensions ($n = 9$) after 2–72 h incubation. This negative result was not surprising, since it is well known that hemoglobin is a powerful NO scavenger and prevents nitrite formation. On the other hand, NO is known to react rapidly with oxyhemoglobin to give nitrate and methemoglobin (45). RBC did not produce nitrate in the extracellular medium ($n = 3$), whereas, in IRBC, net nitrate production in the supernatant was 7.4 ± 1.55 nmol/24 h per 10^7 parasites ($n = 3$), and incubation with 2 mM L-canavanine inhibited nitrate production by 59%.

Citrulline Synthesis (Short-term Incubation). To detect NO synthesis in short periods of time, we measured [³H]citrulline production after cell incubation with [³H]arginine. Within 15 min, [³H]citrulline accumulation was not detectable in the supernatant of human RBC, whereas, in falciparum IRBC (FCR-3 strain), it was clearly present, both in ring ($n = 6$) and trophozoite IRBC ($n = 7$) (Fig. 5). [³H]Citrulline production was significantly higher ($P = 0.02$) in trophozoite IRBC than in ring IRBC. L-Canavanine inhibited [³H]citrulline synthesis in a dose-dependent way (Fig. 5).
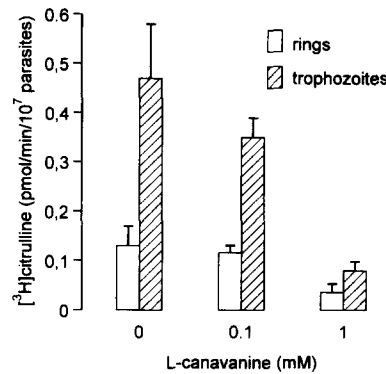

Figure 5. [³H]Citrulline synthesis by ring IRBC ($n = 6$) and trophozoite IRBC ($n = 7$) in the absence (0 mM) or in presence of L-canavanine (0.1 and 1 mM). [³H]Citrulline production was measured in the extracellular medium after 15 min of incubation with [³H]arginine (see Materials and Methods). Data are means ± SEM of experiments performed in duplicate.

The calcium ionophore ionomycin (2 μM) did not influence the [³H]citrulline production in either RBC or IRBC, thus suggesting that NOS activity was not dependent on the increase of intracellular Ca^{++}. A cell-associated [³H]citrulline formation was detectable in IRBC (Fig. 5), but, unlike HUVEC (35), most synthesized [³H]citrulline was extracellular (data not shown). This phenomenon could be attributed to a very fast extrusion of citrulline by IRBC. If IRBC contained pathways to reconvert citrulline into arginine, as the urea cycle in hepatocytes and the arginine–citrulline cycle in endothelial cells (46), this could induce an underestimation of NO synthesis in intact cells. To clarify this point, IRBC were incubated with "cold" citrulline (1 mM) together with L-[³H]arginine. No dilution of [³H]citrulline was observed in these conditions (data not shown), thus suggesting that citrulline is not transformed into arginine by the IRBC.

Citrulline Synthesis (Long-term Incubation). After 24 h of incubation in culture medium, citrulline, measured colorimetrically, was not detectable in human RBC supernatant ($n = 4$) but was clearly measurable in *P. falciparum* IRBC (FCR-3 strain) supernatant. In ring IRBC, citrulline production was 1.55 ± 0.41 nmol/24 h per 10^7 parasites ($n = 3$), and in trophozoite IRBC it was 1.49 ± 0.14 nmol/24 h per 10^7 parasites ($n = 5$). Citrulline production was dependent on substrate availability, since added arginase (2 U/ml throughout the incubation period) inhibited citrulline accumulation by 69 ± 4% ($n = 3$). Colorimetric detection recognizes the presence of the ureido group in citrulline; these results allowed us to exclude that the radioactivity detected in the citrulline fraction of chromatography could be attributed to arginine derivatives (i.e., argininic acid, produced by some bacteria) having an isoelectric point similar to that of citrulline.

