16α-Bromoepiandrosterone, an Antimalarial Analogue of the Hormone Dehydroepiandrosterone, Enhances Phagocytosis of Ring Stage Parasitized Erythrocytes: a Novel Mechanism for Antimalarial Activity

Kodjo Ayi, Giuliana Giribaldi, Aleksei Skorokhod, Evelyn Schwarzer, Patrick T. Prendergast, and Paolo Arese

Department of Genetics, Biology and Biochemistry, University of Torino Medical School, Torino, Italy, and Edenland, Inc., Straffan, Ireland

Received 12 November 2001/Returned for modification 20 December 2001/Accepted 16 July 2002

Dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S), which are the most abundant hormones secreted by the adrenal cortex and are present in plasma at approximately 6 μM, as well as their analogue, 16α-bromoepiandrosterone (EPI), exerted antimalarial activities against two chloroquine-sensitive Plasmodium falciparum strains (Palo Alto, 50% inhibitory concentration [IC50] of EPI, 4.8 ± 0.6 μM; T996/86, IC50 of EPI, 7.5 ± 0.91 μM, and IC50 of DHEA-S, 19 ± 2.6 μM) and one mildly chloroquine-resistant strain (FCR-3, IC50 of EPI, 6.5 ± 1.0 μM). Both EPI and DHEA/DHEA-S are potent inhibitors of glucose-6-phosphate dehydrogenase (G6PD), and G6PD deficiency is known to exert antimalaria protection via enhanced opsonization and phagocytosis of rings, the early forms of the parasite. Plasma-compatible antimalarial EPI concentrations did not inhibit G6PD activity and did not induce ring opsonization by immunoglobulin G and complement fragments, as observed in G6PD deficiency, but nevertheless remarkably stimulated ring phagocytosis. Plasma-compatible, low-micromolar concentrations of EPI induced exposure on the ring surface of phosphatidylserine, a signal for phagocytic removal independent of opsonization. We propose that enhanced ring phagocytosis due to exposure of negatively charged membrane phospholipids may explain the antimalarial activity of EPI.

Recently, it has been shown that 16α-bromoepiandrosterone (EPI), an analogue of the human adrenal steroid hormones dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S), was endowed with antimalarial activity against several strains of Plasmodium falciparum in vitro and against Plasmodium berghei in a mouse model (7). Interestingly, another study (16) has indicated that age-related decreases in the frequency and density of P. falciparum parasitemia were greater during puberty and that the blood level of DHEA-S was a significant predictor of resistance. Both EPI and DHEA/DHEA-S inhibit glucose-6-phosphate dehydrogenase (G6PD) (10) and cell proliferation (13, 24). G6PD deficiency is known to afford antimalaria protection (12), possibly mediated by enhanced phagocytosis of rings, the early parasite stage within the erythrocytes (RBC) (3). Therefore, we explored whether the antimalarial activity of EPI could be related to inhibition of erythocytes G6PD. The present results confirm the antimalarial activities of EPI and DHEA-S and indicate that sub- and low-micromolar EPI concentrations remarkably stimulated ring phagocytosis. EPI did not inhibit G6PD activity and did not enhance deposition of phosphatidic oposisines, but enhanced exposure on the ring surface of phosphatidylserine (PS), a signal for phagocytic removal independent of opsonization (30). We propose that enhanced ring stage phagocytosis due to exposure of negatively charged membrane phospholipids may explain the antimalarial activity of EPI.

MATERIALS AND METHODS

Materials. Buffers, culture media, substrates, enzymes and coenzymes, adenine, heparin, gentamicin, mannitol, dehydroepiandrosterone sulfate (5-androsten-3β-ol-17-one sulfate [DHEA-S]), Tween 20, Triton X-100, 5-amin-2,3-dihydro-1,4-phthahalazinedione (luminol), dimethyl sulfoxide (DMSO), 5,5′-dithio-bis(2-nitrobenzoic acid), methylene blue, rabbit anti-human immunoglobulin G (IgG) second antibodies, rabbit anti-human C3c second antibodies, mouse anti-rabbit second antibodies (all secondary antibodies conjugated to alkaline phosphatase), and annexin V-fluorescein isothiocyanate (FITC) conjugate were from Sigma, St. Louis, Mo. AO M cell culture medium was from Gibco. M450 Pan B and Pan T Dynabeads were from Dynal A.S., Oslo, Norway. Percoll was from Pharmacia, Uppsala, Sweden. D-[1-14C]glucose and [8-3H]hypoxanthine were from Amersham International, Amersham, United Kingdom. Dif-Quik stain was from Baxter Dade AG, Dudingen, Switzerland. Sterile plastics were from Costar, Cambridge, Mass. All other reagents were purchased from common commercial sources. EPI was provided by Edenland, Inc., Straffan, Ireland. In all experiments, EPI or DHEA-S were dissolved in DMSO. All controls were supplemented with DMSO at the same concentration (35 μM final concentration) as in EPI- or DHEA-S-treated samples.

