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Short communication

Inhibition of nitric oxide-dependent activation of soluble guanylyl cyclase by the antimalarial drug, artemisinin

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Abstract

The influence of artemisinin on the activity of human platelet soluble guanylyl cyclase was investigated. Artemisinin (0.1–100 μM) had no effect on the basal activity of the enzyme. Artemisinin inhibited in a concentration-dependent manner the sodium nitroprusside-induced activation of human platelet guanylyl cyclase with an IC_{50} value 5.6 μM. Artemisinin (10 μM) also inhibited (by 71±4.0%) the activation of the enzyme by the thiol-dependent nitric oxide (NO) donor, the derivative of furoxan, 3,4-dicyano-1,2,5-oxadiazole 2-oxide (10 μM), but did not influence the stimulation of soluble guanylyl cyclase by protoporphyrin 1X. Inhibition of guanylyl cyclase activation by NO donors but not by protoporphyrin 1X represents a possible additional mechanism of the pharmacological action of this drug.

Keywords: Guanylyl cyclase; Nitric oxide (NO); Artemisinin

1. Introduction

Soluble guanylyl cyclase (EC 4.6.1.2) catalyzes the biosynthesis of cyclic 3′,5′-guanosine monophosphate (cGMP) — a potent regulator of various cell processes (Walter, 1984). Soluble guanylyl cyclase contains heme and appears to be one of the most important endogenous receptors of nitric oxide (NO). The interaction of NO with the guanylyl cyclase heme results in the formation of the nitrosyl-heme complex which promotes an increase in enzyme activity (Ignarro, 1992) and cGMP accumulation.

Artemisinin, extracted from plant, the *Artemisia annua*, is a potent antimalarial drug. Its molecule has a sesquiterpenoid structure, which contains an endoperoxide group that is essential for antimalarial activity (Klayman, 1985; Ambroise-Thomas, 1999). Artemisinin is effective against malarial strains resistant to 4-aminoquinoline derivatives (Benoit-Vical et al., 1999). No clinically relevant artemisinin-resistant human malaria has yet been reported (Mechnick, 1998).

The mechanism of pharmacological action of artemisinin involves its interaction with hemin (ferriprotoporphyrin IX) or heme (ferroprotoporphyrin IX). Infection of erythrocytes with *Plasmodium falciparum* is accompanied by the parasite-dependent digestion of hemoglobin. The oxidative polymerization of heme leads to the formation of the malarial pigment, β-hematin (hemozoin) (Goldberg et al., 1990). It is suggested that the interaction of artemisinin with heme inhibits hemozoin production and determines the effectiveness of the antimalarial action of artemisinin (Robert and Meunier, 1997).

Later, it was demonstrated that the antimalarial properties of artemisinin correlate with its binding affinity with heme (Paitayatat et al., 1997), although chemical activation of this sesquiterpenoid may also take place through its interaction with non-heme iron (Wu et al., 1999). Therefore, the antimalarial action of artemisinin is due to the ability of the compound to interact with heme. However, the precise mechanism of the antimalarial action of artemisinin has not yet been elucidated.

It should be noted that a number of compounds of different chemical classes, capable of binding with heme, for example, derivatives of 4-aminoacridine and polyhydroxyxanthone (Ignatushchenko et al., 1997) and methylene

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blue, a well-known phenotiasine derivative (Atamna et al., 1996), also possess antimalarial activity. The antimalarial action of methylene blue was first noted by Paul Ehrlich in the 19th century. Methylene blue inhibits the basal activity of soluble guanylyl cyclase and its activation by NO donors (Gruetter et al., 1981).

The role of soluble guanylyl cyclase in malarial infection still requires detailed investigation. Kawamoto et al. (1990) have shown that cGMP may be involved in the induction of exflagellation (formation of malarial microgametes). Treatment of blood from *P. berghei*-infected mice with cGMP or agents, which increase cGMP levels (e.g. nitroprusside, a potent activator of guanylyl cyclase), enhances exflagellation, whereas *N*-methyl-hydroxylamine (guanylyl cyclase inhibitor) inhibits exflagellation. Inhibitors of cGMP formation stop the development of gametocytes (gametogenesis) (Kawamoto et al., 1993).

In spite of a large number of publications, the role of NO in the pathogenesis and development of malaria is not well understood and different views exist (Farre et al., 1999; Dondorp et al., 1998; Jones et al., 1996; Jacobs et al., 1995; Seguin et al., 1994; Asensio et al., 1993; Rockett et al., 1991). Recently, it was shown that human red blood cells infected by *P. falciparum* synthesize a great amount of NO, apparently due to the induction of a specific isoform of nitric oxide synthase (NOS), different from those found in mammalian cells (Ghigo et al., 1995). In this connection, it is especially interesting that the heme moiety of malaria pigment (beta-hematin) mediates the inhibition of excessive NO production (Taramelli et al., 1995). Possibly, this finding can explain the contradictory data on NO production in malaria and the role of nitric oxide in this disease.

