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Nitric Oxide Inhibits Falcipain, the *Plasmodium falciparum* Trophozoite Cysteine Protease[1]

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Nitric oxide (NO) is a pluripotent regulatory molecule possessing, among others, an antiparasitic activity. In the present study, the inhibitory effect of NO on the catalytic activity of falcipain, the papain-like cysteine protease involved in *Plasmodium falciparum* trophozoite hemoglobin degradation, is reported. In particular, NO donors *S*-nitrosoglutathione (GSNO), (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide (NOR-3), 3-morpholinosydnonimine (SIN-1), and sodium nitroprusside (SNP) inhibit dose-dependently the falcipain activity present in the *P. falciparum* trophozoite extract, this effect likely attributable to S-nitrosylation of the Cys25 catalytic residue. The results represent a new insight into the modulation mechanism of falcipain activity, thereby being relevant in developing new strategies for inhibition of the *P. falciparum* life cycle. © 2000 Academic Press

Key Words: **falcipain; trophozoite papain-like cysteine protease; hemoglobinase; nitric oxide; enzyme inhibition; *Plasmodium falciparum*.**

During the erythrocytic stage of their life cycle, *Plasmodium falciparum* trophozoites degrade hemoglobin as a major source of amino acids for malaria parasite protein synthesis. This process includes the transport of hemoglobin from the erythrocyte cytoplasm to the parasite acidic digestive vacuole, the precipitation of heme as a component of the malarial pigment, and the protease catalyzed hydrolysis of hemoglobin to small peptides. Hemoglobin peptide fragments are transported through the membrane of the acidic digestive vacuole to the parasite cytoplasm, where they are degraded to individual amino acids. The initial cleavage of hemoglobin is catalyzed by plasmepsins I and II, two malaria parasite aspartic proteases present in the trophozoite acidic digestive vacuole. Then, falcipain, the *P. falciparum* trophozoite papain-like cysteine protease also present in the acidic digestive vacuole, degrades hemoglobin fragments to small peptides. Finally, exopeptidase activity, present in the malaria parasite cytoplasm, converts small hemoglobin peptides to individual amino acids for *P. falciparum* growth and maturation (1, 2).

Both parasite aspartic and cysteine proteases are promising targets for antimalarial chemotherapy (3–5). In particular, inhibition of falcipain blocks *P. falciparum* development *in vitro* (6). Furthermore, inhibition of the *Plasmodium vinckei* falcipain-homologous cysteine protease cures murine infection *in vivo* (7).

Recently, nitric oxide (NO) has been reported to possess an antiparasitic activity (8). In this respect, an inverse relationship has been observed between malaria severity and NO synthase type-2-induced NO production, in Tanzanian children (9). Of interest, the mosquito *Anopheles stephensi* limits malaria parasite development through an inducible synthesis of NO (10).

In the present study, NO donors are reported to inhibit dose-dependently the falcipain activity present in the *P. falciparum* trophozoite extract, this effect being likely attributable to S-nitrosylation of the Cys25 catalytic residue. These results represent a new insight in the modulation mechanism of falcipain activity, thus suggesting a novel strategy for inhibition of *P. falciparum* life cycle.

Abbreviations used: falcipain, *Plasmodium falciparum* trophozoite papain-like cysteine protease; *P. falciparum*, *Plasmodium falciparum;* Z-Phe-Arg-AMC, *N*-α-benzyloxycarbonyl-L-phenylalanyl-L-arginine-(7-amino-4-methylcoumarin); NO, nitric oxide; GSH, glutathione; GSNO, *S*-nitrosoglutathione; NOC-18, 3,3-bis(aminoethyl)-1-hydroxy-2-oxo-1-triazene; NOR-3, (±)-(*E*)-4-ethyl-2-[(*E*)-hydroxyimino]-5-nitro-3-hexenamide; NOR-3*, NO-deprived NOR-3; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; DTT, dithiothreitol.

[1] This paper is dedicated in memoriam to our friend, Professor Giacomino Randazzo.

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MATERIALS AND METHODS

The following reagents Z-Phe-Arg-AMC, GSH, leupeptin, NOC-18, NOR-3, SIN-1, SNP, and DTT were purchased from Sigma Chemical Co. (St. Louis, MO). NO-deprived NOR-3 (NOR-3*) was obtained storing the NOR-3 solution at alkaline pH and 25.0°C, for 72 h (11). GSNO was prepared by mixing equimolar concentrations of an aqueous solution of $NaNO_2$ and a freshly prepared GSH solution in 2.5×10^{-1} M HCl and 1.0×10^{-4} M EDTA (pH 1.5). The resulting mixture was incubated at 25.0°C for 5 min and then neutralized with NaOH. The GSNO solution was stored at −20.0°C (12). All the other products were obtained from Merck AG (Darmstadt, Germany). All chemicals were of analytical grade and were used without further purification.

