Artemisinin inhibits inducible nitric oxide synthase and nuclear factor NF-kB activation

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Abstract Artemisinin is a natural product used as an alternative drug in the treatment of severe and multidrug-resistant malaria. In the present work we show that artemisinin shares with other sesquiterpene lactones the ability to inhibit the activation of the nuclear factor NF-kB: by this mechanism, artemisinin, as well as parthenolide, inhibits nitric oxide synthesis in cytokine-stimulated human astrocytoma T67 cells. These results suggest that artemisinin, in addition to its antiparasitic properties, could also exert a therapeutic effect on neurological complications of malaria.

Key words: Nitric oxide; Nitric oxide synthase; Artemisinin; Parthenolide; Malaria; NF-kB; IkB-α

1. Introduction

Every year 100 of million people get ill of malaria and about one million die because of neurological complications [1]. As the number of *Plasmodium falciparum* strains resistant to chloroquine and other conventional antimalarial drugs is progressively increased [2], the use of alternative drugs has deserved a growing interest: for instance, artemisinin, a natural product discovered by the traditional Chinese medicine, and its derivatives are deemed to be the most effective and safe drugs for the treatment of severe and multidrug-resistant malaria [3]. The exact mechanism of action of this sesquiterpene lactone endoperoxide, derived from the leafy portions of *Artemisia annua*, is not completely clarified: it has been suggested that its antiparasitic effects involve the generation of an oxidative stress [4].

Several sesquiterpene lactones are known to impair the activity of the transcriptional factor NF-kB [5], by alkylating it [6,7] or preventing the degradation of its inhibitory protein IkB [8–10]. NF-kB controls the expression of genes involved in cytokine production, cellular adhesion, inflammation and apoptosis [11]. The inducible nitric oxide synthase (iNOS) gene is NF-kB-regulated: iNOS catalyzes the generation of nitric oxide (NO), a molecule playing a role in inflammation and immune response [12]. During flogistic processes, the cytokines interleukin-1 beta (IL-1β), interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α) and the bacterial lipopolysaccharide (LPS) induce an increase of iNOS level in macrophages [12]. The circulating levels of TNF-α and IL-1β correlate with the severity of *P. falciparum* malaria, and a high intracerebral level of NO has been proposed to mediate, at least partly, the neurological disorders observed in cerebral malaria [13]. The plasmatic levels of nitrite and nitrate (stable NO derivatives) are increased in patients with *P. falciparum* and *P. vivax* malaria, and are related to the severity of the disease [14], being higher in patients with cerebral malaria [15]. Some sesquiterpene lactones affect the synthesis of NO [9,10,16–18], and in some cases they have been demonstrated to exert this effect via NF-kB inhibition [10].

In our work we have studied the effect of artemisinin and parthenolide on NO synthesis in a human astrocytoma cell line (T67), and investigated the mechanism by which artemisinin and parthenolide modulate NO production.

2. Materials and methods

2.1. Reagents

Fetal bovine serum (FBS) and Dulbecco's minimum essential medium (DMEM) were supplied by Gibco BRL (Paisley, Scotland); L-[2,3,4,5-^{3}H]arginine monohydrochloride (62 Ci/mmol) was obtained from Amersham International (Bucks, UK). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA, USA). The protein content of cell monolayers and cell lysates was assessed with the BCA kit from Pierce (Rockford, IL, USA). If not otherwise specified, other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Aldrich (Milan, Italy). Parthenolide and artemisinin were dissolved in DMSO (final solvent concentration less than 0.1% v/v).

2.2. Cells

Human astrocytoma T67 cells [19] were a gift from Prof. Giuliana Maria Lauro (Department of Biology, University of Rome 3, Rome, Italy). The N11 mouse glial cell line was a gift from Dr. Marco Righi (CNR Insitute of Neuroscience, Section of Cellular and Molecular Pharmacology, Milan, Italy). Cells were cultured up to the confluence in 35- or 100-mm diameter Petri dishes with DMEM supplemented with 10% FBS, then were incubated for 24 h with various reagents, as described in Section 3.

2.3. Measurement of NOS activity and nitrite production

Cells grown at confluence on 35-mm diameter Petri dishes, after a 24-h incubation in the experimental conditions described in Section 3,

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Abbreviations: iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1 beta; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; LPS, lipopolysaccharide; FBS, fetal bovine serum; DMEM, Dulbecco's minimum essential medium; RT-PCR, reverse transcriptase-polymerase chain reaction; EMSA, electrophoretic mobility shift assay; IkB, NF-kB inhibitor

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were lysed and NOS activity was checked as the ability of the cell lysates to convert L-[^{3}H]arginine to [^{3}H]citrulline during 15 min, as previously described [20]. Enzyme activity was expressed as pmol citrulline/min/mg cell protein. In parallel, the nitrite concentration in the culture medium was measured with the Griess method, as previously described [20], and expressed as nmol nitrite/24 h/mg cell protein.

