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Qing-Ping Zeng

Artemisinin and Nitric Oxide

Mechanisms and Implications in Disease and Health

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Preface

Nitric oxide (NO), a physiologically important signaling molecule, was proclaimed "a molecule of the year" in 1992 by the *Science* magazine. The Nobel Prize in Physiology or Medicine in 1998 was awarded for the elucidation of NO's physiological roles. Artemisinin (ART), an effective antimalarial phytochemical monomer, is closely relevant to NO by inhibiting NO synthase (NOS). The discovery of ART led to the winning of the Lasker-DeBakey Clinical Medical Research Award in 2011.

This book introduces the dichotomies of beneficial and harmful effects of NO on human health and diseases in a dose-dependent manner, and also discusses the potential and multifaceted uses of ART in disease interventions and health maintenance. I not only explain why a steady-state relatively low NO level should be kept during development and aging, but also tell how to avoid an extremely high NO level triggered by inflammation. In general, an optimal NO level protects your health, but an extreme NO level hurts your body. So NO is often said to be a "double-edged sword"!

Based on my proposed "a uniform NO threshold theory in disease and health", I intend to reveal a millennium-long mystery of longevity, and also dedicate to decipher the puzzling secrets of aging and aging-associated diseases although they remain largely unknown. Except for the long-lived genetic background, what is the "fountain of youth"? I would say it is NO! Where is the "fountain of youth"? I would say it is hidden in mitochondria! What is the etiological cause of metabolic diseases, cardiovascular diseases, autoimmune diseases, neurodegenerative diseases, and cancer? The answer might be, among others, low-grade inflammation! Where is the origin of low-grade inflammation? The answer might be, at least, derived from the bacterial endotoxin lipopolysaccharide (LPS)!

NO is mainly biosynthesized by three isoforms of NOS in animals and human. A physiological low level of NO that would extend your lifespan is derived from endothelial NOS (eNOS) and neuronal NOS (nNOS), but the pathological high level of NO that might worsen your health is originated from proinflammatory cytokine inducible NOS (iNOS), although it is also responsible for killing the invaded pathogens. A person addicting high-fat diets (HFD) has a raised risk of

developing insulin resistance, type II diabetes, fatty liver, and even liver cancer because his/her iNOS is always activated by low-grade inflammation. In contrast, a person obeying calorie restriction (CR) has a great probability of becoming a centenarian because his/her eNOS and nNOS are higher enough to activate an antioxidative network for scavenging reactive oxygen species (ROS). So iNOS and eNOS/nNOS represent the pivotal targets for improvement of human health!

Why people become obese remains obscure, but obese or lean is most likely modulated by an interaction of nutrients with gut microbiota. A fat-rich food can nurse "meat-addicted microbes" and impede the growth of "fiber-degraded microbes", thereby leading to adipose depots and overweight/obesity. Concisely, while obesity is switched by turning on iNOS for adipogenesis, weight loss is switched by turning on eNOS for adipolysis. Whether obesity is a disease is currently a debating issue. In my opinion, obese persons without chronic inflammation should be healthy and exhibit insulin sensitivity, whereas obese persons with chronic inflammation should be unhealthy and show insulin tolerance and even develop type II diabetes. In an obese person with gastrointestinal dysbiosis due to an overgrowth of Gram-negative bacteria, especially gut mucin-degrading bacteria, LPS within the gut would leak into the bloodstream and trigger a systemic inflammatory response, hence inducing inflammatory diseases. So it could be said that gut bacterial dysbiosis cause LPS leakage, LPS leads to inflammation, and inflammation results in metabolic diseases.

This book was written for medical researchers and students, clinicians, biologists, and other people interested in NO-involved biology and medicine. Mainly based on our own analytic data and the most cited references on ART, NO, and heme-containing proteins, this seven-chapter book has attempted to reconcile the interaction of ART with cytosolic NOS and mitochondrial cytochrome *c* oxidase (COX), and also discuss the current achievements of ART applying in the cutting-edge innovation for antitumor, antibacterial infection, anti-inflammation, and antiaging. As prospects, iNOS and eNOS/nNOS deem the vital targets in drug discovery for disease interventions.

At the very end, I have to say that ART is really an elixir!

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Contents

Abbreviations

3NT	3-Nitrotyrosine
AMP	Ampicillin
AMPK	Adenosine monophosphate-activated protein kinase
ARG	L-arginine
ASC	Artemisinin-sensitizing compound
AT	Aminotriazole
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BIA	Bacteria-induced arthritis
BLA	Betulilic acid
BMI	Body mass index
CII	Collagen type II
CAT	Catalase
CEF	Cefotaxime
CFA	Complete Freund's adjuvant
CIA	Collagen-induced arthritis
CIAA	Complete Freund's adjuvant-induced acute arthritis
CICA	Collagen type II-complete Freund's adjuvant-induced chronic arthritis
CO	Carbon monoxide
COX	Cytochrome *c* oxidase
CR	Calorie restriction
CSC	Cancer stem cell
DM	Diethyl maleate
DNMT	DNA methyltransferase
DNP	2,4-dinitrophenol
DTT	Dithiothreitol
eNOS	Endothelial nitric oxide synthase
EPO	Erythropoietin
G^-	Gram-negative bacteria

G^+	Gram-positive bacteria
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GC	Gualylate cyclase
GLUT	Glucose transporter
GSH	Glutathione (reduced)
HFD	High-fat diet
HIF-1α	Hypoxia inducible factor alpha
HO-1	Heme oxygenase 1
IGF	Insulin-like growth factor
IL	Interleukin
iNOS	Inducible nitric oxide synthase
KEGG	Kyoto Encyclopedia of Genes and Genomes
LA	Lactic acid
LFD	Low-fat diet
LIAA	Lipopolysaccharide-induced acute arthritis
L-NMMA	N^G-monomethyl-L-arginine monoacetate
LPS	Lipopolysaccharide
MA	Mercaptosuccinic acid
ME	Mitochondrial enhancement
mtNOS	Mitochondrial nitric oxide synthase
mTOR	Mammalian target of rapamycin
NAD^+	Oxidized nicotinamide adenine dinucleotide
$NADH+H^+$	Reduced nicotinamide adenine dinucleotide
$NADP^+$	Oxidized nicotinamide adenine dinucleotide phosphate
$NADPH+H^+$	Reduced nicotinamide adenine dinucleotide phosphate
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NG	Nitroglycerine
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NT	Nitroglycerin
PGC-1α	Peroxisome proliferator-activated receptor γ coactivator 1 alpha
PME	Post-mitochondrial enhancement
POX	Peroxidase
RA	Rheumatoid arthritis
RAP	Rapamycin
RIF	Rifampicin
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SCFA	Short-chain fatty acid
SIRT1	Sirtuin 1
SNP	Sodium nitroprusside

SOD	Superoxide dismutase
SpO_2	Saturation percentages of O_2
TERT	Telomerase reverse transcriptase
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
TRF	Telomere restriction fragment
VAT	Visceral adipose tissue
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue

Chapter 1
Background

Abstract ART has been found, for the first time, to alkylate the heme-containing enzymes (hemoenzymes) by covalently conjugating the prosthetic heme. A high dose of ART can kill cancer cells and bacteria through compromising protective NO production. A low dose of ART can mimic CR to extend lifespan and reduce weight by triggering mitochondrial biogenesis. Therefore, ART can exert versatile beneficial effects on human health in addition to antimalaria.

Keywords ART · Hemoenzymes · NO · Target-guided effects

ART (Qinghaosu in Chinese) is an endoperoxide lactone naturally occurring in the medicinal herbal plant *Artemisia annua* L. (Qinghao in Chinese). A Chinese pharmacologist, Ms. You-You Tu, won the Lasker-DeBakey Clinical Medical Research Award in 2011 for her pioneering and excellent contributions to the discovery of ART's antimalarial effects. Although originally developed as an antimalarial drug, ART actually possesses many other therapeutic potentials on human diseases.

As a star molecule, NO was proclaimed "a molecule of the year" in 1992. With the discovery of NO's pleiotropic functions, Mrs. Louis J. Ignarro, Robert F. Furchgott, and Ferid Murad won the 1998 Nobel Prize in Physiology or Medicine. The physiology and pathology of NO are now the fundamentals and hot-spot topics in many biological and medical fields, including neurology, physiology, and immunology.

What is the relevance of ART to NO in health and disease? How to associate ART with NO functionally? These questions reminded me to retrospect our innovative and interesting findings during the past 5 years. In 2010, we found for the first time that ART inhibits the heme-containing protein (hemoprotein) NOS by covalently conjugating the prosthetic heme in tumor cells (Zeng and Zhang 2011) and in bacteria (Zeng et al. 2011). Afterwards, we revealed that ART is able to ameliorate and even abort articular synovitis, an early phase of rheumatoid arthritis (RA), in mice by abrogating NO production (Bao et al. 2012; Wu et al. 2012).

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In recent years, we revealed that ART can mimic CR to extend yeast lifespan and compromise mouse telomere shortening by targeting the mitochondrial respiratory chains. Although NOS is absent in yeast, an alternative hemoprotein COX in mitochondria was observed to produce NO (Wang and Zeng 2014). In a similar pattern with NO, ART enables transient mitochondrial dysfunction through binding to COX, and subsequently plays a longevity-promoting role in yeast (Wang et al. 2015a). Like NO, ART can compromise telomere shortening by targeting COX and evoking antioxidative responses in mouse skeletal muscle cells. Interestingly, the NO donor sodium nitroprusside (SNP), the NO precursor L-arginine (ARG), and the oxidant H_2O_2, in certain concentrations, can also exhibit the CR-like effect in telomere maintenance (Wang et al. 2015b).

It would be considered a huge breakthrough to elucidate the conjugation of ART with COX functionally resembling the interaction of NO with COX. The NO-COX interaction during CR is a prerequisite to trigger antioxidative responses, protect DNA from oxidative damage, compromise telomere shortening, and delay progressive aging. In such a context, the NO-COX interaction is eligible as a promising target for antiaging drug discovery, from which the functional mimetics of CR are expected to emerge or to be developed. Being equally important, validation of ART conjugation to NOS might help to decipher why ART is effective in dealing with so many inflammatory diseases. ART can prevent inflammation-triggered NO burst through inhibiting NOS, by which ART attenuates hypoxia, angiogenesis, hyperplasia, and inflammatory infiltration in the synovial tissue. Therefore, a priority of coping with autoimmune diseases should be better by eradicating infectious pathogens rather than inhibiting inflammation per se.

The discovery of interactions between ART and COX/NOS should pave a path toward penetration into unknown mechanisms involved in the NO's beneficial or harmful effects. From this clue, aging and aging-associated diseases would be no longer tractable and irreversible. It remains, however, an open question regarding whether the powerful oxidant peroxynitrite ($ONOO^-$) that is derived from NO and superoxide anion (O_2^-) would cause carcinogenesis, and it is also unclear if $ONOO^-$ is an initiator of the neurodegenerative diseases via putative protein nitration. The future achievements in this exciting research area should increase our knowledge about aging-related processes, which might eventually facilitate a clinical solution to malignant tumors/cancer, insulin-resistant diabetes, cardiovascular diseases, autoimmune diseases, and neurodegenerative diseases.

1.1 A Brief Story About Discovery on the Pleiotropic Use of ART

Although the mechanism by which ART kills the malarial parasite remains inconclusive, it is gradually evident that ART can preferentially conjugate the heme moiety of hemoproteins among multiple malarial targets. The covalent conjugation

of ART to heme, a process known as heme alkylation, was first observed by Meshnick et al. (1991), who identified the ART-heme adduct by mass spectroscopy. Later, Cazelles et al. (2001) also discovered that ART is capable of alkylating a model heme molecule at α, β, and δ carbon atoms. In mice with malarial infection, Robert et al. (2005) characterized the ART-heme adduct in the spleen and glucuro-conjugated ART-heme derivatives in the urine of ART-treated mice.

After a detailed investigation in tumor cells, on the other hand, Zhang and Gerhard (2009) found that the acceleration of cellular heme biosynthesis increases ART's cytotoxicity, whereas the suppression of endogenous heme biosynthesis decreases ART's cytotoxic activity, underscoring that the heme entity serves as an intracellular enhancer of ART attacking tumor cells. Afterwards, Zhang et al. (2010) observed that heme biosynthesis increases the cytotoxicity of ART-induced free radicals, which are effectively suppressed by O_2^- scavengers. So these results implied that O_2^- is most likely a cytotoxic mediator of ART.

Heme is commonly recognized in presence as a functional constituent of iron-containing metalloproteins including hemoproteins, such as NOS, COX, catalase (CAT), peroxidase (POX), hemoglobin, and myoglobin, etc. In theory, ART can alkylate all hemoproteins and interfere with their functions without selectivity. However, whether ART targets any specific hemoproteins and affects their functions has not been reported until 2011. More strictfully, no individual hemoprotein that interacts with ART and alters its activity was identified before that time. Therefore, no logical cues indicate ART being a NOS inhibitor.

In the first half of 2010, our eyes were absorbed on a published report in *Science*, in which the authors wrote: "because bacterial pathogens use NOS protects them against antibiotics and immune attack, inhibition of NOS could serve as an effective antibacterial intervention" (Gusarov et al. 2009). This startling expectation on a novel antibacterial strategy encouraged us to rationally look for and find out NOS inhibitors as antibiotic synergists. From the previous knowledge of ART reactivity with hemozoin, a pigmented metabolite of hemoglobin in the malarial parasite, we boldly assumed that ART may inhibit NOS by binding to its heme, thereby blocking the inter-conversion between ferrous ion (Fe^{2+}) and ferric ion (Fe^{3+}).

To testify the assumption, we immediately conducted a series of confirming experiments to reveal whether a synergetic effect of ART exists as in combination with common used antibiotics. As our expectation, ART substantially potentates the antibacterial capacity of rifampicin (RIF) in the Gram-positive (G^+) bacteria *Bacilus licheniformis* and ampicillin (AMP) in the G^- bacteria *Escherichia coli*. Surprisingly, we found that ART synchronously inhibits NOS and CAT in *B. licheniformis*. On combined treatment of bacteria by ART with either RIF or AMP, we observed a correlation of suppressed bacterial proliferation with mitigated NO production along with enhanced H_2O_2 burst. So we concluded that ART as a synergist of antibiotics enables the most effective curation of bacterial infections upon inhibiting NOS and CAT, which declines the protective NO level and elevates the lethal H_2O_2 level (Zeng et al. 2011).

Considering the evolutionary conservation of NO signaling among organisms, we assumed that NO may also protect tumor cells from antitumor drugs. Indeed, we observed that ART exerts its coordinated antitumor activity by covalently conjugating NOS in the hepatoma cell line $HepG_2$. We also noticed that the transient heme biosynthesis is synchronous to potent NO burst and correlates with a higher survival rate following incubating $HepG_2$ with 50 μM ART. In contrast, ART at above 100 μM leads to the sharp decline of NO levels and remarkable decrease of survival rates of cultured $HepG_2$ cells. It can be concluded, therefore, that ART plays a beneficial or harmful role to tumor cells in a concentration-dependent pattern. A high level of NO that is induced by 100 μM ART is toxic to tumor cells, whereas a lower level of NO that is induced by 50 μM ART protects tumor cells (Zeng and Zhang 2011).

In 2012, we further discovered that the overload of bacteria, even with nonpathogenic *E. coli*, in mouse gastrointestinal tracts by live bacterial feeding induces inflammatory synovial lesions. The daily feeding of *E. coli* upregulates proinflammatory cytokines, elicits NO burst, drives hypoxic responses, and stimulates angiogenesis and hyperplasia, suggesting that a sustained infection might be, in part, responsible for the onset of synovitis and eventual development to RA (Bao et al. 2012). In mice with bacteria-induced synovitis, anti-inflammation by ART and rapamycin (RAP) can abrogate NO production, mitigate hypoxia induction, and considerably ameliorate or even completely abort synovitis, hence highlighting that NO may serve as an initiator of inflammatory arthritis (Bao et al. 2012; Wu et al. 2012). In 2013, we further observed that both endogenous and exogenous NO enable the tumor-like neoplasia in hypodermal and synovial tissues, which can be blocked by ART and betulilic acid (BLA) (Gao et al. 2015).

In the last year, we validated the covalent conjugation of ART to heme and the synchronous induction of COX in yeast, assuming the inhibition of COX activity and upregulation of its expression. Interestingly, we found with surprise that a trace amount of ART can mimic CR to extend yeast lifespan through an interaction of ART with COX similar with NOS, which initiates a cascade response of metabolic conversions from biosynthesis to degradation (Wang et al. 2015a). It has been documented that COX can be reversibly inhibited by NO via a competitive heme binding, which leads to the so-called metabolic hypoxia, i.e., COX cannot accept O_2 even there is enough O_2 (Xu et al. 2005). An essential consequence of the metabolic hypoxia is mitochondrial uncoupling without energy production albeit electron transport maintaining (Cerqueira et al. 2011).

We also investigated the effect of ART on telomere lengths and expression profiles by CR, NO, and H_2O_2. Intriguingly, all of those treatments downregulate the ubiquitylation pathway genes including tumor suppressor genes and other DNA repair genes, but do not upregulate telomerase reverse transcriptase gene (*Tert*), suggesting that an alleviation of chromosomal DNA damage may predispose the compromise of telomere shortening rather than the elongation of telomeres (Wang et al. 2015b).

Recently, we found that ART, 2,4-dinitrophenol (DNP), and nitroglycerin (NG) exert weight-reducing effects in inflammatory obese mice induced by HFD + LPS. All the examined drug monomers allow a substantial decline of

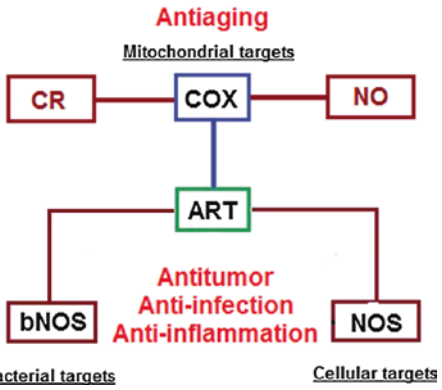

Fig. 1.1 The multifaceted beneficial effects exerted by ART through specifically targeting a variety of hemoproteins. *ART* artemisinin; *bNOS* bacterial nitric oxide synthase; *COX* cytochrome *c* oxidase; *CR* calorie restriction; *NO* nitric oxide; *NOS* nitric oxide synthase

whole body weight, and lead to the recovery of a raised serum insulin level to the normal one seen in the control. The preliminary analysis on obesity versus weight loss indicated that the drugs exert weight-reducing roles accompanied with anti-inflammation and/or antihypoxia. More importantly, we found for the first time that a low dose of LPS rather than a high dose of LPS may be a trigger of the systemic, chronic, and low-grade inflammation (Gao et al. unpublished).

In summary, ART exhibits the multifaceted effects on health and disease through acting on different targets, i.e., hemoproteins. ART can inhibit bacterial NOS (bNOS) and cellular NOS to suppress NO production, thereby mitigating NO's protective role from antibiotics/chemotherapeutics, and exerting anti-infection and antitumor effects. ART can also inhibit CAT in bacteria to enhance H_2O_2 production, which potentates antibiotics' bactericidal ability. Accordingly, ART possesses an anti-inflammatory effect by decreasing a competitive binding of NO to hemoglobin/myoglobin and repressing the metabolic hypoxia. Furthermore, ART is able to mimic CR and NO for alkylating mitochondrial COX and initiating mitochondrial uncoupling/biogenesis, leading to compromised telomere shortening and extended lifespan (Fig. 1.1).

Practically, if you always keep a steady-state NO level derived from eNOS/nNOS by CR or via supplementation with edible NO, you should have a lower chance to have inflammatory diseases. Similarly, if you use ART to inhibit bNOS or iNOS, you should live healthier because of enabling antitumor, anti-infection, and anti-inflammation. Of course, ART is unable to distinguish eNOS/nNOS from iNOS, so the best way to amplify the beneficial role of ART is to inhibit inflammation at first, and then use trace-amount ART.

1.2 A Uniform NO Threshold Theory in Disease and Health

Aging and longevity are constantly the interesting topics in exploring life secrets since ancient civilization. Even through extensive investigations, it remains mysterious how aging is switched and whether longevity can come true. Nevertheless, it

is widely accepted that lifespan is determined by an individual genetic background and multiple environmental factors. Regardless of genetic determinations, it is believed that the natural aging process may be modulated to some extent through artificial interventions such as CR.

For an environmental impact on lifespan, Harman first proposed a free radical theory of aging early in the past century (Harman 1956), and later it was extended to be implicated in the structural and functional adaptation of mitochondria to nutritional stress stimuli (Harman 1972). According to Harman's aging theory, free radicals are supposed to be responsible for biomolecule damage, which results in cellular changes and thus organismal aging. One of the main criticisms to his aging theory is that it only considers the harmful effects of free radicals, but ignores their beneficial roles (Afanas'ev 2010). Nowadays, it is known that ROS in a sublethal concentration have potentials to induce the antioxidative response and provide protection from further oxidative stress. This phenomenon has been called as "hormesis", through which an intrinsic protective potential can be excited by a traceamount of toxic substance (Kaser 2003).

It has been gradually accepted that ROS are "double-edged swords" for living cells. Whether ROS are harmful or beneficial depends on their relative concentrations. In an extremely high concentration, ROS directly cause cell death, whereas in a relatively low concentration, they allow cell survival. So there must have a concentration threshold for ROS exerting a "good" or "bad" effect. Such a concentration-dependent ROS threshold is apparently governed by the homeostasis of cellular oxidation and antioxidation. When the attacking power from oxidants overwhelms the combating capacity by antioxidants, damage to cells and macromolecules must be ensued (Thannickal et al. 2000).

Except for ROS, free radicals also include reactive nitrogen species (RNS), mainly NO, $ONOO^-$, nitric dioxide (NO_2), and trinitric dioxide (N_2O_3). In general, a lower level of NO promotes cell survival and proliferation, while a higher level of NO favors cell cycle arrest, apoptosis, and senescence (Thomas et al. 2004). Because NO can react with O_2^- to produce $ONOO^-$, it means that more ROS predispose more RNS in the case of enhanced NO production. As an essential result, $ONOO^-$ must pose oxidative stress-like nitrosative stress to multiple systems and exert pathogenic effects on subjected organs (Patcher et al. 2007). NO has been shown to increase the mitochondrial levels of O_2^- and H_2O_2 (Poderoso et al. 1996). We also found that NO and H_2O_2 can induce superoxide dismutase (SOD) and catalase (CAT) in yeast (Wang et al. 2015a) as well as in mouse skeletal muscle cells (Wang et al. 2015b).

Mitochondria are major subcellular compartments that generate ROS, which are originated from NO binding to COX. As previously noted, ROS within cells can be modulated by NO-mediated mitochondrial biogenesis (Nisoli and Carruba 2006). NO can thus affect the fate of living cells in either a ROS-independent or dependent manner. Although an extremely high level of NO plays a pathogenic or even lethal role, an appropriate level of NO exerts a beneficial and healthy effect. Such a consideration inspired us suggesting a threshold theory of NO-mediated disease and health effects.

This theory defines why NO has dual impacts on cells and organisms. An optimal level of NO that triggers tolerable doses of ROS can induce antioxidative responses, provide cellular protection, and exert healthy effects. In contrast, when endogenous NO levels are too high (after infection) or too low (due to aging), an abnormal or even pathogenic status may occur and maintain. While high-level NO causes an insufficiency of O_2 supply, low-level NO loses a vasodilating function in blood vessels. NO can also react with O_2^- to generate $ONOO^-$, which would lead to a harmful role to cells through nitrosylating/nitrating the target proteins.

The major annotations on our suggested "a threshold theory of NO-mediated disease and health effects" are summarized as follows:

- **A physiological NO level is distinguished from a pathological NO level**. While NO is beneficial to cells at a low level, or at a physiological level, it is harmful to cells at a high level, or at a pathological level. What is the threshold of NO distinguishing a physiological level from a pathological level? According to previous analytic data provided by other authors, a sustained NO concentration of 10–30 nM allows the phosphorylation of extracellular signal-regulated kinases mediated by cyclic guanosine monophosphate (cGMP), while 30–60 nM NO leads to the phosphorylation of protein kinase B (Thomas et al. 2004). A higher NO concentration reaching 100 nM results in the stabilization of hypoxia inducible factor 1 alpha (HIF-1α) (Thomas et al. 2004). At the concentration of 400 nM, NO enables the phosphorylation and acetylation of the tumor suppressor p53 (Ridnour et al. 2004). Higher levels of NO from 800 nM to 1 μM can cause the nitration of polyadenosine diphosphate-ribose polymerase (PARP), which confers the inhibition of mitochondrial respiration (Borutaite and Brown 2006).
- **NO levels are determined by distinct NOS isoforms and dependent on external stimuli**. While low-level NO (<200 nM) from eNOS or nNOS functions physiologically, high-level NO (>400 nM) from iNOS behaves pathologically (Thomas et al. 2010). Because proinflammatory cytokines are activated by pathogenic infection and immunization, it is implied that a physiological NO level appears under a normal condition, while a pathological NO level occurs in an aberrant state. In theory, NO-mediated pathogenesis can be avoided via anti-infection and/or anti-inflammation. Aging organisms may be subjected to insufficient NO supply due to decreased NO production, especially in the exceptional mitochondrial dysfunction. It was found that NOS activity within mitochondria decreases to as low as 45–75 % in aged mouse brain and hippocampus (Navarro et al. 2008).
- **Low-level NO exerts longevity-promoting effects through triggering oxidative burst and eliciting antioxidative responses**. The competitive binding of NO to COX precludes O_2 binding, thereby leading to mitochondrial dysfunction and respiratory uncoupling (Boveris et al. 2010). Due to the mitochondrial NO-COX interaction, respiratory electron transport is forced to deliver electrons to O_2. Consequently, O_2^- is generated, which activates SOD to produce H_2O_2. Subsequently, H_2O_2 can further induce H_2O_2-degrading enzymes, such as CAT and

POX. An appropriate level of NO, therefore, allows the induction of antioxidant enzymes for ROS scavenging, which enables the mitigation of chromosomal DNA damage and compromise of telomere shortening. It is well known that the length of telomeres is correlated with the lifespan of organisms from yeast to mammals (Vera et al. 2013). Therefore, longer telomeres underlies extended lifespan.

- **High-level NO behaves as an etiological initiator of tumor-like pathogenesis**. The inhibitory effect of high-level NO on O_2 binding to hemoglobin within red blood cells interprets a preanoxic state occurring due to restricted O_2 supply to blood vessel-dispersed tissues. In the case of low O_2 and long distance from the capillary, the inhibition of mitochondrial and cellular O_2 uptake allows O_2 to further diffuse away in the tissue (Poderoso et al. 1996). NO decreases the steepness of the O_2 gradient in the preanoxic border, in which O_2 becomes a rate-limiting factor for O_2 uptake by a low O_2/NO ratio, gradually decreases O_2 uptake, and leaves O_2 to reach cells that would be anoxic (Poderoso et al. 1996). The computational model established by Thomas et al. (2001) has shown that a reversible inhibition of cellular O_2 uptake by NO substantially extends the zone of adequate tissue oxygenation away from the blood vessel. However, when NO is overproduced due to pathogenic infection and immune activation, a tumor-like angiogenesis and hyperplasia may take place in the synovial tissues of joints. The thickened pannus with massive vasculatures and infiltrated lymphocytes was observed microscopically upon the histochemical staining of synovial tissues (Bao et al. 2012; Wu et al. 2012).
- **Interaction of NO with O_2^- promotes the nitrosylation/nitration and inactivation of functional proteins**. As the most potent oxidative reagents, RNS may injure a wide array of biomolecules, including DNA and proteins, as well as biomembranes. At a high level, NO readily reacts with O_2^- to give rise to $ONOO^-$. It is confirmed that $ONOO^-$ is responsible for the modification of proteins by the *S*-nitrosylation of cysteine, 3-nitration of tyrosine, or other nitrosative modifications. Our experimental data disclosed a correlation of high-level NO with large-amount 3-nitrotyrosine (3NT) in mouse synovitis models (Gao et al. 2015). This common modification occurring on a specific protein might cause a series of unexpected changes: protein aggregation and subsequent biological responses; a modulation of protein turnover; an alteration of signaling processes; and an induction of immunological responses (Trujillo et al. 2010). Besides, $ONOO^-$ can in turn "uncouple" the original function of eNOS to let it becomes a O_2^--generating enzyme that contributes to vascular oxidative stress (Forstermann 2010). When the flux of NO exceeds that of O_2^-, NO will react with $ONOO^-$, resulting in the predominant formation of harmful NO_2 and N_2O_3 (Thomas et al. 2010).