RBC preparation. P. falciparum cultivation, and stage separation of parasitized RBC. Freshly drawn blood (Rh+) from hematologically healthy adults of both sexes, anticoagulated with heparin, and kept in heparin-phosphate-dextrose with adenine and mannose, was used. RBC were separated from plasma and leukocytes by centrifugation with an 85% Percoll gradient in phosphate-buffered saline (PBS) and three washings in wash medium (RPMI 1640 medium containing 2 mM glutamine, 24 mM NaHCO3, 25 mM HEPES, 20 mM glucose, and 32 mg of gentamicin per liter [pH 7.30]). P. falciparum strains Palo Alto, FCR-3, and T996/86 (all mycoplasma free) were cultivated at a 0.5% hematocrit and synchronized as described previously (18). Parasite stages were separated on Percoll-mannitol gradients 14 to 18 (rings in the first cycle), 34 to 38 (trophozoites in the first cycle), and 40 to 44 h after administration of the inoculum (schizonts in the first cycle). The parasitemia was usually made up of 30 to 45% rings, >90% trophozoites, and >95% schizonts. To assess total parasitemia and the relative contributions of rings, trophozoites, and schizonts, slides were prepared from cultures at indicated times and stained with Diff-Quik stain, and 400 to 1,000 cells were examined microscopically. All studies, except growth inhibition studies,
were performed with the mycoplasma-free Palo Alto strain. Occasional repeats with the mycoplasma-free FCR-3 strain gave comparable results.

Effects of EPI and DHEA-S on parasite growth. RBC were isolated from plasma and leukocytes, and *P. falciparum* strains Palo Alto and T996/86 (both chloroquine sensitive) and FCR-3 (mildly chloroquine resistant) were synchronized and cultivated at a 0.5% hematocrit. Schizonts (purity, >95%) were isolated on Percoll-mannitol gradients from synchronized cultures. Parasitemia was adjusted to 2% schizonts immediately after inoculum into a fresh RBC suspension at a 0.5% hematocrit. The growth inhibition assay was performed according to the procedure of Desjardins et al. (5). Triplicate readings were performed for each experimental point.

Measurement of PPP flux by 14CO2 production from d-[1-14C]glucose and assay of reduced GSH. Pentosephosphate pathway (PPP) flux was measured by assessing the 14CO2 production from d-[1-14C]glucose in nonparasitized RBC suspended in RPMI 1640 (pH 7.4) at a 10% hematocrit, treated or not with EPI (0.8 to 80 μM, final concentration). After 90 min of preincubation at 37°C with and without EPI, PPP flux was measured (for details, see Materials and Methods). Methylene blue was added at a final concentration of 150 μM. Data (mean values ± standard deviation [vertical bars] of four different experiments) are expressed as production of 14CO2 from d-[1-14C]glucose in counts per s (cPS)/10 μl of packed RBC. The significance of differences is relative to controls. For details, see Materials and Methods.

![FIG. 1. Assay of basal and methylene blue-stimulated PPP flux in nonparasitized RBC treated or not with EPI (0.8 to 80 μM final concentration).](http://aac.asm.org/) After a 90-min preincubation at 37°C with and without EPI, PPP flux was measured (for details, see Materials and Methods). Methylene blue was added at a final concentration of 150 μM. Data (mean values ± standard deviation [vertical bars] of four different experiments) are expressed as production of 14CO2 from d-[1-14C]glucose in counts per s (cPS)/10 μl of packed RBC. The significance of differences is relative to controls. For details, see Materials and Methods.

