Rho Kinase Inhibition in Severe Malaria: Thwarting Parasite-Induced Collateral Damage to Endothelia

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Acute clinical manifestations of falciparum malaria, such as multiorgan failure and cerebral malaria, occur unpredictably and lead to coma and death within hours if left untreated. Despite the emergency administration of effective antimalarial drugs, 15%–20% of patients die. Other therapeutic approaches are therefore urgently needed. There is increasing evidence that endothelial changes play a key role in the pathogenesis of severe malaria. We therefore used coculture models to study interactions between infected erythrocytes and endothelium. We found that adhesion of *Plasmodium falciparum* to endothelial cells in vitro activated the Rho kinase signaling pathway, which is strongly involved in various vascular diseases. When added concomitantly with parasites, the Rho kinase inhibitor fasudil (HA-1077), a drug already in clinical use, decreased both NF-κB activation and endothelial cell apoptosis. Fasudil also helped to restore endothelial barrier integrity after *P. falciparum* adhesion. Rho kinase inhibition thus appears to be a promising adjunctive therapeutic approach to the management of severe human malaria.

An estimated 500 million clinical cases of malaria occur each year, and more than a million people die from complications of *Plasmodium falciparum* infection [1]. Young children and pregnant women in sub-Saharan Africa are particularly susceptible to an acute neurological syndrome called "cerebral malaria" (CM), either in isolation or concomitantly with multiorgan failure syndrome (pulmonary distress, acute renal failure, etc.), the latter of which is mainly seen in adults living in Southeast Asia. Severe malaria cases may then progress to coma and death within hours if left untreated. Despite numerous studies, the pathogenesis of these severe forms is still poorly understood, and there is no way to predict their onset. To date, emergency treatments have consisted mainly of intravenous administration of antimalarial drugs, such as quinine and artemisinin derivatives [2]. However, even though these drugs effectively clear the blood of parasites, 15%–20% of patients still die, and others have permanent sequelae [3, 4]. The current treatments for severe falciparum malaria, which mainly rely on a direct antiplasmodial strategy, appear not to be sufficient, and other additional approaches are needed to improve the outcome of the disease.

The severity of *P. falciparum* infection depends largely on the ability of the parasite to adhere to endothelial cells and to be sequestered in the microvasculature of vital organs [5, 6]. This cytoadherence involves protrusions (knobs) on mature forms of parasitized red blood cells (pRBCs), which can bind to many endothelial adhesion molecules (such as intracellular adhesion molecule [ICAM]–1, CD36, thrombospondin, E-selectin and chondroitin sulfate A [CSA]) that are variably expressed in the organs [7]. In contrast, little is known of signaling triggered by this cytoadherence. Adhesion molecules are known to have signal transduction properties that can trigger changes in endothelial cells, such as cytoskeleton and junction remodeling [8]. Moreover, parasite cytoadherence can occlude capillaries and provoke local

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ischemia, further intensifying host inflammatory processes. Sequestration of pRBC mature forms and secretion of malarial toxins [9], together with the release of proinflammatory cytokines by chemoattracted leukocytes [10], are likely to trigger synergistic events that affect the integrity of the blood-brain barrier and other endothelia [11, 12]. This creates a capillary network environment (where blood-tissue exchanges take place) that is remarkably hostile for endothelial cells. Consequently, the homeostasis of vital parenchyma may be markedly compromised in severe falciparum malaria.

Using coculture models, we have previously shown that *P. falciparum* adhesion to human endothelial cells can specifically trigger proinflammatory gene expression, oxidative stress, and caspase activation [13, 14]. Given that there might be numerous molecular events occurring between pRBC contact and endothelial dysfunction, we assume that strategies interfering with early signaling cascades might therefore be valuable. Members of the Rho family of small GTPases are known to play a pivotal role in the signal transmission of various receptors, including ICAM-1, vascular cell adhesion molecule (VCAM)–1 and selectins involved in cytoadherence. Rho family members act as molecular switches (active and inactive when bound to GTP and GDP, respectively) and are the first intermediates of the intracellular signaling engagement of these receptors with the downstream effector Rho kinase [15].

Here, we focused on the Rho kinase pathway, which has been shown to be highly involved in a variety of vascular diseases when abnormal activation is observed [16–18]. We postulated that adherent *P. falciparum*–infected erythrocytes could trigger this pathway in endothelial cells. We also examined whether fasudil (HA-1077), a Rho kinase inhibitor already in clinical use for cerebral ischemic stroke [19], could prevent *P. falciparum* adhesion–induced endothelial impairment.

MATERIALS AND METHODS

Endothelial cell culture. Primary human lung endothelial cells (HLECs) were isolated after enzymatic digestion and selected using a continuous gradient and immunomagnetic purification technique, as described elsewhere [20]. Endothelial cells of ninth to twelfth passages derived from one batch were used for the experiments. Before use, cells were verified for the expression of ICAM-1, CD36, von Willebrand factor, VCAM-1, CD31, E/P-selectin, and CSA. HLECs were grown in M199 medium (Gibco) supplemented with 10 μg/mL endothelial cell growth supplement (Upstate Biotechnology) and 10% of fetal calf serum (Biowest) at 37°C in 5% CO_2, using 8-chamber Lab-Tek slides (Nunc International), 6-well plates (Becton Dickinson), or Transwell insert supports (Corning LifeSciences).

Plasmodium falciparum *culture.* Three types of parasites—the 3D7 and F12 clones of *P. falciparum* as well as the RAOL strain, a gift from P. Buffet (parasites were isolated from a patient hospitalized at Pitié-Salpêtrière Hospital with CM [according to World Health Organization criteria] and were adapted for in vitro culture)—were used for our experiments. Parasites were maintained in culture in a RPMI 1640 suspension of erythrocytes (Gibco) supplemented with 2.1 g/L NaHCO, 0.1 mg/mL gentamycin, and 0.4% of Albumax II (Gibco, Invitrogen), according to the technique of Trager and Jensen [21]. For each experiment, parasite cultures were enriched in mature forms by use of the plasmagel floating technique [22]. All pRBC suspensions were adjusted to 1% of hematocrit and 50% of parasitemia.

pRBC adhesion assay. HLECs were grown in Lab-Tek slides until confluence. Suspensions of mature pRBCs were deposited onto cells and incubated for 1 h at 37°C with gentle shaking every 10 min. After the incubation, the unbound pRBCs were removed, and the preparation was fixed with 2% glutaraldehyde for 30 min at room temperature before staining with Giemsa. The number of parasites adhering to 700 HLECs was counted by direct observation with a light microscope.

Rho kinase pathway activation assay. The activity of the Rho kinase pathway was assessed using the G-LISA RhoA activation assay (Cytoskeleton). Briefly, HLECs were cultured in 6-well plates until 50%–75% confluence and then exposed to RPMI 1640 suspensions of pRBCs. After 24 h of coincubation, endothelial cells were washed to remove pRBCs before cytoplasm-protein extraction. Endothelial cells were lysed ice cold and were clarified by centrifugation at 9168 *g* (4°C). After measurement of protein concentrations, extracts were equalized with lysis buffer. Cell extracts were incubated in a Rho-GTP affinity plate for 30 min at 4°C. Detection antibodies—primary anti-RhoA antibody and secondary horseradish peroxidase detection reagents—were then applied, and signal was detected by luminometry analysis at 490 nm using a microplate reader (Bio-Tek EL311SX). Purified, constitutively activated RhoA proteins and endothelial cells incubated with cytochalasin D (CytD; 100 ng/mL), a known Rho protein activator, were used as positive controls.