Overall, the threshold theory of NO-mediated disease and healthy effects addresses a "double-edged sword" effect of NO on living cells, and helps to explain why NO can repel the "foes" (pathogens) and also hurt the "friends" (autotissues). Interestingly, this theory can be used to interpret the hormesis (low-dose toxin-exiting) effect on the molecular level. For example, high-level NO and H_2O_2 are usually considered cytotoxic to living cells, but CR-mediated healthy effects are dependent on both low-level NO and H_2O_2. In turn, CR-induced NO triggers ROS

burst, ROS activate antioxidative responses, and antioxidation alleviates oxidative stress. In similar, mitochondrial uncouplers that target the respiratory chain, such as DNP, also provoke ROS generation, induce antioxidative responses, and scavenge ROS, eventually mimic CR's healthy effects.

1.3 The Purpose of Writing the Book

Most recently, a youth-keeping protein, growth differentiation factor 11 (GDF-11), has been identified in the blood of young mice, but whether its antiaging mechanism involves NO signaling is totally unclear. It is known that the "young blood" can activate stem cells and rejuvenate organs and cells in old mice, whereas the "aging blood" can inhibit regenerative capacity in young mice (Hall 2014). To my assumption, GDF-11 might maintain a relative high level in young mice, whereas it might be lower in aging mice. Alternatively, it can be that youngers would keep a natural form of GDF-11, whereas olders would possess a partially denatured form of GDF-11, perhaps due to the $ONOO^-$-mediated nitration in aging mice. Surprisingly, GDF-11 as an authentic youth factor (Loffredo et al. 2013; Sinha et al. 2014) has been currently argued by indicating that myostatin was actually misidentified as GDF-11 (Egerman et al. 2015). However, no response has been given from authors until now.

From a detailed introduction and a deep discussion in this seven-chapter book, I have tried to preliminarily reveal the secrets of longevity and aging-related pathogenesis, and also attempted to validate the threshold mechanism underlying NO-mediated disease and health potentials (Fig. 1.2).

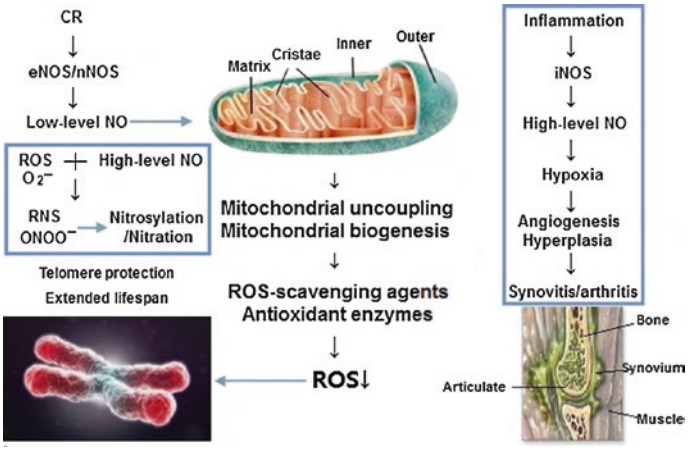

Fig. 1.2 A schematic outline of the threshold theory of NO-mediated disease and health effects showing the dichotomy between physiological low-level NO and pathological high-level NO. *CR* calorie restriction; *eNOS* endothelial nitric oxide synthase; *iNOS* inducible nitric oxide synthase; *LPS* lipopolysaccharide; *nNOS* neuronal nitric oxide synthase; *NO* nitric oxide; *RNS* reactive nitrogen species; *ROS* reactive oxygen species

We are very confident to conclude that ROS should be reversely correlated with lifespan, i.e., a higher level of ROS that has not been scavenged by antioxidants determines a shortened lifespan due to the damage of DNA and telomeres, whereas a lower level of ROS that can induce antioxidants confers an extended lifespan upon the protection of telomeres and chromosomes. Mitochondria are the main organelles responsible for ROS generation, but CR-triggered optimal NO can promote the generation of sublethal ROS and induce the activation of antioxidant enzymes for ROS scavenging.

On the other hand, immune stress from chronic pathogenic infections or by overgrown gut microbiota, especially LPS that is leaked into the bloodstream from G^- bacteria, can activate proinflammatory cytokines and subsequently stimulate iNOS for triggering potent NO bust. High-level NO may competitively occupy the O_2 binding site within hemoglobin/myoglobin, leading to a hypoxic milieu, which can drive angiogenesis, hyperplasia, and inflammatory lesions.

This book is devoted to discuss both beneficial and harmful aspects (so-called "Yin-Yang effects") of NO on the biology and medicine, and is also dedicated to review an up-to-date literature and prospecting the future application of ART in many pathological conditions. I explain why you should keep a steady-state level of aging-suppressed NO and avoid an extremely high level of infection- and inflammation-triggered NO. In the final part of the book, I suggest a hypothesis of healthy/unhealthy obesity and also provide a clue to CSC origin.

The book with a brief description of the multiple implications of ART in combating tumor/cancer, pathogenic bacteria, chronic inflammation, and aging/aging-associated disorders is written for medical researchers and students, clinicians, biologists, and other people interested in NO-driven healthy and pathological effects and ROS-involved aging processes.

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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

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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 O_2 and 1.5 mol of dihydronicotianamide adenine dinucleotide phosphate ($NADPH + H^+$) are consumed, and nicotinamide adenine dinucleotide phosphate ($NADP^+$) is generated, as shown by the equation: $ARG + 3/2\ NADPH + H^+ + 2\ O_2 = CIT + NO + 3/2\ NADP^+$. There are other five cofactors involved in the reaction, including flavin adenine dinucleotide (FAD), flavin mono-nucleotide (FMN), tetrahydrobiopterin (BH_4), calmodulin (CaM), and heme. The sequential electron flow is from $NADPH + H^+$ to FAD, to FMN, to heme, and to O_2. BH_4 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 Ca^{2+} dependence: one is the constitutively expressed and Ca^{2+}-dependent eNOS and nNOS; another is the proinflammatory cytokine-inducible expressed and Ca^{2+}-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, amylnitrite, 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 gualylate 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 lunched 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 amorphadiene synthase (ADS) and cytochrome P450 monooxygenase (CYP71AV1) from *A. annua* L. Consequently, ADS leads to amorpha-4,11-diene production, and CYP71AV1 allows conversion from amorpha-4,11-diene to artemisinic acid (Ro et al. 2006). We have independently reported the in vitro biotransformation of engineered yeast-derived amorpha-4,11-diene 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

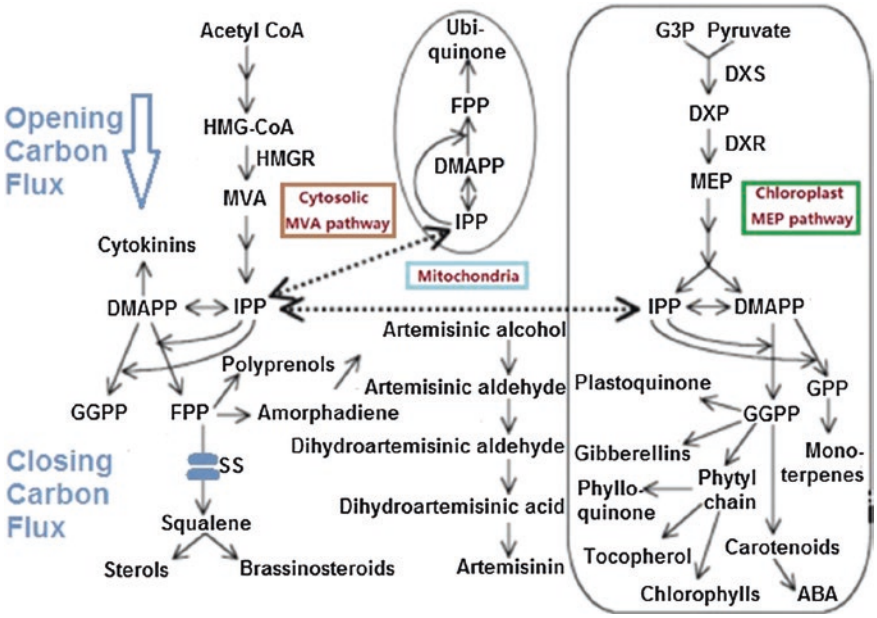

Fig. 2.1 The isoprenoid biosynthesis pathway-based opening/closing carbon flux strategies aiming at ART overproduction in *A. annua* L. *ABA* abscisic acid; *DMAPP* dimethylallyl diphosphate; *DXP* deoxyxylulose 5-phosphate; *DXR* DXP reductoisomerase; *DXS* DXP synthase; *FPP* farnesyl pyrophosphate; *GGPP* geranylgeranyl pyrophosphate; *HMG-CoA* 3-hydroxy-3-l-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 $^1O_2$, suggesting that SA/MJ induces ART overproduction through invoking $^1O_2$ 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, piperaquine, 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 semisynthetic 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 predrugs 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) O_2 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 O_2 sensor), and CooA (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 (His^{105}) 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 O_2. 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 *bc1* 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, ß, and δ carbon atoms (Cazelles et al. 2001) (Fig. 2.2).

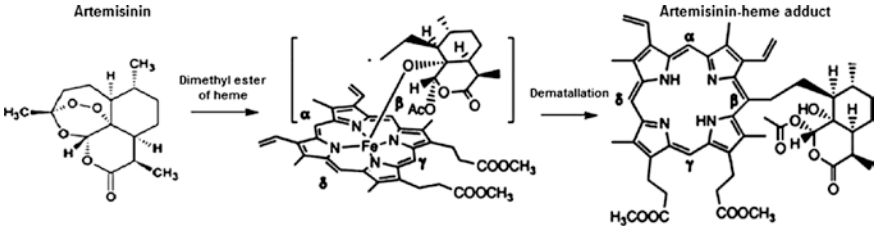

Fig. 2.2 Alkylation of a heme model (dimethyl ester of heme) by a primary carbon-centered radical derived from activated ART. An ART-heme adduct from the β carbon atom is shown, but other adducts are also obtained from α and δ carbon atoms

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). Trioxaquines 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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Chapter 3
ART for Antitumor

Abstract ART acts on tumor cells differentially by the threshold concentrations. High-dose ART kills tumor cells upon the inhibition of NOS, whereas low-dose ART benefits tumor propagation due to induce cytoprotective NO production. Pro-oxidants that antagonize antioxidants can potentiate ART's antitumor capacity. The combination of ART with pro-oxidants should provide an effective solution to the chemotherapy of multidrug resistant tumors.

Keywords ART · NOS · Pro-oxidants · Chemotherapy · Sensitization

3.1 An Overview on Tumor and Antitumor

The malignant tumor, or cancer, severely threatens human health, but the pathogenesis of tumorigenesis/carcinogenesis is poorly understood. The solid tumor might disappear after treatment by a single therapy or a combined regimen of surgery with radiotherapy and/or chemotherapy. For chemotherapy, many options are available for choosing antitumor drugs among alkylating agents, antimetabolites, antimicrotubule agents, topoisomerase inhibitors, and cytotoxic antibiotics (Corrie and Pippa 2008; Lind 2008). The antimalarial compound ART also exhibits antitumor activity although it has not been used for the clinical treatment of cancer. ART's antitumor activity, in an acceptable dose, is lower than commonly used chemotherapeutics. This is perhaps why ART has not been practical for cancer treatment until now. In addition, the mechanism underlying ART killing tumor cells remains unknown, and the specific machinery targeted by ART in tumor cells has not been identified. Nevertheless, it is known that tumor cells resistant to the present antitumor drugs are hypersensitive to ART, suggesting a different tumor-killing mechanism (Efferth et al. 2003).

The cytotoxicity of ART to tumor cells was first evaluated by Woerdenbag et al. (1993). Later, ART's cytotoxicity was shown to be endoperoxide-dependent (Beekman et al. 1998). An iron-dependent antitumor pattern of ART was also

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proposed (Kwok and Richardson 2002). Tumor cells in exposure to ART were found to exhibit a correlation of mitigated proliferation with repressed angiogenesis (Krishna et al. 2008). Recent evidence suggests that ART may share a common feature with the heme-interacting compound coralyne because both exert the cytotoxicity in a heme-dependent manner (Zhang and Gerhard 2009). An involvement of heme in anticancer activity was supported by the findings that cobalt protoporphyrin, an inducer of heme oxygenase, abolishes the activity of ART dimers, whereas tin protoporphyrin, an inhibitor of heme oxygenase, enhances the activity of ART dimers (Wink et al. 1995). Nevertheless, the bona fide targets to which ART binds remain under debate (O'Neill et al. 2010).

The endoperoxide bridge of ART can lead to the generation of carbon-centered free radicals in vivo, causing oxidative stress implicated in the killing of malarial parasites and tumor cells. However, the peroxidized structure is prone to be destroyed by antioxidants abundant in cells. In regard to antimalarial activity, it has been revealed that free radical generators (pro-oxidants) such as riboflavin (vitamin B2) and menadione (vitamin K) are synergetic to ART, whereas free radical scavengers (antioxidants) including tocopherol (vitamin E), ascorbate (vitamin C), glutathione (GSH), and dithiothreitol (DTT) are antagonistic to ART (Senok et al. 1997). Furthermore, ART's antimalarial efficacy would be potentiated when antioxidant enzymes are inhibited (Meshnick et al. 1989). These results imply that ART is more effective in an oxidative milieu.

3.2 ART Diminishes NO-Conferred Anticytotoxicity of Tumor Cells to Chemotherapeutics

3.2.1 Purposes and Significance

The proposed mechanistic patterns or potential targets of ART for attacking the malarial parasite include: (1) heme conjugation; (2) protein alkylation; (3) membrane damage; (4) mitochondrial dysfunction; and (5) energy deficiency due to the inhibition of malarial ATPase (PfATP6) (O'Neill et al. 2010). Although numerous studies have indicated that ART can induce apoptosis and inhibit angiogenesis in tumors, it remains unclear which definitive cellular targets are dependent by ART and derivatives. Nevertheless, ART's targets identified in tumor cells include at least translationally controlled tumor protein (TCTP) (Efferth 2005) and sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (Uhlemann et al. 2005). However, whether such targets are also in action for antitumor remains uncertain.

Recent evidence suggests that ART exhibits similarity with the heme-interacting coralyne because coralyne and ART show a substantial increase in the cytotoxicity when a synthetic heme exists. Detection of the altered absorbance spectrum confirms the presence of a new ART-heme complex, which is consistent with the decomposition of heme porphyrin rings (Zhang and Gerhard 2009). These previous observations encouraged us to suggest that heme might be ART's

preferential mediator for antitumor, and hemoproteins should be ART's targets in tumor cells. Validation of such a suggested idea needs to verify the formation of ART-heme adducts being correlated with the inhibition of a specific hemoprotein and upregulation of the hemoprotein gene. In the present study, we used ART as a heme alkylator to investigate an interaction between ART and hemoproteins in the hepatoma cell line $HepG_2$. By the parallel detections of ART-heme adducts and enzymatic activities, we would confirm which hemoproteins are ART's targets for exerting antitumor activity.

3.2.2 Results and Analysis

3.2.2.1 Identification of ART-Heme Adducts in Tumor Cells

To testify the interaction of ART with hemoproteins, we monitored the dynamic fluctuation of a specific absorbance peak of heme (A_{415}) and a unique absorbance peak of ART-heme adducts (A_{476}) in the tumor cell line $HepG_2$ after incubation with 50 μM ART for 24, 48, and 96 h. To prepare a cell lysate for photometric detection, the in vitro cultured tumor cells were collected and lysed by a simple freeze-thaw cycle (Zhang and Gerhard 2009).

As results, a gradual elevation of A_{476} was observed in cells incubated with ART until 96 h. The readings of A_{476} are from 0.042 to 0.110 after 24 h, to 0.123 after 48 h, and to 0.131 after 96 h. A dual-phase change of A_{415} was also noticed with an elevation (0.145) after 24 h and a decline (0.033 and 0.049) after 48 h and 96 h, respectively (Table 3.1). The increase of heme suggests an enhanced biosynthesis of hemoproteins following inhibition by ART.

3.2.2.2 ART's Threshold Effect and Possible ART-Targeted Enzymes

In the present investigation, we evaluated the effect of different concentrations of ART on the in vitro propagation of $HepG_2$ after incubation for 48 h. It was found

Table 3.1 The fluctuation of A_{415} and A_{476} values of $HepG_2$ after incubation with 50 μM artesunate for 24, 48, and 96 h

Group	A_{415}	A_{476}
Control 24 h	0.044 ± 0.001	0.042 ± 0.004
ART 24 h	0.145 ± 0.002*	0.110 ± 0.003**
ART 48 h	0.033 ± 0.001*	0.123 ± 0.002**
ART 96 h	0.049 ± 0.002*	0.131 ± 0.002**

ART artemisinin. The *singular asterisk* (*) represents statistically significant difference from the control ($P < 0.05$); The *double asterisks* (**) represent statistically very significant difference from the control ($P < 0.01$)

that the survival rate of tumor cells is highly dependent upon ART's concentrations, in which 50 μM ART leads to a higher survival rate accounting for nearly 80 %, whereas 100 and 200 μM ART cause the dramatic cell death, with a survival rate of 40 % for 100 μM ART and of 25 % for 200 μM ART. These results indicated that there exists a threshold effect of ART on the survival/death fate of tumor cells.

Considering that NOS is a hemoprotein and ART enables the alkylation of hemoproteins, we envisaged that ART in an optimal concentration might conjugate the heme-containing NOS to inhibit its activity, and also induce its expression in a feedback manner. Therefore, we should verify an elevation of NO levels after incubation of $HepG_2$ with a sublethal dose of ART. For this purpose, we incubated $HepG_2$ with 50 μM ART for measuring the NO level. As our expectation, a dramatic elevation of the NO level was observed up to 35 μM after incubation for 48 h. In contrast, NO was not detected in $HepG_2$ treated by 100 or 200 μM ART. These results indicated that 50 μM ART can trigger a protective level of NO from tumor cells, but 100 and 200 μM represent the lethal doses of ART.

Because CAT that scavenges H_2O_2 is also a hemoprotein, we measured the H_2O_2 level after incubating $HepG_2$ with 50, 100, or 200 μM ART for 48 h. However, neither low-dose nor high-dose ART results in a significant change of H_2O_2. The level of H_2O_2 in untreated and 50 μM ART-treated tumor cells is equal to 53 mM, and that in 100 or 200 μM ART-treated tumor cells is approximately 57 mM. These results suggested that CAT is unlikely implicated in tumor killing by ART, and H_2O_2-mediated apoptosis may not be a major mechanism by which ART exerts an antitumor effect.

3.2.2.3 Synergistic Effects Through the Combination of ART with Chemotherapeutics

Although high doses of ART (100–200 μM) are effective on tumor killing, it is apparently higher than ART's IC_{50} (70 μM), thereby endowing a nonselective toxicity to normal cells. However, 50 μM ART not only fails to kill tumor cells, but also promotes tumor growth. To obtain a higher antitumor effect using a lower dose of ART, we combined 50 μM ART with 10 μM 5-fluorouracil (FLU) for in vitro antitumor evaluations in $HepG_2$ cells. Consequently, ART exhibits a significant synergistic effect to FLU, in which tumor growth is inhibited by 25–40 %. In contrary, tumor cells treated by 10 μM FLU alone show an inhibition rate of only 8 %. These results suggested that a sublethal dose of ART could effectively kill tumor cells as it is combined with FLU.

It was observed that ART + FLU can dramatically decrease the NO level in $HepG_2$, from 16 μM NO in 50 μM ART-treated tumor cells to 11 μM NO in 50 μM ART + 10 μM FLU-treated tumor cells. At the same time, no significant change in H_2O_2 levels was found in tumor cells incubated with ART alone or ART + FLU. Therefore, ART may not stimulate H_2O_2 for augmenting FLU's antitumor activity.

3.2.3 Discussion

It has been reported that approximately 5–18 % of ART can bind to hemoproteins such as hemoglobin, but does not react with heme-free globins (Meshnick et al. 1991). Among versatile hemoproteins, ART's targets for exerting antitumor activity are currently unidentified. Because NOS was previously proved a cytochrome P450-like hemoprotein (Chinje and Stratford 1991), we planed to examine whether the formation of ART-heme adducts might be correlated with the elevation of NO levels as well as whether they would accompany with the elevation or decline of survival rates in $HepG_2$.

If this is the case, ART conjugation to NOS should lead to its inhibition and induction. Indeed, we detected a dramatic increase of A_{415} following the exposure of $HepG_2$ to ART upon incubation for 24 h, suggesting the de novo biosynthesis of hemoproteins including NOS. As indicated earlier, NO is able to inhibit the progressions of cysteine reduction and Fenton reaction (Gusarov and Nudler 2005). Therefore, NO is likely exploited by bacteria to protect from oxidative damage, and NO confers bacteria resistance to antibiotics (Gusarov et al. 2009).

With equal importance, it has been filed that NO can regulate cancer growth, migration, invasion, survival, angiogenesis, and metastasis in a concentration-dependent manner (Ridnour et al. 2006; Jang and Kim 2002). Evidence supporting a beneficial role of NO to tumor survival comes from a finding that NO levels are higher in cancerous tissues compared to their normal counterparts (Wink et al. 1993). This finding was later deciphered as that NO is harnessed to protect against cellular damage and cytotoxicity from ROS and organic peroxides (Wink et al. 1995).

Our results indicated that a lower concentration (50 μM) of ART allows tumor cells survival with a higher rate, whereas higher concentrations (100–200 μM) of ART cause tumor cell death. Interestingly, 50 μM ART triggers NO burst, but 100–200 μM ART does not. Therefore, it can be deduced that ART-induced protective NO should benefit to tumor cell survival. This conclusion is supported by a previous observation that the cytotoxicity of ART in the mouse macrophage cell line RAW 264.7 is associated with the inhibition of NOS (Konkimalla et al. 2008). Additionally, we did not detect any significant changes of H_2O_2 levels after ART treatment although CAT is also a hemoprotein. This is likely because ART only inhibits the heme-harboring CAT, but does not inhibit the heme-independent GSH-POX, which can detoxify H_2O_2 and may maintain a homeostatic redox state.

In summary, a low dose of ART does not exert an antitumor role because it induces the production of a protective NO level. In contrast, a high dose of ART exhibits a potent antitumor effect by its direct cytotoxicity.

3.2.4 Conclusions

ART kills cancer cells with uncertain mechanisms. Here, we report that ART can exert its antitumor activity by alkylating hemoproteins such as NOS in tumor cells. The conjugation of ART with the heme moiety of hemoproteins was

evident by monitoring the shift of absorbance from heme (A_{415}) to ART-heme adducts (A_{476}). Accordingly, it was observed that a transient elevation of A_{415} is accompanied with a synchronous burst of NO and a higher rate of survival following incubation of tumor cells with 50 μM ART. In contrast, ART at above 100 μM leads to a lower NO level along with a lower survival rate, strongly implying that NOS may represent an important target of ART for killing tumor cells. Although CAT as a hemoprotein also interacts with ART, no remarkable fluctuation of CAT activity was observed after the incubation of tumor cells with 50, 100, or 200 μM ART for 48 h, suggesting that ART kills tumor cells unlikely through the H_2O_2-mediated apoptosis mechanism. Furthermore, we observed that a combination of ART with FLU exhibits a synergistic effect in killing tumor cells, perhaps by abrogating the NO-conferred cytoprotective role. These results should pave an avenue toward the eventual application of ART in clinic antitumor treatment.

3.3 Pro-oxidant Agents Synergize ART in Killing Tumor Cells

3.3.1 Purposes and Significance

Although ART exhibits antitumor activity at some extent, it cannot be comparative to any conventional chemotherapeutics because of its relative lower cytotoxicity. Nevertheless, ART has a potential of killing tumor cells with resistance to common antitumor drugs. The suggested mechanisms of ART combating tumor cells include: (1) antiproliferation and antiangiogenesis; (2) apoptosis; (3) oxidative stress; (4) oncogenes and tumor suppressor genes; and (5) multidrug resistance (Efferth 2006).

Given ART exerting a tumor-killing role via its endoperoxide, any compounds that stabilize the unique structure should serve as synergists, whereas any compounds that destabilize the unique structure might function as antagonists. In other words, a milieu tending to oxidation should enhance ART's antitumor activity, whereas a milieu beneficial to antioxidation should suppress ART's antitumor activity. In fact, the IC_{50} of ART was observed to correlate with the expression levels of 170 genes involved in the oxidative stress response and metabolism in 60 cell lines (Efferth and Oesch 2004).

To test whether oxidative stress benefits ART to exert more potent antitumor effects, we combined ART with pro-oxidants to constitute a drug combination, known as "ART-sensitizing compounds" (ASC), for antitumor evaluations in vitro in the hepatoma cell line $HepG_2$ and in vivo in tumor-bearing nude mice. In addition to ART per se, ASC include three pro-oxidants: the GSH exhauster diethyl maleate (DM); the GSH-POX inhibitor mercaptosuccinic acid (MA); and the CAT inhibitor aminotriazole (AT).

3.3.2 Results and Analysis

3.3.2.1 In Vitro Evaluation of ASC on Tumor Cell Proliferation

The optimal dosages of all components should be lower than their IC_{50}, i.e., 70 μM for ART, 20 μM for DM, 210 μM for MA, and 700 μM for AT. According to this principle, we designed ASC as 50 μM ART, 5 μM DM, 10 μM MA, and 10 μM AT. In the present study, we compared the antitumor effects of ASC with ART through in vitro evaluation tests.

After incubation of $HepG_2$ cells with ART or ASC for 48 h, it was observed that cell proliferation is extraordinarily suppressed. From the absorbance at 490 nm (A_{490}) of 1.55 ± 0.10 for the control, 0.83 ± 0.08 for ART, and 0.50 ± 0.09 for ASC, the growth inhibition rates were calculated to 46.76 % for ART and 67.26 % for ASC. These results indicated that ART and ASC suppress the in vitro propagation of tumor cells, but ASC allows a higher inhibition rate than ART alone.

3.3.2.2 In Vivo Evaluation of ASC on Graft Tumor Growth

Because of their capacity to exhaust GSH and inhibit antioxidant enzymes, pro-oxidants in ASC can attenuate antioxidation and enhance oxidative stress, thereby inducing apoptosis in tumor cells. For evaluating ASC's in vivo antitumor effects, we measured the tumor weight and tumor volume before and after treatment in tumor-bearing nude mice, from which the sensitizing potential of each pro-oxidant to ART for killing tumors should be clarified.

For in vivo evaluations, the optimal dosage for each drug was chosen by referring to 50 % of corresponding lethal dosages (LD_{50}), usually equivalent to 1/10–1/20 LD_{50}. The treatment regimens were divided into a short-term treatment and a long-term treatment. In the latter group, ASC was administered by either subcutaneous injections or intratumor injections. As comparison, FLU was included in the in vivo tests. The results were shown in Table 3.2 and Fig. 3.1.

From above results regarding the tumor weight and tumor volume, it is clear that the pro-oxidants in ASC can certainly sensitize ART to achieve the more effective results of antitumor activity.

3.3.2.3 Mechanisms of Chemosensitizer-Enhanced Antitumor Activity of ART

Upon treatment of $HepG_2$ by ASC or ART alone for 24–48 h, we noticed that the measured GSH content, GSH-POX activity, and CAT activity are dramatically declined (Fig. 3.2).

