

Chapter 2

NO and ART

Abstract NO, mainly synthesized by NOS, is a signal transducer that conveys internal and external stimulations. The isoform of eNOS/nNOS is responsible for stable NO production, while the isoform of iNOS can be induced by proinflammatory cytokines for NO burst. Bacteria also synthesize NO by their own bNOS. ART exerts distinct roles in a dose-dependent manner. Low-dose ART induces and activates eNOS/nNOS, whereas high-dose ART inactivates all isoforms of NOS. ART can also mimic NO to upregulate COX for evoking mitochondrial uncoupling and biogenesis.

Keywords ART • Dose-dependent effects • COX • NO • NOS

2.1 NO and NOS

NO is a gaseous-free radical molecule produced within both prokaryotic and eukaryotic cells. As a messenger involved in the transduction of multifaceted biological signals, NO presents ubiquitously in organisms ranging from bacteria to plants, fungi, and animals including humans. NO is highly reactive, shortly lived, and freely diffusible, which make it possible to convey the signal within an individual cell as well as between adjacent cells. NO can be oxidized to nitrate and further to nitrite in the blood of animals. Under hypoxic conditions, nitrite and nitrate can be reduced back to NO by the multi-types of reduction enzymes. During cooking, unfortunately, the dietary nitrite rich in the pickled meat could react with degraded amino acids to form carcinogenic nitrosamines.

2.1.1 Production of NO in Organism-Dependent Manners

NO exists ubiquitously in all organisms, but it is produced by differential ways. In mammals, fish, birds, invertebrates, and G^+ bacterial, NO is mainly synthesized

from ARG by various NOS (Liu and Gross 1996). Although NO production is also evident in plants, yeast, and G⁻ bacteria, no sequence homologs to mammalian NOS were found in their genomes. In those organisms, it is now convinced that NO is produced alternatively from the reduction of nitrate/nitrite through a type of nitrate/nitrite reductase or other redox partners (Corpas et al. 2004). In *E. coli*, for example, NO is formed from nitrite depending on a nitrite reductase, a NO-sensing regulator, and a flavohemoglobin (Corker and Poole 2003). Also, reduction from nitrite to NO is dependent on cytochrome *c* nitrite reductase alone (van Wonderen et al. 2008). Even in mammals, nitrite reduction by COX can give rise to NO under hypoxic conditions (Castello et al. 2006).

During NOS catalysis, ARG is oxidized to NO and L-citrulline (CIT) via two-step mono-oxygenation reactions. For one mole of NO formation, two moles of O₂ and 1.5 mol of dihydronicotinamide adenine dinucleotide phosphate (NADPH + H⁺) are consumed, and nicotinamide adenine dinucleotide phosphate (NADP⁺) is generated, as shown by the equation: $\text{ARG} + 3/2 \text{NADPH} + \text{H}^+ + 2 \text{O}_2 = \text{CIT} + \text{NO} + 3/2 \text{NADP}^+$. There are other five cofactors involved in the reaction, including flavin adenine dinucleotide (FAD), flavin mono-nucleotide (FMN), tetrahydrobiopterin (BH₄), calmodulin (CaM), and heme. The sequential electron flow is from NADPH + H⁺ to FAD, to FMN, to heme, and to O₂. BH₄ provides an additional electron during a catalytic cycle, which is replaced in the process of turnover.

It is well known that at least three isoforms of NOS including nNOS, eNOS, and iNOS have been identified in mammals (Knowles and Moncada 1994). In humans, nNOS is encoded by *Nos1*, and distributed in the nervous system, skeletal muscles, and plasma membranes; iNOS is encoded by *Nos2*, and distributed in the immune system and cardiovascular system; and eNOS is encoded by *Nos3*, and distributed in the endothelium. The mammalian isoforms can be alternatively divided into two subtypes according to their expression modes and Ca²⁺ dependence: one is the constitutively expressed and Ca²⁺-dependent eNOS and nNOS; another is the proinflammatory cytokine-inducible expressed and Ca²⁺-independent iNOS (Boveris et al. 2010). These subtypes unexceptionally contain four domains in the entity of an entire peptide chain: (1) a variable and tissue specific domain; (2) an oxygenase domain (for heme and ARG binding); (3) a CaM binding domain; and (4) a reductase domain (for FMN, FAD, and NADPH binding).