cGMP Content. cGMP intracellular level in HUVEC is strictly related to endogenous NO production (35, 47). To look for an NO-sensitive guanylate cyclase in normal and infected human RBC, we measured the basal and SNP-stimulated synthesis of cGMP in RBC and trophozoite IRBC
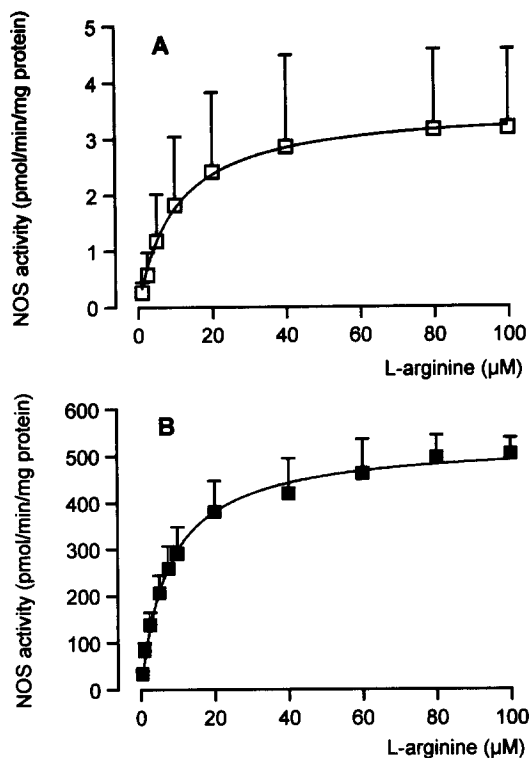
Figure 6. L-Arginine dependence of NOS activity in lysates of HUVEC incubated for 24 h with supernatant from RBC suspensions (A; n = 3) and trophozoite IRBC cultures (B; n = 3). Each point represents mean values ± SEM; the curves were generated as described in the legend to Fig. 1.
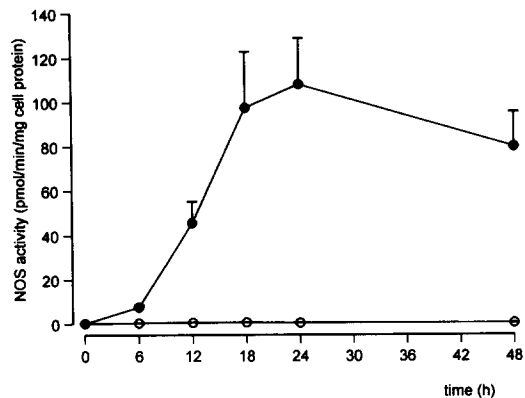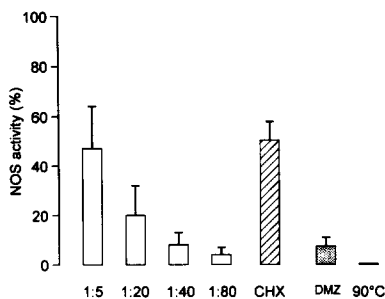

Figure 8. NOS activity in the lysate of HUVEC after 24 h of incubation with IRBC-conditioned medium at different conditions. Conditioned medium was diluted as indicated (1:5–1:80) with fresh growth medium, or supplemented with 10 μg/ml cycloheximide (CHX) or 1 μM dexamethasone (DMZ), or heated at 90°C for 5 min; then the modified conditioned medium was added at a 1:1 (vol/vol) ratio with fresh medium M-199 containing 20% FCS. After 24 h of incubation (or only 6 h for CHX and respective control), HUVEC were washed with PBS, lysed, and checked for NOS activity, as indicated in Materials and Methods, in the presence of 2.5 μCi (0.4 μM) L-[3H]arginine. Conditioned medium was fresh growth medium previously incubated for 24 h with IRBC (10–20% parasitemia). Enzyme activity is expressed as percentage of NOS activity (assumed to be 100% in HUVEC incubated with nondiluted IRBC-conditioned medium). Each column is a mean ± SEM of three different experiments.

(FCR-3 strain). cGMP levels in the two cell types were very low and not significantly different: 0.02 ± 0.01 pmol/10^7 RBC (n = 3) in RBC and 0.03 ± 0.005 pmol/10^7 RBC (n = 3) in trophozoite IRBC (100% parasitemia). SNP (1 mM) stimulation had no effect on cGMP levels in either cell type. Because of the high intracellular content of hemoglobin, it is difficult to state whether the inefficacy of SNP is attributable to the absence of a soluble, NO-sensitive guanylate cyclase or, more likely, to the complete scavenging of exogenous NO by hemoglobin.