This paper, therefore, investigates the influence of artemisinin on human platelet soluble guanylyl cyclase and its effect on the activation of the enzyme by NO-generating compounds of different chemical classes, namely sodium nitroprusside and a derivative of furoxan-3,4-dicyano-1,2,5-oxadiazole 2-oxide.

2. Materials and methods

Human platelets were used as a source of soluble guanylyl cyclase. Platelets were isolated from the blood of donors as described by Chirkov et al. (1987). A suspension of washed platelets in 50 mM Tris–HCl buffer (pH 7.6) containing 0.2 mM dithiothreitol was sonicated in a MSE 5-78 ultrasonic sonicator (UK) for 20 s at 2 °C and centrifuged at $105\,000\times g$ for 1 h. The supernatant was used as human platelet soluble guanylyl cyclase.

Guanylyl cyclase activity was assayed as described by Garbers and Murad (1979). Briefly, the samples (final volume 150 μl) contained 50 mM Tris–HCl buffer (pH 7.6), 1 mM guanosine triphosphate, 4 mM Mg Cl_2, 4 mM creatine phosphate, 20 μg (120–160 units) creatine phosphokinase, 10 mM theophylline and 20 μg of human platelet $105\,000\times g$ supernatant. The effect of artemisinin was studied over the concentration range 0.1–100 μM. Artemisinin was first preincubated (10 min, at 2 °C) with guanylyl cyclase before the NO donors were added. Because of the poor solubility of artemisinin in the buffer solution, it was initially dissolved in dimethyl sulfoxide (DMSO) with subsequent dilution in 50 mM Tris–HCl buffer (pH 7.6) to the required concentration. Control samples contained the same amount of DMSO.

The amount of cGMP formed (15 min, 37 °C) was estimated by Enzyme-Linked Immuno-Sorbent Assay (ELISA) method using Bioimmunogen kits (Russia). The influence of artemisinin on NO release from sodium nitroprusside was measured by NO_2^- formation by the Griess method (Schmidt and Kelm, 1996) according to Kotz et al. (2000).

To study the influence of artemisinin on the activity of NOS, rat brain was used as a source of the enzyme. A 17% rat brain homogenate in buffer containing 20 mM HEPES, 0.5 mM EDTA and 0.1 mM DTT (pH 7.5) was centrifuged at $11\,000\times g$ for 30 min at 4 °C. The supernatant was used for the determination of NOS activity by a spectrofluorimetric method according to Misko et al. (1993). Briefly, the samples (final volume 125 μl) contained supernatant (250 μg of protein), 20 mM HEPES (pH 7.5), 0.1 mM L-arginine, 0.1 mM NADPH and 0.5 mM $CaCl_2$, with or without 5 μM artemisinin. The samples were incubated for 40 min at 37 °C and the reaction was stopped by addition of 125 μl of cold 20 mM HEPES (pH 7.5). NO synthase activity was determined by measuring the rate of nitrite accumulation in acid medium (pH ~2) with 2,3-diaminonaphthalene. The formation of the fluorescent product (naphthalenetriazole) was measured with a spectrofluorimeter Hitachi F-3000 with excitation and emission wave lengths of 365 and 407 nm, respectively. The amount of NO_2^- formed was calculated using $NaNO_2$ as the standard.

Protein was determined by the method of Bradford (1976). The following reagents were used: guanosine triphosphate sodium salt (Fluka, Switzerland), catalase (Calbiochem, Switzerland), artemisinin, superoxide dismutase and other reagents were from Sigma (USA).

Statistical differences were evaluated using the Student's *t*-test.

3. Results

Artemisinin in concentrations from 0.1 to 100 μM had no influence on the basal activity of human platelet soluble guanylyl cyclase. In these experiments, the basal enzyme activity slightly varied from 93 ± 13 pmol cGMP mg^{-1} min^{-1} without artemisinin to 102 ± 10, 93 ± 8, 102 ± 11 and 103 ± 9 pmol cGMP mg^{-1} min^{-1} in the presence of 1, 10, 50, and 100 μM artemisinin, respectively. Fig. 1 shows that artemisinin in a concentration-dependent manner inhibited sodium nitroprusside-stimulated guanylyl cyclase activity with an IC_{50} value of 5.6 μM. NO release from sodium