In a "no-contact" assay, HLECs were cultured in the lower compartment of Transwell inserts (polyester; 0.4-μm pore size). An RPMI 1640 suspension of mature pRBC was then added in the upper compartment and incubated for 24 h at 37°C, before the Rho kinase activity assay was performed as described above.

Quantitative apoptosis assay. Endothelial cell apoptosis was quantitatively assessed by phosphatidylserine uptake of APOPercentage dye (Biocolor). HLECs were cultured in 6-well plates until confluence before exposure with parasite suspension for 24 h. After removal of pRBCs, incubation with the apoptosis purple-red dye (1:20) was performed for 1 h. After rinsing with PBS, apoptosis was quantified by analytical digital photomicroscopy: for each experiment, 4 representative fields per coincubation condition were photographed using an inverted phase-contrast microscope coupled with a digital camera. Images were

then transferred to a computer, and the level of apoptosis was expressed as the number of red pixels, as counted by use of Adobe Photoshop (version 7).

NF-κB subcellular location. HLECs were grown in Lab-Tek slides and were exposed for 6 h to pRBC suspensions. HLECs were then fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and blocked with bovine serum albumin. Cells were incubated for 1 h at room temperature with mouse anti–NF-κB p65 subunit antibodies (Sigma Aldrich). After extensive washing with PBS, goat anti–mouse IgG FITC conjugate (1:500) was applied for 1 h. Slides were mounted with nuclear stain diamidino-phenylindole (DAPI) for fluorescence microscopy analysis. The number of activated HLECs (cells with complete but not partial nuclear migration staining) per each condition ($n = 4$) was counted. Representative fields were photographed and transferred to a computer for assembling in Adobe Photoshop.

Endothelial barrier integrity assay. Confluent endothelial monolayer were obtained by seeding 30,000 HLECs on a Transwell permeable support (polyester; 3-μm pores; 6.5-mm diameter) and grown in M199 medium supplemented as described above for 36 h. Endothelial monolayers were then exposed for 4 h to pRBC suspensions. Tumor necrosis factor (TNF)–α (20 ng/mL) was used as a positive control of in vitro endothelium disruption. In separate experiments, the Rho kinase inhibitor fasudil was applied for 20 h after 4 h of pRBC exposure. Transwell compartments were then washed 3 times, and cell monolayers on Transwell inserts were transferred to a new plate containing PBS. Evans blue dye (0.5 mg/mL; ICN Biomedicals) was then added to the upper compartments. After 5 min of incubation at 37°C in 5% CO_2, material was collected from the lower compartments for optical density (OD) analysis of diffused Evans blue dye (630 nm) by use of a standard microplate reader (Bio-Tek EL311SX).

Reagents. The lyophilized Rho kinase inhibitor fasudil (HA-1077) was purchased from Biaffin and was reconstituted with sterile water in 30 mmol/L stock solution. Recombinant human TNF-α was purchased from R&D Systems, and CytD was purchased from Sigma.

Statistical analysis. Each experiment was conducted at least 3 times independently. Differences between groups were analyzed for statistical significance by use of the Mann-Whitney *U* or Jonckheere-Terpstra tests (StatXact software; Cytel Studio). $P < .05$ was considered to indicate statistical significance.

RESULTS

Induction of the Rho kinase pathway in human endothelial cells by* P. falciparum *adhesion. To determine whether *P. falciparum* could trigger the Rho kinase pathway, we measured the intracellular content of the upstream Rho kinase activator, GTP-bound RhoA, in endothelial cells exposed to the parasite strain 3D7, which is known to adhere to HLECs [13] (figure 1). To determine the direct role of parasite adhesion in this effect, we also incubated endothelial cells with the derivative parasite clone F12, which is isogenic to 3D7 [23] but is adheres poorly to HLECs [13], or with the RAOL strain (adherent *P. falciparum* parasites isolated from a patient with CM and adapted for in vitro culture). For each coincubation experiment, suspensions of mature forms of *P. falciparum*–infected erythrocytes were tested in parallel for their HLEC binding capacity (figure 1). 3D7 and RAOL binding exceeded 1 pRBC/HLEC, whereas F12 binding reached a maximum of 1 pRBC/20 HLECs. The number of uninfected RBCs in control wells was negligible. HLEC coincubation with 3D7 and RAOL led to a significant increase in the concentration of activated RhoA, compared with that for HLEC coincubation with F12, uninfected erythrocytes ($P = .01$), or medium alone ($P = .007$) (figure 2*A*).

HLECs were also coincubated with pRBC in separate compartments of a Transwell diffusion chamber (0.4-μm pore size). HLEC-parasite adhesion was thus prevented (figure 1), whereas macromolecules and medium could diffuse between the 2 compartments (data not shown). HLECs coincubated with 3D7, RAOL, and F12 in no-contact assays did not contain significantly different concentrations of activated RhoA, compared with those for RBC controls. Rho signaling activation thus occurred only when pRBCs adhered directly to endothelial cells (figure 2*A*).

Attenuation of the* P. falciparum *adhesion–induced Rho kinase pathway activation in human endothelial cells due to the Rho kinase inhibitor fasudil. We then tested the effect of fasudil (HA-1077), a potent and selective Rho kinase inhibitor that is already used to treat human acute vascular diseases [19]. HLECs were concomitantly exposed to 3D7 pRBCs and fasudil at 30 μmol/L in a contact assay. The activated RhoA–GTP content of the endothelial cells exposed to pRBCs plus fasudil was significantly reduced, compared with that for the pRBC-exposed controls ($P = .04$) (figure 2*B*). CytD, a known Rho protein activator, was used as a positive control. These experiments showed that Rho kinase pathway induction by *P. falciparum* adhesion could be attenuated by the Rho kinase inhibitor fasudil.

No effect of Rho kinase inhibition by fasudil on parasite growth or adhesion. We then raised the question of whether fasudil decreased the induced Rho signaling pathway activation through cytoadherence or parasite viability–dependent effects. Fasudil had no significant antiplasmodial effects when tested on 3D7 pRBC cultures (figure 3*A*), showing that its effects were not due to an action on parasite viability. Fasudil also had no significant effect on 3D7 parasite binding to endothelial cells (figure 3*B*). The inhibition of Rho kinase activation by fasudil was thus due solely to its endothelial cellular action.