Effect of IRBC-conditioned Medium on NOS Activity in HUVEC

Some pathogens are known to induce NOS activity in white cells (48, 49). Since high levels of NO were detected in malaria infections (21, 22), it was plausible to assume that malarial parasites may also produce NOS-inducing factor(s). If so, the incubation of different cells with conditioned medium from falciparum IRBC cultures should lead to an increase of NOS

Figure 7. Time course of NOS activity induction in HUVEC incubated with RBC- or IRBC-conditioned medium. NOS activity was measured in the lysate of HUVEC incubated for the indicated time periods with conditioned medium from suspensions of RBC (open circles) or IRBC (closed circles). Conditioned medium was fresh growth medium incubated for 24 h with RBC or IRBC (10–20% parasitemia) and then added to HUVEC cultures at a 1:1 (vol/vol) ratio with fresh medium 199 containing 20% FCS. After the indicated time periods, cells were washed and the measurement of enzymatic activity was performed as indicated in Materials and Methods, in the presence of 2.5 μCi (0.4 μM) L-[3H]arginine and 1 μM L-arginine. Each point is a mean ± SEM of three different experiments performed in duplicate.
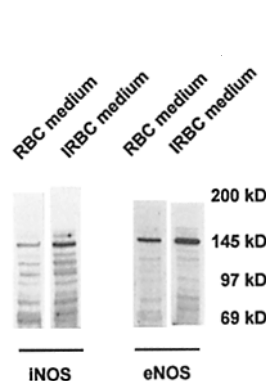

Figure 9. Detection of iNOS and eNOS in HUVEC stimulated with RBC- and IRBC-conditioned media. HUVEC were incubated for 24 h with growth medium (40% vol/vol) from RBC and IRBC suspensions. On the whole HUVEC lysate, a Western blot analysis was performed with an mAb anti-murine macrophage iNOS and mAb anti-human eNOS, respectively. No difference was observed between HUVEC alone and RBC medium-treated HUVEC (data not shown). Representative results from three different experiments.

activity in target cells. HUVEC, chosen as target cells, were incubated for 24 h with trophozoite IRBC (FCR-3 strain)–conditioned medium; thereafter, the HUVEC monolayer was washed and NOS activity was measured in the lysate, prepared as previously described, in the presence of 2.5 μCi (0.4 μM) L-[^{3}H]arginine plus varying concentrations of cold L-arginine (1–100 μM). NOS maximal activity in HUVEC treated with supernatant from RBC was 3.6 ± 1.7 pmol citrulline/min per mg cell protein (n = 3), a value which did not differ from that found in control HUVEC (50). Enzyme maximal activity increased ~150-fold in HUVEC incubated with conditioned medium from trophozoite IRBC (547 ± 47 pmol citrulline/min per mg cell protein, n = 3, P <0.0001); K_m did not significantly differ in the two conditions (10.1 ± 1.5 μM arginine versus 10.6 ± 4.7 μM arginine, respectively). Similar results were obtained with conditioned medium from W2 and D6 P. *falciparum* strains (data not shown). NOS activity was Ca^{++} independent; the substrate dependence of the enzyme activity followed a typical Michaelis-Menten pattern (Fig. 6). The HUVEC enzyme activity was totally removed after ultrafiltration of the lysate with a filter with a cut-off of 100 kD; NOS activity was completely recovered from the filters (data not shown). The increase in NOS activity in HUVEC was already detectable after 6 h of incubation and reached its maximum after 18–24 h (Fig. 7). In the presence of cycloheximide (10 μg/ml), NOS induction at 6 h was inhibited by 49.5 ± 7.5% (n = 3) (Fig. 8). After 24 h preincubation of HUVEC with 1 μM dexamethasone (which is known to inhibit the increase of iNOS), the conditioned medium–induced NOS activity was inhibited by 92.5 ± 3.5% (n = 3). The ability of the conditioned medium to induce NOS in HUVEC after 24 h incubation was still maintained after a 1:40 dilution of the conditioned medium with fresh medium (Fig. 8) but was completely abolished after heating at 60°C for 30 min or at 90°C for 5 min. The NOS-inducing activity was completely abolished when IRBC-conditioned medium was previously filtered through an Ultraspin filter with a 100-kD cut-off; the activity was completely recovered in the filters. Since IRBC cultures were performed in whole blood, it was conceivable that some cytokine(s) delivered from contaminating leukocytes activated by parasites might account for NOS-inducing activity of conditioned medium. This is not likely, since conditioned medium from both cultures prepared with washed RBC and cultures enriched with buffy coat showed the same ability to induce endothelial NOS (data not shown). NOS-inducing activity was not significantly different in the presence or absence of 2.5–10 mM mannose, inositol-1-phosphate, and phosphatidylinositol, alone or in any combination; these compounds are known to inhibit the effects of parasite-derived phospholipid exoantigens on target cells (51, 52). After 24 h incubation of HUVEC with 20 μl packed IRBC, an increase of NOS activity comparable to that obtained with the conditioned medium was measured; half of the same effect was obtained with the lysate of the same amount of IRBC (data not shown). No increase of NOS was detectable in HUVEC after incubation with packed or lysed human normal RBC or RBC from malaria-infected mice. HUVEC were incubated for 24 h with