The 3D7A strain of *Plasmodium falciparum* was cultured according to standard methods (13, 14). The trophozoite extract was prepared from infected erythrocytes (5% parasitemia, 3×10^9 parasites), as previously reported (14). The falcipain catalytic activity present in the trophozoite extract was measured using the fluorogenic substrate Z-Phe-Arg-AMC, as described elsewhere (14). Briefly, Z-Phe-Arg-AMC (1.0×10^{-4} M final concentration) was added to the trophozoite extract (about 10^{-7} M falcipain concentration), and fluorescence (380 nm excitation, and 460 nm emission) was measured continuously over 1 min (14). Falcipain concentration was determined taking into account values of the catalytic parameters for the enzyme hydrolysis of Z-Phe-Arg-AMC (14).

To test the possible activating effect of reducing agents on the falcipain catalytic activity present in the trophozoite extract, the cysteine protease was incubated with DTT at pH 5.5 and 25.0°C. DTT concentration and incubation time ranged between 1.0×10^{-6} M and 1.0×10^{-3} M, and between 20 s and 30 min, respectively. Then, Z-Phe-Arg-AMC was added to the trophozoite extract and the falcipain activity was determined.

The inhibitory effect of NO donors on the falcipain catalytic activity was determined by incubation of the trophozoite extract with the reaction buffer (pH 5.5, 0.1 M sodium acetate) and an appropriate concentration of GSNO, NOC-18, NOR-3, NOR-3*, SIN-1, SNP, or leupeptin (ranging between 5.0×10^{-7} M and 1.0×10^{-3} M), for 30 min at 25.0°C. Then, Z-Phe-Arg-AMC was added to the reaction mixture and the falcipain activity was assayed.

To verify the effect of a reducing agent on the NO-mediated S-nitrosylation of falcipain (i.e., on cysteine protease inhibition), the inactive cysteine protease, obtained by 1.0×10^{-4} M NOR-3-pretreatment, was incubated with an excess of DTT (1.0×10^{-3} M) for 30 min at pH 5.5 and 25.0°C. Furthermore, the trophozoite extract was incubated simultaneously with DTT (1.0×10^{-3} M) and NOR-3 (1.0×10^{-4} M) for 30 min at pH 5.5 and 25.0°C. Then, the falcipain catalytic activity was assayed using Z-Phe-Arg-AMC.

Under all the experimental conditions, the fluorescence change (i.e., the product formation) was linear on the assay time. The slope of fluorescence over time for each inhibitor concentration was compared with that of controls in multiple assays (14).

RESULTS AND DISCUSSION

The catalytic activity of unrelated enzymes is modulated by nitric oxide (NO) through (ir)reversible binding to metal centers as well as by chemical modification of reactive residues (e.g., Cys) (15–17). Note that the NO-mediated S-nitrosylation of the Cys catalytic residue of cysteine proteases, including papain (18), caspases (19–21), cathepsin-B (14, 15, 22, 23), and Coxsackievirus cysteine protease type-3 (24), blocks the enzyme activity. Also, the aspartyl HIV-1 protease activity is inhibited by the NO-mediated S-nitrosylation of Cys regulatory residue(s) (11). On the other hand, the NO-mediated S-nitrosylation of the fibronectin type-1 and epidermal growth factor-like pair of the human tissue-type plasminogen activator endows this serine protease with new potent vasodilatory and antiplatelet properties, without affecting the enzyme catalytic (e.g., fibrinolytic) activity (25).