Table 3.2 Sensitization of ART's antitumor activity by ASC when evaluated in tumor-bearing nude mice

Treatment	Group	Tumor weight (g)	Tumor volume before treatment (mm^3)	Tumor volume after treatment (mm^3)
Short-term injection for 9 days	Control	0.44 ± 0.14	138.69 ± 53.12	460.97 ± 331.21
	ART (40 mg/kg/d), 1/15 LD_{50}	0.43 ± 0.29	273.65 ± 289.38	556.78 ± 554.79
	ASC (40:150:15:55 mg/kg/d), 1/20 LD_{50}	0.41 ± 0.22	208.32 ± 156.39	373.70 ± 302.68
	FLU (13 mg/kg/d), 1/15 LD_{50}	0.42 ± 0.12	116.01 ± 32.27	303.82 ± 33.97
Long-term injection for 18 days	Control	1.35 ± 0.46	29.63 ± 14.86	1050.11 ± 286.49
	ART (40 mg/kg/d), 1/15 LD_{50}	1.18 ± 0.39	51.18 ± 14.45	1091.46 ± 304.98
	ASC (40:200:20:74 mg/kg/d), 1/15 LD_{50} for subcutaneous injection	0.94 ± 0.46	62.96 ± 36.22	782.34 ± 353.23
	ASC (40:200:20:74 mg/kg/d), 1/15 LD_{50} for intratumor injection	0.87 ± 0.18*	48.74 ± 7.12	620.51 ± 116.76
	FLU (18.5 mg/kg/d), 1/10 LD_{50}	0.40 ± 0.16**	75.54 ± 22.88	456.55 ± 167.03

A *single asterisk* (*) represents significant difference from the control ($P < 0.05$); *Double asterisks* (**) represent very significant difference from the control ($P < 0.01$)

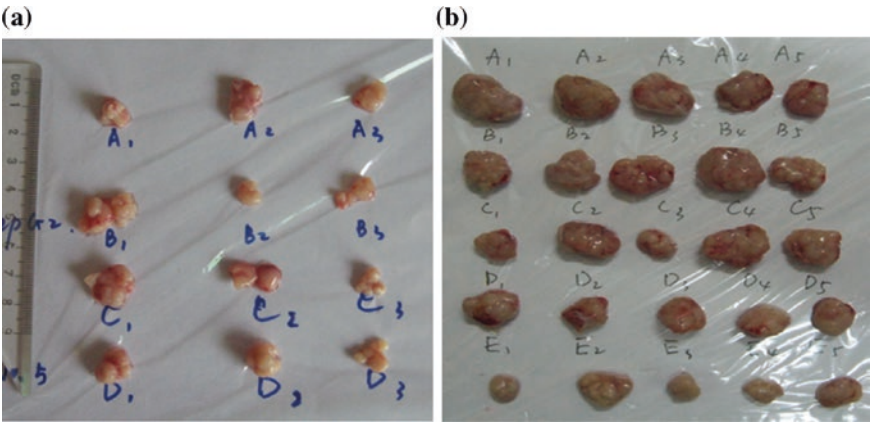

Fig. 3.1 The morphological illustration of grafted tumors on nude mice after short-term injection or long-term injection by antitumor drugs. **a** Tumors in *A1–A3* are from untreated mice; tumors in *B1–B3* are from ART-treated mice; tumors in *C1–C3* are from ASC-treated mice; tumors in *D1–D3* are from FLU-treated mice. **b** Tumors in *A1–A5* are from untreated mice; tumors in *B1–B5* are from ART-treated mice; tumors in *C1–C5* are from ASC1-treated mice; tumors in *D1–D5* are from ASC2-treated mice; tumors in *E1–E5* are from FLU-treated mice

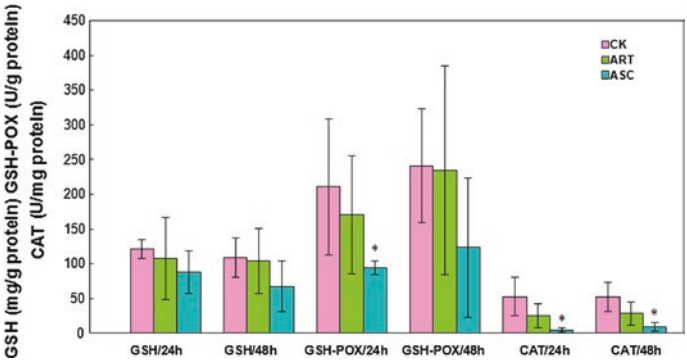

Fig. 3.2 Comparison of antioxidant activities in $HepG_2$ cells after treatment by ART or ASC for 24 and 48 h. *ART* artemisinin; *ASC* artemisinin-sensitizing compound; *CAT* catalase; *CK* control; *GSH* glutathione (reduced); *GSH-POX* glutathione(reduced)-peroxidase. A *single asterisk* (*) represents significant difference from the control ($P < 0.05$)

In the ASC group, CAT activity from 50 U/mg of total proteins in the control drops to 5 U/mg of total proteins as tested after 24 h, and 10 U/mg of total proteins as tested after 48 h. In the ART group, GSH content (100 mg/g of total proteins) and GSH-POX activity (150–230 U/g of total proteins) are slightly lower than those of the control (90 mg/g of total proteins and 200–230 units/g of total proteins, respectively), whereas CAT activity (20 U/mg of total proteins) is significantly lower than that of the control (50 U/mg of total proteins). These results confirmed that pro-oxidants can potentate ART's antitumor activity by suppressing the antioxidative capacity of tumor cells.

3.3.3 Discussion

The sensitizer of radiotherapy, buthionine sulfoximine (BSO), is the most commonly used GSH inhibitor, leading to the decline of GSH levels by inhibiting γ-GSH synthase responsible for GSH biosynthesis (Clark et al. 1984). Depletion of GSH is an important step toward the apoptosis of tumor cells via a ROS-dependent signaling pathway. The rapid proliferation of tumor cells generally leads to the massive production of ROS, but tumor cells can accordingly induce a high level of antioxidant enzymes to scavenge ROS. If GSH is depleted, tumor cells should be unable to scavenge O_2^- via GSH-POX.

In previous work conducted by other authors, ART was combined with transferrins to increase the antitumor capacity of ART because transferrins enable the accumulation of more bioavailable iron and allow a potent burst of ROS (Lai and Singh 2001). It is also known that H_2O_2 can also induce the apoptosis of tumor cells (Simizu et al. 1998). Therefore, the tumor-killing potential of ART should be greatly enhanced when H_2O_2 is increased due to the inhibition of CAT and POX. It is anticipated that ART as an alkylator of hemoproteins including CAT should

inhibit CAT, but not other antioxidant enzymes. In the present study, we found that ART can significantly inhibit CAT but not GSH-POX, leading to a steady-state level of H_2O_2 in mice. In contrast, when ASC was injected into mice, both CAT and GSH-POX are significantly inhibited, suggesting that ASC kills tumor cells more effectively due to a high level of H_2O_2.

Regarding the possible cytotoxicity of each drug in ASC, it is known that MA is equivalent to ART, while DM and AT are less toxic than ART. DM has low toxicity because it only inhibits GSH-POX by depleting GSH, but not affects other POX. MA and AT are also low toxic because they do not interfere with the expression of any antioxidant enzymes.

Taken together, our study has confirmed that ASC confers a sensitizing effect to ART by diminishing the antioxidative response of tumor cells, in which CAT and GSH-POX can be potently inhibited. These results clearly reflected a fact that ART killing tumor cells depends on oxidative stress that can be enhanced by pro-oxidants.

3.3.4 Conclusions

To enhance the antitumor efficiency of ART, we designed the oxidation-enhanced ASC by combining ART with three-type pro-oxidants, including a GSH exhauster, a GSH-POX inhibitor, and a CAT inhibitor. The survival of tumor cells was evaluated in vitro and the size and weight of graft tumors were measured in vivo. Consequently, cell proliferation is substantially inhibited by ASC with a higher inhibition rate (67.26 %) than ART alone (46.76 %). As compared with the control, graft tumors decrease to 30.42–35.58 % in weight and 25.50–37.35 % in volume by ASC. Pro-oxidant treatment can dramatically decrease antioxidant enzyme activities. For example, CAT activity in the control is 50 units/mg of total proteins, while that in ASC or ART treatment is only 5–10 units/mg of total proteins or 20 units/mg of total proteins. Conclusively, pro-oxidants can sensitize ART for exerting more effective antitumor roles through depriving antioxidants in tumor cells.

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Chapter 4
ART for Antibacterial Infection

Abstract In Gram positive bacteria, bNOS-derived NO confers the protection of bacteria from environmental stress such as antibiotic challenges. ART can exert an antibiotic sensitizing role through inhibiting bNOS and CAT. Either in vivo or in vitro, ART can increase the bacteriocidal activity of antibiotics by abrogating the beneficial NO and enhancing the harmful H_2O_2, demonstrating a potential role in combating multidrug resistant bacteria.

Keywords ART · Bacteria · bNOS · H_2O_2 · Multidrug resistance · NO

4.1 An Overview on Bacterial Infection and Antibacterial Infection

Tuberculosis is a kind of leading lethal infectious disease due to infection by *M. tuberculosis*. Currently, the first-line drugs used for treating patients with tuberculosis are conventional drugs invented several decades ago, and the second-line drugs are often expensive but less effective. Following the abuse and irregular use of antibiotics, *M. tuberculosis* has evolved to exhibit the multidrug resistance (MDR) and extensive drug resistance (EDR). Patients with the MDR-type tuberculosis would fail to respond to a standard therapeutic cohort, and the needed antibiotic dosages should increase for more than 1000 folds. The EDR-type tuberculosis exhibits antibiotic resistance similar with the MDR-type tuberculosis, but tolerance to fluoroquinolones and one of three kinds of second-line drugs (Raviglione 2007). Upon the endemic occurrence of MDR and EDR *M. tuberculosis* strains, development of novel antituberculosis drugs becomes urgently needed.

Apart from *M. tuberculosis*, the most popular MDR bacteria (so-called "super bugs") is methicillin-resistant *Staphylococcus aureus* (MRSA) with the plasmid-carrying methicillin-resistant gene *bla* $_{\text{CTX-M-15}}$ (Walsh et al. 2007; Hawkey 2008), where *bla* encodes beta-lactamase, belonging to the group of extended spectrum beta-lactamase (ESBL). MRSA strains first occurred in 1960, and were prevalent

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in 1990. Recently, a new antibiotic resistant gene, *bla* $_{\text{NDM-1}}$, that exists in a plasmid has been identified in G^- bacteria, such as *E. coli* and *Krebsiella pneumoniae*. NDM-1 that represents New Delhi metallo-β-lactamase 1 was nominated because it was identified from antibiotic resistant bacteria in New Delhi, India (Kumarasamy et al. 2010). A recent publication has described that aureusimines, a series of nonribosomal peptidyl secondary metabolites, assist the productive infection by *bla* $_{\text{NDM-1}}$ bacteria (Wyatt et al. 2010).

It is known that G^+ bacteria produce NO by activating bNOS, which can protect bacteria from antibiotics (Gusarov et al. 2009). Ultraviolet irradiation can upregulate bNOS and elevate the protective NO level in bacteria (Patel et al. 2009). Like mammals, NO is also synthesized from ARG in G^+ bacteria. There are three subtypes of bacterial NO-producing enzymes: (1) completely homologous to the mammalian NOS with both a heme-binding region and a reductase domain, such as bNOS in *S. cellulosum* (Agapie et al. 2009); (2) partially homologous to the mammalian NOS with a heme-binding region, but without a reductase domain, including bNOS in *Bacilus*, *Staphylococcus*, and *Streptomyces.* So they need another reductase for NO synthesis (Crane 2008); (3) bNOS-independent enzyme systems as in *E. coli*, encompassing cytochrome *c* nitrite reductase, NO sensitivity regulon, and flavin hemoglobin (Corker and Poole 2003; van Wonderen et al. 2008).

Considering the high cost and long time for developing new antibacterial drugs, we could alternatively find out the inhibitors of bNOS to deprive the protective NO and synergize the insensitive antibiotics. In history, antibiotic synergists were developed to augment the antibacterial capacity of antibiotics. For example, clavulanic acid as an inhibitor of β-lactamase has been widely used in combination with penicillin to kill penicillin-resistant bacteria with an increased β-lactamase activity.

4.2 In Vitro Examination for ART Suppressing NO-Conveyed Bacterial Antibiotic Tolerance

4.2.1 Purposes and Significance

A current challenge of coping with bacterial infection is bacterial pathogens are becoming less susceptible to or more tolerant of commonly used antibiotics. The occurrence of "super bugs" with MDR/EDR predisposes a necessity of solving the more and more severe issue of bacterial resistance to wide-spectral antibiotics. For this purpose, we could consider to sensitize antibiotics by exploiting bNOS inhibitors to abrogate the cytoprotective role played by bacteria-produced NO.

Because either G^+ bacteria or G^- bacteria are capable of generating their own NO, they should be vulnerable to the inhibition of NO production. However, G^+ bacteria synthesize NO depending on bNOS, whereas G^- bacteria are independent on bNOS for NO production. So bNOS inhibitors are only effective on bNOS-harboring G^+ bacteria.

ART possesses pleiotropic functions, including antimalaria, antitumor, and anti-inflammation (Krishna et al. 2008), but there is no evidence regarding ART's

antibacteria role. Here, we report that ART can serve as a novel bNOS inhibitor to synergize antibiotics for more effective antibacterial effects. The combined use of ART with antibiotics might decrease the dosage of antibiotics, and increase their bactericidal efficiency. On the other hand, we also expected that ART could inhibit CAT to elevate the H_2O_2 level for maximizing bacterial apoptosis.

4.2.2 Results and Analysis

4.2.2.1 Induction and Suppression of NO Production in Bacteria

To induce the endogenous NO in bacteria, we cultured the G^+ bacterium *Bacilus licheniformis* under oxidative stress conditions including hypoxia and cold, from which we investigated the influence of ART on oxidative stress-induced NO production. Consequently, ART was found to substantially mitigate the production of NO from *B. Licheniformis* under the oxidative stress condition. A gradual increase of NO production was noticed in bacterial cultures standing without agitation at the room temperature (as the hypoxia group) or without agitation at 4 °C (as the hypoxia + cold group). High levels of NO were measured in both treatments, whereas the control has only a negligible NO level. Accordingly, hypoxia and cold-triggered NO can be repressed by ART and L-NMMA (Fig. 4.1).

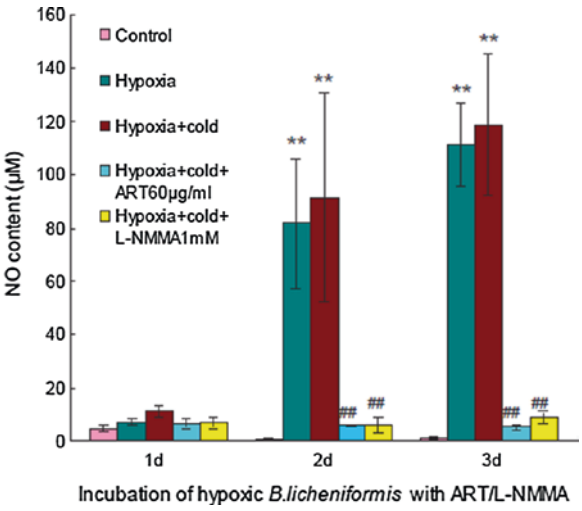

Fig. 4.1 Induction of NO production in *B. licheniformis* upon acclimatization to oxidative stress and suppression of oxidative stress-inducible NO. *ART* artemisinin; *L-NMMA* N^G-monomethyl-L-arginine monoacetate; *NO* nitric oxide. *Double asterisks* (**) represent very significant difference from the control ($P < 0.01$); *Double pound signs* (##) represent very significant difference from the hypoxia + cold group ($P < 0.01$)

These results indicated that bacteria can synthesize NO for adaptation to oxidative stress circumstance, and ART can inhibit the synthesis of NO in bacteria, hence representing a novel inhibitor of all kinds of NOS including bNOS. Thus, we could expect that ART would sensitize those antibiotics that pose oxidative stress to bNOS-dependent bacteria.

4.2.2.2 Attenuation of NO-Conferred Antibiotic Tolerance by ART: In Vitro Study

We first tested whether bacterial NO burst under a hypoxic condition confers *B. licheniformis* tolerance to RIF. After cultured for 12 h with RIF (20, 40, 60, or 120 μg/ml), hypoxia-acclimatized bacteria proliferate at a higher rate, whereas nonacclimatized bacteria exhibit no proliferation. Similarly, hypoxia-acclimatized bacteria also show accelerated proliferation in the presence of cefotaxime (CEF) (15, 25, or 50 μg/ml). These results demonstrated that hypoxia-induced NO allows bacteria to thrive in exposure to RIF/CEF, suggesting that NO confers *B. licheniformis* tolerance to those antibiotics.

By attenuating the protective NO production in bacteria, ART was anticipated to reverse NO-mediated protection of bacteria from antibiotics. Indeed, a combination of ART (60 μg/ml) with RIF (20–120 μg/ml) leads to a lower rate of bacterial growth than RIF alone, implying that ART potentates RIF by abrogating bacterial NO production. We observed that enhanced NO burst, at a maximal level of 80 μM, occurs in bacteria in exposure to RIF (20–120 μg/ml), whereas a decreased NO level (20 μM) or even a negligible NO level was measured in bacteria cotreated by ART (60 μg/ml) with RIF (20–120 μg/ml).

As comparison, we also detected the NO level in bNOS-free *E. coli*, either with or without CEF, but no correlation of bacterial proliferation with NO production was established. The growth dynamics of *E. coli* under CEF (100 μg/ml) are similar with those under ART (60 μg/ml) + CEF (100 μg/ml). The maximal NO levels are only 4–8 μM under CEF (100 μg/ml) or ART (60 μg/ml) + CEF (100 μg/ml). From the extremely low level of NO in *E. coli*, we could infer that its own NO may not be sufficient to detoxify antibiotics.

4.2.2.3 Bacterial Production of H_2O_2 Due to CAT Inhibition by ART

As an antioxidant hemoprotein, CAT was expected to interact with ART and to exhibit a lower enzymatic activity. If this is true, we could deduce that there should be a similar mechanism by which ART binds to CAT and blocks the conversion of toxic H_2O_2 to nontoxic H_2O or other detoxified compounds. To confirm this deduction, we measured the CAT activity of *B. licheniformis* after incubation with RIF (20–120 μg/ml) or RIF (20–120 μg/ml) + ART (60 μg/ml). The results showed that CAT activity is considerably reduced once ART was included in the culture (Fig. 4.2). For example, after 24 h, CAT activity in bacteria treated by RIF

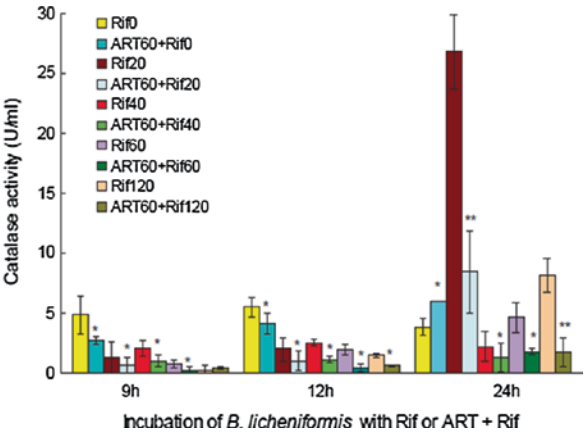

Fig. 4.2 CAT activity in *B. Licheniformis* upon exposure to RIF or ART + RIF. *ART* artemisinin; *Rif* rifampicin. A *single asterisk* (*) represents significant difference in ART + RIF from RIF ($P < 0.05$); *Double asterisks* (**) represent very significant difference in ART + RIF from RIF ($P < 0.01$)

(20 μg/ml) is as threefold as that in bacteria treated by RIF (20 μg/ml) + ART (60 μg/ml).

It means that enhanced H_2O_2 production following CAT inhibition by ART should facilitate the bactericidal effect of antibiotics. Therefore, ART can abrogate the protective NO, on the one hand, and also promote the harmful H_2O_2, on the other hand, thereby exhibiting a dual synergistic effect as the inhibitors of both bNOS and CAT.

4.2.2.4 Identification of ART-Bacterial Heme Conjugates

Based on our assumption, ART would bind to bNOS, CAT, and other hemoproteins, hence prohibiting the interconversion between Fe^{3+} and Fe^{2+}. We monitored the dynamic fluctuations of heme and ART-heme conjugates. As results, A_{415} that reflects the absorbance of heme and A_{476} that represents the absorbance of ART-heme conjugates were observed to have higher readings ($A_{415} = 0.3$; $A_{476} = 0.2$) after adding ART for three hours. As comparison, the lower readings ($A_{415} = 0.18$; $A_{476} = 0.06$) were determined in bacterial cultures without ART supplementation. After elevations by three-hour incubation, both A_{415} and A_{476} decline to readings equal to the control by incubation for 6–9 h.

The elevation of A_{415} was assumed to the de novo biogenesis of hemoproteins, while the elevation of A_{476} could be a hint indicating the formation of ART-heme conjugates. The increases of hemoproteins including bNOS and CAT need the overexpression of corresponding genes, because they should be inducible after the inactivation of those enzymes due to heme alkylation by ART. The double decline of A_{415} and A_{476} is likely attributed to bacterial death upon ART's bactericidal effects.

4.2.3 Discussion

The nonpathogenic *B. subtilis* and pathogenic *B. anthracis* as well as other G^+ bacteria generate NO by their own bNOS that contains a prosthetic heme moiety for the reduction of a catalytic center from [Fe^{3+}] to [Fe^{2+}] (Gusarov et al. 2008). Our present research showed that *B. licheniformis* can accumulate nitrate and nitrite, the oxidized products of NO, in the media under oxidative stress conditions including hypoxia and hypoxia + cold. This finding is supported by a previous report describing that NO is not consumed and can accumulate in the microenvironment of human tissue at lower O_2 concentrations (Taylor and Moncada 2010). Furthermore, we also found that bacterial NO production can be repressed by ART or L-NMMA, suggesting that bNOS is involved in the oxidative stress-induced NO production.

Although *E. coli* strains generating NO are independent on bNOS, they have an enzyme complex encompassing nitrite reductase, flavohemoglobin, and NO-sensing regulator responsible for NO production (Corker and Poole 2003). Indeed, we observed an elevation of NO levels in *E. coli* overnight cultures. The NO level, either in the presence or absence of ART or ART + CEF, was detectable, but relatively low (below 12 μM), much lower than that in *B. licheniformis* (80–120 μM), suggesting that such a low NO level in *E. coli* is insufficient to protect bacteria from antibiotic attacking. ART may be directly cytotoxic to bacteria because ART can be converted to carbon-centered free radicals in vivo. The inhibition of a bacterial multidrug efflux pump system, of course, should represent an alternative mechanism of ART sensitizing antibiotics in *E. coli* (Li et al. 2011).

Given that heme alkylation by ART has been verified by identifying the ART-heme adducts in malaria-infected mice (Robert et al. 2005), and considering that an ART-heme interaction has been also confirmed in tumor cells (Zhang and Gerhard 2009), we proposed that ART should also interact with bacterial hemoproteins. In addition to bNOS, CAT is also a hemoprotein in *Bacilus* bacteria (Bol and Yasbin 1994). In the present study, we did detect the presence of ART-heme conjugates in *B. licheniformis* after incubation with ART for three hours. Later on, however, ART-heme conjugates cannot be detected, probably because of the degradation of conjugates following bacterial death. This situation is similar with our observation in tumor cells (Zeng and Zhang 2011).

Until recently, there has no literature describing the impact of ART on bacterial CAT. Actually, our data showed that CAT activity is considerably reduced once ART was included. For example, a higher CAT activity (25 units/ml) for 24 h-cultures was measured in 25 μg/ml RIF, but it is sharply declined to below two units/ml when 25 μg/ml RIF was combined with 60 μg/ml ART. Mechanistically, CAT is believed to be activated by NO in bacteria through diminishing the rate of cystine reduction to cysteine, which drives the Fenton reaction and simultaneously inhibits CAT activity (Gusarov and Nudler 2005). Due to the CAT inhibition, hydroxyl radicals derived from an excess H_2O_2 would exhibit an extreme toxicity to bacterial DNA through base modifications and strand breaks (Woodmansee and Imlay 2002).

It can be concluded that ART facilitates the bactericidal effect of antibiotics by synchronously inactivating bNOS and CAT, which provide a cost-effective strategy against lethal bacterial infections (Zeng et al. 2011). The development of more potent antibiotics should prohibit the lethal pathogens from worldwide transmission, but it is costly, time consuming, and technically difficult (Patel and Crane 2010). If an antibiotic synergist like ART could be beneficial to antibiotics for fighting bacteria, it would minimize the antibiotic dosage and cost. Importantly, antibacteria with antibiotics and synergists should impede the rapid incidence and transmission of MDR bacteria.

4.2.4 Conclusions

Oxidative stress-acclimatized bacteria were found to thrive in the presence of RIF by generating NO, which was evident from accelerated bacterial growth under RIF, but can be repressed by ART or L-NMMA. Suppressed bacterial proliferation was found to correlate with mitigated NO production upon the combination of ART with RIF/CEF. The detection of ART-heme conjugates and accordingly declined bNOS and CAT activities indicated that ART renders bacteria susceptible to antibiotics by alkylating hemoproteins. By compromising NO-mediated protection from antibiotics and triggering harmful H_2O_2 burst, ART serves as a promising antibiotic synergist for killing MDR bacteria.

4.3 In Vivo Evaluation on ART as a Synergist of Antibiotics Against Bacterial Infection

4.3.1 Proposes and Significance

Although some antibiotics and toxins might lead to bacterial DNA damage and cell death due to the induction of oxidative stress, bacteria can survive through the cytoprotective effect mediated by NO. Such effects are integrated from (1) direct detoxification; (2) CAT activation; (3) SOD induction, and (4) a suppressed Fenton reaction (Gusarov et al. 2009). In the in vitro test, we gained a partial supporting result regarding ART synergistic to antibiotics. For further evaluating the sensitization of antibiotics by ART in vivo, we combined ART with some commonly used antibiotics for antibacterial tests in mice.

As mentioned earlier, the G^+ bacterium *B. Licheniformis* should carry bNOS, so ART would inhibit its NO production. In contrast, the G^- bacterium *E. coli* should not possess bNOS, so ART cannot affect its NO production. Nevertheless, it is worthy of noting that mouse gastrointestinal tract is an extraordinarily complex organ because there has an interaction of gut microbiota with intestinal mucosa cells. First, not only bacteria produce the protective NO, but also host

cells release the bactericidal NO. Second, although NO production in G^- bacteria is unaffected by ART, G^+ bacteria among cohabitats can be influenced by ART. Third, ART can inhibit bacterial CAT, either G^+ or G^-, to exert an enhanced apoptotic role via H_2O_2.

To induce the gastrointestinal bacterial infection, we fed mice daily with live bacteria, either *B. Licheniformis* or *E. coli* collected from overnight bacterial cultures. To estimate the degree of bacterial infection, we determined mouse serum NO levels and counted the colony numbers on solid media plating with diluted stool cultures. These simple measurement methods enable the convenient investigation on the effect of ART on antibiotics' bactericidal capacity. However, the available data of serum NO levels and bacterial colony numbers were all derived from both endogenous (host residing) and exogenous (daily feeding) bacteria. To distinguish the endogenous bacteria from the exogenous bacteria, we introduced a plasmid carrying a selective marker (antibiotic resistance) into bacteria, which could mimic antibiotic resistant bacteria.

4.3.2 Results and Analysis

4.3.2.1 Indirect and Direct Monitoring of Bacterial Infection in Gastrointestinal Tracts of Mice Fed with Live Bacteria

Except for the production of NO from mouse gut microbiota, daily fed live bacteria can also activate mouse mucosa immune systems to trigger a burst of NO into mouse gastrointestinal tracts. In consequences, only a low-level (4–6 μM) NO was measured in the serum of control mice, whereas a high-level (22–25 μM) NO was determined in the serum of mice after feeding with *E. coli* for three days. These results implied that live bacterial feeding, even nonpathogenic, can lead to bacterial infection, macrophage activation, and NO production. Therefore, a raised serum NO level may represent an indirect index indicating bacterial infection.

To directly monitor the gastrointestinal bacterial infection, we established a correlation of bacterial infection with colony formation upon plating a fecal dilution of mice fed with bacteria. After cultured overnight on the plate containing AMP (100 μg/ml), we did observe a few colonies occurring on the fecal plate prepared from bacteria-fed mice, whereas no colony was formed on the fecal plate prepared from control mice. The combined use of a plasmid-harboring bacterial strain with selective cultural plates ensures only fed bacteria rather than host bacteria forming colonies because the plasmid carries AMP resistance gene (Amp^r).