Attenuation of* P. falciparum*–induced endothelial apoptosis and activation due to Rho kinase inhibition by fasudil. We then studied the role played by the Rho kinase pathway in parasite-induced endothelial responses. *P. falciparum* adhesion is also

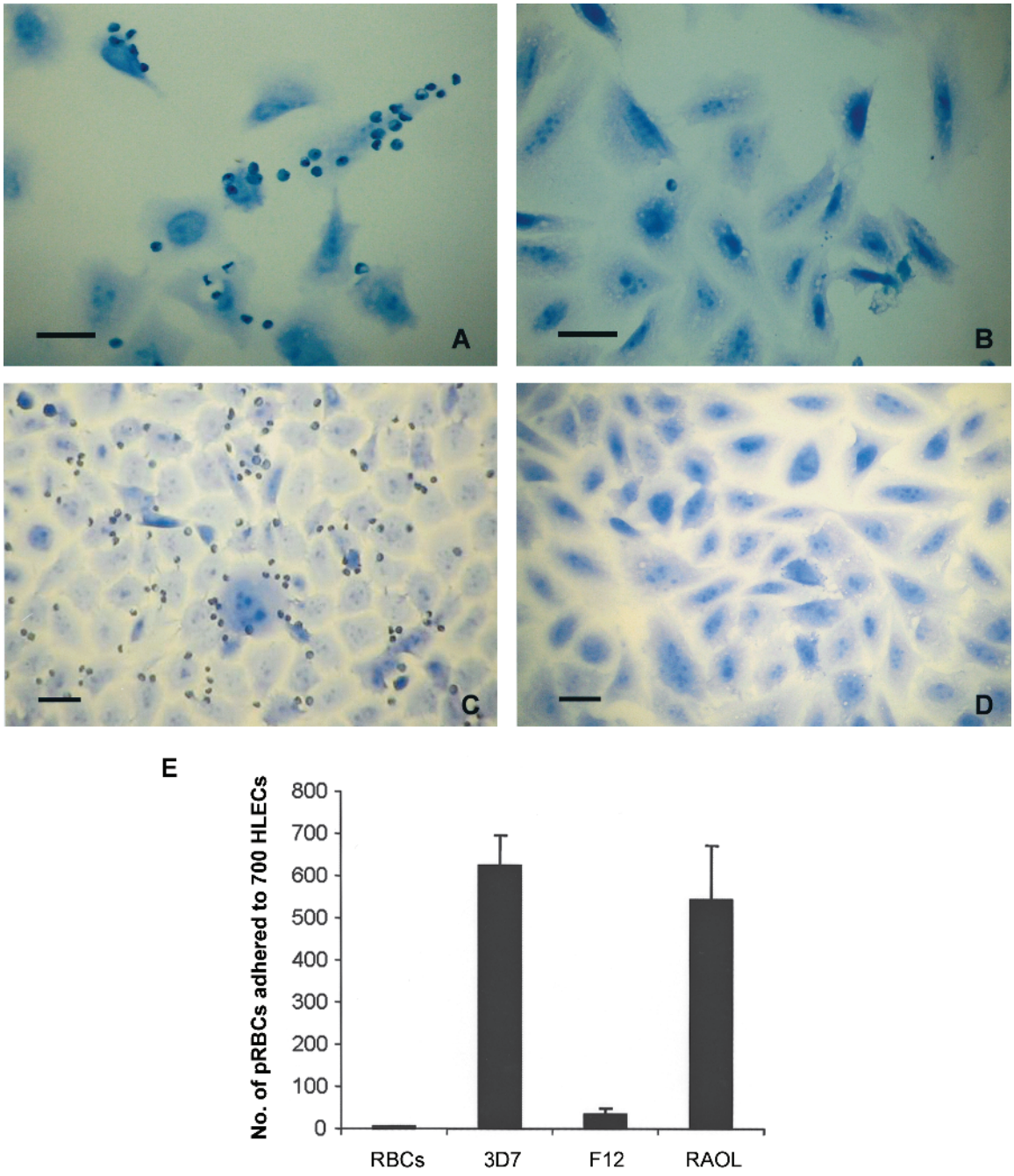

Figure 1. *Plasmodium falciparum* cytoadherence tests on human lung endothelial cells (HLECs). Endothelial cells were grown in 8-chamber Lab-Tek slides. Suspensions of mature forms of *P. falciparum*–infected erythrocytes were then deposited onto HLECs. After removal of unbound erythrocytes, cells were stained with Giemsa, and the no. of parasitized red blood cells (pRBCs) adhering to 700 HLECs was counted. Comparative binding of 3D7 and F12 pRBCs on nonconfluent HLECs are shown in the representative photographs in panels A and B respectively, whereas panels C and D show representative fields of confluent endothelial cells that were put into contact with 3D7 pRBCs or that had no contact with 3D7 pRBCs, respectively (bar shows 40 μm). Data in the bar graph *(E)* are mean $\pm$ SD values for pRBC cytodherence ($n = 6$).

known to induce endothelial cell apoptosis [13]. To determine the role played by Rho kinase in this effect, HLECs were concomitantly exposed to 3D7 mature forms and fasudil. As expected, pRBCs induced phosphatidylserine apoptosis dye uptake by endothelial cells (9.5-fold increase, compared with that in uninfected RBCs) (figure 4). Compared with the untreated cells, pRBC-induced apoptosis was reduced 1.4-fold by 1 μmol/L fasudil (HF1) and 4.1-fold by 30 μmol/L fasudil (HF2). Thus, the Rho kinase inhibitor fasudil strongly protected endothelial cells from pRBC-induced apoptosis.

Like other stimuli, such as TNF-α, *P. falciparum* cytoadherence is known to induce proinflammatory endothelial responses via NF-κB–dependent cascades [9, 24]. Several lines of evidence point to an