RBC- or IRBC (FCR-3)-conditioned medium, and their lysates were then tested for iNOS and eNOS proteins by Western blot analysis. IRBC medium–treated HUVEC showed an apparent increase of a 140–145-kD protein reacting with both eNOS and iNOS antibodies (Fig. 9). To evaluate the effect of conditioned medium on NO synthesis in intact HUVEC, we incubated endothelial cells (grown at confluence in 35-mm dishes) with RBC- and IRBC-conditioned medium for 8 h, followed by 16 h of incubation with fresh medium. After this time, nitrite concentration in the supernatant was 1.65 ± 0.8 nmol/24 h per mg cell protein in RBC-conditioned HUVEC (n = 4) and 26.52 ± 4.3 nmol/24 h per mg cell protein in IRBC-conditioned HUVEC (n = 4) (P <0.01). When short-term [^{3}H]citrulline synthesis was performed in the same conditions, in RBC medium–treated HUVEC, intracellular synthesis was 0.057 ± 0.01 pmol/min per mg cell protein (n = 4) versus 0.293 ± 0.036 (n = 4) in IRBC medium–treated HUVEC (P <0.002).

Discussion.

NO has been implicated in different aspects of P. *falciparum* malaria infection (53). The ability of cytokine-treated macrophages, neutrophils, and hepatocytes to kill plasmodia seems to correlate with their NO production (21, 22, 24, 25, 27, 29), and NO derivatives inhibit the growth of P. *falciparum* in vitro (23, 44). On the other hand, the neurological symptoms in malaria patients have been attributed to increased plasma levels of TNF (18, 19). This cytokine is able to induce in many tissues the synthesis of NO, which could be the real effector of cerebral malaria. TNF produced in response to malaria infection could induce NOS activity in endothelium, smooth muscle, and leukocytes (19). NO might diffuse to the brain and damage its functions by acting in different ways. As a cytotoxic agent, it could cause lipoperoxidation and inhibition of glycolysis and cell respiration (15, 17); neuronal death after ischemia and hypoglycemia has been attributed to an abnormal NO firing by nonadrenergic noncholinergic neurons (6). As a neurotransmitter (6), NO may disturb neuronal functions, and, as a vasodilator, it can contribute to the increase of intracranial pressure and to systemic hypotension observed in malaria patients (19). Until now, NO production in malaria infection has been thought to be the consequence of an increased secretion of cytokines by the host in response to the parasite (20, 22, 24, 25).

In the present work, we demonstrate that Plasmodium itself may generate NO and produce some soluble factor(s) able to evoke NO synthesis in host tissues.

NOS activity, measured as both citrulline and nitrate production, is present in human RBC parasitized with P. *falciparum*, is associated to the intracellular parasite, and is present both in ring and trophozoite IRBC. The enzyme content (which is related to Vmax) in the two blood stages is similar, whereas affinity for the substrate differs. NOS activity is shared by any strain of P. *falciparum* studied but is not a general property of Plasmodium IRBC, as murine erythrocytes infected by 10 different strains of rodent parasites do not exhibit NOS

activity. [³H]Citrulline synthesis in the *P. falciparum* IRBC lysate is attributable neither to urea cycle–related enzymes nor to an arginine deiminase, and it is impaired by NOS inhibitors as L-canavanine, L-NAA, and L-NNA (Fig. 3). The parasite NOS is independent from Ca^{++} concentration in the reaction mixture and is not impaired by calmodulin inhibitors, similar to the inducible isoform found in mammalian macrophages. L-Canavanine is the most effective inhibitor, and it has been reported that this compound is relatively more specific for the inducible, Ca^{++}-independent NOS (12, 37). Indeed, L-canavanine is a weak inhibitor of HUVEC cNOS; IC_{50} for L-canavanine is 1.52 mM in HUVEC (50) versus 0.07 and 0.08 mM in ring and trophozoite IRBC, respectively. The IRBC NOS has an apparent molecular mass lower than mammalian NOSs, and it is recognized by both anti-eNOS and anti-iNOS mAbs; thus, it is likely to belong to a new class of NO-synthesizing enzymes.