4.3.2.2 In Vivo Study in Mice Fed with *E. Coli*

By overnight incubation, no colony occurs on the plate covering a layer of fecal dilution prepared from *E. coli*-fed mice injected with 100 μg/ml AMP + 60 μg/ml

ART, or 200 μg/ml AMP + 60 μg/ml ART. In contrast, 30 or 3 colonies form on the fecal plate that covers a layer of stool dilution prepared from *E. coli*-fed mice injected with 100 or 200 μg/ml AMP. These results indicated that AMP with combination with ART cannot allow colony growth, highlighting that ART is able to potentate the antibacterial capacity of AMP in vivo.

Even AMP-resistant bacteria that carry Amp^r plasmids were used for feeding, only a few colonies are formed from the fece of mice injected with CEF (25 μg/ml), whereas no colony appears from the fece of mice injected with CEF (25 μg/ml) + ART (60 μg/ml). It was likely that ART can potentate CEF via mitigating the NO-mediated protection, but we cannot, at this moment, definitely exclude a possibility of ART exerting the direct cytotoxicity.

4.3.2.3 In Vivo Study in Mice Fed with *B. Licheniformis*

The daily feeding of mice with *B. Licheniformis* also elicits NO production in mice, in which threefold increase of NO levels were observed after bacterial feeding for three days. In control mice, the serum NO level is only 1.88 ± 0.242 μM, but in the bacteria-fed mice, the serum NO level is 6.67 ± 2.42 μM. It was also noted that 20 μg/ml RIF fails to decline the NO level (8.21 ± 2.42 μM), whereas 40 μg/ml RIF can decline the NO level (3.42 ± 2.42 μM). Interestingly, the NO level (3.42 ± 2.42 μM) in bacteria-fed mice injected with RIF (40 μg/ml) is equivalent to the NO level (3.93 ± 1.21 μM) in bacteria-fed mice injected with RIF (20 μg/ml) + ART (60 μg/ml). These results suggested that ART can synergize RIF for more effective antibacteria and decrease the dosage of RIF.

4.3.3 Discussion

One possibility of NO becoming cytotoxic is when it meets O_2^-. NO and O_2^- interact to form $ONOO^-$, one of the powerful RNS toxics to living cells. Alternatively, peroxynitrous acid that also kills cells can form as a result of the interaction between nitrite and H_2O_2 under mildly acidic conditions (Kono et al. 1994). The induction of NOS in phagocytes is only one form of host defense reliant upon NO (Vallance and Charles 1998).

Our in vivo tests ascertained that ART + AMP, ART + CEF, or ART + RIF can suppress the bacterial infection occurring in mouse gastrointestinal tracts. During the antibacterial process, a combination of ART with one kind of antibiotics can remarkably decline the serum NO levels. Why NO is protective for bacteria? A recent research might answer this question, in which NO has been described to modulate bacterial biofilm formation through a multicomponent cyclic-di-GMP signaling network (Plate and Marletta 2012).

In theory, the decline of NO levels may be resulted either from bNOS or from iNOS because ART inhibits all kinds of NOS without discrimination. While bNOS-derived low-level NO can protect bacteria, iNOS-derived high-level NO must kill bacteria. Although ART can help antibiotics to kill bacteria through blocking the protective NO from bNOS, it can also decrease the bactericidal NO from iNOS. Fortunately, ART can also elevate the H_2O_2 level by inhibiting CAT, which directly causes bacterial death. Therefore, a net outcome of ART-antibiotic combination is beneficial to enhance antibiotics' antibacterial capacity.

In summary, ART synchronously inhibits bNOS and CAT, which can cause the decrease of protective NO and simultaneous increase of bactericidal H_2O_2 in G^+ bacteria. In G^- bacteria without bNOS, ART still inhibits CAT and increases H_2O_2, which also enhance antibiotic toxicity because $ONOO^-$ accumulation from the reaction of NO with O_2^- would be augmented.

4.3.4 Conclusions

By choosing *B. licheniformis* and *E. coli* as representative G^+ and G^- bacteria, and through stool culture and colony counting or serum NO determination, we evaluated the in vivo sensitization of antibiotics by ART in mice with gastrointestinal infections via daily live bacterial feeding. While bacteria protect themselves from antibiotics through releasing NO, ART enhances the sensitivity of bacteria to antibiotics upon inhibiting bNOS and CAT. Consequently, ART can accelerate the antibacterial efficiency of CEF and RIF against bacteria. Importantly, ART can assist AMP to repress the propagation of drug tolerant bacteria, which should release an exciting news for helping combat superbug's antibiotic resistance.

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Chapter 5
ART for Anti-inflammation

Abstract Chronic/acute synovitis, an early phase of RA, can be experimentally induced in mice by autoantigen/bacterial antigen challenging and live bacterial feeding. While a donor of NO replicates the modeling of synovitis, an inhibitor of NOS blocks the progression of synovitis, suggesting inflammation-triggered NO burst represents an etiological cause of synovitis. ART, via inhibiting iNOS, can ameliorate the synovial inflammation by mitigating NO-driven hypoxia, angiogenesis, and hyperplasia.

Keywords ART · Antigen · Bacteria · NO · Synovitis

5.1 An Overview on Synovitis and Antisynovitis

RA is a chronic and progressive inflammatory disease that mainly destroys cartilage and bone (Toes and Huizinga 2009). RA is recognized initially by synovial inflammation (synovitis) in multiple joints, and eventually leading to the localized destruction of articulates (arthritis). RA also affects lungs, pleura, pericardium, sclera and subcutaneous tissue, so patients with RA have a raised risk developing cardiovascular diseases, such as arteriosclerosis and myocardial infarction (van Zonneveld et al. 2010). In a histopathological view, RA is characterized by a pronounced synovial hyperplasia, or called the pannus, a thickened membrane-like covering of the inflammatory granulation tissue over the articular cartilage. Like a malignant tumor, the pannus can invade and destroy cartilage and bone by secreting matrix proteases such as metalloproteinases and aggrecanases (Laragione and Gulko 2010). The synovitis-associated synovial hyperplasia and pannus formation can also promote the excretion of proinflammatory cytokines (Smith 2011).

Although the etiological cue initiating RA remains undefined, several kinds of disease-modifying antirheumatic drugs have been applied to the clinical treatment of RA for over 60 years. There was a significant explosion of such

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symptom-targeted disease-modifying antirheumatic drugs (DMARDs) in the last decade. Recently, the monoclonal antibody-based biological antirheumatic agents (BARAs) that block tumor necrosis factor α (TNFα), including etanercept, infliximab, adalimumab, golimumab, and certolizumab, have been licensed for clinical use (Schett et al. 2011; Tak and Kalden 2011). Although anti-TNFα therapies allow the disease improvement and joint erosion prevention in patients with RA, nearly 40 % patients who accept TNFα antibodies are nonresponders. Importantly, inactivation of TNFα will interfere with the innate immune defense and predispose an elevated risk of the pathogenic infection (Walsh et al. 1991; Rahman and McFadden 2006; FDA alerts 2011). Moreover, joint repair and erosion healing rarely occur in spite of an intensive treatment by TNFα inhibitors (Smolen et al. 2007; Biniecka et al. 2011; Schett et al. 2011). The therapeutic regimens that are sensitive, effective, and suitable for nonresponders are currently unavailable, so further endeavors aiming at the discovery of novel prophylactics and therapeutics for RA are urgently needed.

It is understandable that microbial pathogens can stimulate the release of TNFα for orchestrating the antimicrobial response. It is also reasonable that TNFα blockers or antagonists can ameliorate RA if the onset of RA is attributed to microbial pathogens. In fact, microorganisms are believed to cause many rheumatic diseases, even though currently no evidence supports such an involvement (Sherbet 2009). Most recently, however, a novel finding about the relevance of an autoimmune disease to bacterial infection has changed this situation. The commensal segmented filamentous bacteria can drive autoimmune diseases in K/BxN mice, which are abrogated under bacteria-free conditions, but restored after colonization with bacteria (Wu et al. 2010). It was suggested that gut microbiota-induced interleukins might spill into systemic circulation and promote autoimmune attacks at distant sites such as joints (Cua and Sherlock 2011). We further assumed that gut bacterial infection-induced interleukins and other proinflammatory cytokines might be linked to the inflammatory articular lesions including the early phase synovitis and the late-phase arthritis.

We argue, however, alternative inducer(s) must exist as initiator(s) of pannus formation because interleukins are unlikely relevant to the tumor-like hyperplasia. A central role of NO in the pathogenesis of RA has been suggested, although an underlying mechanism they mentioned is restricted in NO-mediated immune dysfunction (Nagy et al. 2007, 2010). The clinical data indicated that the inflamed synovium is a predominant site generating NO, and T cells in patients with RA produce 2.5 times more NO than healthy T cells (Farrell et al. 1992; Nagy et al. 2008). Further evidence shows that TNFα blockade leads to a downregulation of iNOS in human peripheral blood mononuclear cells (Perkins et al. 1998). An engineered peptide of the growth factor progranulin, Atsttrin, ameliorates mouse inflammatory arthritis through binding to TNF receptors (TNFR) and inhibiting TNFα-dependent NO production in macrophages (Tang et al. 2011). The triptolides extracted from *Tripterygium wilfordii* Hook F are effective for treatment

of experimental arthritis, probably by inhibiting iNOS (Wang et al. 2004). From above results and other references regarding bacterial infection-induced iNOS in human neutrophils (Wheel et al. 1997) and NO-driven angiogenesis (Lala and Chakraborty 2001; Muntan and De la Mat 2010), we proposed that NO might be a candidate mediator initiating synovial hyperplasia.

Owing to synovial hyperplasia resembling tumor malignancy, it can be expected to halt the synovial hyperplasia by administering apoptosis inducers. Indeed, treatment of the adjuvant-induced rat arthritis by phytol, an inducer of apoptosis, blocks both acute and chronic phases of arthritis by increasing oxidative stress and decreasing autoimmune responses (Hultqvist et al. 2006). On the other hand, ART is also evident to induce apoptosis of tumor cells (Efferth et al. 2001, 2003; Du et al. 2010), so it is reasonable to use ART as an antiarthritic agent. The potential of ART in antitumor has been convinced from the finding that ART inhibits the proliferation of fibroblast-like synoviocytes by blocking the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the P13 kinase/Akt signal pathway (Motzer et al. 2008). ART also downregulates the angiogenesis-related functional factors, such as HIF-1α, and vascular endothelial growth factor (VEGF) (Hess et al. 2009). Besides, ART is also known to ameliorate the experimental arthritis in DBA/1 mice by suppressing an inflammatory Th17 response (Yao et al. 2011).

The mammalian target of rapamycin (mTOR) is upregulated in several types of cancer (Xu et al. 2007; He et al. 2009; Wang et al. 2008), and the mTOR inhibitor RAP (sirolimus or everolimus) has been approved for treatment of advanced renal cell carcinoma (An et al. 2009), refractory mantle cell lymphoma (Zhou et al. 2010), and progressive advanced pancreatic neuroendocrine tumors (Pantuck et al. 2007). From this, we could predict that RAP is also effective on RA. Indeed, it has been reported that the mTOR inhibitor sirolimus induces osteoclasts and suppresses bone resorption through inhibiting the phosphorylation of S6 protein, S6 kinase, and 4E-BP1 in osteoclasts (Glantschnig et al. 2003). Other reports on RAP's antiarthritis have also demonstrated as follows: (1) Everolimus prevents ovariectomy-induced bone loss in rats via inhibiting cathepsin K expression and osteoclasts activity (Kneissel et al. 2004). (2) RAP decreases the articular invasion by fibroblast-like synoviocytes in pristine-induced arthritis rats, resulting from an attenuation of the phosphorylation of mTOR and substrates, p70S6K1 and 4EBP (Laragione and Gulko 2010). (3) Sirolimus or everolimus reduces synovial osteoclast formation and protects against local bone erosions and cartilage loss by downregulating digestive enzymes and enhancing osteoclast apoptosis in human TNF-transgenic mice with a chronic inflammatory and destructive arthritis (Cejka et al. 2010).

We would introduce some findings that might help to answer the questions involving the unsolved mechanisms causing RA: what is the de novo etiological initiator of RA? Whether such an initiator can be used to replicate RA? Are there alternative therapeutic options targeting the pathogenic initiator?

5.2 ART Mitigates Bacteria/Collagen-Induced Synovitis

5.2.1 Purposes and Significance

Although the monoclonal antibody against TNFα is approved for clinical use in RA patients, the desired therapeutic regimens suitable for nonresponders are still unavailable because etiological initiators leading to RA remain enigmatic and unidentified. Therefore, previously pooled data on the pathogenesis of RA are far from an entire revelation of primary initiators leading to RA, and all current therapies tackling RA cannot eradicate the inflammatory origin.

We assumed that a primitive initiator of RA is probably the sustained pathogen infection-activated immune responses, which lead to the large-scale production of proinflammatory cytokines. In turn, a chronic inflammatory state can activate iNOS, thereby triggering potent NO burst, and can eventually driving synovial hypoxia, angiogenesis, and hyperplasia. Until recently, however, cumulative evidence concerning NO-induced tumor-like synovial hyperplasia is trivial, and the bona fide mechanism behind how NO drives synovial hyperplasia remains completely undefined.

To figure out a possible association of gastrointestinal bacterial infection with the inflammatory synovitis, we established a mouse model of bacteria-induced arthritis (BIA) that simulates collagen-induced arthritis (CIA) by daily live bacterial feeding. The dynamic changes of serum NO and LA levels, along with the saturation percentages of O_2 (SpO_2) were compared between BIA and CIA mice. Furthermore, we quantified 40 kinds of proinflammatory cytokines and angiogenesis-relevant HIF-1α and VEGF during modeling. The NO donor compound SNP was used to replicate the acute mouse synovitis seen in BIA and CIA. Finally, we explored whether synovial inflammation could be compromised when the antibiotic CEF and/or the immunosuppressant RAP or ART were administered.

The present study pays attention to the elucidation of whether a sustained gastrointestinal bacterial infection would represent one of the pathogenic initiators toward RA, and to answer how NO could serve as a pivotal signal that conveys bacterial infection to articular inflammation. On the basis of elucidating the pathogenesis of RA, we suggested a novel pathogenesis-based therapeutic strategy for RA.

5.2.2 Results and Analysis

5.2.2.1 Correlation of Inflammatory Synovitis with Synovial Hyperplasia and Lymphocytic Infiltration

After four-week daily feeding with the overnight cultures of nonpathogenic *E. coli* DH5α bacteria (about 10^8), BIA mice display some morphological changes, such as red and swollen paws, manifesting a classic character of the early phase inflammatory arthritis. In similar, intradermal injection with collagen type II-complete

Freund's adjuvant (CII-CFA) also leads to paw redness and swelling within four weeks in CIA mice. Although it seems that CIA mice develop the inflammatory symptoms more significant than BIA mice, establishment of such a novel experimental model with a typical, albeit mild, inflammatory synovitis/arthritis phenotype might help elucidate the pathogenic mechanism of RA.

To further identify the pathological alterations occurring in the synovium, we conducted the histopathological analysis parallelly in BIA and CIA mice. From the histochemical staining of tissue sections prepared from the inflamed synovial and articular tissues, we did not found any forms of the articular damage and subintimal fibrosis, but observed some extents of the multilayer intimal hyperplasia and mild synovial infiltration. The lymphocytic infiltration is nearly identical in BIA and CIA mice, but a higher degree of intimal hyperplasia occurs in CIA mice. The phenotypical manifestations of inflammatory lesions are, therefore, mirrored by histological alterations in the synovial hyperplasia.

An alternative mouse synovitic model, so-called BIA-CIA mice, that was established by live bacterial feeding and synchronous intradermal CII-CFA injection, was found to eventually develop a typical synovitis after four weeks, but with only mild morphological lesions and less histological damage than CIA mice. Cotreatment of mice by intra-articular CII-CFA injection with live bacterial feeding also lead to the alleviated synovial inflammation and histological alterations within three days. While CIA mice show the more severe lymphocytic infiltration, BIA-CIA mice exhibit the mild lymphocytic infiltration. These results implied that there might be immune suppression or immune protection in BIA-CIA mice, which could be originated from the inhibition of innate immune functions by bacterial products.

5.2.2.2 Global Cytokine Upregulation and Enhanced NO Production upon Bacterial Infection and/or CII-CFA Immunization

To follow up the immunological profile of inflammatory pathogenesis, we analyzed as many as 40 kinds of proinflammatory cytokines among modeling mice. As results, almost all proinflammatory cytokines are upregulated in BIA and CIA mice, among which IFNγ, interleukins, and colony-stimulating factors are significantly upregulated. However, BIA-CIA mice show an overall downregulation of proinflammatory cytokines including TNFα, IFNγ, IL-1β, IL-6, and IL-10. These results revealed a direct association of the up/downregulation of proinflammatory cytokines with the enhancement/attenuation of inflammatory lesions.

To monitor the dynamic change of NO during bacterial feeding and/or CII-CFA injection, we determined the time course fluctuations of serum NO levels in modeling mice. As results, bacterial feeding allows the gradual elevation and maintenance of steady-state high NO levels, whereas CII-CFA injection leads to the formation of double NO peaks, occurring immediately after the primary challenging on the 1st day and boosting on the 21st day. Interestingly, BIA-CIA mice also exhibit the double NO peaks similar with CIA mice, but the NO levels are

relatively lower than CIA mice. The maximal NO level was detected in CIA mice (40 mM) than in BIA-CIA mice (25 mM) or BIA mice (20 mM) during modeling. These results clarified that CII (alloantigen) and live bacteria or dead bacteria in CFA (xenoantigen) can more or less produce NO.

5.2.2.3 NO-Correlated Hypoxic Consequences Following Infection or Immunization

To confirm the possibility of potent NO burst leading to hypoxia, we directly monitored a fluctuation of SpO_2 in modeling mice with erythematous and edematous paws. A dramatic decrease of SpO_2 was determined within the hind legs of CIA mice, whereas only a slight decrease of SpO_2 was measured in those of BIA-CIA and BIA mice. Interestingly, a lower SpO_2 was found to perfectly correlate with a higher NO level and vice versa. For instance, 7 mM NO corresponds 80 % SpO_2 in control mice, while 17 mM NO accords 50 % SpO_2 in CIA mice. Control mice have a lower NO level (7.14 mM) and a higher SpO_2 (83.5 %), but BIA mice have a higher NO level (11.03 mM) and a lower SpO_2 (69.5 %) (Fig. 5.1).

To find out further evidence confirming NO-driven hypoxia, we evaluated whether NO correlates with lactic acid (LA) in modeling mice. Consequently, a higher NO level (10 μM) has relevance to a higher LA level (12 mM) in mice fed with bacteria for 28 days or injected by CFA for two days. In control mice, both NO and LA kept the stable levels. These results indicated that NO-driven hypoxia might promote the anaerobic degradation of carbohydrates by glycolysis, leading to the accumulation of LA in blood and tissues.

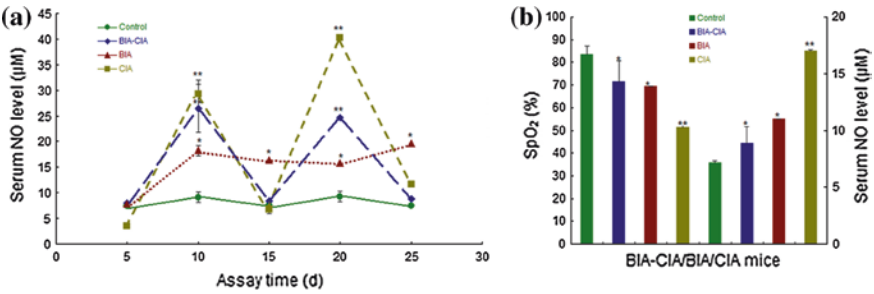

Fig. 5.1 Dynamic monitoring of NO production and comparative analysis of relationship between NO and SpO_2 in BIA, CIA and BIA-CIA mice. **a** Time course detection of serum NO levels in model mice. *BIA* bacteria-induced arthritis; *CIA* collagen-induced arthritis; *NO* nitric oxide; *SpO_2* saturation percentages of O_2. Sampling and detection were conducted every five days and until 25 days during modeling. **b** Measurement of SpO_2 and NO in BIA, CIA, and BIA-CIA mice. Sampling and detection were conducted after modeling for three days in CIA and BIA-CIA mice or after modeling for 28 days in BIA mice. The *singular asterisk* (*) represents statistically significant difference from the control ($p < 0.05$), and *double asterisks* (**) indicate statistically very significant difference from the control ($p < 0.01$)

Furthermore, we quantified the expression of angiogenesis-responsible and hypoxia-induced genes in CIA mice. In consequences, we noticed the upregulation of HIF-1α and VEGF in the synovial tissue of mice after intra-articular CFA injection. Also, the positive staining against HIF-1α or VEGF was remarkably enhanced in the hypodermal tissue injected with 20 μg (100 μg/ml) SNP, accounting for 7-fold increases for HIF-1α staining and 4-fold increases in VEGF staining. From these results, it is clear that either endogenous or exogenous NO can equally induce the synovial angiogenesis via upregulating HIF-1α and VEGF.

5.2.2.4 Replication of Inflammatory Synovitis by Administration of SNP

To address NO as an initiating factor of inflammatory synovitis, we simply administered mice with SNP by intra-articular injection. Fascinatingly, a single injection with SNP can lead to the significant edema on the paw after only one day. As expectation, coinjection with a mixture of SNP and CII-CFA induces a severe inflammatory phenotype, confirming that NO legitimates a causative effector leading to inflammatory lesions. The exogenous NO-induced acute synovitis, therefore, represents a rapidly constructed early phase model of RA in mice.

As a critical hypoxic parameter, SpO_2 is very low (55–57 %) in mice after injection with SNP regardless of treatment with or without CII-CFA. At the same time, we also found that CII-CFA induces the moderate SpO_2 (62–66 %), which are lower than control mice (82 %). It is worthy of noting that SpO_2 seems not to correlate the severity of inflammation. For example, we saw that the inflammatory signature is more severe in CII-CFA-injected mice with high SpO_2 (62 %) than SNP-injected mice with lower SpO_2 (55–57 %). These results implied that CII-CFA-triggered immune activation, in addition to NO, should be implicated in the acceleration of inflammatory progression.

5.2.2.5 Abrogation of NO Production and Termination of Hypoxic Induction Through Antibacteria and/or Inhibiting INOS

To validate bacterial infection as the origin of NO generation, we determined the serum NO level in BIA mice after subcutaneous injection of CEF, ART, or ART + CEF for three days (twice a day). As results, either treatment can considerably decline the NO level lower than the control. Conceivably, ART declines the NO level because it inhibits iNOS even though infection still exists. CEF allows an equal NO level to the control because it kills bacteria and blocks infection. The combined treatment leads to a lower NO level owing to the dual effects of CEF-suppressed infection and ART-inhibited NO production. These results verified that persistent gastrointestinal infection and potent NO burst can be abrogated by antibacteria and/or iNOS inhibition.

We can conveniently evaluate the therapeutic efficacy of CEF, phytol, alcohol, or a drug combination on BIA by measuring the hypoxic parameters, including NO, LA, and SpO_2. From these measurements, we noticed that CEF injection or diluted alcohol drinking can eradicate the hypoxia-derived consequences, but phytol only partially normalizes the hypoxic parameters (Table 5.1).

In CIA mice established by intradermal or intra-articular injection of CII-CFA, on the other hand, administration of ART or RAP enables a dramatic decrease of the NO level, even lower than the control. A combination of ART with RAP, or RAP with alcohol in BIA-CIA mice leads to the considerable repression of NO production. In particular, an extremely lower NO level (0.448 mM) was detected in an ART-injected CIA mouse. In contrast, untreated BIA-CIA and CIA mice exhibit higher NO levels, up to 10 mM in the maximum.

Intriguingly, CIA mice without any treatment can give rise to a low NO level almost equal to that of the control, which might be attributed to the decay of CII-CFA-triggered NO after a longer duration of postimmunization. Actually, three weeks have been passed from CII-CFA boosting to NO detection.

Table 5.1 Measurement of hypoxic parameters for evaluation of potential antiarthritic drugs in BIA mice

Treatment	NO (μM)	LA (mM)	SpO_2 (%)
Control	7.15 ± 0.30	7.88 ± 0.13	83.00 ± 1.83
BIA (nontreatment)	9.87 ± 0.48*	12.58 ± 0.19**	70.50 ± 2.08*
Injection of BIA with 15 μg/ml CEF for 3 days	2.97 ± 0.13**	7.83 ± 0.07	80.50 ± 0.71
Injection of BIA with 15 μg/ml CEF for 5 days	3.12 ± 0.15**	7.91 ± 0.05	82.50 ± 0.71
Injection of BIA with 60 μg/ml phytol for 3 days	5.14 ± 0.13*	6.79 ± 0.05	69.50 ± 0.71*
Injection of BIA with 60 μg/ml phytol for 5 days	4.62 ± 0.15*	11.50 ± 0.05**	78.50 ± 0.71
Injection of BIA with 15 μg/ml CEF + 60 μg/ml phytol for 3 days	7.57 ± 0.26	14.36 ± 0.05**	75.50 ± 0.71
Injection of BIA with 15 μg/ml CEF + 60 μg/ml phytol for 5 days	4.84 ± 0.15*	13.57 ± 0.05**	80.50 ± 0.71
Injection of BIA with 60 μg/ml phytol and feeding with 15 % alcohol for 3 days	7.30 ± 0.13	6.79 ± 0.05	76.50 ± 0.71
Injection of BIA with 60 μg/ml phytol and feeding with 15 % alcohol for 5 days	5.91 ± 0.15*	8.32 ± 0.05	76.50 ± 0.71

CEF cefotaxime (subcutaneous injection); *LA* lactic acid; *NO* nitric oxide; *SpO_2* saturation percentages of O_2. Measurements were conducted in BIA mice after drug administration for 3 days or 5 days. The injected volume of each drug or a drug combination is 200 μl in all groups of treatment. The *singular asterisk* (*) represents statistically significant difference from the control ($p < 0.05$), and *double asterisks* (**) indicate statistically very significant difference from the control ($p < 0.01$)

5.2.2.6 Evaluation of ART and/or RAP-Mediated Amelioration of Synovial Inflammation

According to the morphological and histochemical identifications after treatments, it can be confirmed that articular synovitis would be ameliorated in antiarthritic drug-treated mice, although their merits are not on an average level. In mice with synchronous intra-articular CII-CFA injection and ART or RAP administration, no intimal hyperplasia and subintimal fibrosis occur in the synovial tissue of mice. However, dispersed synovial infiltration by inflammatory lymphocytes was observed in ART-treated mice, but not in RAP-treated mice. These results indicated that ART as an iNOS inhibitor only suppresses hyperplasia, while RAP as an immunosuppressant that blocks immune activation-dependent NO production can ameliorate synovial hyperplasia and lymphocytic infiltration.

Among CIA mice, postmodeling treatment by ART and RAP aborts both intimal hyperplasia and subintimal fibrosis, but fails to block the mild and dispersed inflammatory infiltration into synovial tissues by mononucleated cells. Coadministration of CIA mice with RAP and alcohol cannot hamper the synovial progression to local hyperplasia and lymphocytic infiltration. Postmodeling injection of BIA-CIA mice with ART and RAP abolishes the subintimal fibrosis and inflammatory infiltration, but intimal hyperplasia cannot be blocked completely. In contrast, RAP and alcohol can remit synovial hyperplasia, but do not inhibit the eventual progression to the mild and dispersed inflammatory infiltration.

All above results from different regimens demonstrated that pretreatment prior to NO generation is more effective than posttreatment after NO generation. In other words, synovial damage made prior to drug administration cannot be ameliorated by any regimens of posttreatment. These results conclusively indicated that NO should represent one of the most important initiators leading to RA-like synovitis and arthritis. NO is mainly responsible for synovial hyperplasia, so NO-initiated synovial lesions may be irreversible by iNOS inhibitors.

5.2.3 Discussion

Through pathogenic infection-triggered NO production, iNOS is involved in an immune attack against active invaders. For example, bacterial infection of human colon epithelial cells has been reported to allow upregulated iNOS expression and enhanced NO production (Witthoft et al. 1998). Apart from the gastrointestinal infection following live bacterial feeding, immunization of mice with CII-CFA also provokes potent NO burst, hence suggesting that NO production is dependent on the immune activation regardless of the pathogenic infection. This is the reason why live bacterial feeding and CII-CFA injection can equally induce the synovial inflammation.