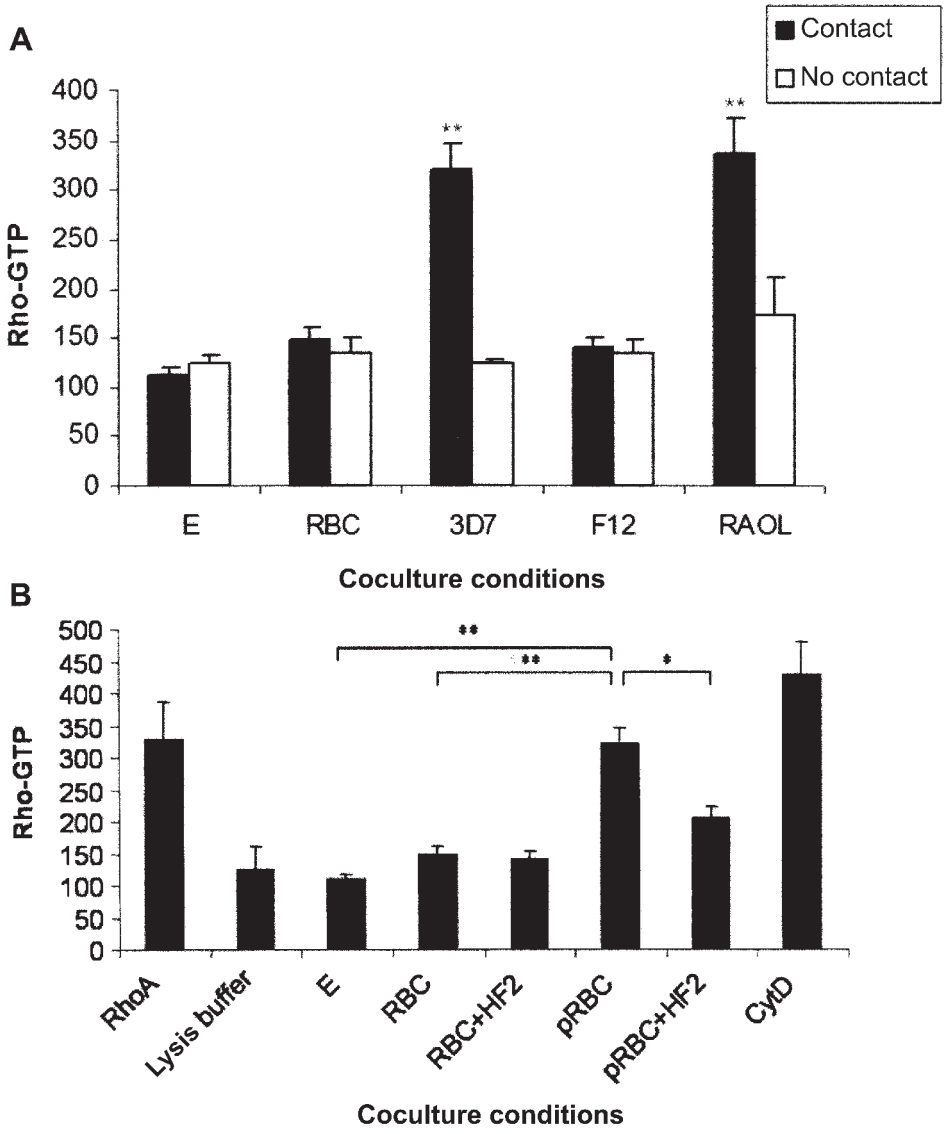

Figure 2. *A,* Requirement of *Plasmodium falciparum* adhesion on human lung endothelial cells (HLECs) for RhoA activation. HLECs were cultured in 6-well plates and exposed for 24 h to a suspension of mature forms of parasitized red blood cells (pRBCs) (adjusted to 1% of hematocrit and 50% of parasitemia). Endothelial cells were then analyzed for their activated RhoA-GTP cytoplasmic content. In the contact assay, HLECs and pRBCs were incubated in the same compartment, whereas, in the no-contact assay, parasites and endothelial cells were separated by a polyester porous membrane (0.4-μm pore size). 3D7, F12, and RAOL, exposed to *P. falciparum* 3D7-, F12-, or RAOL-infected erythrocytes; E, incubated with medium alone; RBC, exposed to uninfected erythrocytes; Rho-GTP, extracted activated RhoA content of endothelial cells. *B,* Effect of fasudil on *P. falciparum*–induced RhoA activation. HLECs were concomitantly exposed to pRBCs (3D7 clone) and the Rho kinase inhibitor fasudil at 30 μmol/L (HF2). CytD, incubated with cytochalasin D (100 ng/mL); E, extracts from endothelial cells incubated in medium only; lysis buffer, extraction buffer; RBC, exposed to uninfected red blood cells; RhoA, purified, constitutively active RhoA protein; pRBC, exposed to parasitized red blood cells. Data are mean ± SD optical density values from 3 independent experiments. $^*P < .05$; $^{**}P < .01$.

essential role for Rho-dependent signaling in NF-κB phosphorylation and nuclear translocation [25]. We therefore examined whether fasudil could also modulate *P. falciparum*–induced endothelial cell activation. When exposed to adherent pRBCs, 91% (SD, 11.3%) of HLECs were activated, compared with only 45% (SD, 12%) when HLECs were coincubated with fasudil ($P = .003$). All HLECs were activated by TNF-α (20 ng/mL), used as a positive control, whereas <1% of cells were activated by uninfected RBCs (figure 5). Thus, Rho kinase inhibition by fasudil also decreases *P. falciparum*–induced NF-κB endothelial cell activation.

Reversion of* P. falciparum*–induced endothelial barrier dysfunction due to Rho kinase inhibition by fasudil. Given that one major problem in severe cases of falciparum malaria is that the onset of the disease remains unpredictable, a relevant adjunctive drug treatment should be efficient in the late stages of pathology. HLECs were cultured to confluence on Transwell in-