**RESULTS**

Effects of EPI and DHEA-S on parasite growth. Parasite growth was assessed by measuring the incorporation of [3H]hypoxanthine in growing trophozoites starting at 20 to 30 h post-invasion. Both EPI and DHEA-S had antimalarial effects on two chloroquine-sensitive strains (Palo Alto and T996/86) and on one mildly chloroquine-resistant strain (FCR-3). DHEA-S was only tested with strain T996/86. The 50% inhibitory concentration (IC50) of EPI and DHEA-S were assessed with a growth inhibition assay as described by Desjardins et al. (5) with microtiter plates predosed with various concentrations of EPI and DHEA-S. The following results were obtained (shown as means ± standard deviations of triplicate readings for each experimental point). The EPI IC50 for strains Palo Alto, T996/86, and FCR-3 were 4.8 ± 0.68, 7.5 ± 0.91, and 6.5 ± 1.01 μM, respectively. The DHEA-S IC50 for strain T996/86 was 19.2 ± 2.6 but was not determined for strains Palo Alto and FCR-3. Note that the IC50 for EPI were in the low-micromolar range and were comparable to IC50 recently reported for other strains (7). The antimalarial concentration of the physiological hormone DHEA-S was higher, but still not far from the plasma drug concentrations in young adult males (16).

Effect of EPI on PPP flux and GSH level in nonparasitized RBC. PPP flux was estimated by measuring the 14CO2 production from d-[1-14C]glucose. This assay is considered to reflect G6PD activity in the intact RBC much better than measurement of G6PD activity in the cell lysate (3, 8). For this reason, PPP flux was measured in unstimulated and methylene blue-stimulated intact RBC at various EPI concentrations. As shown in Fig. 1, significant inhibition of methylene blue-stim-
ulated flux was observed only at 80 μM EPI. No significant changes were observed at lower EPI concentrations. The levels of GSH were measured in nonparasitized RBC treated or not with different EPI concentrations (data not shown). The GSH level did not change at 0.8 and 8 μM EPI and decreased nonsignificantly at 80 μM EPI. In conclusion, only 80 μM EPI was able to induce a moderate reduction of PPP flux not accompanied by any significant decrease in GSH level.

**Effect of EPI on phagocytosis of nonparasitized and stage-separated parasitized RBC.** As shown in Fig. 2A, EPI significantly enhanced ring phagocytosis. The effect was evident already at 0.5 μM EPI and was present roughly dose dependently at higher EPI concentrations (5 to 10 μM final concentration). At all EPI concentrations studied, differences from homogeneous controls were statistically significant. EPI did not significantly modify phagocytosis of trophozoites (Fig. 2B).

**Effect of EPI on deposition of autologous IgG and complement C3 fragments in rings.** As shown in Fig. 3, treatment of rings with 1 to 2 μM EPI did not further increase the deposition of the opsonins autologous IgG (Fig. 3A) and complement fragment C3c (Fig. 3B) and did not change the aggregation state of band 3 (data not shown).

**Effect of EPI on PS exposure.** As shown in Fig. 4 and Table 1, treatment of rings with EPI (2 μM final concentration) induced an approximately 62% increase in mean emitted fluorescence intensity (MFI) after labeling with annexin V-FITC, a marker for exposure of PS (17). MFI in trophozoites was remarkably higher than in rings and was not further increased by EPI treatment, indicating maximal exposure of PS in advanced parasite stages irrespective of the presence of EPI.
DISCUSSION

This study confirms recently published data (7) on antimalarial activity exerted by EPI against P. falciparum in vitro and in a mouse model. With strains Palo Alto, T996/86, and FCR-3, effective antimalarial concentrations of EPI inducing 50% growth inhibition (IC50) were in the low-micromolar range, while the physiological hormone DHEA-S also exerted antimalarial activity, but was somewhat less effective. EPI is an analogue of DHEA and DHEA-S, the most-abundant steroid hormones secreted by the adrenal cortex and present in adult male plasma at an approximately 6 μM concentration (20, 21). At postpubertal plasma-compatible concentrations, DHEA-S, DHEA, and EPI are potent and noncompetitive inhibitors of G6PD (10). Reports indicate that inhibition of G6PD activity by DHEA, EPI, or other analogs induced growth arrest, decreased DNA synthesis, or both in a variety of normal or tumor cell lines and tissues (11, 13, 24, 28).