The modeling of RA in mice generally requires the heat-killed *M. tuberculosis*-containing CFA in addition to CII, implying that anti-CII responses are

insufficient to induce the classic arthritic lesions in mice. Some authors thought that anti-CII reactivity might be a consequence of inflammation rather than the cause (Courtenay et al. 1980). This is the reason why so many kinds of autobodies can be detected in the blood of RA patients or experimental arthritic rodents, such as those against citrullinated proteins, glucose-6-phosphate isomerase, integrin, and fibrin, etc. (McDevitt 2000; Wilder 2002; Humby et al. 2009). So we assumed that CII is likely dispensable for modeling RA in mice. Indeed, we successfully induced acute arthritis in mice by intra-articular injection with CFA alone instead of CII-CFA. Therefore, any immune activators including autoantigens and bacteria, either live or dead, can evoke the immune responses and make the inflammatory lesions by provoking the NO-driven hypoxia.

The mucosal response to an enteric infection includes the production of chemoattractant cytokines (chemokines), anti-inflammatory cytokines, and proinflammatory cytokines, in which TNFα is an important proinflammatory cytokine that amplifies the epithelial immune response to the bacterial infection (Eckmann and Kagnoff 2005). TNFα, IL-1β, and other proinflammatory cytokines can upregulate iNOS in chondrocytes and synovial cells of osteoarthritis (Abramson 2004). In the present study, we observed a global activation of chemokines, cytokines, and corresponding receptors in BIA and CIA mice, which drives a complicated and chronic inflammatory process (Cejka et al. 2010). The unexpected outcome that TNFα is downregulated in CIA mice seems puzzling, but it is most likely because the attenuation of immune responses to CII-CFA after several weeks of immunization. In fact, sampling for cytokine chip profiling was carried out after 28 days of modeling.

Although the implication of NO in the pathogenesis of experimental arthritis was poorly understood, CII-CFA-triggered NO burst has been noticed in CIA mice (Cannon et al. 1996). We have observed the formation of double NO peaks after primary challenging and boosting with CII-CFA, and also noticed a stable NO level higher than the control during daily live bacterial feeding. Albeit in distinct manners, either CII-CFA or bacteria can provoke NO production, permanently or transiently, in BIA and CIA mice. Surprisingly, we also found that cotreatment by CII-CFA with bacteria allows a global downregulation of proinflammatory cytokines, suggesting the bacteria-conferred suppression to CII-CFA-activated immune responses. In consistence with our findings, other authors have previously reported that *Schistosoma japonicum* infection can significantly attenuate the clinical signs, reduce the histological damages, alter the humoral immune responses, and inhibit the splenocyte proliferation in CIA mice (Song et al. 2011).

Additionally, immunosuppression by bacteria has been addressed in a recently published review (Kelly et al. 2012). Because arthritic development is mostly dependent on the systemic immune activation, immunosuppression can, of course, alleviate the inflammatory arthritis. RAP is a well-known immunosuppressant that reduces pannus formation, cartilage erosion, and joint damage in rats with adjuvant-induced arthritis (Teachey et al. 2009). ART also plays an antiarthritic role in CIA mice (Wang et al. 2008). Even though the pharmacological mechanisms of RAP and ART as arthritic therapeutics are thought to be multifaceted, it is

unambiguous that both drugs can suppress NO production, and administration of RAP and/or ART can effectively block the onset of synovitis. So there should be an association between the increase of arthritic inflammation and the decrease of NO production.

Antibacteria by CEF, anti-inflammation by diluted alcohol, proapoptosis by phytol, or a combination of multidrugs in live bacterial feeding mice improves the inflammatory feature of synovial tissues, which could be reflected by the normalization of hypoxic parameters. CEF can dramatically decrease those hypoxic parameters to the values comparative to the control. Phytol, alcohol, or their combinations also normalize, more or less, the hypoxic parameters. Support evidence of alcohol beneficial to antiarthritis is from a clinical cohort demonstrating that alcohol consumption is inversely associated with the risk and severity of RA (Maxwell et al. 2010). On the other hand, phytol as an oxidative burst inducer was used for treatment of experimental arthritis in rats, by which autoimmune responses are suppressed and both acute and chronic arthritis ameliorated (Hultqvist et al. 2006).

We observed the remarkable synovial hyperplasia and angiogenesis from the histochemical analysis of articular sections. Angiogenesis is an early event in the inflammatory joint, which enables the activated monocytes entering the synovium and expanding them throughout a pannus via the recruitment of endothelial cells, eventually resulting in cartilage degradation and bone destruction (Kennedy et al. 2010). Hypoxia can induce the expression of angiogenesis-related genes including HIF-1α and VEGF (Kasuno et al. 2004). NO can also activate HIF-1α under the normoxic conditions (Natarajan et al. 2003). So we concluded that NO is eligible as a hypoxic inducer capable of initiating angiogenesis and hyperplasia.

The synovium itself is a relatively hypoxic tissue, in which O_2 tension in cartilage ranges from 7 % (53 mmHg) in the superficial layer to less than 1 % (7.6 mmHg) in the deep zone (Fermor et al. 2007). NO can also accelerate its own consumption by increasing its entry into red blood cells (Han et al. 2003). NO inhibits the mitochondrial enzyme COX in competition with O_2, leading to so-called a "metabolic hypoxia" situation, in which cells cannot use O_2 although it is available (Xu et al. 2005). High levels of NO inhibit cell respiration by binding to COX, whereas slow and small-scaled NO release can stimulate mitochondrial biogenesis in diverse cell types (Nisoli and Carruba 2006). Our results also indicated that high NO levels are correlated with low SpO_2, hence validating NO conveying signals for angiogenesis and hyperplasia.

Due to the hypoxic induction, blood sugars are anaerobically catabolized and necessarily converted to LA by glycolysis, which can be accumulated in the bloodstream unless O_2 supply is rehabilitated. By monitoring the dynamic changes of NO and LA, we observed a proportional fluctuation of NO with LA in arthritic modeling mice. The high LA level is a new and simple parameter for quantifying hypoxia and indicating transversion from normoxia to hypoxia. Furthermore, it is known that hypoxia can activate HIF-1α, which in turn binds to the promoter of downstream hypoxia-inducible genes such as *VEGF* for starting transcription and translation (Olson and van der Vliet 2011). We detected the overexpression of

HIF-1α and VEGF in the inflamed synovial tissue of CIA mice. When SNP was injected into the hypoderm of mice, HIF-1α and VEGF are expressed in higher levels, thereby confirming a relevance of NO-driven overexpression of HIF-1α and VEGF with synovial angiogenesis during hyperplasic induction.

In view of different roles playing by NO and proinflammatory cytokines, we believe both of which are likely important in the initiation and progression of inflammatory arthritis in mice. But it is possible that NO is mainly responsible for synovial hyperplasia, whereas proinflammatory cytokines are apparently relevant to inflammatory infiltration. NO may induce synovial angiogenesis and hyperplasia by hypoxia, and can subsequently guide proinflammatory cytokines penetrating deeply into the synovium along with the newborn blood vessel (Ng et al. 2010). Our results indicated that NO promotes synovial angiogenesis by activating HIF-1α and VEGF, but then enhanced angiogenesis mitigates glycolysis. These results would become a solid basis for future arthritic treatment by inhibiting NO-driven angiogenesis. Currently, published data have demonstrated that anti-VEGF treatment by bevacizumab reduces blood supply, increases glycolytic metabolites, and promotes tumor metastasis in glioblastoma (Keunen et al. 2011), underlining that no amelioration would be reached if inflammation-originated hypoxia is not yet alleviated.

Our study has answered a long-term unanswered question about the association of distal or systemic infection with inflammatory arthritis: gastrointestinal infection can serve as an etiological initiator of inflammatory arthritis by dually upregulating proinflammatory cytokines that allow lymphocytic infiltration and triggering NO to drive synovial hypoxia and hyperplasia. These achievements should shed light on the prophylactic and therapeutic interventions of RA and other human autoimmune diseases in the future.

5.2.4 Conclusions

BIA that simulates CIA was developed in mice upon daily live bacterial feeding. The morphological lesions of paw erythema and edema together with the histological alterations such as synovial hyperplasia and lymphocytic infiltration emerge as the early phase manifestation of RA. Bacteria and collagen induce the global upregulation of proinflammatory cytokines, accompanying with the elevation of serum NO levels and decline of SpO_2. NO-driven hypoxia is evident from the accumulation of LA, an end product from glycolysis. Upregulation of HIF-1α and VEGF validates hypoxia-induced angiogenesis. The administration of SNP also causes articular inflammation by inducing synovial hypoxia. Antibacteria by CEF and/or immunosuppression by RAP or inhibition by ART can abrogate NO production, mitigate hypoxia, and considerably ameliorate or even completely abort synovitis, hence highlighting NO may serve as an initiator of inflammatory arthritis. Taken together, bacteria can mimic collagen to enable synovial lesions via upregulating proinflammatory cytokines, triggering NO production, driving

hypoxic responses, and inducing synovial angiogenesis and hyperplasia, suggesting sustained infection might be, in part, responsible for the onset of synovitis and arthritis in mice.

5.3 ART Alleviates Adjuvant/LPS-Induced Synovitis

5.3.1 Purposes and Significance

The conventional arthritic models in rats or mice are usually constructed by intradermal injection of the mixture of CII with CFA, which is CII-CFA-induced chronic arthritis (CICA). However, a previous research has shown that anti-CII reactivity is a consequence of inflammation rather than the cause (Courtenay et al. 1980). Our study also implies that CII may be dispensable for the modeling of RA because SNP as an exogenous NO donor can replicate the early phase morphological features of CICA. So we used CFA instead of CII-CFA to establish a new kind of mouse arthritic model designated as CFA-induced acute arthritis (CIAA). Considering the major immunogen of CFA is the preparations of dead bacteria, we also attempt to establish another mouse arthritic model by the purified bacterial LPS denominated as LPS-induced acute arthritis (LIAA).

Because synovitis is characterized by the tumor-like hyperplasia, we suggested a novel concept of 'treating synovitis as tumor' (Wu et al. 2012). For this purpose, we choose three kinds of candidate antitumor drugs, ART, RAP, and BLA for treatment of acute mouse synovitis. As references, we would replicate the acute arthritic model by injecting SNP. Additionally, we also use the iNOS inhibitor L-NMMA to block the CFA-triggered NO burst and correlate this enzyme inhibition to tissue protection, thereby confirming that immune responses can elicit the synovial inflammation involving NO.

We expected that the in vivo pharmacological assessment of ART, RAP, and BLA would help to confirm the tumor-like nature of synovitis and arthritis, and it should benefit to further evaluation for the clinical treatment of tumor/cancer and RA in the future.

5.3.2 Results and Analysis

5.3.2.1 Morphological, Histological, and Immunological Assessments on the Replication of CIA Modeling

After intradermal injection with CII-CFA for 28 days, CICA mice exhibit a series of early phase arthritic phenotypes, mainly erythema and edema on mouse paws, remarkable hyperplasia with multilayer synoviocytes, and lymphocytic infiltration with dispersed and chronic inflammation. Accordingly, CIAA mice established by

intra-articular CFA injection and LIAA mice established by intra-articular LPS injection also show the morphological and histological lesions that resemble CICA mice established by intradermal CII-CFA injection. In general, synovitis occurs in CICA mice within weeks after twice injections with CII-CFA, but it occurs in CIAA or LIAA mice only within three days upon only one injection with CFA or LPS. These acute synovitis/arthritis models should represent the most rapid modeling procedure of RA in mice so far.

To elucidate the relevance of arthritic modeling with immune activation, we investigated the cytokine microarray profiles in CIAA mice upon pre- and postimmunization. Consequently, 31 cytokines are upregulated in CIAA mice, including the proinflammatory cytokines IFNγ (1.5-folds), TNFα (2.24-folds), and IL-1β (1.46-folds). In similar, LPS injection also increases the serum levels of TNFα to 1.691 ± 0.07 pg/ml in LIAA mice, which is very significantly different from control mice (1.128 ± 0.07 pg/ml). These results clearly demonstrated that intra-articular injection with CFA or LPS can activate the critical proinflammatory cytokines and initiate the global inflammatory responses.

5.3.2.2 NO Production, Hypoxia, and Angiogenesis in CIAA and LIAA Mice

After intra-articular injection of mice with CFA, we observed the generation of NO after only 4 h, and determined the accumulation of NO after one day. Potent NO burst occurs on the 2nd day, and the NO peak (above 10 μM) slightly declines on the 3rd day. Accordingly, CIAA mice exhibit a dramatic decrease of SpO_2, immediately after CFA injection. While control mice have a maximal SpO_2 above 80 %, CIAA mice have a minimal SpO_2 below 60 %. In LIAA mice, NO also reversely correlates with SpO_2, in which the NO level elevates from 2 μM (control mice) to 8 μM, and SpO_2 declines from 98.33 ± 0.58 % (control mice) to 67.00 ± 1.73 %. The elevation of NO is followed by the decline of SpO_2, implying that a high NO level might lead to a low SpO_2 upon modeling.

To reveal whether CFA would affect the expression of HIF-1α and VEGF, we performed the immunohistochemical analysis on synovial sections prepared from CIAA mice. Consequently, both of which are significantly upregulated after CFA injection, although HIF-1α shows a higher expression level than that of VEGF. Furthermore, CFA was observed to promote the significant synovial angiogenesis. In LIAA mice, HIF-1α and VEGF are markedly upregulated, in which the immunohistochemical staining strength of HIF-1α is 99.96 ± 1.17 (11.81 ± 1.95 in control mice), and that of VEGF is 110.68 ± 4.55 (9.04 ± 0.08 in control mice) (Fig. 5.2). These results indicated that immunization-triggered NO and NO-driven hypoxia can upregulate HIF-1α and VEGF. Accordingly, we observed the elevation of serum LA levels on the 1st day of CFA injection, but they gradually decline latter, suggesting that hypoxia-mediated glycolysis is inhibited and angiogenesis is activated following the induction of HIF-1α and VEGF.

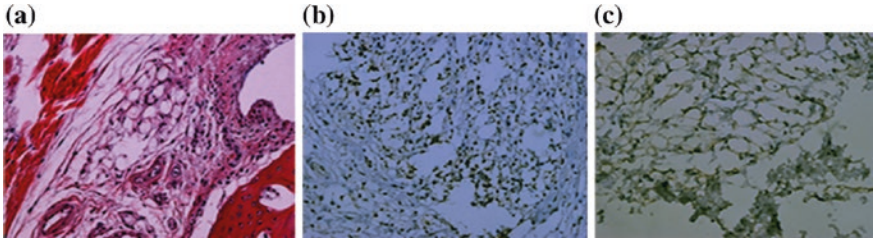

Fig. 5.2 Induction of accelerated angiogenesis (**a**), enhanced HIF-1α expression (**b**), and enhanced VEGF expression (**c**) in the synovial tissue of CIAA mice (200×). *CIAA* collagen-induced chronic arthritis; *HIF-1α* hypoxia-inducible factor α; *VEGF* vascular endothelial growth factor

5.3.2.3 Simulation of CFA by SNP and Suppression of CIAA by L-NMMA

To make sure that NO is an initiator of synovitis, we used SNP for intra-articular injection to simulate CFA-induced CIAA. Consequently, we noticed that SNP (0.5–1.0 mg/ml) can mimic CFA (0.5–1.0 mg/ml) to cause erythema and edema in both injected and noninjected paws. From the results of histochemical analysis, we confirmed that SNP can simulate CFA to induce the synoviocyte propagation and inflammatory infiltration. Among 84 hypoxia signaling genes, SNP downregulates *NOS2* for 2.98-folds, *HIF-1α* for 2.28-folds, and *HYOU-1* for 4.01-folds, whereas CFA upregulates *NOS2* for 2.04-folds, *HIF-1α* for 1.24-folds, and *HYOU-1* for 1.83-folds. These data suggested that SNP-released NO could repress the expression of hypoxia-sensitive genes, whereas the overexpression of those genes occurs upon induction by CFA-activated proinflammatory cytokines.

The intra-articular injection of mice by CFA causes the erythematous and edematous paws, which are similar with those seen in mice injected by CII-CFA. When CFA was coinjected with L-NMMA, however, no significant erythema and edema occur in mouse paws. Accordingly, CFA injection allows a higher SpO_2, whereas CFA + L-NMMA exhibits a lower SpO_2 (Table 5.2).

Table 5.2 Effects of L-NMMA on CFA-induced synovial hypoxia in mice

Treatment	SpO_2 (%)
CFA (0.5 mg/ml) into a left hind leg	$77.33 \pm 1.20^*$
CFA (1 mg/ml) into a right hind leg	$72.67 \pm 1.55^*$
L-NMMA (0.5 mg/ml) + CFA (0.5 mg/ml) into a right hind leg	86.67 ± 2.87
L-NMMA (0.5 mg/ml) + CFA (1 mg/ml) into a left hind leg	96.75 ± 3.10
CFA (0.5 mg/ml) injection into a right hind leg	$72.00 \pm 1.96^*$
CFA (0.5 mg/ml) + L-NMMA (0.5 mg/ml) into a right hind leg	90.75 ± 2.02
Control	98.33 ± 0.58

CFA complete Freund's adjuvant; *L-NMMA* N^G-monomethyl-L-arginine monoacetate; *SpO_2* saturation percentages of O_2. Intra-articular injection was performed using 100 μl of drug/drug combinations. The *singular asterisk* (*) represents different from the control ($p < 0.05$)

By the enzyme-linked immunosorbent assay, we detected the antibody against 3-nitrotyrosine (3NT) that reflects the extent of $ONOO^-$-mediated nitration of proteins, and we also detected the antibody against cyclic citrulline peptide (CPP) that indicates the occurrence and progression of RA. The results indicated that either combination with L-NMMA or not, CFA/CII-CFA elevates the anti-3-nitrotyrosine antibody levels, suggesting CFA/CII-CFA induces the production of both NO and O_2^- by activating the immune responses, and thereby accelerates the reaction of NO with O_2^- to give rise to $ONOO^-$, which can directly enhance protein nitration. In contrast, SNP alone or in combination with CFA/CII-CFA leads to a lower anti-3NT antibody level. This is likely because SNP only releases NO but does not generate O_2^-. Intriguingly, a reverse correlation of anti-3NT antibody with the anti-CCP antibody was noticed in mice after CFA/CII-CFA injection, suggesting that the 3-nitrosyl group on the tyrosine residue is most likely from ARG of proteins because the denitration of L-arginine might result in conversion to citrulline, which induces the anti-CCP antibody due to absence in native proteins.

5.3.2.4 Modulation of Immune Responses and Hypoxia Consequences in CIAA/LIAA Mice by ART, RAP, and/or BLA

To test if administration of ART or RAP before or during CIAA modeling suppresses the immune responses and mitigate the hypoxia consequences, we designed a pretreatment regimen by injecting mice with 60 μg/ml ART or 30 μg/ml RAP on four hours prior to immunization and continuous injection in twice a day for three days. As results, proinflammatory cytokines were found to be remarkably downregulated. For example, ART and RAP decrease TNFα for 1.24- and 1.11-folds, respectively, compared to 2.24-folds in untreated CIAA mice. RAP seems to have a more potent immunosuppressive effect than ART because the expression levels of proinflammatory cytokines are lower in the RAP group than those in the ART group. In LIAA mice, ART (60 μg/ml) can decrease TNFα content from 1.691 ± 0.07 pg/ml (control mice) to 1.462 ± 0.07 pg/ml, although it is lower than that after treatment by 0.5 mg/ml L-NMMA (1.079 ± 0.15 pg/ml).

To observe the effects of different administration procedures on disease improvement in modeling mice, we determined the serum NO levels before or after injection with ART or RAP in a preinjection group (four hours before CFA immunization) and a postinjection group (three days after CFA immunization). While pretreatment by ART (60 μg/ml) or RAP (30 μg/ml) for three days decreases the NO level to that comparative to the control, posttreatment by RAP (50 μg/ml) for five days causes a dramatic decline of the NO level. For LIAA mice, ART (60 μg/ml) can decline the NO level from 8 μM (control mice) to 5 μM, albeit slightly lower than that in L-NMMA (0.5 mg/ml)-treated LIAA mice (4 μM). Accordingly, ART (60 μg/ml) or L-NMMA (0.5 mg/ml) can downregulate the expression level of iNOS, represented by the weaker immunohistochemical staining from the gray scale values, 92.67 ± 4.75 (LIAA mice) to 8.54 ± 2.03 or 21.46 ± 4.30.

As comparison with untreated CIAA mice, pretreatment by ART (60 μg/ml) or RAP (30 μg/ml) for three days, or posttreatment by ART (60 μg/ml) or RAP (50 μg/ml) for five days leads to SpO_2 increase to a normal reading. These results demonstrated that ART and RAP can mitigate NO-mediated hypoxia, either in pretreatment or in posttreatment. After treatment of LIAA mice with 60 μg/ml ART or 0.5 mg/ml L-NMMA, SpO_2 elevates to as high as 79.67 ± 2.08 % or 81.67 ± 2.89 %, higher than LIAA mice (67.00 ± 1.73 %), but lower than control mice (98.33 ± 0.58 %).

5.3.2.5 Effects of ART, RAP, and/or BLA on Synovial Angiogenesis, Glycolysis, and Inflammation

The upregulation of HIF-1α and VEGF in CIAA mice were reversed by pretreatment or posttreatment with ART or RAP, in which a more effectively inhibitory effect on the induction of HIF-1α and VEGF was observed in pretreatment mice than in posttreatment mice. For example, posttreatment by RAP (50 μg/ml) upregulates HIF-1α for 2-folds. While HIF-1α is downregulated by pretreatment, VEGF remains unchanged. LIAA mice treated by ART (60 μg/ml) show the lower expression levels of HIF-1α and VEGF than untreated LIAA mice. In similar, L-NMMA (0.5 mg/ml) also downregulates HIF-1α and VEGF. The therapeutic effects of ART and ART + BLA on the expression of HIF-1α, VEGF, and iNOS in LIAA and CIAA mice were listed in Table 5.3.

Table 5.3 Immunohistochemical analysis of HIF-1α, VEGF, and iNOS in LIAA and CIAA mice treated by ART or ART + BLA

Group	Immunohistochemical staining strength ($n = 3$)		
	HIF-1α	VEGF	iNOS
Control	11.81 ± 1.02	9.04 ± 2.00	13.45 ± 3.12
LIAA mice treated by 60 μg/ml	13.82 ± 1.22*	20.05 ± 3.11**	51.72 ± 4.48*
LIAA mice treated by 100 μg/ml BLA	22.83 ± 1.98*	11.27 ± 3.87**	18.45 ± 1.65**
LIAA mice treated by 60 μg/ml ART	75.19 ± 3.03	60.63 ± 4.51*	36.55 ± 5.25*
LIAA mice	99.96 ± 4.71$^{\#}$	110.68 ± 4.55$^{\#\#}$	92.67 ± 4.75$^{\#\#}$
CIAA mice treated by 60 μg/ml	32.16 ± 2.69**	7.01 ± 3.56*	23.45 ± 5.33*
CIAA mice treated by 100 μg/ml BLA	51.24 ± 3.95*	10.67 ± 1.98	64.73 ± 1.98*
CIAA mice treated by 60 μg/ml ART	26.50 ± 5.53*	9.45 ± 4.15*	8.13 ± 1.08*
CIAA mice	100.21 ± 5.88$^{\#\#}$	19.35 ± 3.79$^{\#}$	156.85 ± 5.55$^{\#}$

ART artemisinin; *BLA* betulilic acid; *CIAA* collagen-induced acute arthritis; *HIF-1α* hypoxia-inducible factor 1α; *iNOS* inducible NOS; *LIAA* LPS-induced acute arthritis; *VEGF* vascular endothelial growth factor. The *singular asterisk* (*) represents different from the model ($p < 0.05$); *Double asterisks* (**) represent very different from the model ($p < 0.01$); The *singular pound sign* (#) represents different from the control ($p < 0.05$); *Double pound signs* (##) represent very different from the control ($p < 0.01$)

We noticed that either CFA or LPS upregulates HIF-1α, VEGF, and iNOS, and ART or ART + BLA downregulates HIF-1α, VEGF, and iNOS to a certain extent. However, it seems that BLA is more effective than ART or ART + BLA in suppressing HIF-1α, VEGF, and iNOS in LIAA mice but not in CIAA mice.

As an indicator discriminating the normoxia or hypoxia state, HIF-1α and VEGF were found to reversely correlate the serum LA levels. It was observed that LA levels are unchanged or slightly elevated after CFA immunization, but dramatically elevated following the suppression of angiogenesis by ART or RAP. Both ART and RAP elevate the LA levels in the pretreatment group, whereas only RAP but not ART declines the LA levels in the posttreatment group.

For preinjection, ART (60 μg/ml) or RAP (30 μg/ml) causes the insignificant signs of erythema and edema on both injected and noninjected hind legs, and ART (60 μg/ml) + RAP (30 μg/ml) allows the erythematous and edematous paws. For postinjection, ART (60 μg/ml)-treated CIAA mice show erythema and edema on the injected hind leg, but RAP (50 μg/ml) or ART (60 μg/ml) + RAP (50 μg/ml) exhibits a distinguishable alleviation of inflammatory symptoms. These results indicated that ART, RAP, and ART + RAP could exert prophylactic and therapeutic effects on experimental arthritis, in which the pretreatment cohort seems superior to the posttreatment one.

5.3.2.6 Antiarthritic Role of ART, RAP, and/or BLA in Mitigation of Synovial Hyperplasia and Inflammatory Infiltration

No significant synovial hyperplasia or only mild matrix neutrophilic and lymphocytic infiltration was observed in CIAA mice pretreated by ART (60 μg/ml) or RAP (30 μg/ml). The synovial hyperplasia into 1–2 layers and dispersed inflammatory infiltration were seen in CIAA mice pretreated by ART (60 μg/ml) + RAP (30 μg/ml). Posttreatment of CIAA mice by ART (60 μg/ml) leads to a double-layer synovial hyperplasia, and the dispersed inflammatory infiltration. The posttreatment of CIAA mice by RAP (50 μg/ml) or ART (60 μg/ml) + RAP (50 μg/ml) gives rise to a mild or no significant synovial hyperplasia with inflammatory infiltration. It is clear that pretreatment by ART (60 μg/ml) or RAP (30 μg/ml), or posttreatment by RAP (50 μg/ml) or ART (60 μg/ml) + RAP (50 μg/ml) demonstrates an effective remission of synovial hyperplasia and inflammatory infiltration, thereby representing the interventional options for the experimental arthritis. In LIAA mice, a single-dose of ART (60 μg/ml) or the combined use of ART (60 μg/ml) with BLA (100 μg/ml) can alleviate synovial hyperplasia and decrease inflammatory cells, in which the efficacy of antisynovitis seems better in ART + BLA than in ART alone.

5.3.3 Discussion

The rapid turnover of a RA-simulated animal model is urgently needed for mechanism-based high-throughput screening of candidate antiarthritic drugs. Unlike rats,

CIAA modeling in mice not only requires heat-killed bacteria-containing CFA to potentate CII's immune stimulation, but also spent a longer duration (3–4 weeks) to manifest the pathological alterations (Luross and Williams 2001). It is anticipated that much shorter modeling time will be needed when intradermal injection is changed to intra-articular injection. Indeed, intra-articular CII-CFA injection allows the synovial hyperplasia and lymphocytic infiltration as short as three days in our studies. We further assumed that CFA alone instead of CII-CFA might also induce synovitis due to anti-CII reactivity as only a consequence but not a cause leading to RA (Courtenay et al. 1980). As expectation, CFA enables the phenotyping of CIAA mice within three days as same as CII-CFA. Furthermore, LPS as the bacterial endotoxin can also establish the LIAA model that assembles CIAA mice. The applications of CFA and LPS for rapid arthritic modeling have not been reported at this moment.