NOS activity in the lysate cannot be equated to the actual NO production in intact cells. Its measurement is performed in a reaction mixture containing saturating concentrations (2 mM) of NADPH, which are much higher than those observed in intact RBC (about 30 μM) (54); moreover, cell disruption may abolish any enzyme compartmentalization, which could limit the availability of the substrate and/or cofactors. To gain information about the NO production in intact cells, it is advantageous to monitor the accumulation of nitrite or citrulline in the bathing medium after a 24-h incubation of IRBC in fresh culture medium; furthermore, short-term production of [³H]citrulline from [³H]arginine is the only way to check the NO production soon after a stimulus, and it allows the measurement of the Ca^{++}-dependent component of NO synthesis in intact cells. Production of citrulline from arginine was observed in intact IRBC, but not in control RBC, both with radiometric (Fig. 5) and colorimetric methods. Citrulline production was not modified by ionomycin, a calcium ionophore. It was inhibited by L-canavanine in a dose-dependent way and depended on availability of extracellular L-arginine, since pretreatment with arginase blocked its accumulation in the medium. The values of citrulline accumulation after 24 h in IRBC cultures were markedly lower than those predictable from NOS activity in vitro. This suggests that the parasite enzyme operates normally at a rate that is a small fraction of its Vmax; such a restraint of the actual activity may be due to a limited availability of substrate and/or cofactors. Alternatively, it is possible that most of the citrulline produced is promptly metabolized by the parasite, but our data from short-term radiometric measurements suggest that, to the contrary, citrulline is rapidly extruded from the IRBC. Nitrite accumulation could not be detected in IRBC culture medium, most likely because of high levels of hemoglobin, a powerful NO scavenger, in IRBC. Oxyhemoglobin elicits conversion of NO into nitrate. When IRBC were incubated in a low nitrate culture medium, an accumulation of nitrate in the supernatant could be observed, and its amount was similar to extracellular citrulline production. However, RBC did not release nitrate as well as citrulline in the supernatant. In HUVEC, NO production can be indirectly monitored by the cGMP intracellular levels (35,

47). In human RBC, a synthesis of cGMP is measurable (54, 55), although it is not clear whether a soluble guanylate cyclase contributes to its synthesis. In RBC from mouse spleen, a membrane-associated guanylate cyclase, activated by lysophospholipids and fatty acids, has been detected (56). cGMP has been found in virtually all cell types and phyla examined (13). In plasmodia, the guanylate cyclase activity has not been extensively studied, but cGMP is detectable and its increase seems to play a key role in exflagellation of *P. berghei* and *P. falciparum* (57). Our results show that, in control RBC, cGMP levels are very low and similar to reported values (55). The same cGMP concentrations are found in IRBC, and neither are influenced by SNP treatment. It is difficult to state whether these negative results are due to the absence of an NO-sensitive guanylate cyclase in RBC and IRBC or to the hemoglobin-mediated scavenging of endogenous and exogenous NO.

Our data are in agreement with reports showing NO synthesis in species phylogenetically distant from mammalian ones, suggesting a very ancient origin for NO production in the biological systems (58). It is hard to say which could be the role of NO in *Plasmodium*, since it is conceivable that the vast majority of this free radical is scavenged by hemoglobin before it crosses the RBC membrane and reaches the extracellular environment. It is possible that *Plasmodium*-derived NO exerts its action in the host during the exoerythrocytic stage (sporozoite, merozoite), perhaps participating in the mechanisms of cell invasion. However, since parasite growth is inhibited by the NOS inhibitors L-canavanine and L-NNA, and the effect of these compounds seems to correlate with their ability to inhibit *Plasmodium* NOS activity, a physiological role for NO production should exist. At the present time, only speculations for such role can be made. NO could react with intraparasitic molecules, as pigment or thiols; NO could participate in the assembly of free heme into hemozoin or favor the formation of S-nitrosothiols. It is well known that hemozoin accumulation is indispensable for parasite survival, as soluble heme can damage biological membranes and inhibit a variety of enzymes (59). Thus, the inhibition of parasite growth by L-canavanine may suggest that NO is necessary to heme detoxification. S-Nitrosothiols can store NO in a relatively stable form at acidic pH; it has been suggested that macrophages and other cells could store S-nitrosothiols within the acidic lysosome environment, and after exocytosis these molecules spontaneously decompose by homolytic cleavage to NO and the corresponding disulfide (2). The acidic milieu of the food vacuole in *P. falciparum*, could store as well part of NO as nitrosothiols, allowing the parasite to release NO in a second step. This could be of great importance for the cytotoxic effect exerted by the ingested IRBC on phagocytosing macrophages (60).