Production of NO in mitochondria was observed as early as in 1997, but no mitochondrial NOS (mtNOS) has been isolated until 2002, when the purification of an authentic mtNOS from rat liver extracts was proclaimed (Elfering et al. 2002). From which a mitochondrial inner membrane integral protein was identified from a spliced transcript of nNOS. Later, it was detected that the expression level of mtNOS is declined in the rat gastrocnemius upon electroporation of the small interfering RNA (siRNA) of nNOS (Finocchietto et al. 2008). Nevertheless, some authors were unable to detect the expression of mtNOS in rat or mice (Brooks et al. 2003; Tay et al. 2004). As a whole, whether mtNOS really exists remains inconclusive and waits for further findings.

Two structural types of bNOS were characterized in G⁺ bacteria. In the bNOS of *B. anthracis* and *B. subtilis*, only an oxygenase domain that shares sequence

homology with mammalian NOS was confirmed, but no reductase domain identified (Gusarov et al. 2008). Later, an alternative bNOS homolog that contains both oxygenase and reductase was characterized in *Sorangium cellulosum* (Agapie et al. 2009).

2.1.2 NO's Medical Use: From Nitroglycerin to Viagra

NO is considered an effective antianginal drug for alleviating ischemic pain (angina) because it causes vasodilation and decreases cardiac workload. NO-releasing drugs such as nitroglycerin (NG) can lower arterial pressure and left ventricular filling pressure by dilating the veins. NG has been used for over 130 years as a vasodilator for treatment of angina and chronic heart failure. Although NG was known for long time to be converted to NO, the conversion-responsible enzyme mitochondrial aldehyde dehydrogenase has not been identified until 2005 (Chen et al. 2005). Another drug, amyl nitrite, can also release NO in vivo and serve as a NG-like vasodilator. Besides, a sequential reduction of dietary nitrate from nitrate-rich vegetables, such as spinach, arugula, and beetroot, can also provide NO to reduce the blood pressure in prehypertensive persons (Webb et al. 2008).

The endothelium of blood vessels uses NO to direct surrounding smooth muscles relaxation and vasodilation, thereby increasing blood flow. NO was therefore originally known as an “endothelium-derived relaxing factor”. NO activates guanylate cyclase (GC) that catalyzes the formation of cGMP. As a secondary messenger, cGMP can activate protein kinase G that accelerates Ca^{2+} reuptake. Low-level Ca^{2+} ensures that myosin is no longer phosphorylated by myosin light chain kinase, hence halting the cross-bridge cycle and leading to smooth muscle relaxation (Rhoades and Tanner 2003).

Sildenafil citrate, popularly known by its trade name *Viagra*, stimulates penis erection by inhibiting cGMP-specific phosphodiesterase type 5, an enzyme that promotes the degradation of cGMP. It is clear that both NO and *Viagra* can maintain a high level of cGMP even though NO accelerates cGMP production, whereas *Viagra* inhibits cGMP degradation (Webb et al. 1999). Since becoming commercially available in 1998, *Viagra* has been used as a prime drug for improving erectile function in men.

2.1.3 A “Double-Edged Sword”: Physiological and Pathological Views on NO

NO contributes to vessel homeostasis by inhibiting vascular smooth muscle contraction and growth, platelet aggregation, and leukocyte adhesion to the endothelial tissue (Roszer 2012). The constitutively expressed eNOS is a primary NO

generator for controlling vascular tone, insulin secretion, and airway function, in particular, being involving in the regulation of cardiac function and angiogenesis. The constitutive nNOS is engaged in the development of nervous system, and functions as a retrograde neurotransmitter important in long-term potentiality in memory and learning. The environment-activated iNOS produces high-level NO in response to proinflammatory cytokines released upon stimulation by parasite invasion, bacterial infection, and tumor growth (Green et al. 1994). Therefore, NO from eNOS and nNOS mainly plays roles on signaling, while NO from iNOS aids the immune system to kill pathogens and tumor cells. Phagocytes (monocytes, macrophages, and neutrophils) are armed with iNOS, from which high-level NO is lethal to bacteria and intracellular parasites because it damages DNA and degrades iron sulfur centers into iron ions and iron-nitrosyl compounds (Green et al. 1990).