In regard to the implication of NO in the incidence of arthritis, it is reported that knockout of iNOS confers resistance to IL-1-induced bone resorption in mice (Perkins et al. 1998). Most recently, it has also been demonstrated that hyaluronan, a disaccharide polymer that is widely used for the treatment of patients with osteoarthritis, limits erosive damage of cartilage and bone in adjuvant-induced arthritis rats by suppressing the synovial iNOS (Chou et al. 2011). Previous references have mentioned a central role of NO in the pathogenesis of RA, but these authors have only addressed the NO-induced immune dysfunctions (Nagy et al. 2010) or NO-regulated mitochondrial activity (Cillero-Pastor et al. 2011). As our knowledge, the present investigation on the NO's pathogenic roles that initiate inflammatory lesions is the first mechanistic association of immunization-triggered NO with the hypoxia, angiogenesis, and hyperplasia (Bao et al. 2012; Wu et al. 2012). We have revealed that the endogenous induction of NO by CII-CFA, CFA, or LPS and the exogenous supplementation of NO by SNP can equally allow the onset of synovitis. In contrast, the suppression of immune activation by RAP, inhibition of NO production by ART or L-NMMA, or downregulation of HIF-1α and VEGF by BLA can alleviate and even ameliorate the inflammatory lesions occurring in synovial tissues.

As noted in patients with RA, a finding of a direct correlation of partial O_2 pressures with inflammatory lesions in the synovial tissue has led to a proposition that hypoxia seems a primary driver of the inflammatory process seen in arthritic joints (Ng et al. 2010). How can NO drive the hypoxia? It is noted that NO can accelerate its own consumption by increasing its entry into red blood cells under hypoxic conditions (Han et al. 2003). The synovial tissue is a relatively hypoxic one, in which the partial O_2 pressure in the cartilage ranges from 6 % in the superficial layer to less than 1 % in the deep zone, so potent NO burst should aggravates the local hypoxia in the synovial tissue by allowing NO's occupation in the O_2-binding site of hemoglobin and myoglobin (Fermor et al. 2007). In this study, we found that CFA injection can lead to a higher NO level and a lower SpO_2 value, but coinjection with CFA and L-NMMA enables a lower NO level, but a higher SpO_2 value. Furthermore, SNP injection can dramatically decline SpO_2. A positive

relationship between NO and SpO_2 established in our study strongly supports that NO is a driver to hypoxia. Besides, a recent study has demonstrated that IL-17A is a key mediator in inflammatory arthritis, and an association of hypoxia with IL-17A expression appears to be indirect, probably through hypoxia-induced proinflammatory pathways and leukocyte influx within the joint microenvironment (Moran et al. 2011).

In the present study, we also found that SNP does not upregulate any hypoxia signaling pathway genes, otherwise downregulates *NOS2* gene. In contrast, CFA/CII-CFA injection can upregulate a lot of hypoxia signaling pathway genes including *NOS2*. It is possible that the activation of hypoxia pathway genes needs the perpetual NO production, and the induction of *NOS2* is the prerequisite mediating hypoxia signaling activation. Nevertheless, frequent SNP injections can upregulate both HIF-1α and VEGF. Under a hypoxic condition, HIF-1α and VEGF were found to be upregulated, thereby initiating angiogenesis (Xu et al. 2005), during which Toll-like receptor 2 promotes angiogenesis, cell adhesion, and invasion (Saber et al. 2011). As a similar observation in the present study, serum LA levels were found to be affected by angiogenesis progression. At first, LA levels elevate due to NO-driven hypoxia, and then LA levels decline after angiogenesis. It can be interpreted that hypoxia leads to enhanced glycolysis and overproduced LA, whereas angiogenesis results in compromised hypoxia, suppressed glycolysis, and lowered LA levels. Besides, CFA not only triggers NO production, but also promotes 3-nitrotyrosine formation, suggesting CFA also enhances the synchronous production of O_2^-. Interestingly, we noticed that the anti-3-nitrotyrosine antibody levels are reversely proportional to the anti-CCP antibody levels.

Although anti-TNFα-based biologic therapies are approved for clinic use in patients with RA (Biniecka et al. 2011), as many as 40 % RA patients are nonresponders to anti-TNFα antibodies (Schett et al. 2011). Why is there nonresponders to anti-TNFα? As known, NO initiates synovial angiogenesis and hyperplasia, and leads to acute synovitis and finally to chronic arthritis. Because NO is triggered by not only TNFα, but also IL-1β and other proinflammatory cytokines (Bakker et al. 2009), inhibition of TNFα alone but not others should be partially responsive among patients with RA. Based on such a consideration, it can be predicted that blockage of TNFR by engineered peptides (Tang et al. 2011) or antisense technologies (Paquet et al. 2011) may also provide a restrictive protection from RA even among responders.

The novel concept of 'treating synovitis as tumor' suggested by ours seems supported by the evaluation of potential antitumor agents, ART (Zhou et al. 2007), RAP (Motzer et al. 2008; Hess et al. 2009; Yao et al. 2011), and BLA (Zuco et al. 2002; Robert and Samir 2004), as candidate antisynovitic drugs. Actually, ART (Wang et al. 2008) and RAP (Kneissel et al. 2004; Laragione and Gulko 2010; Cejka et al. 2010) exhibit certain antiarthritic roles, but no documents describing BLA's antiarthritic effects. We found that CFA-induced synovitis can be considerably ameliorated by preadministration or postadministration with ART or RAP through reversing the inflammation-triggered NO signaling process. Pretreatment by ART or RAP can block the onset of synovitis, whereas

posttreatment by ART or RAP can only partially alleviate the severity of inflammation. Interestingly, combined posttreatment by ART and RAP can thoroughly abrogate the incidence of synovitis in CIAA mice. In similar, ART and/or BLA exhibits the comparative improved effects on the synovitis of LIAA mice, which was confirmed from morphological, histopathological, biochemical, and functional genomic assays.

This work has further highlighted a pathogenic process of synovitis, either in CIAA or LIAA mice from systemic immune activation to NO-driven synovial hypoxia, angiogenesis, hyperplasia, and inflammatory lesions. We have also revealed that the pharmacological mechanisms of ART, RAP, and BLA in the prevention of synovial and articular tissues from inflammatory lesions, which should enable the innovation of the concept regarding the pathogenesis and treatment of RA from the conventional symptom-based therapies to the novel pathogenesis-based therapies.

5.3.4 Conclusions

To disclose the therapeutic mechanisms underlying ART, RAP, and/or BLA against synovial inflammation, we established two acute arthritis models in mice by intra-articular injection of CFA and LPS separately. The global upregulation of proinflammatory cytokines, dramatic burst of NO, overexpression of HIF-1α and VEGF, and eventually synovial angiogenesis and hyperplasia were observed in modeling mice. Articular injection of mice with the NO donor SNP also causes articular erythema and edema, whereas coadministration of CFA with the NO synthetic inhibitor L-NMMA abrogates articular inflammation. Preinjection and postinjection of acute arthritis mice with ART, RAP, and/or BLA can reverse the overexpression of HIF-1α and VEGF, synovial angiogenesis, tumor-like hyperplasia, and lymphocytic inflammatory infiltration. Conclusively, ART by blocking NO generation, RAP by downregulating proinflammatory cytokines, and BLA by suppressing the transcriptional factor, HIF-1α-induced expression of VEGF, hold potential promise in prophylactic and therapeutic interventions of RA.

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Chapter 6
ART for Antiaging

Abstract ART can mimic CR to extend yeast lifespan, during which both ART and CR-triggered NO can activate antioxidative responses and convert the metabolic pattern from biosynthesis to degradation. ART also mimics CR to compromise mouse telomere shortening by upregulating antioxidative enzymes for effective ROS scavenging, which is followed by the alleviation of DNA damage and downregulation of tumor suppressors. This is the first time for having found ART exerting an antiaging role.

Keywords Antioxidation · ART · CR · Lifespan · Telomere

6.1 An Overview on Aging and Antiaging

CR, limited food intake without malnutrition, is an extensively reproducible intervention in lifespan extension among organisms ranging from yeast to mammals (Koubova and Guarente 2003; Spindler 2010). Owing to evolutionary conservation, CR-enhanced longevity has been frequently described in yeast (Jiang et al. 2000), nematodes (Walker et al. 2005), fruit flies (Rogina and Helfand 2004), and mammals (Youngman et al. 1992). In yeast, the chronological lifespan (CLS) and the replicative lifespan (RLS) are extended by glucose reduction from 2 to 0.5 %. While CLS is more relevant to postmitotic cell aging, RLS is closely related to replicative aging seen in stem cells (Skinner and Lin 2010). In primates, when daily diets are restricted to 40 % as ad libitum (AL) feeding, some commonly occurred age-related diseases, including cancer, diabetes, atherosclerosis, neurodegenerative disorders, and respiratory failures, can be mostly delayed (Kenyon 2005; Colman et al. 2009; Mattison et al. 2012). CR in humans also greatly reduces the subjected risks of cancer, diabetes, and cardiovascular diseases (Fontana and Klein 2007).

CR is believed to exert a longevity-promoting effect through enhanced mitochondrial biogenesis (López-Lluch et al. 2006; Civitarese et al. 2007), which is initiated by NO generated from eNOS (Nisoli et al. 2003, 2005) and/or nNOS/mtNOS (Giulivi et al. 1998; Finocchietto et al. 2008). In mammals, CR is known to induce

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cGMP for activating NOS and producing NO, through which mitochondrial biogenesis is evoked and lifespan extended (Nisoli et al. 2004; Nisoli and Carruba 2006). As supporting evidence, the NO donor S-nitrosoglutathione confers yeast an extended lifespan (Li et al. 2011). Nevertheless, NOS homologs have not been identified in yeast although NO is always detected in budding yeast (*Saccharomyces cerevisiae*) (Lewinska et al. 2011) and fission yeast (*Schizosaccharomyces pombe*) (Kig and Temizkan 2009). However, it is believed that mitochondrial COX might be responsible for NO production from nitrite reduction (Castello et al. 2006).

CR-enhanced mitochondrial biogenesis seems an acceptable conclusion for lifespan extension because it has been reported that an incremental respiratory activity corresponds an extended lifespan in cells and animals (Lanza and Nair 2010). Accompanying with longevity, augmented respiration occurs in yeast, fruit flies, mice, and rats (Lin et al. 2002; Civitarese et al. 2007). However, a recent report has argued that a life-long CR only preserves mitochondrial functions other than enhances mitochondrial biogenesis (Lanza et al. 2012). Other new findings have also questioned if CR really elicits mitochondrial biogenesis (Hancock et al. 2011; Miller et al. 2012).

To decipher such a discrepancy, we suggested here that a respiratory burst might occur only in the early phase of CR (a short-term or acute CR), whereas a respiratory delay might kept in the late phase of CR (a long-term or chronic CR). In consistence with this deduction, a systemic research has revealed that mitochondrial translation and O_2 consumption are enhanced only during the logarithmic and early postdiauxic growth stage in yeast cultures (Pan et al. 2011). An "uncoupling to survival" hypothesis suggests that CR could increase the respiratory capacity through mitochondrial uncoupling (Brand 2000). Indeed, several uncoupling strategies do lead to lifespan extension in model organisms including yeast (Barros et al. 2004), nematodes (Lemire et al. 2009), and fruit flies (Humpherey et al. 2009). A low dose of DNP, a mitochondrial uncoupler, allows lifespan extension in mice (Cerqueira et al. 2011).

An alternative conclusion has been drawn that CR leads to the oxidized nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase Sirtuin 1 (SIRT1)-dependent autophagy in human cells in vitro and in nematodes in vivo (Morselli et al. 2010). However, it has been debated about a correlation of autophagy with longevity because autophagy is unlikely sufficient to extend lifespan although CR-induced antiaging effects might be linked to autophagy (Hansen et al. 2008). For instance, RAP inhibits polyglutamine aggregation independently of autophagy by reducing protein synthesis is essential for longevity (King et al. 2008), and translation inhibition alone is sufficient to extend lifespan (Hands et al. 2009). Currently, an implication of SIRT1 in CR-induced lifespan extension in nematodes and fruit flies are being questioning (Blagosklonny 2010; Burnett et al. 2011), but the SIRT1 activator resveratrol can induce the beneficial metabolic changes in obese persons by mimicking CR's effects (Timmers et al. 2011).

An evolutionarily conserved protein kinase, mTOR, is being extensively investigated in regard to its antiaging effects. The activation of mTOR homologs limiting the extension of lifespan has been filed in yeast (Pan and Shadel 2009),

nematodes (Vellai et al. 2003), fruit flies (Kapahi et al. 2004), and mice (Selman et al. 2009). By inactivating mTOR, RAP can extend mouse lifespan (Harrison et al. 2009). However, it has been demonstrated that NO can prolong life expectancy through upregulating mTOR activity (Pervin et al. 2007). Similarly, branched-chain amino acids can promote the survival of middle-aged mice by activating mTOR partly through enhancing NO generation (D'Antona et al. 2010). Furthermore, it remains unclear which aspects of the mTOR signaling pathway contribute to the aging or aging-delaying process because mTOR controls the multifaced aspects of cell physiology, including ribosome biogenesis, translation, autophagy, and proliferation (Wullschleger et al. 2006).

A well-documented association of aging with telomeres suggests a possible correlation of lifespan extension with chromosome protection. Since the theory depicting metabolic suppression mitigates DNA damage was suggested in the last decade (Koubova and Guarente 2003), a novel model of CR-conferred DNA protection seems commonly accepted. According to this model, starvation can alleviate DNA damage and confer a tissue-protective phenotype through repressing the cellular metabolism and activating the mitochondrial functionality (Longo and Fontana 2010). Most recently, CR is shown to maintain/elongate telomeres and synergize with telomerase for extending mouse longevity (Vera et al. 2013). Although CR-triggered NO is believed to relevant to longevity, it remains unsolved whether NO also maintains/elongates the length of telomeres. Interestingly, it has documented that CAT inactivation or H_2O_2 induction can extend yeast lifespan (Mesquita et al. 2010). These questionable issues encourage us to explore a plausible relationship between NO and H_2O_2 during aging, and decipher how can NO and H_2O_2 exert their antiaging effects.

How can CR cause mitochondrial uncoupling is an open question, and why CR-mediated mitochondrial uncoupling enables lifespan extension is yet to be elucidated. Furthermore, it is indefinable for the mechanistic explanation of CR-exerted effects on longevity by current theories because enhanced respiratory activity is apparently conflicted with limited food supply during CR. It is also confusing for the concept of mitochondrial biogenesis or mitochondrial activation. Additionally, autophagy is likely a result rather than a cause for CR-extended lifespan, and the molecular event involving the interaction between SIRT1 and resveratrol as well as between mTOR and RAP needs further verification.

6.2 ART Extends Yeast Lifespan via NO Signaling

6.2.1 Purposes and Significance

Because NO is known to reversibly bind to the prosthetic heme moiety of COX (Mason et al. 2006), we proposed that CR-triggered NO may interact with COX to result in mitochondrial uncoupling and respiratory enhancement. To verify this preposition, we chose ART that alkylates hemoproteins (Zhang and Gerhard 2009)

including COX to simulate the NO-COX interaction. It is believed that CR enables the synchronous decreases of ATP and reduced nicotinamide adenine dinucleotide (NADH), which can separately activate adenosine monophosphate-activated protein kinase (AMPK) and SIRT1 (Rodgers et al. 2005; Canto et al. 2009). In turn, AMPK and SIRT1 can coordinately activate peroxisome proliferator-activated receptor γ coactivator 1 alpha (PGC-1α) for mitochondrial biogenesis in mammals (Lee et al. 2006). It is unambiguous that NAD^+ and Sir2 (yeast homolog of mammalian SIRT1) are required for yeast lifespan extension (Lin et al. 2000).

Here, we report CR-based "dual-phase responses", which is composed of a phase of mitochondrial enhancement (ME), or a respiratory burst phase, and a phase of postmitochondrial enhancement (PME), or a respiratory decay phase. The ME phase that is characterized by an enhanced antioxidative response is transient and occurs at an acute CR stage, whereas the PME phase that is represented by an attenuated metabolic activity is maintained throughout a chronic CR stage. Because CR can trigger NO and H_2O_2 production, we assumed that ART and H_2O_2 may follow the dual-phase pattern to mimic CR for yeast lifespan extension. To enable a systematical evaluation on CR-mediated antioxidative augments and metabolic alterations, we analyzed the global expression profiles of CR-treated yeast by whole transcriptomic microarray. Among which some transcripts of critical genes were further confirmed by quantitative polymerase chain reaction (qPCR) or enzyme activity determination. Comparatively, we parallelly examined the expression levels after treatment by ART or H_2O_2 for comparison. We expect that all those data should shed light into the elucidation of bona fide mechanisms underlying CR prolongs yeast lifespan through NO- and H_2O_2-involved signaling cascades.

6.2.2 Results and Analysis

6.2.2.1 Mimicry of CR by ART and H_2O_2 to Extend Lifespan in a Dose-Dependent Manner

To replay the episode of yeast lifespan extension by CR, yeast cells were incubated on the cultural plates containing 0.5 % glucose. In parallel, yeast cultural media were supplemented with different doses of ART. As compared with the control, or calorie nonrestriction, low-dose (0.1 and 0.5 μm) ART was observed to allow a significant increase of survived cells when colonies were counted after treatment until 3 weeks. In contrast, high-dose (5, 25, and 50 μm) ART decreases the survived cell numbers when observed after treatment for 2 weeks. From much higher to relatively lower, CR, low-dose ART and calorie nonrestriction give rise to the distinct percentages of viable cells. These results indicated that low-dose ART extends yeast lifespan as a CR-mimetic, whereas high-dose ART shortens yeast lifespan due to causing cell death.

To disclose the involvement of H_2O_2 in yeast lifespan extension, we treated yeast cells by different concentrations of H_2O_2. Consequently, H_2O_2 in low concentrations (0.1, 0.5, and 20 μm) slightly extends lifespan, but H_2O_2 in high concentrations (above 20 μm) renders rapid cell killing. These results demonstrated that H_2O_2 also promotes yeast longevity in a dose-dependent manner, and implied that oxidative stress-induced antioxidative responses may be engaged in antiaging effects.

6.2.2.2 Induction of Deferentially Expressed Genes and Up/Downregulation of Specific Pathway Genes by CR and Mimetics

To provide evidence supporting that ART or H_2O_2 may exhibit a similarity with CR in deferentially expressed yeast genes, we compared the CR and mimetics-inducible transcripts from whole-transcriptome yeast expression profiling. In a hierarchical clustering map based on all target values, those transcripts induced by CR and mimetics exhibit significant difference from calorie nonrestriction. According to cluster analysis, it is clear that transcripts from CR are partially common to those after treatment by mimetics, whereas transcripts from treatment by mimetics are more similar than those from CR. While the inducible transcripts are common between CR and H_2O_2 as well as between CR and ART, other inducible transcripts are unique for CR. Among those deferentially expressed genes, CR induces more genes than ART. Additionally, the common regulated genes are less for CR versus ART than for CR versus H_2O_2.

According to the definition on yeast metabolic pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG), we analyzed the gene oncology data and identified some regulated pathways among CR and mimetic treatments. In all KEGG pathways with twofold up/downregulation, CR down/upregulates more pathways, whereas ART down/upregulates less ones. For all treatments, the ribosome pathway and MAPK signaling pathway are downregulated, whereas meiosis pathway upregulated (Wang et al. 2014).

6.2.2.3 Induction of Metabolic Alterations from Biosynthesis to Degradation of Metabolites

Although the microarray data of inducible pathway genes are available for different treatments, some have not been depicted in gene ontology diagrams because of insignificance (less than twofold up/downregulation) as comparison with calorie nonrestriction. To address that ART and H_2O_2 mimic CR in metabolic modulation-related gene expression, we parallelly quantified the metabolic pathway transcripts responsible for the metabolisms of glucose, fatty acids, amino acids, and nucleotides in CR and mimetic groups (Table 6.1).

Table 6.1 Up/downregulation of genes responsible for anabolism/catabolism of some major metabolites by CR and mimetics

Metabolites	Pathways and genes	Up/downregulation folds		
		CR	ART	H_2O_2
Glucose	Glycolysis			
	Acs2	−17.28	−2.63	−3.02
	Adh6	−6.18	−1.54	−3.06
	Ald3	−1.49	−1.83	2.13
	Eno1	−1.75	−1.38	−2.26
	Pdc6	−4.88	−2.16	−3.71
	Pgm2	−1.68	−1.74	−2.02
	Pyk2	−1.27	−1.72	−2.20
	Tdh2	−4.32	−4.17	−12.62
	Tdh3	−1.49	−2.47	−10.36
	Citrate cycle			
	Aco1	−1.71	−1.43	−1.63
	Cit2	1.04	1.11	1.06
	Fum1	−2.55	−1.16	−1.20
	Idh1	−3.26	1.08	1.06
	Kgd1	−1.58	−1.26	−1.28
	Kgd2	−1.51	−1.15	−1.29
	Lat1	1.00	1.32	1.39
	Lsc2	−1.63	1.03	−1.32
	Mdh3	1.55	1.39	1.39
	Pck1	−2.21	−1.19	−1.26
	Pdb1	−1.60	−1.04	−1.14
	Pyc2	−1.73	1.46	1.33
	Sdh4	−1.58	−1.12	−1.17
Fatty acids	Biosynthesis (including unsaturation and elongation)			
	Acc1	−2.46	1.50	−1.21
	Fas1	−2.54	−1.00	−1.53
	Fas2	−2.29	1.19	−1.22
	Fen1	−2.03	1.59	1.54
	Ifa38	−2.02	−1.22	−1.30
	Sur4	−4.07	−2.47	−3.54
	Tsc13	−2.37	−2.15	−2.85
	Ole1	−2.94	−1.03	−1.12
	Degradation (β-oxidation)			
	Dit2	2.86	2.61	2.82
	Faa2	3.31	1.38	−1.06
	Faa4	2.13	1.20	−1.16
	Pot1	4.56	1.60	1.56
	Pox1	5.99	2.31	1.78

(continued)

Table 6.1 (continued)

Metabolites	Pathways and genes	Up/downregulation folds		
		CR	ART	H_2O_2
Amino acids	Cysteine and methionine biosynthesis			
	Aro8	−4.17	−1.46	−1.54
	Cys3	−3.91	−2.54	−4.85
	Hom2	−2.09	−1.19	1.04
	Irc7	−14.85	−1.89	−2.98
	Met6	−2.24	−1.68	−1.88
	Sah1	−7.18	−1.29	−1.29
	Sam1	−4.11	−1.94	−1.43
	Sam4	−4.94	−2.27	−2.64
	Spe2	−2.84	−1.38	−1.32
	Spe4	−2.02	−1.31	−1.19
	Utr4	−2.71	−1.02	−1.05
Nucleotides	Purine and pyrimidine degradation			
	Apa1	2.73	1.84	2.40
	Cdc8	2.25	1.11	−1.19
	Dal3	2.70	4.26	4.10
	Dbp3	2.24	3.34	4.70
	Dbp4	3.28	1.47	1.65
	Gud1	2.37	1.90	2.00
	Hnt2	2.66	2.82	3.20
	Met14	3.28	1.46	1.74
	Prs2	2.14	1.50	1.61
	Rnr3	2.02	1.02	1.30
	Rpa12	2.08	1.11	2.07
	Rpa34	2.50	−1.20	1.78
	Rpa43	2.83	1.29	2.46
	Rpc31	2.24	1.10	1.08
	Rpc53	2.75	1.41	2.06
	Trr2	3.18	1.24	1.26

ART artemisinin; *CR* calorie restriction

As a major kind of energy metabolites, glucose is regenerated through gluconeogenesis and degraded through glycolysis. Because gluconeogenesis and glycolysis are inter-reversible, it is reasonable that gluconeogenesis/glycolysis pathway genes are downregulated by CR due to glucose restriction. Regarding to fatty acids, another family of major energy metabolites, it is also understandable that fatty acid biosynthesis pathway genes are downregulated, whereas fatty acid degradation pathway genes upregulated in all treatment groups. In similar, biosynthesis pathway genes for two kinds of

amino acids, cysteine and methionine, are downregulated, whereas degradation pathway genes for two kinds of nucleotide bases, purine and pyrimidine, upregulated. From above results, we can predict that the biosynthesis of both proteins and nucleic acids should be attenuated or even halted because of food shortage and nutrition insufficiency in CR, which can be mimicked by ART and H_2O_2.

6.2.2.4 Downregulation of Genes for Protein Biosynthesis and Upregulation of Genes for Protein Degradation by CR and Mimetics

To further reveal the effect of CR and mimetics on protein biosynthesis and degradation, we examined the expression levels of yeast ribosomal protein large subunit genes (*Rpl*) and ribosomal protein small subunit genes (*Rps*) after treatment by CR and mimetics. As the consequence, more *Rpl*/*Rps* and other related genes are downregulated by CR and mimetics. During treatment, a dramatic decreased protein concentration was noticed in all tested samples. These results indicated that the biosynthesis of ribosomal proteins is inhibited, and ART or H_2O_2 can simulate CR in the modulation of ribosome genes and hence their expression.

Accordingly, ribosome biogenesis genes that accelerate the ribosomal protein biosynthesis were found to be highly upregulated in all groups, implying a feedback response of ribosomes from various stress conditions. Not surprisingly, upregulation of ribosome biogenesis genes is more remarkable for the CR group because its ribosome genes are extremely downregulated. At the same time, we also analyzed the impact of different treatments on the controlled degradation of proteins through ubiquitylation. Consequently, some ubiquitylation genes (*Ubi*) are upregulated, but others downregulated. These results proved that selective protein degradation is enhanced after treatments, by which amino acids that are released from degraded proteins can be re-utilized. Furthermore, it was also found that most of autophagy genes (*Atg*) are upregulated by each treatment albeit a few of which are downregulated. It is clear that CR and mimetics can partially augment autophagy in order to help yeast coping with environmental stress (Fig. 6.1).

6.2.2.5 Mitohormesis: Modulation of Transporter Gene Expression Profiles and Lipid Metabolic Patterns by CR and Mimetics

Genome-wide expression profiling was used in the present study to compare the effects of CR and mimetics on ATP-binding cassette transporter gene expression profiles and lipid metabolic patterns. The dynamic changes of SOD

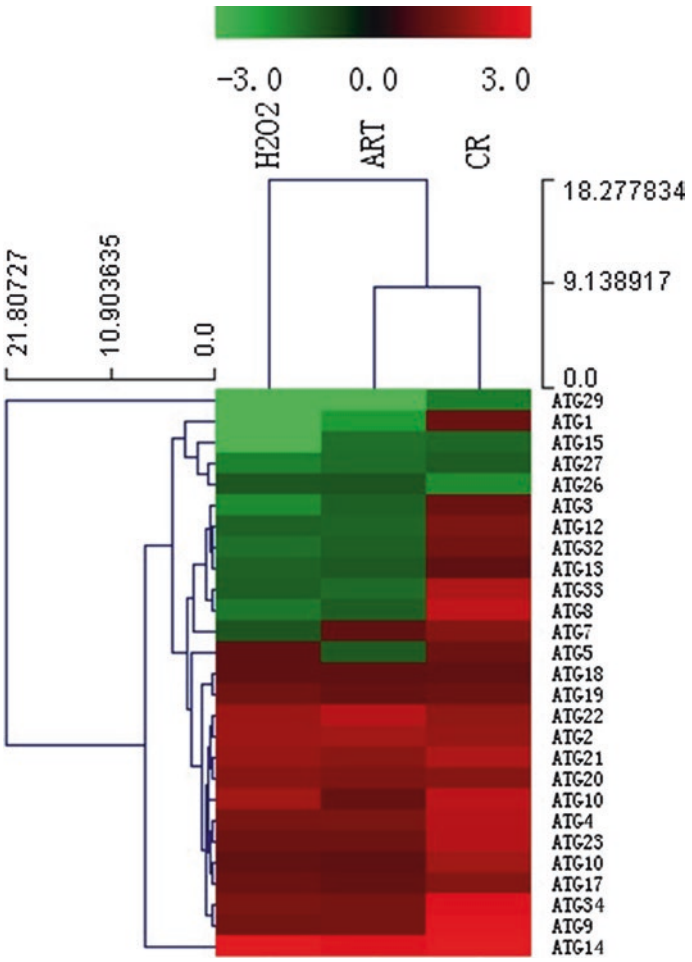

Fig. 6.1 Transcriptome-wide profiling of selective protein degradation-related autophagy pathway genes. *ART* artesunate; *CR* calorie restriction. The *red color* indicates upregulation, and the *green color* represents downregulation

activity were monitored in pretreated and post-treated yeast cells. After treatments, cellular detoxification-related ATP-binding cassette transporter genes are downregulated or unchanged, whereas the peroxisomal ATP-binding cassette transporter genes *Pxa1* and *Pxa2* that enhance long-chain fatty acid transport as well as the plasma membrane ATP-binding cassette transporter genes *Aus1* and *Pdr11* that accelerate sterol uptake are considerably upregulated (Table 6.2).