2.4. Western blot

Whole cell extracts, obtained as previously described [21], containing equal amounts of proteins (30 μg) were electrophoresed in a 8% polyacrylamide gel, transferred to PVDF membrane sheets (Immobilon-P, Millipore) and probed with anti-iNOS (from mouse; diluted 1:500 in PBS–BSA 1%, Transduction Laboratories, Lexington, KY, USA), anti-β-actin (from rabbit; diluted 1:250 in PBS–BSA 1%, Sigma) or anti-IkB-α (from rabbit, diluted 1:200 in PBS–BSA 1%, Santa Cruz Biotechnology, DBA, Italy) antibodies; after a 1-h incubation, the membrane was washed with PBS–Tween 0.1% and subjected for 1 h to a peroxidase-conjugated anti-mouse or anti-rabbit IgG (sheep; Amersham International, diluted 1:1000 in PBS–Tween with Blocker Non-Fat Dry Milk 5%, Bio-Rad). The membrane was washed again with PBS–Tween and proteins were detected by enhanced chemiluminescence (Amersham International).

2.5. Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was obtained as previously described [21] and real-time RT-PCR was carried out using the prepared cDNA as a template, as described in [22]. The same cDNA preparation was used for the quantification of iNOS and β-actin, used as a housekeeping gene. Human iNOS-specific primers and probes were designed with the Beacon Designer Premier Biosoft International Software (Palo Alto, CA, USA) using published cDNA sequences (Accession No. L09210) and were custom-made by Invitrogen (Milan, Italy). The sequences of iNOS primers for quantitative RT-PCR are 5′-CAGCGGGATGACTTTCCAAG-3′, 5′-AGGCAAGATTTGGACCTGCA-3′; cycling: one cycle at 50°C for 2 min, one cycle at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and annealing/extension at 60°C for 1 min. To perform the relative quantitation of each sample, the iNOS PCR product was compared with the β-actin product and expressed as Relative Expression using the Bio-Rad Software Gene Expression Quantitation.

2.6. Electrophoretic mobility shift assay (EMSA) of NF-kB and AP-1

Cells were plated in 100-mm diameter dishes at confluence and all procedures for nuclear protein extraction were performed at 4°C with ice-cold reagents. Cells were mechanically scraped in PBS and washed, then 1×10^7 cells were resuspended in 500 μl of lysis buffer A (15 mM KCl, 10 mM HEPES, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM PMSF, 1 mM dithiothreitol (DTT), 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.1% NP-40, pH 7.6). Cell suspensions were then incubated for 10–15 min on ice with occasional vortexing, and centrifuged for 30 s to pellet nuclei, which were rinsed with wash buffer B (2 mM KCl, 25 mM HEPES, 0.1 mM EDTA, 1 mM PMSF, 1 mM DTT, 10 μg/ml aprotinin, 2 μg/ml leupeptin, pH 7.6) and incubated at 4°C for 20 min. Nuclear extracts were then prepared by centrifugation at $20\,000\times g$ for 15 min in buffer C (25 mM HEPES, 0.1 mM EDTA, 20% glycerol, pH 7.6) and stored at −80° until used for EMSA. The probes contained the NF-kB or the AP-1 oligonucleotide consensus sequence and were labeled with γ-^{32}P (Amersham Life Science) (3000 Ci/mmol, 250 μCi) using T4 polynucleotide kinase (Boehringer Mannheim). The sequences of oligonucleotides used were (binding sites underlined): NF-kB [5′-AGTTGAGGGGACTTTCCAGG-3′] (Promega); AP-1 [5′-CGTTTGATGAGTCAGCCGGAA-3′] (Promega). 10 μg of extracts was incubated for 20 min with 20 000 cpm of ^{32}P-labeled double-stranded oligonucleotide at 4°C in a 25 μl reaction mixture containing: 10 μg/ml BSA, 10× buffer D (100 mM KCl, 20 mM HEPES, 0.5 mM EDTA, 2 mM DTT, 0.1 mM PMSF, 20% glycerol, 0.25% NP-40, pH 7.6), 5× buffer F (300 mM KCl, 100 mM HEPES, 10 mM DTT, 100 μM PMSF, 20% Ficoll, pH 7.6) and 1 μg/μl poly(dI-dC). In competition assays, 100× cold NF-kB or AP-1 competitor was added. The DNA–protein complex was separated on a non-denaturating 4% polyacrylamide gel in TBE buffer (Tris–HCl, boric acid, EDTA 2 mM, pH 8.0). After electrophoresis, the gel was dried and autoradiographed by overnight exposure to X-ray film.