nitroprusside (100 μM) was not reduced in the presence of artemisinin (5 μM) (data not shown). Artemisinin (in the final concentration 10 μM) also inhibited activation of the enzyme by another NO donor (Ferioli et al., 1995), a derivative of furoxan, 3,4-dicyano-1,2,5-oxadiazole 2-oxide. Recently, Kotz et al. (2000) demonstrated that 3,4-dicyano-1,2,5-oxadiazole 2-oxide in the final concentration 10 μM in aqueous solution in the presence of 500 μM reduced glutathione generates only NO. Under our experimental conditions, 10 μM artemisinin attenuated the activation of human platelet soluble guanylyl cyclase by 3,4-dicyano-1,2,5-oxadiazole 2-oxide (10 μM, in the presence of 500 μM reduced glutathione) by 71±4%: the enzyme activity with and without 10 μM artemisinin was 313±18 and 1066±85 pmol cGMP mg^{-1} min^{-1}, respectively. The basal guanylyl cyclase activity was 66±5 pmol cGMP mg^{-1} min^{-1}.

The interaction of artemisinin with heme involves the reductive activation of the endoperoxide group of this sesquiterpenoid, leading to the formation of dioxygen-derived radicals (Jefford et al., 1996; Cumming et al., 1997). To elucidate whether oxygen-derived radicals are involved in the inhibitory effect of artemisinin, we have studied the influence of superoxide dismutase and catalase on the inhibition by artemisinin of sodium nitroprusside-induced guanylyl cyclase activation. Table 1 shows that neither superoxide dismutase nor catalase had any influence on the inhibitory effect of artemisinin. The inhibitory effect of artemisinin (10 μM) without and with superoxide dismutase (167 units ml^{-1}) or catalase (127 units ml^{-1}) was 60±3.6%, 58±2.9% and 63±4.0%, respectively (Table 1). Inhibition of NO-dependent guanylyl cyclase activation by artemisinin was also confirmed in a series of independent experiments with protoporphyrin 1X-induced soluble guanylyl cyclase activation.

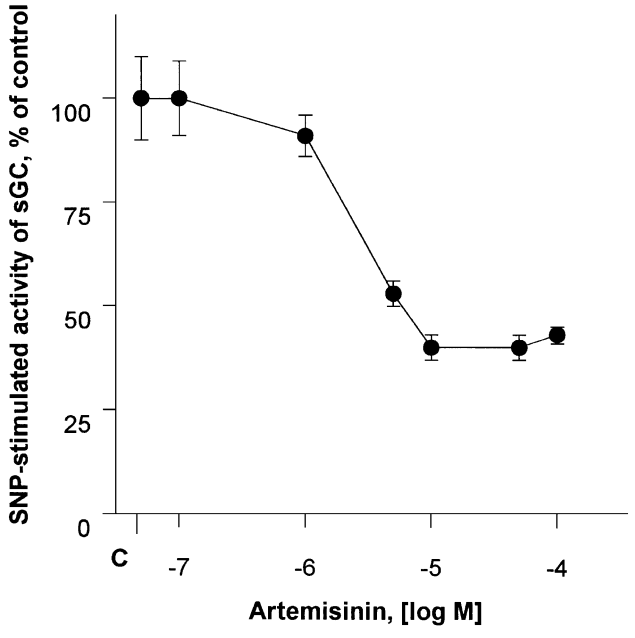

Fig. 1. Effect of artemisinin on sodium nitroprusside-stimulated soluble guanylyl cyclase activity from human platelets. Soluble guanylyl cyclase (sGC) activity was determined in the presence of 100 μM sodium nitroprusside (SNP) and in the absence (con) and presence of artemisinin (0.1–100 μM). Abscissa: artemisinin concentration in the sample (log *M*). Ordinate: sodium nitroprusside-stimulated activity in the absence of artemisinin (con) was taken as 100%. Basal guanylyl cyclase activity was 93±13 pmol cCMP mg^{-1} min^{-1}. Guanylyl cyclase activity in the presence of 100 μM sodium nitroprusside was 1088±110 pmol cCMP mg^{-1} min^{-1}. Data represent means±S.D. of four independent experiments.

Table 1
Effect of superoxide dismutase (SOD) and catalase on the inhibition by artemisinin (10 μM) of sodium nitroprusside (SNP, 100 μM)-stimulated activity of human platelet soluble guanylyl cyclase (sGC)

Compounds and additives	SNP-stimulated activity of sGC (pmol cGMP mg^{-1} min^{-1})
SNP	642±60
SNP+artemisinin	255±15*
SNP+artemisinin +SOD (167 units ml^{-1})	270±14*
SNP+artemisinin +catalase (127 units ml^{-1})	240±17*
SNP+500 μM glutathione	1426±15**
SNP+500 μM glutathione +artemisinin	355±21**
SNP+500 μM glutathione +artemisinin+SOD (167 units ml^{-1})	385±19**

Data represent means±S.D. of three independent experiments.
*$P<0.01$ compared with SNP.
**$P<0.01$ compared with SNP+500 μM glutathione.