However, persistent NO burst is followed by a sustained chronic inflammation implicated in the metabolic diseases including insulin resistance and type 2 diabetes (Wellen and Hotamisligil 2005; de Luca and Olefsky 2008), cardiovascular disease, autoimmune disease, and neurodegenerative disease (Lin et al. 2009). It is still unknown if NO is a major mediator of chronic inflammation, but we found that the NO donor SNP can induce the articular synovitis in mice (Bao et al. 2012). In contrast, N^G -monomethyl-L-arginine monoacetate (L-NMMA) can otherwise ameliorate the disease by inhibiting iNOS (Wu et al. 2012).

An important biological interaction of NO with proteins is S-nitrosylation, a reversible conversion of the thiol groups of cysteine, which leads to the formation of S-nitrosothiol as the cellular reservoir of NO. S-nitrosylation represents a mechanism for the posttranslational regulation of all major proteins (van Faassen and Vanin 2007). As a product of tyrosine nitration mediated by $ONOO^-$, 3NT was also detected in large numbers of pathological conditions (Mohiuddin et al. 2006; Pacher et al. 2007), and in numerous disease-affected tissues (Buddi et al. 2002). The 3NT-mediated nitrosative stress may participate in the pathogenesis of diabetes and other aging-related disorders (Pacher et al. 2005).

Another important reaction is the alkylation of transition metal ions, which involves the binding of NO to a transition metal ion. The typical case is alkylating the prosthetic heme in hemoproteins, in which NO might behave as either an inhibitor or an activator to affect a homoprotein's function (van Faassen and Vanin 2004).

2.1.4 Conclusions

The free radical NO exists in all organisms but is produced deferentially. In G^+ bacteria and animals, NO is mainly synthesized from ARG. In G^- bacteria, yeast, and plants, NO can be reduced from nitrate/nitrite. NO serves as an endogenous signaling transducer and exerts either physiological or pathological effects. By reversibly binding to GC, NO can enhance cGMP production, but *Viagra* can repress cGMP degradation, both of which are employed for medical use. The

S-nitrosylation of cysteine and 3-nitration of tyrosine are two major types of post-translational modifications of many proteins, which possess huge physiological and pathogenic potentials.

2.2 ART and Derivatives

Artemisia annua L., a Latin nomenclature of Qinghao in Chinese or sweet wormwood in English, is a well-known medicinal herbage containing ART (Qinghaosu in Chinese) (Acton and Klayman 1985). *A. annua* L. is a unique economic source for ART isolation, but ART is only rich in some local varieties, for example, grown in the narrow districts of Southwest China. This ecological distribution of high-yield *A. annua* L. might be determined by biotic/abiotic stress environments (He et al. 2015).

While ART's poor blood solubility limits its effectiveness, several chemical modifications greatly improve its bioavailability. As a start molecule, ART has been modified to a series of soluble and effective derivatives, including artesunate, artemether, arteether, and dihydroartemisinin.

2.2.1 The History of ART Discovery

The herbaceous *A. annua* L. was decocted in ancient China for treating skin illness. The earliest therapeutic use of *A. annua* L. was dated back to 200 BC, which had been recorded in the ancient medicinal book of “*Fifty-two Prescriptions*”. The application of *A. annua* L. in *da-bai-zi*, a fever perhaps from malaria, was first described in “*The Handbook of Prescriptions for Emergencies*” edited in the middle of the 4th century. Since then, a leachate of *A. annua* L. had become a folk antimalarial remedy for thousands of years.