These results indicated that mitohormesis depends on ATP-binding cassette transporter-mediated lipid transport and subsequent lipid degradation and reutilization (Wang and Zeng 2014).

Table 6.2 ABC transporter gene expression profiles in yeast treated by CR and mimetics

ABC transporter gene	CR	H_2O_2	ART
Plasma membrane			
Pdr5	−4.57	−2.33	−1.29
Snq2	1.65	−1.08	1.17
Pdr12	−3.19	2.49	2.96
Pdr15	−3.16	−2.42	−1.44
Aus1	1.31	1.42	1.05
Pdr11	2.43	2.41	2.02
Yor1	−1.12	−1.36	1.02
Ste6	−2.08	−2.12	−2.82
Vacuole membrane			
Bpt1	2.23	1.56	1.36
Ybt1	1.06	1.34	−1.11
Ycf1	1.26	1.63	1.54
Mitochondrial membrane			
Atm1	2.2	1.15	1.14
Mdl1	−1.37	1.36	1.26
Mdl2	1.18	1.46	1.28
Peroxisome membrane			
Pxa1	5.47	−1.66	−1.43
Pxa2	4.11	2.00	1.98

ART artemisinin; *CR* calorie restriction

6.2.2.6 Expression Fluctuations of Oxidative Phosphorylation and Antioxidation Responsible Genes: Dual-Phase Responses During CR and Mimetic Exposure

The metabolic flux of glucose flows from the cytosol to mitochondria. Because the cytosolic glycolysis pathway genes are globally downregulated by CR and mimetics, it could be predicted that the mitochondrial oxidative phosphorylation pathway genes should be accordingly downregulated by each treatment. Indeed, it was found that those genes encoding COX (complex IV), F_1F_0-ATP synthase (Complex V), and other associated signature proteins are downregulated in every group, although the expression levels of those genes are much lower in the CR group than in the mimetic groups.

The analytic data of transcriptomic microarray indicated that H_2O_2 degradation-responsible antioxidant genes, such as *Cat1* encoding mitochondria-localized CAT and *Gpx2* encoding cytosolic GSH-POX, are upregulated by CR and mimetics. In contrary, H_2O_2 generation-responsible antioxidant genes, such as *Sod1* and *Sod2* coding for cytosolic copper-zinc SOD (Cu/Zn-SOD) and mitochondrial manganese SOD (Mn-SOD), are downregulated by CR and mimetics, implying that the main type of ROS in this stage is H_2O_2 other than O_2^-. Besides, it was also seen that other antioxidant enzyme-encoding genes are either upregulated (*Trx3*), or downregulated (*Gto3* and *Dot5*), or unchanged in different treatment groups.

As experimental evidence supporting above suggestion of H_2O_2 as a main type of ROS, we investigated the expression modes and activity dynamics of mitochondrial signatures including antioxidant enzymes. In ME yeast cells incubated for 12 h, CR increases both Cu/Zn-SOD and Mn-SOD activities. In contrast, in PME yeast cells incubated for several days, CR decreases both Cu/Zn-SOD and Mn-SOD activities. So CR-based "dual-phase responses" can be typically distinguished by the "up-and-down" modes of antioxidant responses.

Additionally, an elevation of the expression level of peroxisome genes in all treatment groups seemed to support above deduction that H_2O_2 has become the major kinds of ROS, which can be thoroughly scavenged by the cooperation of CAT, GSH-POX, and peroxisomal enzymes. More importantly, upregulation of peroxisomal β-oxidation enzymes contributes to energy generation by the oxidation of fatty acids released by the degraded storage lipids and recycled cellular components during carbon starvation.

6.2.2.7 Differential Regulation of Protein Kinase Genes in Logarithmic and Postlogarithmic Stages

It is noted that a repression of the respiratory activity should lead to the decrease of ATP and NADH, i.e., the elevation of AMP/ATP and NAD^+/NADH ratios, which can in turn activate AMPK and SIRT1 separately. In postlogarithmic yeast cells, only a slight upregulation of *Snf1* (coding for a yeast homolog of AMPK) and *Sir2* (coding for a yeast homolog of SIRT1) was observed in CR treatment, but no significant changes of those genes were noticed in mimetic treatment. Additionally, *Sch9*, encoding a yeast homolog of mammalian ribosomal S6 kinase 1 (S6K1), was also found to be weakly upregulated in the CR group but unchanged in mimetic groups.

It was shown that mitogen-activated protein kinase (MAPK) pathway genes (*Kss1* and *Fus3* are yeast homologs of MAPK) are mostly downregulated by CR and mimetics, indicating a common modulation pattern in the PE phase. As a critical gene controlling cell fate and determining lifespan, *Tor* is upregulated in either treatment group, in which *Tor1* is considerably upregulated for many folds. Because TOR is activated via phosphorylation, we do not expect that TOR activity is correlated with its transcript abundance. Nevertheless, it seemed that the inhibition of TOR activity may induce Tor expression perhaps in a feedback manner.

We further quantified the induced expression levels of *Snf1*, *Tor1*, and *Kss1* in logarithmic yeast among treatment groups. It is very clear that their expression patterns are distinct in the logarithmic stage from those in the postlogarithmic stage. For example, Tor1 exhibits downregulation in the logarithmic stage, but shows upregulation in the postlogarithmic stage. In the logarithmic yeast, the overall expression levels of *Snf1* and *Kss1* that are induced by CR, ART, or H_2O_2 are typically lower than those in calorie nonrestriction. These results further strengthened that protein kinase genes are regulated in a dual-phase mode.

6.2.2.8 Covalent ART-Heme Conjugation and Synchronous COX Induction

Until currently, no direct evidence informs how CR elicits ROS and induces antioxidant enzymes. Considering the previous results demonstrating that ART can alkylate the heme moiety and hence inactivate heme-harboring proteins, we proposed that ART may covalently conjugate COX and permanently inhibit its activity, which in turn induces COX and increases its activity. Indeed, we found that ART really increases yeast COX activity in different concentrations and incubated for a varying duration. This finding might be well interpreted by an induction of *Cox* after ART conjugation to COX.

As our finding, both heme and ART-heme adducts increase after treatment for 3 h with low-dose ART, but both decrease by high-dose ART. The increase of A_{415} (heme) and A_{476} (ART-heme adducts) readings following exposure of yeast to low-dose ART for 3 h means de novo hemoprotein biosynthesis, and the subsequent decline of both A_{415} and A_{476} under a low-does ART indicates hemoprotein degradation. In high-dose ART groups, neither heme induction nor ART-heme conjugation were observed, probably because yeast cells are killed by an overdose of ART (Wang et al. 2015a).

Upon the inhibition of COX activity, it should be expected to detect the increase of ART-induced *Cox1* expression during the initial phase. The microarray data indicated that all COX components become downregulated after exposure to ART for only 1 h. Accordingly, mitochondrial respiratory chain genes responsible for oxidative phosphorylation were also found to be globally downregulated. Therefore, ART can functionally mimic CR-triggered NO to enhance ROS production and induce antioxidant responses, which may be the consequence of mitochondrial biogenesis or compensatory enhancement of mitochondrial function. Accelerated ROS generation may be attributed to COX inhibition by NO as well as ART, whereas reduced ROS emission can be the result of antioxidant induction upon mitochondrial enhancement.

6.2.3 Discussion

CR is convinced in delaying aging/prolonging lifespan among examined eukaryotes, either in yeast (Skinner and Lin 2010) or in mice (Nisoli et al. 2003). At present, how can CR exert beneficial effects on health and lifespan remains debating, and some conclusions about the mechanism underlying CR-driven longevity are apparently controversial (Piper et al. 2011). An implication of mitochondrial biogenesis in CR-mediated lifespan extension was suggested in last decade (Nisoli et al. 2003), but challenges to it are frequently raised (Hancock et al. 2011; Lanza et al. 2012; Miller et al. 2012). Mitochondrial biogenesis is characterized by an enhanced respiratory activity, which means more glucose should be consumed and much ATP produced for compensating energy

deficiency during CR. However, this is an apparent paradoxical phenomenon for CR because glucose supply is restricted during CR.

As an urgent response to the suddenly happened nutritional stress, of course, a transient respiratory burst just at the start of glucose restriction should be possible because the unexpected insufficiency of ATP and NADH must be compensated by this emergent response. It has reported that yeast can respond robustly to the decreasing glucose levels by shifting their metabolic state from one that favors fermentation to one that favors respiration (Kaeberlein et al. 2007). It is demonstrated that respiratory burst is by no means a prerequisite for longer life expectancy of yeast because CR promotes the longevity in respiratory-deficient yeast strains (Kaeberlein et al. 2005a, b). Considering previous results regarding CR-mediated mitochondrial biogenesis, we suggested a dual-phase mode of CR-impacted mitochondrial functions: a ME phase representing the "short-term CR" or "acute CR" and a PME phase characterizing the "long-term CR" or "chronic CR". Indeed, we observed an enhanced mitochondrial respiratory activity in the ME phase, whereas an attenuated one in the PME phase.

To provide the experimental evidence supporting the suggestion of dual-phase modes, we investigated the CR-mediated expression fashions and activity dynamics of mitochondrial signatures, including those involved in oxidative phosphorylation and antioxidation. Consequently, CR-induced *Sod2* overexpression was found to correlate increased Mn-SOD activity in ME yeast cells. In contrast, downregulated *Sod2* expression and accordingly decreased Mn-SOD activity were measured in PME yeast cells. The "dual-phase responses" of CR, therefore, can be distinguished based on the "up-and-down" modes of mitochondrial signatures. In particular, PME during chronic CR allows the downregulation of biosynthesis pathway genes and the synchronous upregulation of degradation pathway genes involved in major common metabolic pathways. In consistence with our findings, it has been previously described that a shift of the carbon metabolism may account for the extension of yeast lifespan responsive to CR (Lin et al. 2002).

Except for the upregulation of ubiquitylation genes responsible for selective protein degradation and amino acid recycle, CR also allows the overexpression of autophagy genes responsible for re-utilization of cellular constituents. It is known that yeast can augment autophagy during entry into stationary phase, presumably as an adaptive response to starvation (Kamada et al. 2004). Consistent with this observation, it has been demonstrated that yeast mutants defective for autophagy do exhibit short-lived phenotypes (Powers et al. 2006). We also found that autophagy-involved *Atg* genes are upregulated by all treatments, thereby validating that CR and mimetic treatments should involve autophagy. Interestingly, our study revealed that ribosome biogenesis genes are induced to counteract the downregulation of ribosome genes, hence highlighting that translation occurring in the ribosomes is controlled by an interactive way. Besides, we also observed the upregulation of peroxisomal β-oxidation genes responsible for the oxidation of fatty acids in CR and mimetic-exposed yeast. A most recently published report has indicated that CR-activated peroxisomal β-oxidation systems contribute to energy regeneration by stored lipids and recycled cellular components (Lefevre et al. 2013).

In the context of signaling, it is clear that AMPK (encoded by *Snf1* in yeast) and SIRT1 (encoded by *Sir2* in yeast) are responsible for the coactivation of PGC-1α, which leads to the initiation of transcription and translation (protein biosynthesis) essential for mitochondrial biogenesis. However, no significant fluctuations of both *Snf1* and *Sir2* were observed in our study, so we had to alternatively consider the expression alterations of other signal transducers. Consequently, we did notice a marked downregulation of *Kss1* and *Fus3* by CR or mimetics. Both of which belong to MAPK pathway genes, and their encoding products serve as critical checkpoints for protein biosynthesis (Gustin et al. 1998; Pearson et al. 2001). In other words, if MAPK is inactivated, protein biosynthesis should be suppressed (Son et al. 2011). After exposure of PME yeast cells to CR or mimetics, we observed the downregulations of MAPK pathway genes and ribosome genes, indicating protein biosynthesis being hampered in PME yeast cells. In similar, a previous report has also described that inhibition of mRNA translation extends nematode lifespan (Pan et al. 2007).

Why MAPK pathway genes are downregulated in PME cells? To answer this question, we should remember that ROS is almost thoroughly scavenged due to potent antioxidative responses provoked by CR and mimetics. While *Sod1* and *Sod2* are induced in ME cells, *Cta2* and *Gpx1* are induced in PME cells. Also, it is noted that ROS is a powerful activator of all MAPK subfamilies (ERK, JNKs, and p38-MAPK) (Gaitanaki et al. 2003). Indeed, we did not observed the upregulation of *Sod1*, *Sod2*, and other antioxidant genes (except for *Cta2* and *Gpx1*) in PME cells, suggesting a low ROS level due to potent antioxidation. Therefore, it seems that MAPK may not be necessarily activated due to ROS deprival. However, why inactivation of MAPK leads to suppression of MAPK pathway genes remains unexplained, it is likely a consequence of nonphosphorylated MAPK being more than phosphorylated MAPK. Although other protein kinases such as *Tor1* and *Sch9* are also implicated in yeast longevity (Fabrizio et al. 2001; Thomson et al. 2008; Pan et al. 2011; Leprivier et al. 2013), we only observed the significant upregulation of *Tor1* in PME cells, as same as the case described in fission yeast (Rollis et al. 2013). It is unclear whether the upregulation of *Tor1* is due to inhibition of Tor per se in yeast underlying the condition of CR and mimetics.

Numerous investigations on CR paradigms have revealed markedly elevated NO levels although no NOS homologues are found in yeast until now (Kig and Temizkan 2009). However, COX is proposed to produce NO from nitrite under hypoxic conditions (Castello et al. 2006; Taylor and Moncada 2010). We really detected potent NO burst from yeast during CR, but no elevation of NO levels were detected when the NOS substrate ARG was added in yeast cultures (data not shown). On the other hand, it is shown that the mitochondrial uncoupler DNP significantly increases the expression of mitochondrial biomarkers, indicating that DNP-mediated mitochondrial uncoupling promotes mitochondrial biogenesis (Cerqueira et al. 2011). Our results describing the inducible expression of *Cox1* in yeast cells upon exposure to ART seem to support such a preposition that NO-COX interaction can be mimicked by ART-COX conjugation. The covalent binding of ART to COX was monitored by the time-course assay of ART-heme adducts, along with the synchronous measurement of increased COX activity.

A correlation of enhanced respiration by potent antioxidation as one of the mechanisms of longevity is established in yeast, nematodes, and mice. A growing body of literature has indicated that mitochondrial ROS can act as a mediator exerting adaptive/hormetic effects on yeast lifespan (Agarwal et al. 2005; Kharade et al. 2005; Piper et al. 2006). A recent study has also shown that CR elevates H_2O_2 levels in an early stationary phase, and induces SOD activity to help extending lifespan (Mesquita et al. 2010; Schulz et al. 2007). Our results showing that H_2O_2 can mimic CR to extend yeast lifespan provides support to the putative mechanism. Supporting evidence also comes from a recent work indicating that a mitochondrial superoxide signal triggers an increased longevity in nematodes (Yang and Hekimi 2010).

Nevertheless, how antioxidative responses delay aging is still largely unknown. We found that CR-exposed yeast cells exhibit no remarkable induction of *Sod2* genes but significant activation of *Sod2*-encoded Mn-SOD in PME. In this aspect, a recent report provides a possible explanation: the activation of Mn-SOD needs SIRT3 for deacetylating two critical lysine residues (Qiu et al. 2010), suggesting that CR may modulate Mn-SOD activity post-translationally. Additionally, ART and H_2O_2 mimic CR in fully different ways from previously described CR mimics. While RAP acts as an inhibitor of mTORC1 (Harrison et al. 2009), and resveratrol serves as an activator of SIRT1 (Borra et al. 2005) or as an inhibitor of cAMP phosphodiesterases (Park et al. 2012), ART and H_2O_2 do behave like mitochondrial uncouplers such as DNP (Cerqueira et al. 2011).

We proposed here a mechanistic model of CR-triggered NO and ART-driven lifespan extension by dual-phase responses (Fig. 6.2). In the ME phase, mRNA and protein synthesis are increased, respiration is enhanced by accelerated mitochondrial biogenesis. In the PME phase, mRNA and protein synthesis are decreased, respiration is decayed by suppressed mitochondrial biogenesis.

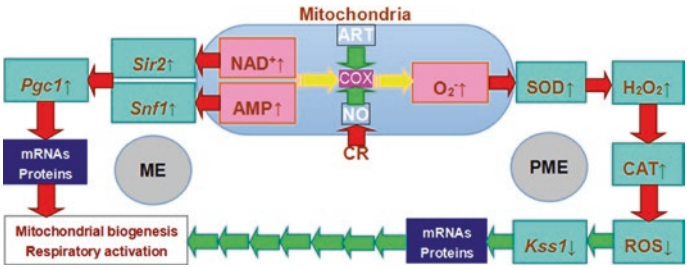

Fig. 6.2 A putative signaling pathway responsible for yeast lifespan extension involved in the mode of dual-phase responses. The *red arrows* indicate a promotion role (positive control), the *green arrows* indicate a repression role (negative control), and the *yellow arrows* represent the direction of electron transport along the mitochondrial respiratory chain. The *up arrow* (↑) within a frame shows an elevation level, and the *down arrow* (↓) within a frame shows a decline level. *AMP* adenosine monophosphate; *ART* artemisinin; *CAT* catalase; *COX* cytochrome c oxidase; *CR* calorie restriction; *ME* mitochondrial enhancement; NAD^+ oxidized nicotinamide dinucleotide; *NO* nitric oxide; *PME* postmitochondrial enhancement; *ROS* reactive oxygen species; *SOD* superoxide dismutase

CR exerts a role in yeast lifespan extension experiencing two stages: the one is enhanced antioxidation to allow the mitigation of ROS generation in the ME phase via respiratory burst; and another is attenuated metabolism to allow the adaption to food shortage in the PME phase via respiratory decay. CR-extended lifespan can be partially mimicked by ART and H_2O_2 via the induction of antioxidant responses and alteration of metabolic responses, which are mirrored by global transcriptome profiling and other sensitive detection procedures.

6.2.4 Conclusions

Although CR-prolonged lifespan is suggested to associate with mitochondrial biogenesis leading to enhanced respiratory activity, it is apparently controversial for that accelerated energy consumption occurs regardless of insufficient nutrient intake. In reconciling the contradiction of less food supply versus much metabolite dispense, we surprisingly noticed a specific mode of CR-based "dual-phase responses" that encompass the phases of ME and PME, which can be distinguished by the expression modes and activity dynamics of mitochondrial signatures. The PME phase is characterized by the downregulation of biosynthesis pathway genes and corresponding upregulation of degradation pathway genes. While protein biosynthesis-gated ribosome genes are downregulated, protein degradation-tuned ubiquitylation genes upregulated. CR-exerted aging-delaying effects can be mimicked by ART, suggesting that ART-heme conjugation functionally resembles NO-heme interaction and therefore establishing a correlation of ART-heme conjugates with increased COX activity. Like CR and ART eliciting antioxidative responses and initiating metabolic modulations, exogenous H_2O_2 also induces antioxidant genes and alters respiratory rhythms, thereby extending yeast lifespan. In conclusion, the available data would provide a perfect explanation to the paradoxical outcome of yeast mitochondrial respiratory activity during CR.

6.3 ART as a NO Mimetic Compromises Mouse Telomere Shortening

6.3.1 Purposes and Significance

CR is known to extend lifespan among distinct organisms with the putative mechanism underlying NO-driven enhanced mitochondrial biogenesis, but whether NO also protects the chromosomal telomeres from erosion remains elusive and inconclusive. Telomeres protect chromosomal ends from degradation, and DNA repair activities, therefore, are essential for chromosome stability (Chan and Blackburn 2002). The telomere length is considered a "molecular clock" of progressive

cellular aging of somatic cells, especially in blood white cells. A longer telomere may reflect less DNA damage and more chromosome integrity. Because CR can prolong lifespan among eukaryotes, we are interested in whether CR would elongate telomere lengths in mice. We believed that this investigation should be beneficial to eventually elucidate aging-involved and longevity-related mechanisms in mammals and humans as well.

According to the findings that CR activates eNOS (Cerqueira et al. 2011) and NO binds to COX (Mason et al. 2006), we proposed that CR-triggered NO may interact with COX to initiate mitochondrial uncoupling/biogenesis and induce antioxidative responses, thereby alleviating oxidative stress, attenuating DNA damage, and eventually mitigating telomere attrition. To provide evidence supporting this proposition, we injected mouse skeletal muscles individually with a kind of NO generators, including the NO inducer ART that inhibits NOS activity and induces NOS expression in a feedback manner (Zeng and Zhang 2011), the NO donor SNP that releases NO in vivo, or the NO precursor ARG that generates NO through NOS catalysis. In the present study, all NO generators were called as CR mimetics because they can partially mimic CR's effects. We intended to disclose the implication of NO derived from CR mimetics in telomere maintenance/elongation, and reveal a possible correlation of mitochondrial biogenesis with telomere maintenance.

6.3.2 Results and Analysis

6.3.2.1 CR Mimetics-Accelerated Antioxidative Responses for Attenuation of Oxidative Stress

Upon competitive occupation within the heme-localized cavity of COX (Mason et al. 2006), NO should block the transport of electrons along the respiratory chain, trigger the burst of ROS, and induce the corresponding responses from the ROS-scavenging networks. To reveal the effect of NO on the elicitation of antioxidative responses, we evaluated the activities of major antioxidants after injection of ART, SNP, and ARG separately into mouse skeletal muscles. At the same time, we compared their antioxidant activities with those in mice exposed to CR. As one of the most common types of ROS, H_2O_2 was also included in the comparison. Consequently, all tested mouse samples exhibit the simultaneous activation of two types of antioxidant enzymes, SOD and CAT, and one type of the antioxidant peptide GSH in a time-dependent manner. Interestingly, ARG confers the highest activities of SOD, CAT, and GSH (Wang et al. 2014). These results indicated that CR mimetics can activate antioxidative responses by synchronously activating the high-molecular-weight antioxidant enzymes and low-molecular-weight antioxidant peptides, which can be repeated by CR per se and exogenous H_2O_2.

Except for activation of total superoxide-scavenging enzymes including Cu/Zn-SOD and Mn-SOD, CR and mimetics also specifically activates Mn-SOD and its

activator SIRT3. ARG leads to the highest Mn-SOD activity, suggesting a synergistic manner of antioxidants located in different compartmental space. However, ARG does not give rise to the highest SIRT3 activity although SIRT3 still shows activation in a time-dependent manner. Accordingly, as an essential consequence of ROS scavenging by coordinated antioxidation, a significant decline of ROS was measured in mouse skeletal muscles treated by CR and mimetics (Wang et al. 2015b). These results indicated that all treatments exhibit the hormetic effects through triggering antioxidative responses and in turn scavenging ROS thereafter.

6.3.2.2 Global Downregulation of Ubiquitylation Genes by a Low Oxidative Stress Milieu

To make sure the plausible relevance of CR to autophagy, we set out to investigate whether CR would affect autophagy-related ubiquitylation genes (*Ubi*) that are involved in regulated protein degradation. Along with exposure of mice to CR, four kinds of CR mimetics (ART, SNP, ARG, and H_2O_2) were separately injected into mouse skeletal muscles for expression profiling of *Ubi* pathway genes. Surprisingly, almost all examined *Ubi* genes are notably downregulated among different treatment groups. It could be observed that the ubiquitin-activating enzyme (E1)-encoding gene, *Atg7*, and *Ube2c* gene that encodes a ubiquitin-conjugating enzyme (E2) are downregulated much in CR but less in other treatments. It was also noticed the upregulation of two ubiquitin-protein ligase (E3) genes, *Hecw1* and *Rnf148*, in mice treated by CR and H_2O_2, but not in other samples. These data demonstrated that *E1* and *E2* genes are downregulated by ART, SNP, or ARG, but *E3* genes are upregulated by CR or H_2O_2 (Wang et al. 2015b). These results suggested that regulated protein degradation is unlikely for mice treated by ART, SNP, or ARG, but likely for mice treated by CR or H_2O_2.

Among the expression profiles of *Ubi* genes, one of the most markedly downregulated genes is the well-known tumor suppressor gene *Brca1*, whose expression level declines down to approximately 25-folds in all samples of mouse skeletal muscles treated by CR and mimetics. Another tumor suppressor gene, *Bard1*, which encodes BARD1 interacting with BRCA1, is also downregulated much by ART, SNP, or ARG, but less by CR. The famous tumor suppressor gene, *Trp53*, is slightly downregulated in all treatment groups. BRCA1 is known to interact with other partner proteins for DNA repair, so we further analyzed the expression levels of *Brca2*, *Rb*, *Myc*, *Rad50*, and *Rad51* in mouse skeletal muscles after treatment by CR per se and mimetics. It is clear that all quantified DNA repair genes are either downregulated or unchanged in the CR group. In other treatment groups, *Brca2*, *Rad50*, and *Rad51* are downregulated or unchanged, but *Rb* and *Myc* are mildly upregulated (Wang et al. 2014). These results indicated that CR only allows the existence of low-level DNA repair proteins, whereas CR mimetics partially mimic CR for downregulation of DNA repair genes. The global downregulation of tumor suppressor genes and other DNA repair genes might reflect less DNA damage, higher chromosomal integrity, and thereby longer telomeres. If this deduction is correct, CR and mimetics should increase the lengths of telomeres within treated cells.

6.3.2.3 Predisposition of Downregulation of DNA Repair Genes on Compromise of Telomere Shortening

We found CR keeps longer telomeres compared with AL. Surprisingly, it was also shown that ART, SNP, ARG, or H_2O_2 enables the elongation/maintenance of telomeres in mouse skeletal muscle cells. It is clear that the longest main bands of telomere restriction fragments (TRF) occur in SNP-treated mice, whereas the shortest main TRF bands appear in AL mice. Interestingly, treatment of mice by ART, SNP, or ARG leads to the longer main TRF bands than CR, and CR even gives rise to the shortest single TRF bands. This is likely because CR mice are older than other treated mice for 3 months when they were employed for measurement of telomere lengths.

To further validate whether the longer telomeres are originated from the increased activity of telomerase reverse transcriptase (TERT), we also quantified *Tert* mRNA in samples collected from CR and mimetics-treated mice. As results, while ARG gives rise to a relatively lower *Tert* mRNA level, other treatments lead to unchanged levels, suggesting that longer telomeres are likely due to attenuated DNA damage rather than enhanced telomerase expression.

Nevertheless, it can be expected that a steady-state telomerase activity should keep the normal telomere lengths. We observed the coordinated downregulation of *Brca1* and *Tert* mRNAs, and also noticed the coexistence of BRCA1 and TERT with similar abundance (Wang et al. 2014). Overall, it was suggested that longer telomeres are not related with high-level telomerase, but both *Tert* mRNA and TERT protein are downregulated by underlying treatments.

6.3.2.4 Initiation of Dual-Tunnel NO Signaling upon NO-Driven Enhanced Expression of Mitochondria-Targeted Proteins

Our previous work showed that ART can alkylate hemoproteins including NOS and CAT by conjugating heme (Zheng and Zhang 2011; Zeng et al. 2011). Furthermore, COX is also a kind of hemoprotein within mitochondria, so we assumed that ART should also inhibit COX activity and block electron transport, thereby initiating a wide-range responses. Surprisingly, we found that ART can induce and activate eNOS, which are mirrored by the synchronous accumulations of both eNOS and phosphorylated eNOS (p-eNOS^{Ser1177}). Interestingly, SNP and ARG can also lead to the coordinated accumulation of eNOS and p-eNOS^{Ser1177}, implying a common signaling pathway for eNOS induction and activation among ART, SNP, and ARG (Wang et al. 2014). These results provided strong support to our assumption of ART capable of inducing and activating eNOS.

To identify the cascade of ART-initiated signaling, we further evaluated the impact of ART on the expression and phosphorylation of AMPK and Akt/PKB. As results, the synchronous accumulations of AMPK and Akt as well as their phosphorylated forms, p-AMPK^{Thr172} and p-Akt^{Ser473}, were observed in mouse skeletal muscles injected with ART. In similar, SNP and ARG also give rise to the high levels of AMPK/p-AMPK^{Thr172} and Akt/p-Akt^{Ser473}. Furthermore, we also

noticed that ART, SNP, or ARG can lead to the upregulation of mitochondrial biomarkers, such as mitofusin 2, cytochrome *c*, and COX4, which are coordinated with their upstream activators, including SIRT1 and PGC-1α. Besides, an activated phosphorylation form of mTOR, p-mTOR^{Ser2448}, was also detected upon treatment by ART, SNP, or ARG (Wang et al. 2014).