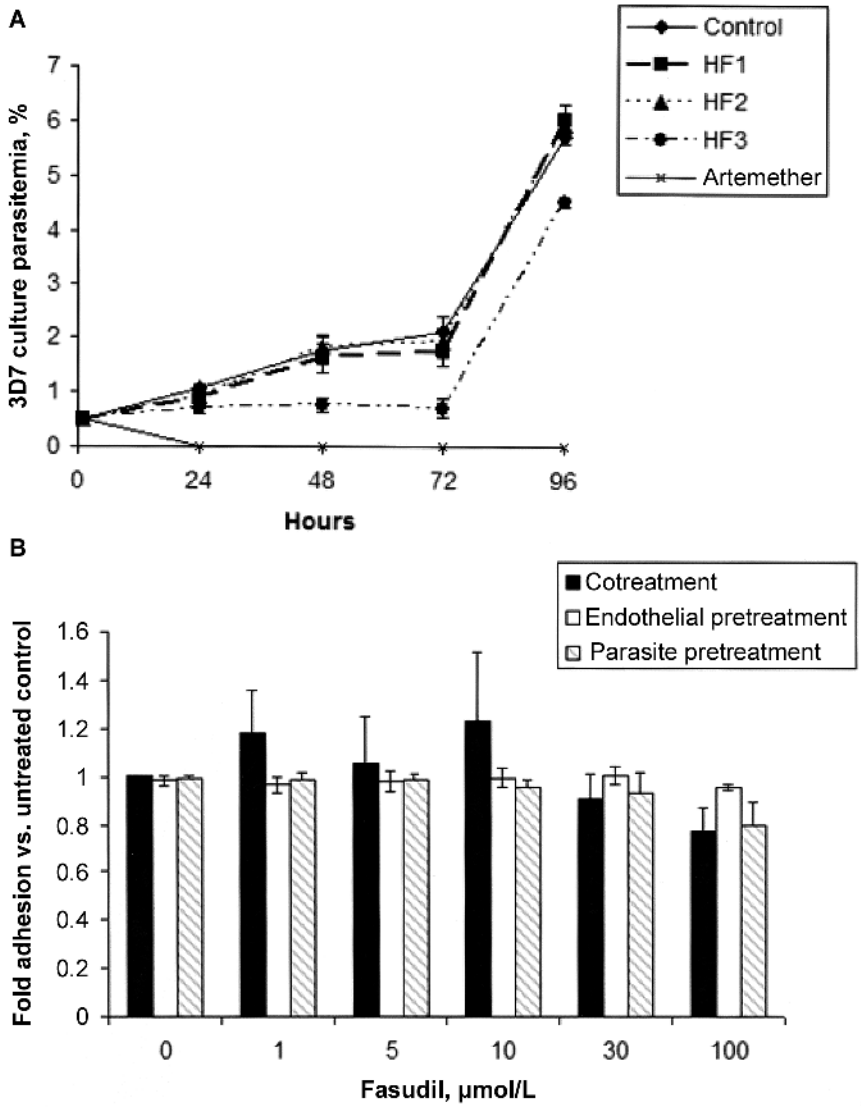

Figure 3. *A,* Effect of fasudil on *Plasmodium falciparum* growth. The Rho kinase inhibitor fasudil (HF1, 1 μmol/L; HF2, 30 μmol/L; HF3, 100 μmol/L) was added to aliquots of asynchronous 3D7 parasite culture (75% ring forms and 25% mature trophozoites and schizonts), which had been adjusted to 0.5% of parasitemia. Parasitemia was then quantified every 24 h on Giemsa-stained thin blood smears. The antimalarial drug artemether at 5 nmol/L was used as a positive antiplasmodial control. Data are mean ± SD values for counted culture parasitemia ($n = 3$). *B,* Effect of fasudil on *P. falciparum* cytoadherence. For endothelial pretreatment, human lung endothelial cells (HLECs) were grown in 8-chamber Lab-Tek slides until confluence and were pretreated for 24 h with fasudil (1–100 μmol/L) before the 3D7 *P. falciparum* adhesion assay was conducted. For parasite pretreatment, parasitized red blood cells (pRBCs) were harvested from cultures pretreated for 24 h with fasudil (1–100 μmol/L) before the adhesion assay was conducted. For cotreatment, fasudil (1–100 μmol/L) was added concomitantly with pRBCs during the *P. falciparum* adhesion assay. After removal of unbound erythrocytes, the preparation was fixed and stained with Giemsa. The no. of pRBCs adhering to 700 HLECs was counted by use of a light microscope. Data are mean ± SD fold pRBC adhesion values vs. the untreated control ($n = 4$).

serts and then exposed for 4 h to mature forms of pRBC. Endothelial monolayer integrity was determined by measuring the OD of Evans blue dye collected from the lower compartments. The OD of Evans blue dye from wells with cells exposed to TNF-α (20 ng/mL), which is known to disrupt vascular endothelium in vitro, was substantially higher than that with cells exposed to uninfected RBCs or medium alone. The OD of Evans blue dye was also significantly increased in adherent pRBC-exposed endothelial monolayer assays compared with RBC-exposed controls ($P < .0001$) (figure 6*A*). We then examined whether fasudil also had an effect when added after adherent pRBC exposure. HLECs were subjected to 3D7 or RAOL parasite adhesion for 4 h, as described above, before fasudil was added (figure 6*B*). The OD of the Evans blue dye was significantly lower in pRBC+HF2–exposed endothelial monolayers than in pRBC-exposed untreated HLECs ($P = .002$) and was similar to that in RBC+HF2–exposed controls. The difference in Evans blue dye between pRBC+HF1 and pRBCs without fasudil was not significant.

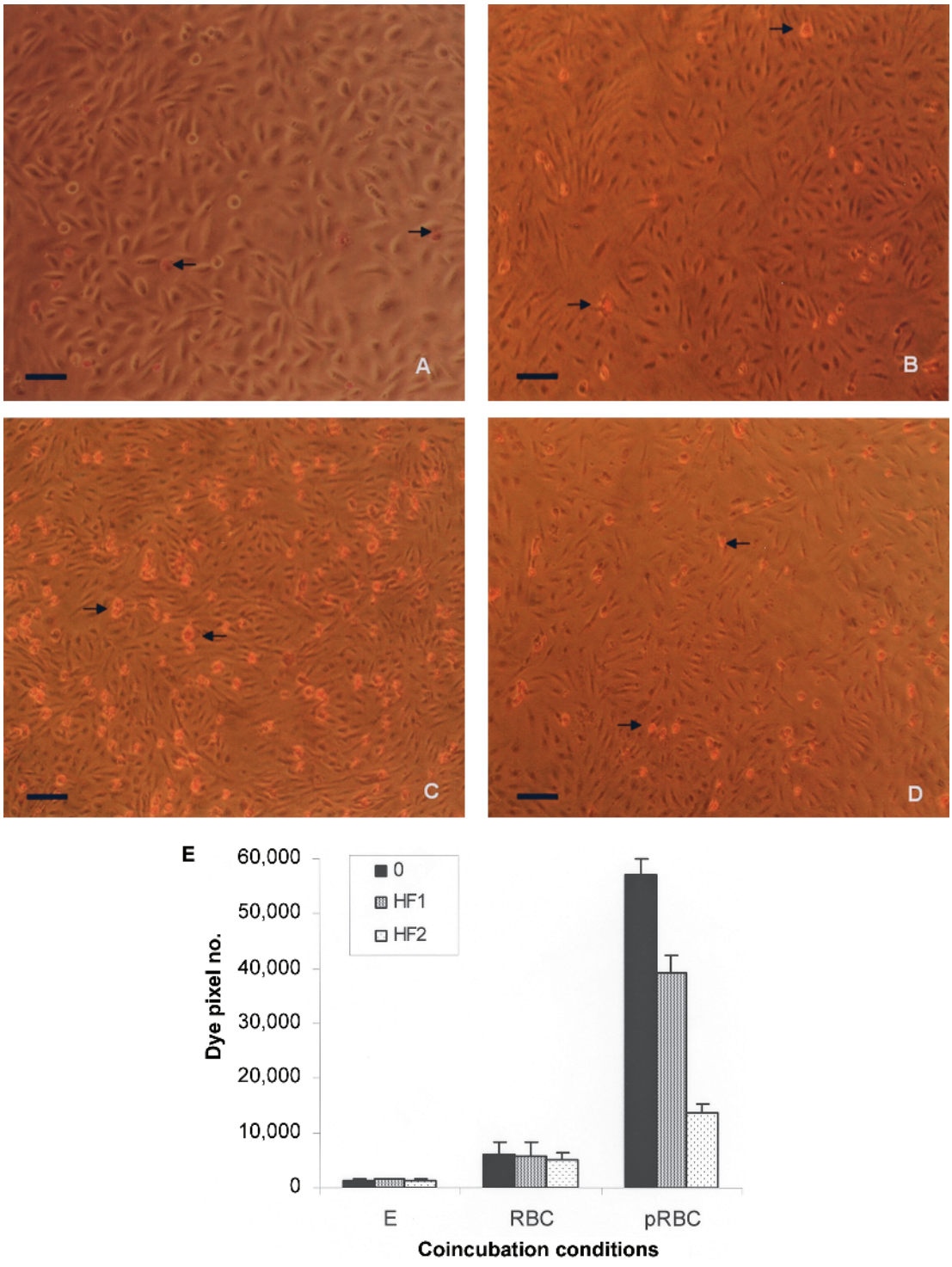

Figure 4. Effect of fasudil on *Plasmodium falciparum*–induced endothelial cell apoptosis. Human lung endothelial cells (HLECs) were cultured in 6-well plates and exposed for 24 h to a suspension of mature forms of parasitized red blood cells (pRBCs; 3D7 clone; adjusted to 1% of hematocrit and 50% of parasitemia) or to control RBCs with or without the Rho kinase inhibitor fasudil. Endothelial apoptosis was then assessed by phosphatidylserine uptake of purple-red APOPercentage dye *(bottom).* After the incubation, HLECs were photographed using an inverted phase-contrast microscope coupled with a digital camera. *A,* Nonexposed endothelial cells. *B,* RBC-exposed endothelial cells. *C,* 3D7 pRBC–exposed endothelial cells. *D,* Fasudil (HF2)–treated 3D7 pRBC–exposed endothelial cells. Arrows indicate purple dye uptake by apoptotic cells (bar shows 100 μm). *E,* Endothelial apoptosis quantified by analytical digital photomicrospy. For each well, 4 photographs of a representative field were taken. The no. of purple pixels was counted using Adobe Photoshop. Data are mean ± SD pixel nos. from 3 independent experiments. 0, untreated cells; E, HLECs incubated with medium alone; HF1, cells treated with 1 μmol/L fasudil; HF2, cells treated with 30 μmol/L fasudil; RBC, HLECs exposed to uninfected red blood cells; pRBC, HLECs exposed to parasitized red blood cells.