In the late 1960s, a nation-wide cooperative task, “Project 523”, was launched by Chinese government and army in order to look for efficient antimalarial drugs that combat the endemic chloroquine-resistant malarial parasite in Southeast Asia at the request from North Vietnam in the war. Based on the recorded medicinal plants in the ancient literature of traditional Chinese medicine and the thousand-year experience from ancient Chinese practitioners, Chinese scientists pioneered an extensive screening and evaluation toward the discovery of antimalarial candidates (Zhang 2007).

After as many as 5000 medicinal plant preparations were tested using animal malarial models, a research team led by Ms. You-you Tu in the Pharmaceutical Research Institute of China Academy of Traditional Chinese Medicine fortunately isolated and purified ART from *A. annua* L. in 1972. In the original nomenclature, ART was known as arteannuin in English. ART was subsequently identified as a sesquiterpene lactone with an endoperoxide bridge (Liu et al. 1979). Upon

analysis by nuclear magnetic resonance, mass spectroscopy, and X-ray crystal diffraction, ART was eventually characterized with the molecular weight (282.3), chemical formula ($C_{15}H_{22}O_5$), and ventral configuration (O–O) (Li 2007). As a representative scientist principally contributed to the discovery of ART, Ms. Tu was awarded the prestigious Lasker-DeBakey Clinical Medical Research Award in 2011.

Nowadays, ART is becoming a cardinal component in ART-based combination therapies (ACTs) recommended by the World Health Organization (WHO) for combating the chloroquine-resistant malarial parasite frequently occurring in the malarial endemic districts worldwide (WHO 2001). Three kinds of ART derivatives, including artesunate, artemether, and arteether, have been listed in the “*International pharmacopoeia*” (WHO 2003) and “*WHO Model List of Essential Medicines*” (WHO 2005).

2.2.2 Production of ART in Transgenic Plants and Engineered Microbes

Although the biosynthesis of ART is unique in *A. annua* L., its “upstream” biosynthetic pathway is ubiquitous among eukaryotes including microorganisms. Therefore, it is possible to re-establish a “downstream” ART biosynthetic pathway in yeast (Zeng et al. 2008a). In the past decade, ART biosynthetic genes were successfully cloned and introduced into yeast, resulting in the production of ART precursors including artemisinic acid and dihydroartemisinic acid (Zeng et al. 2008b, 2012). However, those ART precursors cannot be automatically converted into ART in yeast due to the lack of an optimized volatile oil phase with the involvement of singlet oxygen (1O_2) (Yang et al. 2008, 2010).

A team led by Jay Keasling at the University of California, Berkeley had engineered yeast to produce artemisinic acid, which can be chemically converted to ART. Using a modified mevalonate pathway in yeast, they expressed the encoding genes of amorphaadiene synthase (ADS) and cytochrome P450 monooxygenase (CYP71AV1) from *A. annua* L. Consequently, ADS leads to amorphadiene production, and CYP71AV1 allows conversion from amorphadiene to artemisinic acid (Ro et al. 2006). We have independently reported the in vitro biotransformation of engineered yeast-derived amorphadiene by cold-acclimated *A. annua* L. cell-free extracts, which gives rise to the considerably elevated ART content up to 0.647 %, accounting for 15-fold increase as *A. annua* L. cell-free extracts without cold acclimation (0.045 %) (Zeng et al. 2012).

Because *A. annua* L. currently remains a sole source for the large-scale commercial production of ART and derivatives, there has been a continuing need for the genetic improvement of *A. annua* L. In 2010, *Nicotiana benthamiana*, an alternative species of tobacco (*N. tabacum*), was engineered to produce artemisinic acid (van Herpen et al. 2010). Chinese scientists have obtained many high-yield