Protoporphyrin IX, an immediate heme precursor, is an endogenous stimulator of guanylyl cyclase activity. However, in contrast to NO, which requires the guanylyl cyclase heme for activation, the latter is not involved in the protoporphyrin IX-induced stimulation of the enzyme (Ignarro, 1992). The addition of 10 μM of artemisinin (final concentration) did not influence human platelet guanylyl cyclase activation by protoporphyrin IX (5 μM) (guanylyl cyclase activity with and without artemisinin was 371±30 and 393±20 pmol cGMP mg^{-1} min^{-1}, respectively; the basal guanylyl cyclase activity was 46±7 pmol cGMP mg^{-1} min^{-1}).

Artemisinin had no influence on rat brain NOS activity. There was no significant difference in enzyme activity in the absence and presence of 5 μM artemisinin (11.64±0.81 and 10.52±0.41 pmol NO_2^- mg^{-1} min^{-1}, respectively, $P>0.05$). NOS activity in the presence of 500 μM L-(N^G-nitro) arginine methyl ester (standard inhibitor of the enzyme) was 1.81±0.02 pmol NO_2^- mg^{-1} min^{-1} ($P<0.05$).

4. Discussion

Artemisinin is a drug used for the treatment of malaria. However, the mechanisms of its antimalarial effects are not

completely understood. The data presented here demonstrate for the first time that artemisinin is a rather potent inhibitor of NO-dependent activation of soluble guanylyl cyclase by sodium nitroprusside and 3,4-dicyano-1,2,5-oxadiazole 2-oxide, but it has no influence on protoporphyrin IX-induced enzyme activation.

The ability of artemisinin to inhibit the activation of guanylyl cyclase by NO donors but not by protopophyrin IX suggests the involvement of the guanylyl cyclase heme in this process. It is possible that the action of artemisinin described here is mediated by the interaction of NO with oxygen radicals generated from artemisinin during its reaction with the guanylyl cyclase heme. However, the lack of an effect of superoxide dismutase and catalase on the inhibitory action of artemisinin excluded this possibility (Table 1).

Artemisinin had no influence on rat brain NOS activity. Therefore, the possible interaction of artemisinin with the heme of NOS cannot account for the inhibitory activity of this compound with respect to nitroprusside-induced guanylyl cyclase activation.

The resistance (about 40%) of nitroprusside-induced cGMP production to high artemisinin concentrations (Fig. 1) is possibly due to oxidation (by artemisinin) of guanylyl cyclase heme and the lack of thiols in the samples, which may prevent this oxidation. An increase in the thiol concentration in the samples by addition of 500 μM reduced glutathione led to the enhancement of the inhibitory effects of artemisinin (10 μM) from 60±3.6% to 75±4.5% (Table 1), which may explain (at least partly) the resistance observed.

The molecular mechanism of the artemisinin inhibition is not yet clear and needs further investigation.

The data in the literature on the role of NO in malaria do not allow us to conclude which biochemical mechanism is responsible for the therapeutic action of artemisinin. Nevertheless, it should be noted that the inhibition of NO-dependent activation of soluble guanylyl cyclase by artemisinin occurs within the range of its therapeutic concentrations ranging from 0.2 to 12.8 μM (Schildbach et al., 1990). The IC_{50} value determined in the present study, 5.6 μM, is just within this range. This suggests that the action of the antimalarial drug involve the inhibition of NO-dependent activation of soluble guanylyl cyclase. It is interesting to note that sometimes the development of falciparum malaria is characterized by pathological changes associated with potent systemic and pulmonary vasodilation, with a possible lethal outcome (Charoenpan et al., 1990; Bruneel et al., 1997). This pathological state has much in common with septic shock accompanied by a sharp increase in NO-dependent guanylyl cyclase activation (Hobbs, 1997). The hypotension in septic shock can be eliminated by methylene blue, which increases blood pressure (Driscoll et al., 1996; Brown et al., 1996). The possibility exists that artemisinin may have an analogous action.

Thus, the data on artemisinin inhibition presented here demonstrate for the first time a new biochemical effect of artemisinin. This finding provides an additional mechanism of pharmacological action of this drug and substantiates the necessity of taking into account the influence of antiparasitical preparations on the activity of soluble guanylyl cyclase.

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