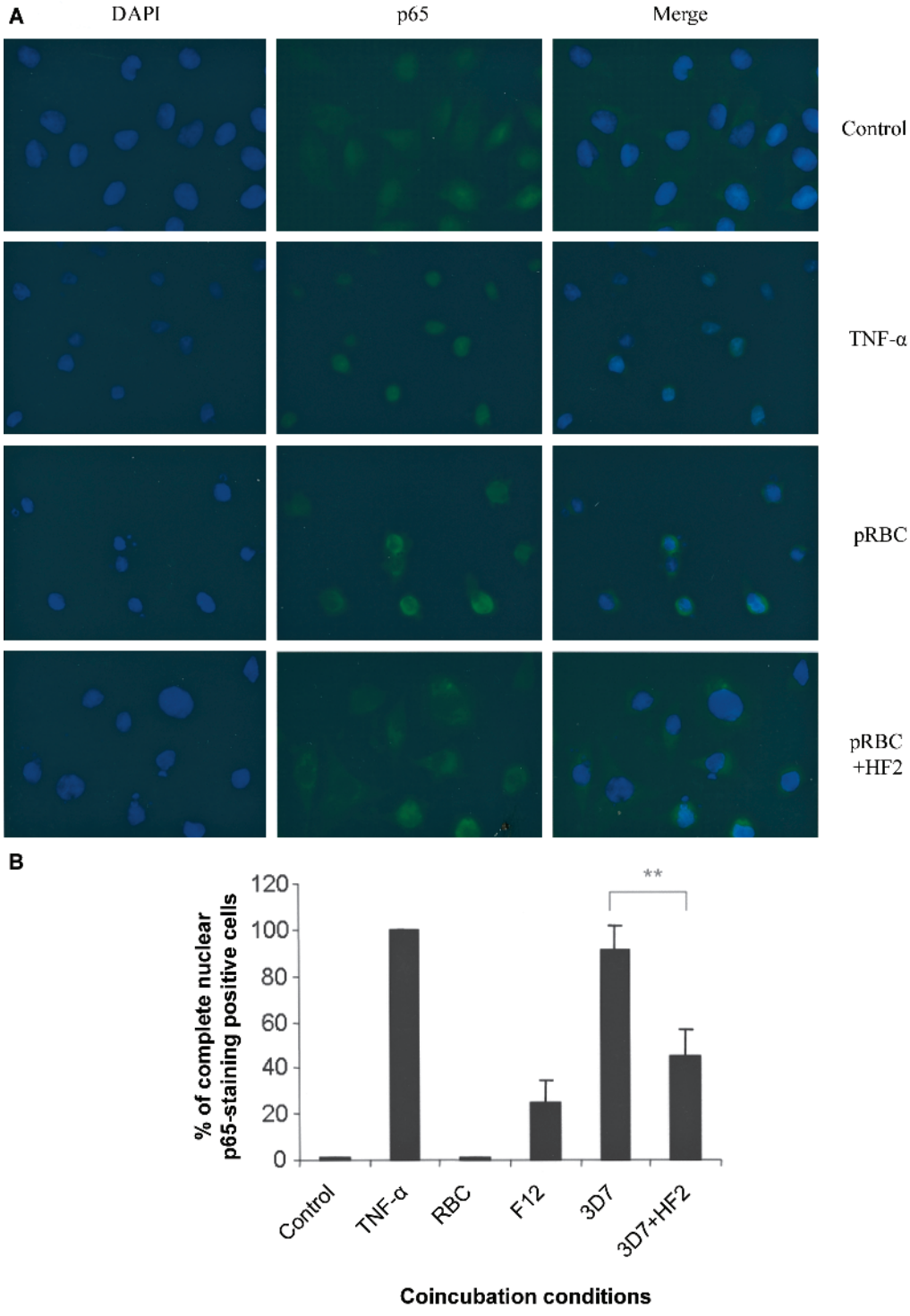

Figure 5. Effect of fasudil on *Plasmodium falciparum*–induced NF-κB endothelial activation. Human lung endothelial cells (HLECs) were grown in 8-chamber Lab-Tek slides. After parasitized red blood cell (pRBC) exposure for 6 h with or without fasudil treatment (HF2, 30 μmol/L), cells were fixed and permeabilized for immunostaining with primary anti–NF-κB p65 subunit antibody and secondary fluorescein isothiocyanate–anti–mouse IgG antibody. Nuclei were stained with diamidino-phenylindole (DAPI). Control indicates endothelial cells incubated with medium only. Tumor necrosis factor (TNF)–α (20 ng/mL) was used as a positive control for nuclear NF-κB translocation. *A,* Representative photographs of each condition. *B,* Activated HLECs (cells with complete but not partial migration nuclear p65 staining) for each condition ($n = 4$). Data are mean ± SD percentages of positive nuclear p65 cells. $^{**}P < .005$.