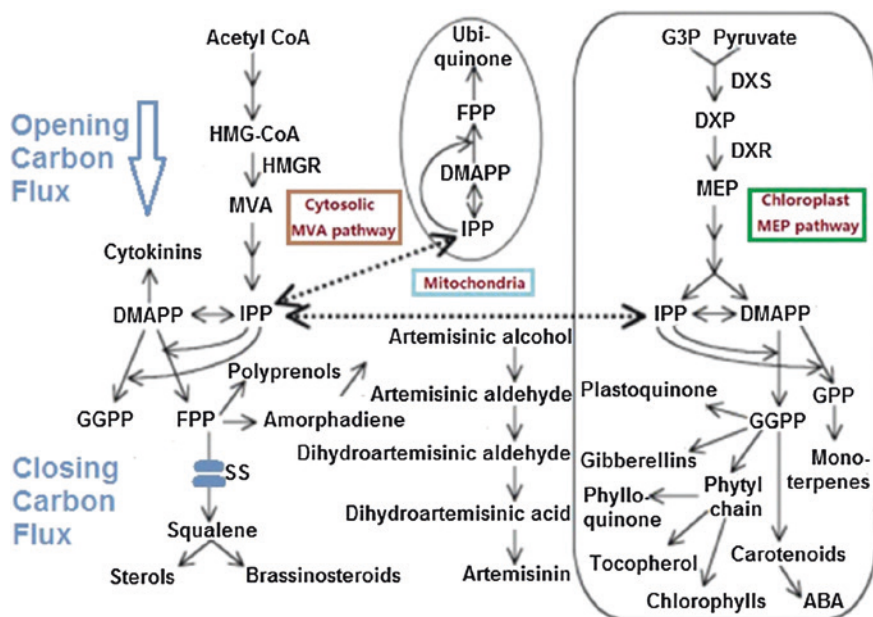


Fig. 2.1 The isoprenoid biosynthesis pathway-based opening/closing carbon flux strategies aiming at ART overproduction in *A. annua* L. ABA abscisic acid; DMAPP dimethylallyl diphosphate; DXP deoxyxylulose 5-phosphate; DXR DXP reductoisomerase; DXS DXP synthase; FPP farnesyl pyrophosphate; GGPP geranylgeranyl pyrophosphate; HMG-CoA 3-hydroxy-3-methylglutaryl coenzyme A; HMGR HMG-CoA reductase; G3P glyceraldehyde 3-phosphate; GPP geranyl pyrophosphate; IPP isopentenyl pyrophosphate; MEP methylerythritol 4-phosphate; MVA mevalonic acid; SS squalene synthase

transgenic *A. annua* L. plants using the integrated genetic engineering strategies of “opening carbon flux” toward ART and “closing carbon flux” forward other products such as squalene (Fig. 2.1).

We introduced an antisense squalene synthase gene (*asSS*) into the genome of *A. annua* L., and observed a well correlation of low-level *SS* mRNA with low-content steroids and high-yield ART. We also obtained enhanced ART production in transgenic *A. annua* L. plants upon cold stress or storage, accounting for 3.7-fold and 5.2-fold increases of ART content, respectively (Feng et al. 2009). These studies should shed light on the exploration of accelerated and sustainable ART supply.

One of the rate-limiting steps of ART biosynthesis was identified at the final nonenzymatic reaction (Zeng et al. 2009). As a *cis* element responsive to oxidative stress, the promoter of *ADS* from *A. annua* L. was found to be inducible by salicylic acid (SA) and methyl jasmonate (MJ). SA/MJ-treated *A. annua* L. exhibits a correlation of the upregulation of *ADS* with the emission of $^1\text{O}_2$, suggesting that SA/MJ induces ART overproduction through invoking $^1\text{O}_2$ burst (Guo et al. 2010; Zeng et al. 2011). Taking together, these results are implicated in simulating a

natural cellular environment to enable an accelerated accumulation of ART. Those achievements should pave a wide path toward a practical solution to currently limited ART supply.

2.2.3 The Pharmaceutical Values and Toxicological Concerns of ART

ART is a potent antimalarial agent that effectively kills chloroquine-resistant *Plasmodium falciparum* and other multidrug resistant malarial parasite strains. Administration of ART would allow the curation of 90 % malarial patients within 48 h although escaped parasites might recur shortly. A best way to prohibit the recurrence of malaria is to combine the rapidly but shortly effective ART with the long half-life antimalarial drug, such as mefloquine, lumefantrine, amodiaquine, piperazine, or pyronaridine (Krudsood et al. 2010).