We explored the possibility that *P. falciparum* IRBC may release a soluble factor able to induce NOS in host cells. Indeed, incubation with conditioned medium from trophozoite IRBC (FCR-3 strain) induces in HUVEC a 150-fold stimulation of NOS activity in a dose- and time-dependent way (Fig. 6). Such an effect could be reproduced also with the conditioned medium from human RBC infected with other strains of *P. falciparum.*, In parallel, both nitrite and citrulline

synthesis by intact HUVEC were significantly increased in cells incubated with IRBC-conditioned medium (FCR-3 strain). The increase of NOS activity was at least partly attributable to de novo enzyme synthesis, as suggested by the time dependence of such an increase, the sensitivity to cycloheximide and dexamethasone, the higher Vmax value, and the increased level of a 140–145-kD protein reacting in the Western blot with anti-eNOS and anti-iNOS antibodies. These results suggest that IRBC-conditioned medium induces both the eNOS and iNOS isoforms in HUVEC; the induced activity was not attributable to an IRBC-like NOS, since it showed an apparent molecular mass >100 kD, as expected for a mammalian NOS. The active factor in this conditioned medium showed a high molecular mass (>100 kD) and was thermolabile, suggesting that it is a protein or a glycoprotein. We suggest naming this factor Plasmodium-derived NOS-inducing factor (PNIF).

It has been demonstrated that human and rodent malaria parasites release exoantigens (61), which stimulate macrophages to produce reactive nitrogen intermediates (62) and to secrete TNF in vitro (63). The macrophage-activating component of such exoantigens seems to be a phospholipid (64). A glycosylphosphatidylinositol toxin purified from P. falciparum induces TNF and IL-1 production by macrophages and regulates glucose and lipid metabolism in adipocytes (52). Phosphatidylinositol and inositolmonophosphate have been demonstrated to possess an inhibitory effect on exoantigen-induced production of TNF by murine macrophages (51); mannose and inositolmonophosphate inhibit the lipogenesis and the glucose oxidation evoked by glycosylphosphatidylinositol in

adipocytes (52). Our preliminary data seem to rule out the possibility that PNIF is a phospholipid exoantigen, because its NOS-inducing activity in HUVEC is not impaired by the presence of high concentrations of phosphatidylinositol, inositolmonophosphate, or mannose. On the other hand, PNIF is unlikely to be an LPS-like molecule or a cytokine such as IL-1, IL-6, TNF, oncostatin M, IFN-γ, or GM-CSF; in fact, NOS induction in HUVEC is not triggered by any of these substances, alone or in different combinations (unpublished data). This suggests that PNIF cannot be identified with one or more of these cytokines. A not yet well-defined NOS-inducing activity is released by different cells in the culture medium: rat colon carcinoma cells (49), P1HTR tumor cells (48), and murine endothelioma cells (50).

In conclusion, our results suggest that, during malarial infections, the increase of NO production, which has been invoked in the pathogenesis of neurological and cardiovascular complications of the disease, may not need the intervention of host-derived cytokines. P. falciparum IRBC release into the extracellular environment a soluble factor able to induce NOS activity in the host cells and in the parasite itself. A protracted activation of NOS in HUVEC could support the persistent hypotension and neurological damages observed in malaria patients (6, 19).

Our efforts are presently addressed to purifying both NOS and PNIF molecules from the P. falciparum-infected erythrocytes and to verifying whether their activity is detectable in other human and murine Plasmodium species, by extending our observations to malaria patients and murine models of cerebral malaria.

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