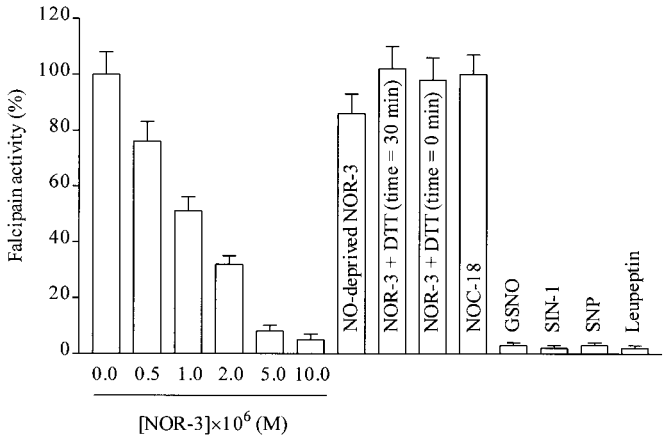

FIG. 1. Effect of NO on the catalytic activity of freshly prepared falcipain, the *P. falciparum* trophozoite papain-like cysteine protease. Dose-dependent inhibitory effect of NOR-3 on the catalytic activity of falcipain. NO-deprived NOR-3 (NOR-3*; 1.0×10^{-5} M) does not affect falcipain activity. Addition of DTT (1.0×10^{-3} M) to inactive falcipain, as obtained by preincubation with NOR-3 (1.0×10^{-4} M) for 30 min, restores the cysteine protease activity (time = 30 min). The simultaneous addition of DTT (1.0×10^{-3} M) and NOR-3 (1.0×10^{-4} M) to active falcipain prevents the cysteine protease inhibition (time = 0 min). GSNO, SIN-1, and SNP (1.0×10^{-5} M) block falcipain action. On the contrary, NOC-18 (1.0×10^{-5} M) does not affect the trophozoite cysteine protease catalytic activity. As a control, leupeptin (1.0×10^{-6} M), a typical falcipain inhibitor (14), suppresses the cysteine protease activity. Each bar represents the mean ± SEM of at least four experiments. All data were obtained at pH 5.5 and 25.0°. For further experimental details, see text.

Trophozoite extract displays catalytic properties very similar to those reported for the purified native falcipain (26). DTT does not affect significantly the catalytic properties of the freshly prepared trophozoite cysteine protease over the whole reducing agent concentration range explored (i.e., between 1.0×10^{-6} M and 1.0×10^{-3} M) and the time scale investigated (i.e., between 20 s and 30 min).

As shown in Fig. 1, NO and related reactive nitrogen intermediates, released from NO donors GSNO, NOR-3, SIN-1, and SNP (12, 27–29), inhibit falcipain action. However, the cysteine protease activity is essentially unaffected by the NO donor NOC-18 (see Fig. 1). In this respect, it may be observed that the half time for NO release from GSNO, NOR-3, SIN-1, and SNP is in the same time scale (12, 27–29) as the activity assay. On the contrary, NOC-18 releases NO very slowly, the half time being about 2 days (30).

Falcipain inhibition by NO results to be dose-dependent, as shown for NOR-3 in Fig. 1. Moreover, as

expected, NO-deprived NOR-3 (NOR-3*) does not affect falcipain activity (see Fig. 1). As a positive control of enzyme inactivation, leupeptin, a typical falcipain inhibitor (14), induces the complete suppression of the trophozoite cysteine protease action (see Fig. 1).

To verify that falcipain inactivation may occur via S-nitrosylation, the reversibility of enzyme inhibition has been investigated. As expected (22), the incubation of inactive NOR-3-treated falcipain with DTT completely restores the enzyme activity (see Fig. 1). In addition, the simultaneous incubation of DTT and NOR-3 with the trophozoite extract prevents the cysteine protease inhibition (see Fig. 1). Although falcipain inactivation by chemical modification of Tyr and Trp cannot be ruled out, nitrosylation of these residues is less likely to occur, given their much lower reactivity with respect to Cys residues (15–17).

Based on the NO-mediated inhibition mechanism of cysteine proteases (31), the inspection of the three-dimensional model of falcipain (32–33), and the analysis of the amino acid sequence of parasite papain-like cysteine proteases and related enzymes (34), we suggest that the Cys25 catalytic residue of falcipain may undergo S-nitrosylation.

As a whole, the present data indicate that falcipain is inactivated by NO donors (e.g., GSNO, NOR-3, SIN-1, and SNP), probably through S-nitrosylation, thus representing a novel intriguing approach for the inhibition of *P. falciparum* life cycle. Note that GSNO and nitroso-L-cysteine have been reported to kill *P. falciparum in vitro* (35). In particular, the EC_{50} value for parasite killing by GSNO and nitroso-L-cysteine (approximately 4.0×10^{-5} M) (35) is consistent with the concentration of GSNO, NOR-3, SIN-1 and SNP (ranging between 5.0×10^{-7} M and 1.0×10^{-3} M) used in the present study for the inhibition of falcipain action. Therefore, the use of known NO-releasing drugs, which are employed in the treatment of coronary artery disease with limited toxicity (29), appears to be a potential, useful tool in the therapeutic treatment of malaria. Since cysteine proteases are critical for virulence or replication of many viruses, bacteria, fungi, and parasites (5, 36), NO-mediated S-nitrosylation of pathogen cysteine proteases may represent a general mechanism of antimicrobial host defences (37).

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