Except for *P. falciparum*, ART can also kill other helminth parasites, such as *Schistosoma japonicum*, *Clonorchis sinensis*, *Theileria annulatan*, and *Toxoplasma gondii*. The schistosomes of *S. japonicum* cause schistosomiasis, another prevalent parasitic infection as the second to malaria. ART and its derivatives are potent anthelmintics (Xiao 2005).

Since the last decade, on the other hand, an early-phase research wave of ART's anticancer activity has been initiated. We and other authors previously found ART's significant anticancer effects on human hepatoma cell lines (Chen et al. 2000; Hou et al. 2008). Additionally, ART was also observed to exhibit antiarrhythmic activity (Wang et al. 1998) and antihepatitis B virus activity (Romero et al. 2005). Besides, artemisinic acid was shown to have antibacterial activity (Roth and Acton 1989).

In the cytotoxicity assays of ART and derivatives to Ehrlich ascite tumor cells, Woerdenbag et al. (1994) estimated that the half inhibitory concentrations (IC_{50}) of ART, artesunate, artemether, and arteether are 12.2–29.8 μM , the IC_{50} of artemisitene is 6.8 μM , and the IC_{50} of dihydroartemisinin dimmers is 1.4 μM . Later, Zheng et al. (1994) and Jung (1997) determined the cytotoxicity of ART and semi-synthetic analogs on tumor cells, including L-1210, P-388, A-549, HT-29, MCF-7, and KB lines. Beekman et al. (1997, 1998) also investigated the stereochemistry-dependent cytotoxicity of ART and derived analogs.

ART is generally well tolerated at the dose used for malarial patients. The side effects of ART are nausea, vomiting, anorexia, and dizziness. A rare but serious adverse effect is allergic reaction (Taylor and White 2004). The drugs other than ART used in ACTs are believed to contribute to some adverse effects. It was also noted that adverse effects in malarial patients tend to be higher when treated with ART derivatives (Price et al. 1999).

Although the neurotoxicity of ART occurs at high doses in experimental animals, no significant clinic toxicity appears in patients when administered in a therapeutic dosage. A high dose of ART was shown to induce fatal resorption in

animals, but no mutagenic and teratogenic roles of ART were found in malaria-infected pregnant women.

2.2.4 The Plausible Mechanisms of ART's Actions

Currently used ART derivatives are actually prodrugs of dihydroartemisinin, a biologically active metabolite of ART, which is effective when parasites reside inside red blood cells. Although there is no consensus regarding the mechanism by which ART kills the malarial parasite, several lines of evidence have indicated that ART may exert antimalarial effects by perturbing the redox homeostasis in the parasite. After infection into a red blood cell, malarial parasites consume hemoglobin in their digestive vacuoles, a process that generates oxidative stress (Ginsburg and Atamna 1994). The activity of malarial cysteine protease in digestive vacuoles can be inhibited by ART (Pandey et al. 1999), and the membranes of digestive vacuoles can be damaged soon after parasites are exposed to ART (del Pilar Crespo et al. 2008). The digestive vacuole is already re-established by a mid-ring stage of the parasite's blood cycle, a stage sensitive to ART derivatives but not to other antimalarial drugs (Abu Bakar et al. 2010).

A study to investigate the action mode of ART using a yeast model has demonstrated that ART targets mitochondria, in which electron transport chains can activate ART, generate ROS, and depolarize mitochondrial membranes (Li et al. 2005). Subsequent studies have confirmed that ART can attack the mitochondria of parasites but not mammalian cells. Different from atovaquone, ART acting on mitochondria does not inhibit the electron transport during respiration. Therefore, an action specificity of ART derivatives might arise from its own activation (Wang et al. 2010).

It is also thought that when the peroxide lactone of ART comes into contact with a high titer of iron commonly seen in cancerous cells, ART might become unstable and release ROS. Recent pharmacological evidence has demonstrated that dihydroartemisinin targets human metastatic melanoma cells and induces the mitochondrial apoptosis (Cabello et al. 2011).

