Brief Communication

Artesunate mitigates proliferation of tumor cells by alkylating heme-harboring nitric oxide synthase

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ABSTRACT

Artesunate (ART), a semi-synthetic derivative of antimalarial artemisinin, kills cancer cells with uncertain mechanisms. Here, we report for the first time that ART may exert the anti-tumor activity by conjugating the prosthetic heme of hemoproteins in a hepatoma cell line, HepG2, which was evident by monitoring the shift of absorbance from heme (A415) to the ART-heme adduct (A476). Accordingly, a transient elevation of A415 was observed with a synchronous burst of nitric oxide (NO) and a high rate of survival following incubation of HepG2 with 50 μM ART. In contrast, ART at above 100 μM led to an abrogation of NO generation and a decline of the survival rate in HepG2. These data implied that heme-containing nitric oxide synthase (NOS) may represent a major cellular target of ART in killing tumor cells.

1. Experimental procedures

1.1. Cell culture and ART treatment

A cell suspension was prepared by inoculating HepG2 cells into a low-glucose DMEM medium (Gibco) containing 10% fetal bovine serum (TBD), and subsequently cultured at 37 °C in an incubator with 5% CO2. After subculture for several generations, cells were digested by 0.25% trypsin (TBD), collected by centrifugation and resuspended with a fresh medium in a density of 0.1–0.5 × 10^5/ml. The 96-well plate with each well being filled by 200 μl of the diluted cell suspension was placed at 37 °C in an incubator with 5% CO2. After the medium was replaced by a fresh one supplemented with ART, cells were continuously cultured at 37 °C in an incubator with 5% CO2.

1.2. Spectrophotometric monitoring

HepG2 cells from the control group and ART treatment group were collected on 24, 48 and 96 h and lyzed by a freeze–thaw cycle. The absorbance values at 415 and 476 nm were monitored as described [7].

1.3. Determination of the NO level

For plotting a standard curve, 1 M NaNO3 was dissolved into a series of dilutions by the DMEM medium containing 10% fetal bovine serum (TBD). Each dilution was mixed with Griess Reagent I and II in the proportion of 1:1:1 (v/v) for measurement of the absorbance at 540 nm. For quantitative analysis of the NO level, 150 μl of the diluted cell suspension from the control group or ART treatment group was mixed with Griess Reagent I and II for measurement of the absorbance at 540 nm.

1.4. MTT assay

HepG2 cells were plated into a 96-well plate filled with different concentrations of ART. 20 μl aliquots of MTT solution (5 mg/ml) were added to each well. The medium was removed and replaced by 150 μl of 100% DMSO to dissolve the formazan crystal with agitation for 10 min on a shaker.

2. Results and discussion

The cytotoxicity of artemisinin and semi-synthetic analogues such as sodium artesunate (ART) to tumor cells was first found in 1993 [1]. Later then, artemisinin-mediated cytotoxicity was shown to be endoperoxide-dependent [2]. Tumor cells as exposure to artemisinin exhibit diminished proliferation with repressed angiogenesis as the consequence of enhanced oxidative stress-induced apoptosis [3]. Importantly, multi-drug resistant tumor cells are also hypersensitive to artemisinin, suggesting a different mechanism from the commonly used cancer therapeutics [4]. Although the major target of artemisinin towards tumor cells are still under debate [5], an iron-dependent mechanism of artemisinin in anticancerogenesis has been proposed [6]. Recent evidence suggests that artemisinin has a similar mechanism to a heme interacting...
Nitric oxide synthase (NOS) is dimeric, calmodulin-dependent or calmodulin-containing cytochrome P450-like hemoprotein that combines reductase and oxygenase catalytic domains in one dimer [11,12]. A beneficial role of NO in carcinogenesis comes from the finding that NO presents in a higher level in cancerous tissues compared to their normal counterparts [13]. It has been also documented that NO can protect against cellular damage and cytotoxicity from reactive oxygen species (ROS) and organic peroxides [14,15].

In contrast, NO generation from HepG2 cells was nearly abrogated by 100 or 200 μM ART, which might represent a lethal ART level capable of highly inhibiting the NOS activity. This conclusion has been supported by an observation that the cytotoxicity of ART is associated with the inhibition of NOS in RAW 264.7 mouse macrophage cells [16].

Fig. 1. Dynamic fluctuations of $\lambda_{415}$ and $\lambda_{476}$ representing heme and ART $+$ heme by the interaction of ART with hemoproteins. A single asterisk (*) and double asterisks (**) represent significant difference from the control (CK) ($P < 0.05, n = 3$) and very significant difference from the control (0 h) ($P < 0.01, n = 3$), respectively.

Fig. 2. The correlation of HepG2 survival with NO generation upon treatment by ART. (A) The survival percentage of HepG2 cells following incubation with 50, 100, 200 μM ART for 48 h. Double asterisks (**) represent very significant difference from the control (CK) ($P < 0.01, n = 8$). (B) The NO content of HepG2 cells following incubation with 50, 100, 200 μM ART for 48 h. Double asterisks (**) represent very significant difference from the control (CK) ($P < 0.01, n = 3$).
NO was also utilized by bacteria for protection from oxidative damage by transiently inhibiting cysteine reduction and potently suppressing the Fenton reaction [17]. Recently, NO generation from bacteria has been confirmed to confer resistance to antibiotics [18].

In conclusion, ART may exert a potent anti-tumor activity through simply alkylating hemoenzymes and subsequently inhibiting the activity of NOS, thereby mitigating NO generation and abrogating protection from anti-tumorigenesis.

Competing interests

The authors declare that they have no competing interests.

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