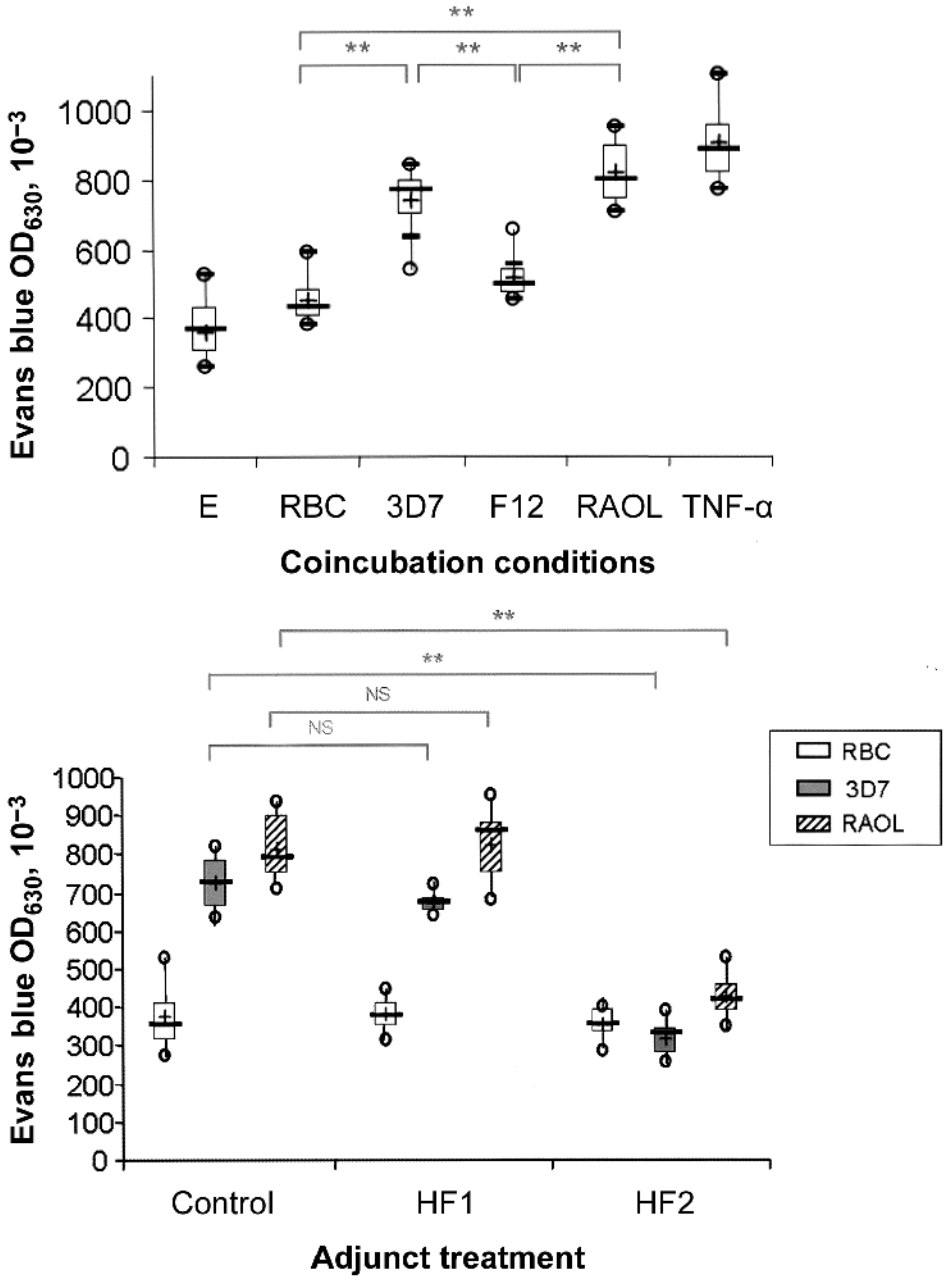

Figure 6. Effect of adjunct fasudil treatment on the integrity of the *Plasmodium falciparum*–exposed endothelial monolayer. Human lung endothelial cells (HLECs) were grown on Transwell inserts to form a cell monolayer and were exposed for 4 h to 3D7-, F12-, or RAOL-parasitized red blood cells (pRBCs; adjusted to 1% of hematocrit and 50% of parasitemia), control RBCs, or tumor necrosis factor (TNF)–α (20 ng/mL) *(A).* Adjunct treatment with the Rho kinase inhibitor fasudil was applied, after 4 h of pRBC exposure, at 1 μmol/L (HF1) or 30 μmol/L (HF2) for 20 h *(B).* Endothelial barrier integrity was then assessed by means of passage of Evans blue dye into lower compartments and optical density analysis (630 nm). 3D7 and RAOL, endothelial cell/3D7 or RAOL pRBC coincubation; E, endothelial cells; HF, fasudil; RBC, endothelial cell/RBC coincubation. Data are median values *(solid lines)* with interquartile ranges, mean values (+), and maximum and minimum values for the data set. NS, not significant. $^{**}P < .005$.

We thus show that endothelial barrier integrity can be restored after pRBC exposure with appropriate fasudil treatment.

DISCUSSION

The aim of the present study was to reveal endothelial signal cascades induced by *P. falciparum* cytoadherence that could be exploited therapeutically. Our results clearly show that infected-erythrocyte adhesion to endothelial cells in vitro directly triggers Rho signaling activation. Parasite-derived soluble factors may not participate in this phenomenon, as shown in the no-contact assays, in which pRBC adhesion was prevented but macromolecule or medium passage was not. This was performed so as to study *P. falciparum*–induced endothelial signaling effects with-