2.2.5 Conclusions

ART, a sesquiterpene lactone with a unique endoperoxide bridge, is extracted from the medicinal plant species *A. annua* L. Although ART was originally discovered as a malaria-killing drug, it actually exhibits a wide range of therapeutic potentials, especially in antitumor. However, ART's applications are often restricted by its availability and cost. Fortunately, ART biosynthetic genes have been cloned and ART precursors are produced in engineered yeast. The re-established or modified ART biosynthetic pathway in either plants or microbes could eventually lead to the large-scale industrial production of ART and derivatives.

2.3 Interactions of Heme with NO and ART

GC is a major receptor for NO binding, by which the enzyme is activated to catalyze the generation of cGMP, thereby playing a pivotal role in cellular function and metabolic regulation (Hobbs and Stasch 2010). On the other hand, NO behaves as a reversible inhibitor of COX to exert a signaling effect (Boveris et al. 2010). Both GC and COX are hemoproteins, whose prosthetic heme moieties are targeted by NO. In theory, all kinds of hemoproteins can interact with NO through the heme-NO interaction albeit resulting in either activation or inhibition.

Based on their functions, hemoproteins can be classified into four subtypes: (1) O₂ transport: hemoglobin, myoglobin, neuroglobin, cytoglobin, and leghemoglobin; (2) electron transfer: cytochrome *a*, cytochrome *b*, and cytochrome *c*; (3) catalysis: COX, NOS, CAT, POX, cytochrome P450s, ligninase, and tryptophan oxygenase; (4) sensory: GC, FixL (an O₂ sensor), and CoxA (a carbon oxide sensor).

2.3.1 Activation of GC by the NO-Heme Interaction

The hemoprotein GC is a heterodimer composed of one α subunit and one heme-binding β subunit. The mammalian GC contains one heme per heterodimer. NO binding to heme results in the activation of a C-terminal catalytic domain and the production of cGMP. NO leads to at least 200-fold increase of GC activity (Wolin et al. 1982), in which NO displaces the axial histidine (His¹⁰⁵) by direct competition at the proximal side of heme (Lawson et al. 2000). However, the exact mechanism by which NO-mediated changes in the heme coordination resulting in the activation of GC has not yet been established.

Under oxidative stress conditions, GC can be oxidized and lose its heme. A heme-free enzyme no longer responds to NO, but it can still respond to other activators because they can bind to the empty pocket of heme in a similar manner with NO. In addition, GC contains an allosteric site to which some stimulators can bind. These allosteric agents can potentate the NO-GC signaling, thereby allowing even a suboptimal NO level also maximally activating GC.

2.3.2 Inhibition of COX by the NO-Heme Interaction

The hemoprotein COX is a large transmembrane protein complex found in bacteria and mitochondria in eukaryotes. It is a downstream enzyme complex on the respiratory chain. It receives an electron from each of four cytochrome *c*, and transfers them to O₂. COX accepts four protons from the inner aqueous phase,

hence converting one molecule of O_2 to two molecules of H_2O . Additionally, COX translocates four protons across the mitochondrial membrane to establish a cross-membrane electrochemical potential that allows the production of adenosine triphosphate (ATP) via catalysis by the proton-driven ATP synthase (H^+ -ATPase).

In mammals, COX composes 14 protein subunits, in which 7 subunits are encoded by the nuclear genome, and 3 subunits are originated from the mitochondrial genome (Balsa et al. 2012). This complex contains one cytochrome *a*, one cytochrome *a*₃, and two copper centers, *Cu*_A and *Cu*_B (Tsukihara et al. 1995). Cytochrome *a*₃ and *Cu*_B form a binuclear center for O_2 reduction. Cytochrome *c* can be reduced by a preceding component on the respiratory chain, cytochrome *bc*1 complex (complex III). The ferrous cytochrome *c* (Fe^{2+}) passes an electron to the *Cu*_A binuclear center and is oxidized to the ferric cytochrome *c* (Fe^{3+}). An electron from the reduced *Cu*_A binuclear center is first passed to cytochrome *a*, and then to the cytochrome *a*₃-*Cu*_B binuclear center.