Since the parasite growth-inhibitory concentrations of EPI were in the same range as the inhibition constant of EPI for G6PD, we checked whether EPI treatment induced decrease of PPP flux, a reliable way to check G6PD activity in the intact cell. EPI up to 80 μM did not inhibit basal PPP flux in the intact RBC and inhibited nonsignificantly methylene blue-stimulated flux. These data make it very unlikely that G6PD inhibition was responsible for the inhibition of parasite growth.

In contrast to the lack of G6PD inhibition, as little as 0.5 μM EPI significantly enhanced phagocytosis of rings (Palo Alto strain). Ring phagocytosis is advantageous to the human host, because phagocytosed rings are digested rapidly, and the process is repeated without loss of efficiency (3, 25). In contrast, more mature forms of the parasite, although actively phagocytosed, hinder repetition of the phagocytic process because the malarial pigment hemozoin, abundantly present in mature parasite forms, is indigestible and severely affects important functions of the monocyte (25). Increased phagocytosis of rings will reduce parasitemia and allow lower numbers of rings to mature to trophozoites or schizonts, which adhere to cerebral endothelia and may cause cerebral malaria, a major cause of malaria mortality (1). Previous studies (9, 29) have shown that phagocytic recognition of rings growing in normal RBC is mostly mediated by deposition of IgG with anti-band 3 specificity and complement fragments. In contrast, phagocytosis of trophozoites is mediated by other phagocyte receptors, such as scavenger receptors and, possibly, mannose receptors (29; unpublished data).

The present data do not support the initial working hypothesis that EPI might inhibit the G6PD activity of RBC and thus transform EPI-treated rings into cells phenotypically similar to rings developing in G6PD-deficient RBC (3). The membrane alterations and opsonization that occur in senescent, oxidatively damaged or ring stage parasitized RBC (19, 26, 29) were apparently not operating in EPI-treated rings. In contrast, low-micromolar EPI induced externalization of PS in rings, but not in nonparasitized RBC. A large number of data indicate that exposure of PS occurs in senescent and pathological RBC (reviewed in reference 30) and in RBC parasitized by late P. falciparum forms (27), as well as in a variety of activated, altered, or apoptotic cells (14). Here we show that exposure of PS was quite remarkable in late forms and could not be further enhanced by EPI. Loss of phospholipid asymmetry and exposure

![FIG. 4. Effect of EPI on cell-surface exposure of PS as measured by FACS. Shown are the results of FACS analysis of EPI (2 μM final concentration)-treated (open areas) or untreated (shaded areas) nonparasitized RBC (A), rings (B), and trophozoites (C) stained with FITC-annexin V. The data are representative of three independent experiments, all showing similar results.](http://aac.asm.org/)

![TABLE 1. Effect of EPI on cell-surface exposure of PS as measured by MFI](http://aac.asm.org/)
of PS are a well-known mechanism for recognition of effete or damaged cells by scavenger receptors of phagocytic cells (14). Exposed PS is recognized by monocyte scavenger receptor CD36 and other receptors that trigger phagocytosis (14).

Two studies indicating interaction of DHEA and DHEA-S with RBC membrane and modification of phospholipid organization may explain EPI-induced exposure of PS. A first study (6) has shown that both DHEA-S and DHEA destabilized the bilayer gel phase and lowered the gel to the fluid phase transition temperature. DHEA-S, a close analogue to EPI because of PS are a well-known mechanism for recognition of effete or stressed adult (4). Correspondingly, levels of DHEA-S in fetal blood are remarkably elevated (22). Thus, despite the lack of steroid by the fetus is up to eightfold that of a non-pregnant 3184 AYI ET AL. ANTIMICROB. AGENTS CHEMOTHER. term are as large as the adult ones, and the total daily production of steroids in fetal blood is remarkably elevated (22). Thus, despite the lack of contact between maternal and fetal circulation, high levels of steroids with antimalarial activity may offer additional protection to the fetus and newborn.

ACKNOWLEDGMENTS

This work was supported by a research grant from Edenland, Straf- fan, Ireland, to K. Ayi. Assistance by Elena Valente and Mauro Prato with the parasite cultures and some of the experiments is gratefully acknowledged.

REFERENCES