2.7. Statistical analysis

Each experimental point has been performed in duplicate or triplicate per experiment; all data in the text and figures are given as means ± S.E.M. Statistical analysis was carried out using the Student's *t*-test for unpaired data.

3. Results and discussion

3.1. Artemisinin and parthenolide inhibit nitrite production and NOS activity and expression

Micromolar doses of sesquiterpene lactones have already been shown to inhibit nitrite levels, NOS activity and expression [10,16]. We investigated the effect of artemisinin and parthenolide in T67 cells, possessing an iNOS isoform which is known to be stimulated by the treatment with LPS and cytokines [23]. Cells were incubated for 24 h with artemisinin or parthenolide, in the presence or absence of a mix containing LPS, IL-1β, IFN-γ, and TNF-α: the nitrite level in the culture medium was not modified by artemisinin or parthenolide alone, while both molecules significantly reverted the increase of nitrite induced by the LPS/cytokines mix (Fig. 1). The same effect was observed when NOS activity was measured in the lysates of cells previously incubated with either sesquiterpene lactone for 24 h (Fig. 1). Therefore artemisinin, similarly to parthenolide, was able to inhibit the LPS/cytokine-induced increase of NOS activity, measured as both nitrite accumulation in the extracellular medium and ability of the cell lysate to convert [^{3}H]arginine to [^{3}H]citrulline. The measurement of NOS in the cell lysate provides information about the content of catalytically active enzyme: indeed, citrulline production in the lysate is an index of the maximal NOS activity, i.e. measured in the presence of saturating concentrations of substrate and in the absence of the cell regulatory control. Put differently, the nitrite accumulation in the supernatant of cultured cells depends on the actual NO synthesis by whole, viable cells.

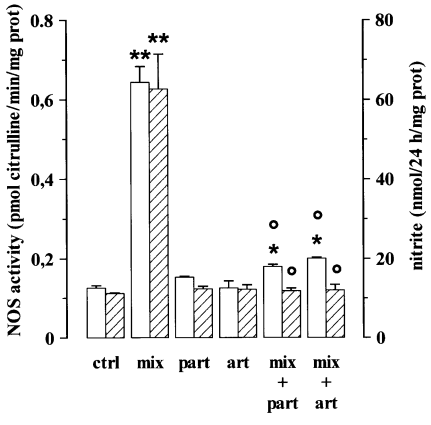

Fig. 1. NOS activity (open bars) and nitrite levels (hatched bars) in T67 cells incubated for 24 h in DMEM+10% FBS in the absence (ctrl) or presence of a LPS/cytokines-containing mix (mix: 20 μg/ml LPS, 50 ng/ml TNF-α, 100 ng/ml IFN-γ, 10 ng/ml IL-1β), 10 μM parthenolide (part) or 10 μM artemisinin (art), alone or in combination with the mix. After this time, NOS activity was measured in the cell lysates and extracellular medium was checked for nitrite concentration (see Section 2). Measurements were performed in duplicate, and data are presented as means ± S.E.M. ($n=5$); vs. ctrl: $^{**}P<0.02$, $^{*}P<0.05$; versus mix: $^{\circ}P<0.002$.