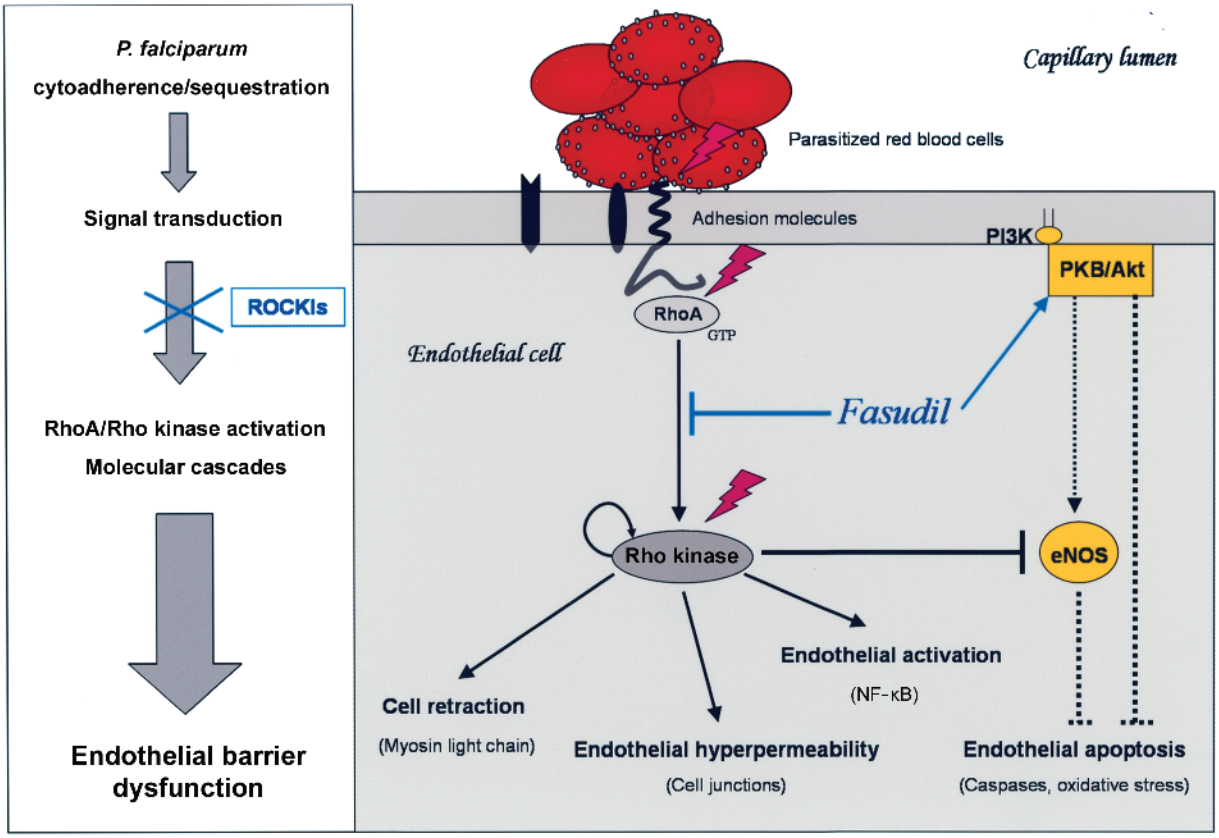

Figure 7. Rho kinase inhibitor–based intervention against *Plasmodium falciparum*–induced endothelial signaling: a potential adjunctive therapeutic strategy for severe human malaria. Emergency treatments for cerebral malaria and multiorgan failure syndrome are not always effective. Rho kinase inhibition might preserve and even restore the function of the blood-brain barrier and other endothelia. Indeed, *P. falciparum* virulence may depend in part on its cytoadherence specificity, where parasitized red blood cell (pRBC) clones have different binding affinities for a specific pattern of readily available endothelial surface adhesion molecules in deep microvascular beds. Some of these receptors, including intracellular adhesion molecule 1 in the brain, have an intracellular domain coupled to G-proteins and Rho signaling. The signal transduction then involves GDP/GTP exchanges and the activation of RhoA, which in turn activates its downstream effector Rho kinase. The RhoA/Rho kinase pathway is involved in endothelial dysfunction when it is abnormally activated. This leads to cell retraction and endothelial hyperpermeability by modulating the phosphorylation of myosin light chain and tight junctions, respectively. Rho kinase is also known to inhibit the PI3K/Akt/eNOS cell survival pathway by down-regulating eNOS expression and attenuating Akt phosphorylation. Hence, Rho kinase inhibitors such as fasudil would have dually beneficial effects on endothelial resistance to pRBC adhesion by (1) preventing parasite activation of rho-dependent cascades and (2) promoting endothelial cell survival in the presence of other proinflammatory agonists.

out the use of blocking antibodies, the ligation of which on endothelial cell receptors is known to activate molecular cascades and mimic adhesion effects [26]. Our results have to be seen in the light of previous studies, which have shown that pRBC adhesion is required to induce endothelial cell apoptosis [13]. We found that the Rho kinase inhibitor fasudil, a drug already in clinical use, could strongly protect endothelial cells from pRBC-induced apoptosis. Rho kinase inhibitors are known to improve endothelial function through a dual mechanism involving the rapid enhancement of the PI3K/Akt/endothelial nitric oxide synthase (eNOS) cell survival pathway [27] and the inhibition of Rho kinase phosphorylation activity [28]. Interestingly, the eNOS antiapoptotic pathway was recently shown to be one of the main targets of Rho kinase down-regulation [29], reflecting the antagonistic aspect of crosstalk between these 2 pathways. Rho kinase can also activate various cascades through its ability to phosphorylate multiple substrates (including other kinases), thereby increasing NF-κB activity and endothelial cell activation [30]. NF-κB is a family of transcription factors that regulate the expression of genes, many of which (including those encoding adhesins and cytokines, e.g., TNF-α and interleukin-1), are strongly involved in the etiology of severe falciparum malaria. Adhesion of pRBCs has also been shown to induce reactive oxygen species (ROS) production by endothelial cells, leading to cell death [14, 31]. Both superoxide anion and hydrogen peroxide are known to be strong inducers of NF-κB activation [32]. Thus, the inhibitory effects of fasudil on NF-κB migration might also be mediated by the PI3K/Akt/eNOS triplet, which inhibits apoptosis through the prevention of mitochondrial ROS release. However, whether *P. falciparum*–induced endothelial ROS production results from Rho kinase signaling has still to be determined.

More importantly, we showed that the Rho kinase inhibitor fasudil restored endothelial barrier integrity after HLEC exposure to pRBCs in vitro. This indicates that fasudil could efficiently target endothelial pathogenic mechanisms even after their onset by pRBCs. *P. falciparum*–induced endothelial barrier dysfunction was likely due to apoptosis, but Rho kinase is also known to up-regulate endothelium hyperpermeability by cytoskeleton and junction rearrangements (figure 7) [33].

There is increasing evidence that endothelial cells are involved in the pathology of human severe malaria. In addition to postmortem histological studies showing blood-brain barrier dysfunction and endothelial activation (reviewed in [34]), clinical studies support the idea of targeting endothelial dysfunction as part of emergency patient management. Compared with Malawian children with uncomplicated malaria, those with CM on hospital admission have higher blood levels of circulating endothelial microparticles [35], which are released on cell activation or apoptosis and have procoagulant and proinflammatory properties [36]. Moreover, vascular leakage can persist for several days after drug-induced *P. falciparum* clearance [37]. Plasma homocysteine levels were recently shown to correlate positively with malaria severity, ranging from mild malaria (fever and myalgias) to severe malaria (prostration, lethargy, and anemia) [38]. Indeed, severe hyperhomocysteinemia is associated with endothelial hyperpermeability. Prolonged exposure to homocysteine creates a prothrombic state and subjects the endothelial compartment to oxidative stress and apoptosis [39]. The endothelium can also suffer damage through the action of polymorphonuclear cells and monocytes [40], the presence of which is associated with *P. falciparum* disease severity and poor clinical outcome in infected children [41]. Interestingly, neutrophil-stimulated endothelial hyperpermeability and microvascular leakage in ischemic and inflammatory heart diseases are mediated by RhoA/Rho kinase signaling [42].

It has been postulated that sequestration is an essential part of the *Plasmodium* survival strategy, because it avoids passage through the spleen, where infected red cells would otherwise be destroyed. Organ sequestration involves various adhesion molecules expressed on the microvascular endothelial surface. Nevertheless, adhesion molecules are also known to trigger signaling cascades within endothelial cells for leukocyte diapedesis during inflammation process [43]. ICAM-1 is a major *P. falciparum* cytoadherence receptor in the brain [44] and is also involved in immune cell homing. Cytoadherence of pRBCs is likely to act not only by mimicking leukocyte recruitment [45] but also by hijacking host responses and thereby causing "collateral damage" to the endothelium (figure 7). Interestingly, *P. falciparum* was recently shown to down-regulate endothelial tight junction mRNAs (occludin and zonula occludens–1) [46]. All *P. falciparum* infections do not lead to severe malaria complications, whereas all *P. falciparum* parasite isolates do cytoadhere. Therefore, cytoadherence/sequestration might be essential but not sufficient in the pathogenesis of severe falciparum malaria, and parasite virulence might rely on signal-triggering specificity [47].

Small GTPases and the downstream effector Rho kinase have been strongly implicated in vascular and neurological diseases when abnormal activation was reported, opening recent research into Rho kinase inhibitors for vascular protective therapy. Endothelial dysfunction has been linked to various Rho kinase–dependent inducers of inflammation, such as hypoxia, thrombin, homocysteine, and endothelin-1 [17], some of which are also clinically related to malaria severity. Fasudil is currently the only Rho kinase inhibitor approved for clinical use. It has been widely used in Japan since 1995 to prevent cerebral vasospasm after subarachnoid hemorrhage. It was also recently shown to be effective and well tolerated in phase 2 trials in patients with stable angina [48] and cerebral ischemic stroke [19]. Fasudil has been shown to improve hemodynamic function, tissue oxygenation, and regional cerebral blood flow [49]. This latter property would be beneficial in lowering intracranial hypertension and thereby reducing the poor outcome of CM in African children, as suggested by Newton et al. [50].

The findings presented here strongly suggest that the Rho kinase pathway could be involved in pathogenic interactions between endothelium and *P. falciparum* during human CM and multiorgan failure syndrome. They also provide insights into how the simple presence of specific, adherent *P. falciparum* clones in the capillary network may compromise endothelial function and cause such drastic symptoms. Rho kinase inhibition appears, then, to be a relevant additional strategy in the management of severe malaria, and fasudil, a well-tolerated drug already in use in human disease, could be a worthwhile adjunctive molecule to be tested in a clinical study of patients with acute manifestations of malaria.

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