NO inhibits COX by excluding O_2 binding in the range of 80–200 nM (Cleeter et al. 1994; Brown and Cooper 1994) although NO is a multisite inhibitor of mitochondrial complexes IV, III, and I. At a lower O_2 tension, NO interacts predominantly with the fully reduced (ferrous/cuprous) center and competes with O_2 . As the O_2 tension is raised, a reaction with the oxidized COX becomes increasingly important. There is no requirement for NO to bind to the singly reduced binuclear center, but it interacts with either the ferrous heme iron or oxidized copper (Mason et al. 2006).

2.3.3 Conjugation of ART with Heme

This was first reported by Meshnick et al. (1991, 1994), who identified the ART-heme adduct by mass spectrometry. The *in vitro* reaction of ART with heme in the presence of red cell membranes was shown to cause the oxidation of protein thiols (Meshnick et al. 1993). ART was also known to alkylate the heme model molecule at α , β , and δ carbon atoms (Cazelles et al. 2001) (Fig. 2.2).

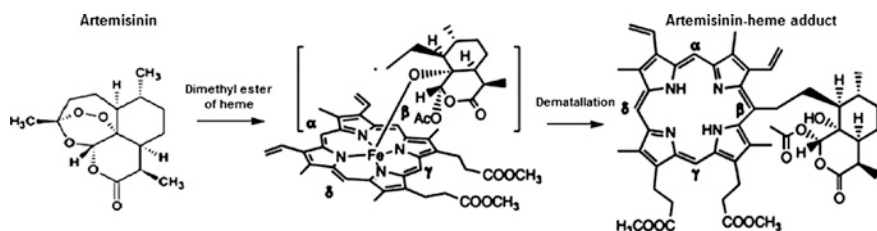


Fig. 2.2 Alkylation of a heme model (dimethyl ester of heme) by a primary carbon-centered radical derived from activated ART. An ART-heme adduct from the β carbon atom is shown, but other adducts are also obtained from α and δ carbon atoms

Studies with totally synthetic trioxolanes and tetraoxanes support the alkylation of heme identified by mass spectrometry (Creek et al. 2008). ART-heme adducts were also detected in the spleen and urine of *Plasmodium vinckei*-infected and ART-treated mice. The hydroxylated and glucuronyl derivatives of ART-heme adducts in the urine were identified by combined liquid chromatography with mass spectrometry (Robert et al. 2005). Trioxaquinines as ART-mimicked hybrids containing an aminoquinoline moiety and a synthetic 1,2,4-trioxane entity were also shown to form heme-drug adducts similar with those detected in the spleen of *Plasmodium*-infected mice (Bousejra-El et al. 2008).

Above results have suggested that the interference of ART with the accumulation of hematin might represent a plausible mechanism underlying ART killing malarial parasites. In the studies with infected mice, detection of heme-drug adducts (as glucuronyl-conjugated derivatives) indicated that ART-mediated alkylation might be a key factor that exerts antimalarial activity in vivo. Because the progression of malarial infection ultimately leads to hematin deposition in the liver and spleen of infected mice, it is of course unable to rule out a possibility of the heme-drug adducts being derived from the interactions of parasites with mouse organs (O'Neill et al. 2010).

2.3.4 Conclusions

As a common signaling molecule, NO conveys external stimuli mainly by activating the hemoprotein GC through the NO-heme interaction. Alternatively, NO can also competitively bind to mitochondrial COX to cause metabolic hypoxia. ART can alkylate hemoproteins by covalently conjugating their heme moieties and serve as a functional mimetic of persistently bound NO. An effect of the ART-heme interaction can be simply investigated by monitoring the formation of ART-heme adducts and synchronously detecting the activity change of a specific hemoprotein. The alkylation of hemoproteins by ART has deep implications in the modulation of protein function and gene expression.

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