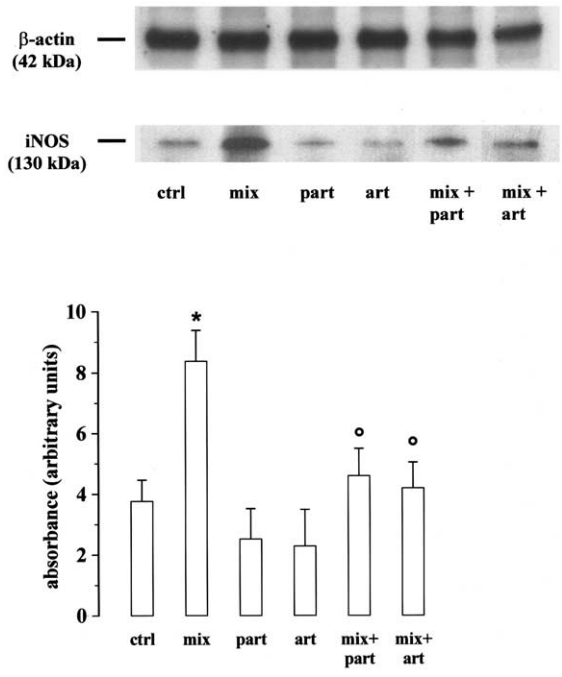

Fig. 2. Western blot detection of iNOS in T67 cells, incubated for 24 h in the absence (ctrl) or presence of the LPS/cytokines mix (mix: see legend of Fig. 1), 10 μM parthenolide (part) or 10 μM artemisinin (art), alone or in combination with the LPS/cytokines mix. The experiment in the upper panel is representative of three similar experiments. Western blot has also been performed in the same cell lysates with antibodies against the housekeeping protein β-actin, as a control of the gel protein loading. The protein bands have been quantitated densitometrically; the values, expressed as arbitrary units, are represented in the lower panel as means ± S.E.M. ($n = 3$); vs. control: $^{*}P < 0.05$; versus mix: $^{\circ}P < 0.05$.

Western blot experiments showed that a 24-h incubation with the LPS/cytokines mix induced a strong increase of iNOS protein expression: this effect was completely abrogated by the co-incubation with both artemisinin and parthenolide (Fig. 2). In real-time RT-PCR experiments, the mix strongly induced the iNOS mRNA transcription; this increment was blunted when LPS-stimulated cells were incubated with artemisinin or parthenolide (Fig. 3).

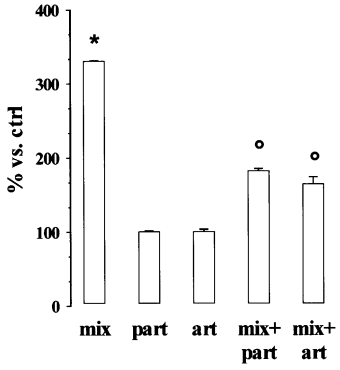

Fig. 3. Real-time RT-PCR of iNOS mRNA in T67 cells incubated in the absence (ctrl) or presence of the LPS/cytokines mix (mix: see legend of Fig. 1), 10 μM parthenolide (part) or 10 μM artemisinin (art), alone or in combination with the LPS/cytokines mix ($n = 3$). The RT-PCR efficiency was controlled by amplifying a β-actin fragment, used as a housekeeping gene. The iNOS PCR product was compared with the β-actin product and expressed as relative expression versus β-actin, in terms of percentage of control (assumed as 100%). Vs. ctrl: $^{*}P < 0.01$; vs. mix: $^{\circ}P < 0.02$.

3.2. *Artemisinin and parthenolide block the LPS/cytokine-induced activation of NF-kB*

NF-kB is a heterodimeric cytosolic protein, which in resting cells is kept inactive by the binding with a member of the IkB inhibitor protein family [24]. After phosphorylation, IkB is ubiquitinylated and degraded by a proteasome system and NF-kB can translocate to the nucleus promoting iNOS transcription [25]. Our EMSA experiments, performed in nuclear extracts, showed that NF-kB was activated by a 24-h stimulation with the LPS/cytokines mix, and translocated into the nucleus (Fig. 4). Both artemisinin and parthenolide completely reverted the effect of the mix (Fig. 4). To evaluate the specifity of the drug's effects, we investigated the nuclear translocation of another transcription factor, AP-1, in the same extracts and at the same experimental conditions: in this case both artemisinin and parthenolide did not significantly modify the activation of AP-1 (Fig. 4). As LPS/cytokine-elicited NF-kB activation is mediated by the phosphorylation and subsequent proteolytic degradation of the inhibitory protein IkB [26], we investigated whether artemisinin and parthenolide could interfere with this step. Western blot analysis showed that the level of IkBα was significantly reduced in LPS/cytokine-treated cells, and both artemisinin and parthenolide restored IkBα expression when incubated together with the LPS/cytokines mix (Fig. 5). These results suggest that artemisinin, as well as parthenolide, prevents the LPS-induced proteolytic degradation of IkBα protein and the consequent activation and translocation of NF-kB, thus accounting for the suppression of iNOS gene expression and NO synthesis in cells stimulated with LPS/cytokines. Parthenolide (10 μM for 1 h) has already been shown to inhibit activation of NF-kB in Jurkat T leukemia cells [8], and NF-kB translocation and iNOS transcription in LPS/IFN-γ-stimulated rat aortic smooth muscle cells [9]; in both cases the drug prevented the degradation of IkB [8,9]. On the other hand, in

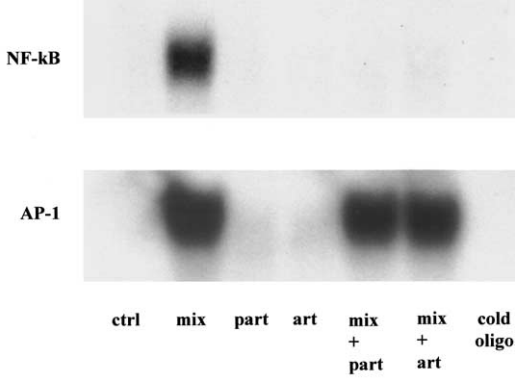

Fig. 4. EMSA detection of NF-kB (upper panel) and AP-1 (lower panel) activation. Cells were incubated for 24 h in the absence (ctrl) or presence of the LPS/cytokines mix (mix: see legend of Fig. 1), 10 μM parthenolide (part) or 10 μM artemisinin (art), alone or in combination with the LPS/cytokines mix. Nuclear extracts were subjected to EMSA as described in Section 2; in the last lane of each experiment the sample incubated with LPS/cytokines was mixed not only with the labeled oligonucleotide probe but also with 100× cold oligonucleotide as specific competitor (cold oligo). These experiments are representative of three similar experiments per each transcription factor.

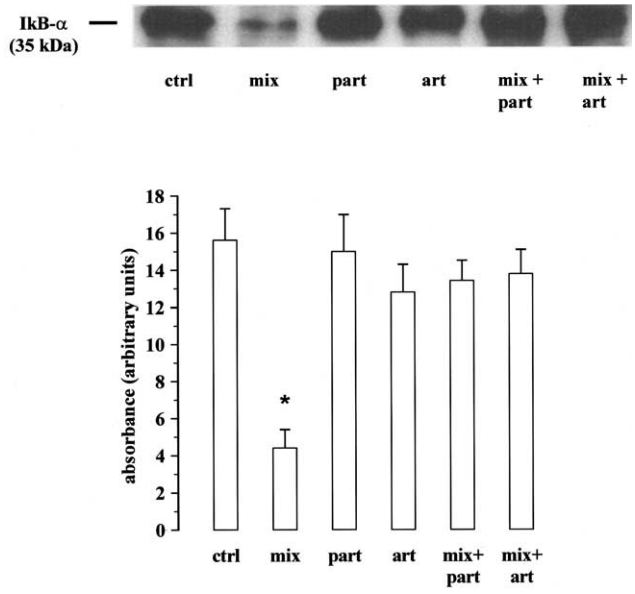

Fig. 5. Western blot detection of IkBα. After a 24-h incubation in the absence (ctrl) or presence of the LPS/cytokines mix (mix: see legend of Fig. 1), 10 μM parthenolide (part) or 10 μM artemisinin (art), alone or in combination with the LPS/cytokines mix, cellular extracts were analyzed by Western blot with an anti-IkB-α antibody (see Section 2 for details). The experiment in the upper panel is representative of three similar experiments. The protein bands have been quantitated densitometrically; the values, expressed as arbitrary units, are represented in the lower panel as means ± S.E.M. ($n = 3$); vs. control: $^{*}P < 0.05$.

primary cultures of rat microglial cells parthenolide inhibited the LPS-elicited NO synthesis and iNOS induction, but it did not prevent the LPS-induced activation of NF-kB and IkBα degradation [18]. The differences in experimental conditions and glial cell strains could account for this discrepancy, but we cannot exclude other mechanisms of action. Anyway, the effects we have observed in this work are not specific of T67 human astrocytoma cells: indeed, we were able to reproduce the same experimental results also in the N11 mouse glial cell line, using LPS as an iNOS inducer (data not shown).

Cerebral malaria has been attributed to vessel occlusion by infected erythrocytes [27] and/or to excessive TNF-α levels, generated by endothelial cells and stimulated macrophages [28]. TNF-α can induce NO overproduction in the central nervous system, which has been related to neuronal dysfunction in several diseases, including malaria [13]. Our data suggest a new mechanism of action for artemisinin: its well-known positive effect on severe malaria could be accounted for not only by its ability to reduce the parasite burden, but also by its capacity to suppress NF-kB-mediated NO synthesis. Furthermore, owing to this property, artemisinin could be studied for its potential role as an anti-inflammatory agent, like parthenolide and other sesquiterpene lactones.

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