To follow up the time-course changes of mitochondrial signatures, we examined the expression dynamics of AMPK, PGC-1α, and cytochrome *c* in mouse skeletal muscles treated by ART, SNP, or ARG. When compared with the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH), we found that AMPK reaches its maximal level within 3 h and subsequently maintains a stable-steady level. Differently, PGC-1α and cytochrome *c* exhibit a time-dependent feature in their expression, namely short-time treatment allows a relative lower level, long-time treatment leads to the highest level (Wang et al. 2014). These results indicated that the signal is conveyed from AMPK to PGC-1α, and finally to cytochrome *c* rather than vice versa.

To verify whether enhanced mitochondrial protein expressions also ensure the according increase of their activities, we measured the activities of eNOS and COX4 after treatment of mouse skeletal muscles by ART, SNP, or ARG. Consequently, all treatments can really lead to the increase of eNOS and COX activities, indicating that an enhanced expression necessarily confer an increased activity for eNOS and COX4 (Wang et al. 2015b). Besides, upregulation of SIRT3, a mitochondria-specific deacetylase, was also monitored after the same treatments as above-mentioned, which may be considered an indirect hint implying mitochondrial biogenesis.

6.3.2.5 Association of CR Mimetics-Derived NO with Mitochondrial Biogenesis

The expression data have revealed the induced upregulation of eNOS by CR mimetics, but direct evidence confirming the elevation of NO levels is still lacking. So we determined the NO level in skeletal muscles of mice injected by CR mimetics. As results, NO burst was seen after treatment for 6 h although a decline trend was observed after treatment for 3 days (Fig. 6.3a), addressing that all kinds of CR mimetics used in this study play their roles upon NO signaling. Furthermore, we also measured the ATP levels in the skeletal muscles of mice injected by CR mimetics. The results as depicted in Fig. 6.3b indicated that ATP is increased after treatment for 6 h, but maintains a steady-state higher level after treatment for 3 days. These results provided support to CR-enhanced mitochondrial functions.

At last, we scrutinized whether the density of mitochondria are changed in mouse skeletal muscle cells exposed to ART, SNP, ARG, or H_2O_2. As compared with one-layer and linear-arrayed mitochondria seen in AL-exposed cells, SNP-treated cells, or H_2O_2-treated cells (Fig. 6.3c–e) show a remarkable mitochondrial proliferation with multilayer mitochondria, and ART-treated cells or ARG-treated cells (Fig. 6.3f and g) also possess more mitochondrial layers than AL-exposed cells after treatment for 6 h.

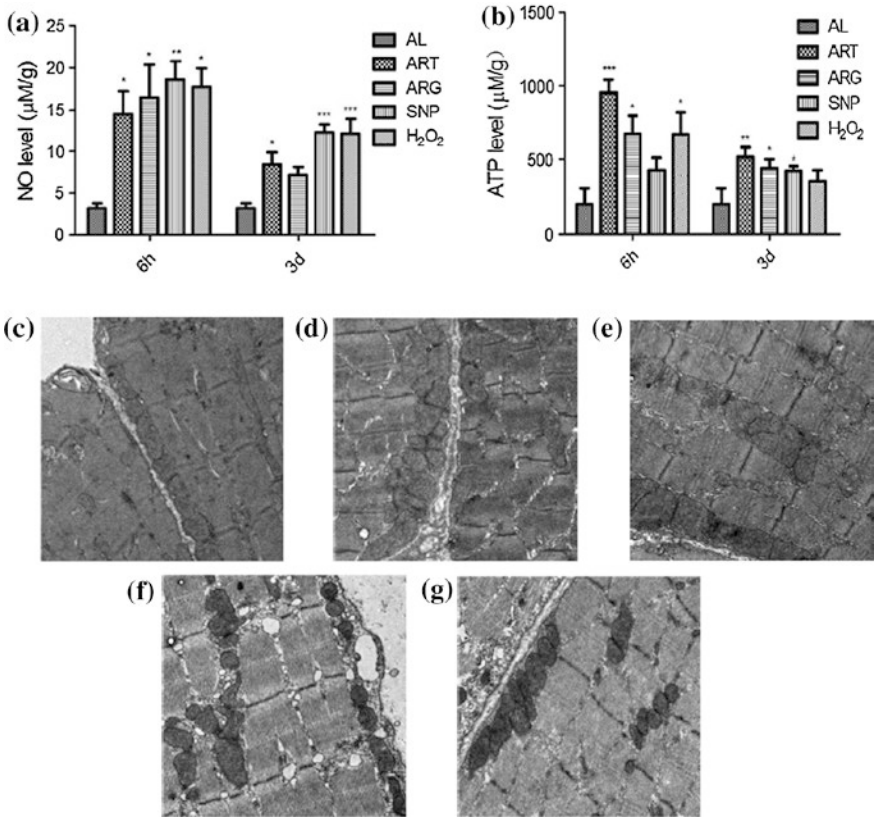

Fig. 6.3 Determination of NO and ATP levels and electronic microscopic phenotyping of mitochondria in mouse skeletal muscles injected by ART, ARG, SNP, or H_2O_2. **a** The elevation of NO levels upon treatment by CR mimetics. **b** The elevation of ATP levels upon treatment by CR mimetics. **c–g** Mitochondrial density and structure in mice treated by ART, ARG, SNP, H_2O_2, and in AL mice, respectively. Samples were collected from mouse skeletal muscles by one injection after 6 h or by daily injection by 260 μm ART, 67 μm SNP, 5.7 mm ARG, or 200 μm H_2O_2 (50 μl injection volume/20 g body weight)

These results unambiguously indicated that CR and mimetics can produce NO, drive mitochondrial biogenesis, and recover energy supply in mice during a short period, for example, within 6 h as examined in the present study.

6.3.3 Discussion

CR-triggered NO production is repeatedly demonstrated in mice (Nisoli et al. 2003, 2005), and we also detected an increase of eNOS activity in mice exposed to CR as well as treated by CR mimetics. Considering the previous conclusion

that mitochondrial uncoupling-elicited increases of AMP/ATP and NAD^+/NADH ratios can separately activate AMPK and SIRT1 (Rodgers et al. 2005; Lee et al. 2006), we assumed that at least two NO-involved signaling pathways exist for sensing such decreases of ATP and NADH levels in mitochondria: one is the AMP-dependent and AMPK-sensed pathway, and another is the NAD^+-dependent and SIRT1-sensed pathway. It has been documented that both AMPK and SIRT1 can coordinately activate PGC-1α, a pivotal modulator for mitochondrial biogenesis (Lee et al. 2006; Suwa et al. 2006). The activated AMPK is known to phosphorylate PGC-1α at threonine and serine residues, thus enabling deacetylation by SIRT1 and, therefore, activation (Canto et al. 2009). Furthermore, AMPK is also informed to catalyze the phosphorylation of TSC2 and regulatory-associated protein of mTOR (Raptor), which lead to the rapid inhibition of mTORC1 activity (Shaw 2009). A most recent finding has indicated that CR decreases ATP levels in nematodes through the tricarboxylic acid cycle intermediate α-ketoglutarate, by which the subunit β of ATP synthase (complex V) is targeted and inhibited (Chin et al. 2014).

It is known that ART inhibits NOS activity and induces NOS overexpression for enhanced NO production (Zeng and Zhang 2011), and SNP, ARG, and H_2O_2 were confirmed to elevate the serum NO levels in treated mice, demonstrating that those NO generators can mimic CR to trigger an elevated NO level. Therefore, CR and mimetics enable the coordinated accumulations of some relevant kinases, acetylases, and mitochondrial signatures/biomarkers. Importantly, we did detect the remarkable elevation of ATP levels and synchronous increase of mitochondria in mice treated by CR mimetics.

According to the earlier findings that CR activates eNOS via Akt (Cerqueira et al. 2011), we suggested the following interactive NO signaling pathways: (1) Akt-eNOS-AMPK-PGC-1α axis, in which AMPK senses the increase of AMP levels; (2) Akt-eNOS-SIRT1-PGC-1α axis, in which SIRT1 senses the increase of NAD^+ levels. The activity of eNOS is known to be regulated by phosphorylation at multiple sites, in which the activation site Ser1177 and the inhibitory site Thr495 are the most thoroughly studied sites (Chen et al. 1999). It should be understandable for the above described scenario because AMPK and Akt are responsible for the phosphorylation of Ser1177 of eNOS in response to various stimuli (Dimmeler et al. 1999; Fulton et al. 1999). Therefore, it should be that a dual signaling cascade may be operated step-by-step: CR activates Akt for phosphorylation of eNOS; Akt activates eNOS for NO production; NO interacts with COX leading to decreases of ATP and NADH; and high-ratio AMP/ATP and NAD^+/NADH eventually activate AMPK and SIRT1. Most recently, the AMPK activator metformin has been revealed to extend the lifespan of fruit flies for 30 %, during which *Atg1* is activated, autophagy initiated, and lifespan prolonged (Ulgherait et al. 2014).

Evidence is emerging to support a concept of mitochondrial hormesis (mitohormesis), which suggests that ROS triggers defense responses, thus leading to increased stress resistance and extended lifespan (Schriner et al. 2005; Schulz et al. 2007). As another supporting evidence, mitochondrial superoxide was found

to increase longevity in nematodes by triggering the response to ROS (Yang and Hekimi 2010). Moreover, H_2O_2-induced antioxidative capacity was proven to extend yeast lifespan (Mesquita et al. 2010). Our findings have validated that CR and mimetics can induce SOD, CAT, and GSH for effective ROS scavenging. In particular, our findings revealing the synchronous induction of mitochondrial Mn-SOD and SIRT3 by CR and mimetics are in consistence with a recent report describing that CR dramatically reduces oxidative stress by inducting SIRT3-activated Mn-SOD (Qiu et al. 2010).

According to previous work done by others, BRCA1 and BRCA2 are believed to interact with RAD51 during repairing DNA double-strand breaks (Boulton 2006; Badie et al. 2010). BRCA1 is recruited to the telomere in a RAD50-dependent manner and may regulate telomere length and stability, in part through its presence at the telomere (Ballal et al. 2009). BRCA1 and BARD1 also constitute a heterodimeric RING finger complex with ubiquitin ligase (E3) activity (Hashizume et al. 2001). Therefore, it seemed that ROS-mediated DNA damage is repaired by BARD1, BRCA1, BRCA2, RAD50, and RAD51. We noticed that CR and mimetics can lead to the global downregulation of almost all examined *Ubi* genes including DNA repair genes, hence implying a less extent of DNA damage. Because BRCA1 is involved in DNA repair (Starita and Parvin 2003), we are confident to conclude that the dramatic downregulation of DNA repair genes implies that extensive DNA repair is unnecessary because of scarce DNA damage.

Telomeres are recently shown to be a favored target of persistent DNA damage in aging and stress-induced senescence (Hewitt et al. 2012). Downregulation of DNA repair genes is an important hint indicating attenuated DNA damage due to potent ROS scavenging from inducible antioxidant networks. Indeed, the longer telomeres were detected among treatments, in which ARG that leads to the longest telomeres may be implicated in human health beneficial (Gad 2010). Whether longer telomeres are due to compromised telomere shortening or enhanced telomere extension is unclear although CR is shown to synergize with telomerase in extending mouse telomeres (Vera et al. 2013). Our data verified that both *Tert* mRNA and TERT are downregulated in similar with BRCA1, and addressed that longer telomeres are attributed to less DNA damage, leading to mitigated telomere shortening.

From above results, we may draw the following conclusion as that CR-triggered NO can enhance the respiratory capacity, as described in yeast (Barros et al. 2004), nematodes (Lemire et al. 2009), fruit flies (Humpherey et al. 2009), and mice (Cerqueira et al. 2011). The accelerated respiration is attributed to NO signaling, which initiates augmented ROS burst, enhanced antioxidative responses, mitigated DNA damage, and downregulated DNA repair genes, finally leading to the compromise of telomere shortening. Interestingly, we have noticed a dual-phase mode of respiratory modulation in budding yeast, i.e., a phase of respiratory burst during acute CR followed by a phase of respiratory decay during chronic CR (Wang et al. 2015a). We should examine if such a dual-phase mode of respiratory modulation exists in mice exposed to long-term CR.

After treatment by CR mimetics for 6 h, we determined an elevated ATP level in mouse skeletal muscles, which is followed by/accompanied with an elevation of serum NO levels. Importantly, all CR mimetics-treated samples were found to display a typical characteristic denoting mitochondrial biogenesis: the large numbers and multiple layers of mitochondria are distributed along the fibrous muscle tissues. In contrast, a nontreated sample shows only one-layer mitochondrial distribution (Wang et al. 2015b). So we can draw a preliminary conclusion that incremented mitochondrial functions might be originated from mitochondrial biogenesis, which should be preceded by a putative process of mitochondrial uncoupling. However, we are currently unable to ensure whether mitochondrial signatures are highly expressed after transient respiratory dysfunction.

It is worthy of indicating that ART was found to simulate the lifespan-prolonging effect of CR in the present work. In this regard, two possibilities for ART-mediated effects may exist: one is a direct effect, and another is an indirect effect. To exert a direct role, ART may bind the heme moiety of COX. To act indirectly, ART may firstly conjugate the NOS's heme, and secondly induce NOS overexpression, NO production, and NO-COX interaction. However, we are unable to exclude these two possibilities at the moment because we have not yet distinguished ART-NOS from ART-COX structurally. Nevertheless, we actually detected the upregulation of eNOS and COX4 followed by the increases of eNOS and COX activities after injecting ART into mouse skeletal muscles, suggesting that ART may simultaneously target eNOS and COX, thereby inhibiting their activities, and subsequently induce their expression in a feedback manner.

The present study is mainly focused on the engagement of AMPK and SIRT1 in mitochondrial biogenesis through PGC-1α although we detected the overexpression of p-mTOR^{Ser2448} induced by CR mimetics. The implication of AMPK and other protein kinases in metabolic modulations via mTOR signaling is of much importance (Shaw 2009). So our future work would be focused on identifying whether there is also an mTOR-specific mode for metabolic alterations in response to CR, from which we might reconcile the apparent controversial of enhanced respiration leading to extended lifespan.

In summary, we have revealed, for the first time, a mechanistic detail of telomere maintenance mediated by CR and mimetics. We have also provided a direct message supporting the debating hormesis hypothesis by validating CR mimetics' beneficial roles on DNA protection. Therefore, our study should shed light on the discovery of new targets and development of new drugs for antiaging and toward longevity.

6.3.4 Conclusions

We report here that three-types of NO generators, ART as a NO inducer, SNP as a NO donor, and ARG as a NO precursor, can mimic CR to enable the compromise of mouse telomere shortening by eliciting antioxidant responses and inducing mitochondria-targeted and SIRT3-activated Mn-SOD, followed by the global

downregulation of protein turnover-relevant ubiquitylation pathway genes, including DNA repair-responsible tumor suppressors such as BRCA1. Surprisingly, exogenous H_2O_2 can also correlate the repression of oxidative stress with the mitigation of telomere attrition, suggesting an involvement of antioxidation in the attenuation of DNA damage and DNA repair. Indeed, telomeres are kept almost intact without the upregulation of *Tert* gene and TERT enzyme, implying telomere maintenance rather than telomere elongation. As a functional NO mimetic, ART may exert its role directly through binding to COX leading to mitochondrial uncoupling, or indirectly via first binding to NOS for NO production and then binding to COX leading to mitochondrial uncoupling. We also established a solid link between NO signaling and mitochondrial biogenesis by monitoring the increased numbers of mitochondria followed by the elevated levels of NO and ATP upon exposure to CR mimetics. In conclusion, CR-triggered NO promotes mitochondrial biogenesis, leading to the activation of whole antioxidative networks and alleviation of telomere erosion, thereby maintaining the stability and integrity of chromosomes, which are the hallmarks of longevity.

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Chapter 7
Prospective

Abstract The exact mechanisms behind aging-related diseases remain unknown. Oxidative/nitrosative stress-mediated posttranslational modifications leading to protein misfolding and structural/functional abnormality have been supposed to be the plausible pathogenic initiators of neurodegenerative diseases. Obesity might be an outcome of the activation of iNOS and inactivation of eNOS, leading to mitochondrial loss and adipose whitening without adipose burning. Inflammation-driven mitochondrial dysfunction might be the intrinsic effector of Warburg effects showing potent ROS burst, which should enhance mutagenesis and tumorigenesis/carcinogenesis.

Keywords Aging-related disease · Cancer · Obesity · Warburg effect

As an endogenous signaling molecule, NO is critical for metabolic homeostasis and cell cycle among multifaceted functions. NO not only vasodilates blood vessels, but also delays aging and extends lifespan. Intriguingly, NO is employed to assist the immune system to kill invaders. In the presence of O_2^-, NO is converted to the potent oxidant $ONOO^-$, which would cause a series of adverse consequences if sustained, mainly including DNA damage and gene mutation as well as protein misfolding and dysfunction.

Whether NO is a "friend" or a "foe" depends on its origination and relative levels, among which the physiological NO generates in a low level from eNOS or nNOS, whereas the pathological NO bursts in a high level from iNOS (Ignarro 2010). Therefore, low-level NO usually exerts a beneficial effect and physiological function, while high-level NO is beneficial to attacking pathogens but harmful by causing tissue damage. During aging, eNOS and nNOS activities gradually decline, and mtNOS activity maintains for only 45–75 % (Boveris et al. 2010), suggesting NO in older persons is insufficient to maintain the normal physiological function.

Environmental stresses originated from ROS and RNS are major pathogenic side effectors that potentially initiate physiological disorders. By a dependent and interactive way, ROS and RNS not only cause DNA damage, but also modify

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proteins. Gene mutations and chromosome instability are implicated in tumorigenesis and carcinogenesis, while protein modifications may interfere with enzymatic functions and signal transduction due to denatured proteins/enzymes. So ROS and RNS are most likely among the etiological initiators of many disorders and syndromes, including aging and aging-related diseases (Bondy and Maiese 2010).

Cumulative evidence has shown that ROS, RNS, and their interactions are seemingly implicated in the pathogenesis of inflammatory diseases. For example, high-level NO-driven hypoxia induces angiogenesis, hyperplasia, and inflammatory infiltration in the synovial tissue during progression to RA (Bao et al. 2012; Wu et al. 2012); NO-derived $ONOO^-$ mediates nitrosylation/nitration of proteins as a possible etiological initiator of protein misfolding-related diseases; and NO-derived $ONOO^-$ initiates DNA damage and gene mutation with an implication in the origin of CSC. The following prospects would share with peers the most recent achievements on $ONOO^-$'s pathogenic potentials and suggest further endeavors to be pursued in the future.

7.1 Nitrosylation/Nitration in the Active Center of Proteins: A Universal Initiator of Aging-Related Disorders?

Autophagy, or autophagocytosis, is a lysosomal clearance process that recycles cellular components and reallocates nutritional intermediates to ensure homeostasis between anabolism and catabolism within living cells. In response to diverse environmental signals such as growth factors, amino acids, energy currency, and starvation, autophagy is actively implicated in many physiological and pathological aspects. While aging is often associated with the reduced autophagy, longevity is attributed to the controlled autophagy in animals and mammalian cells. Knockdown of the autophagy inhibitor p53 can induce the degeneration seen in aging animals. Autophagy triggers lifespan extension, whereas impaired autophagy is often accompanied with the loss of longevity-promoting effects conferred by resveratrol, RAP, and CR (Rubinsztein et al. 2011). Perhaps the most primordial function of autophagy is adaptation to nutrient deprivation, but new evidence also reveals the autophagy's crucial roles in immunity and inflammation, thereby protecting from infectious, autoimmune, and inflammatory diseases (Levine et al. 2011).

In mammals, autophagy is dually tuned by SIRT1 and mTORC1. While SIRT1 knock in induces autophagy, SIRT1 knockout or knockdown prevents the induction of autophagy by resveratrol and nutrient deprivation in human cells (Morselli et al. 2010). Autophagy is negatively regulated by mTORC1, which can be inhibited by RAP or starvation. Under glucose starvation, AMPK promotes autophagy by directly activating the mammalian autophagy-initiating kinase Ulk1 through the phosphorylation of serine 317 and serine 777. Under nutrient sufficiency, high mTOR activity prevents Ulk1 activation by the phosphorylation of Ulk1 at serine

757 and disrupting the interaction of Ulk1 with AMPK (Kim et al. 2011). SIRT1 deficiency results in elevated mTOR signaling, and the SIRT1 activator resveratrol reduces mTOR activity in a SIRT1-dependent manner. SIRT1 interacts with TSC2, a component of the mTOR inhibitory complex upstream to mTORC1, and regulates mTOR signaling in a TSC2-dependent manner (Ghosh et al. 2010). Paradoxically, RAP extends life span and increases insulin sensitivity, but the chronic administration of RAP causes glucose intolerance and insulin resistance. This phenomenon is now deciphered by that RAP disrupts a second mTOR complex, mTORC2, which is required for the insulin-mediated suppression of gluconeogenesis. Suppression of mTORC1 signaling is sufficient to extend life span independently from glucose homeostasis, so the effects of RAP on glucose homeostasis and longevity can be uncoupled (Lamming et al. 2012).

ROS and RNS can regulate autophagy, but how they are engaged in autophagy remains obscure. Sarkar et al. (2011) have revealed a surprising autophagy-modulating pattern by $ONOO^-$-mediated S-nitrosylation of target proteins in mammalian cells. $ONOO^-$ inhibits the autophagic flux by S-nitrosylation of the cysteine residue of JNK1 or IKKβ, which leads to the failure of JNK1/IKKβ phosphorylation, and thereby allows the decrease of JNK1 activity and the abrogation of Bcl-2 phosphorylation, or the activation of mTORC1 upon inactivation of IKKβ, AMPK, and TSC2 (Zeng 2013). From this knowledge, an integrated autophagy-controlling network comprising both mTORC1 and SIRT1 signaling pathways can be summarized (Fig. 7.1).

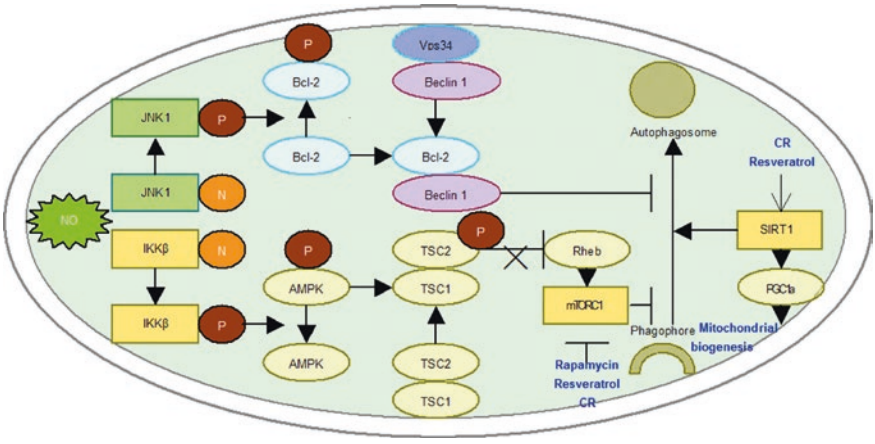

Fig. 7.1 The mechanism underlying NO-repressed autophagy. NO inhibits JNK1 phosphorylation by S-nitrosylation. Decrease of phospho-Bcl-2 and increase of Bcl-2-Beclin1 interaction disrupt Vps34-Beclin1 association. NO also inhibits IKKb phosphorylation by S-nitrosylation. Decrease of phospho-AMPK and TSC2 activity alleviate the inhibitory effect of TSC1/2 on Rheb (denoted by "×"), thereby allowing Rheb to activate mTORC1 for inhibition of autophagy. RAP induces autophagy by inhibiting mTORC1, whereas resveratrol and CR induces autophagy by activating SIRT1 or inactivating mTORC1. SIRT1 accelerates mitochondrial biogenesis by activating PGC-1α

While the overexpression of NOS impairs autophagic flux, the inhibition of NO production induces autophagy. The NOS inhibitor L-NMMA also induces autophagy but is independent on mTORC1 activity or Bcl-2 phosphorylation. Interestingly, deprival of NO by L-NMMA or the endogenous dominant-negative NOS regulator NOS_4 reduces mutant huntingtin aggregation and neurodegeneration in the fruit fly Huntington disease model.

Accumulation of amyloid-β and tau is an invariant feature of Huntington disease, but RAP-induced autophagy allows a significant reduction in amyloid-β levels (Caccamo et al. 2010). Although S-nitrosylation is able to interpret the NO-enhanced autophagy and alleviated neurodegeneration, it has not determined whether S-nitrosylation is a general mechanism of NO-impaired autophagy or only represents a randomized event. Cho et al. (2009) have demonstrated that the S-nitrosylation of dynamin-related protein 1 (DRP1) rich in the brains of Alzheimer's disease patients increases GTPase activity and mediates β-amyloid-related mitochondrial fission and neuronal injury. However, Bossy et al. (2010) have refuted that the S-nitrosylation of DRP1 does not affect GTPase activity and is not specific to Alzheimer's disease. This discrepancy may be attributed to the reversible feature of S-nitrosylation, but another possibility is the alternative modifications of DPR1 or downstream proteins by S-nitrosylation.

NO can also lead to the nitration of tyrosine and other amino acid residues through modification by $ONOO^-$ except for the S-nitrosylation of cysteine. Treatment of rats or mice by LPS in vivo or $ONOO^-$ in vitro significantly promotes the formation of 3-nitrotyrosine in insulin receptor substrate 1 (IRS-1) and reduces the insulin-dependent tyrosine phosphorylation, eventually leading to the insulin resistance of skeletal muscles (Pilon et al. 2010). The enhanced 3NT modification of mitochondrial proteins occurs with aging, in which the nitration of F1-ATPase at tyrosine 269 leads to ADP binding to the enzyme's active center, and is associated with a moderate impairment of the mitochondrial function (Lam et al. 2009). If the nitration of tyrosine occurs globally, it seems reasonable to interpret the results of Sarkar et al. (2011) by the nitrosative inactivation of separated signaling proteins of the autophagy machinery. Besides, L-NMMA abrogates neurodegeneration perhaps through directly blocking the misfolding and aggregation of proteins such as Huntingtin due to S-nitrosylation or 3-nitration.

Indeed, S-nitrosylation of protein-disulfide isomerase or the E3 ubiquitin ligase parkin is found to initiate protein misfolding and aggregation in Parkinson's disease (Gu et al. 2010). Previously, overexpression of nNOS was detected in the brains of Parkinson's disease patients (Eve et al. 1998). The existence of 3NT was found in the core of Lewy bodies, the pathological hallmark of Parkinson's disease progression (Good et al. 1998). The observation of nNOS was correlated with the presence of 3NT in circulating neutrophils from Parkinson's disease patients (Gatto et al. 2000). Recent studies have shown that α-synuclein is one of the major building blocks in Lewy bodies (Ischiropoulos 2009). It is apparent that the majority of Lewy bodies and protein inclusions contain nitrated and oxidized α-synuclein, indicating that oxidation is participated in the formation of these inclusions. Additionally, nitration or nitrosylation of ubiquitin E3-ligases can result

in the defective transfer of proteins to ubiquitin. In the proteosome, mutations of the E3-ligase parkin explain the appearance of juvenile Parkinson's disease by the less degradation of NOS and aggravation of excess NO-mediated mitochondrial damage and complex I abrogation (Boveris et al. 2010).

Due to reaction with the excessive O_2^- leading to generation of $ONOO^-$, the scarcely available NO should exist during aging. Indeed, senescent endothelial cells display higher mTORC1 activity, increased O_2^- production and decreased bioactive NO levels than young endothelial cells. This is contributed by the so-called "uncoupling" of NOS that does not produce NO but O_2^-. Silencing mTORC1 in senescent cells reduces O_2^- generation and enhances NO production, whereas the overexpression of a constitutively active mTORC1 mutant in young endothelial cells mimics endothelial dysfunction of senescent cells through NOS uncoupling and induces premature cellular senescence. RAP and resveratrol, by inhibiting mTORC1 signaling, result in the decrease of O_2^- levels, but increase of NO levels in the senescent cells as well as in the aortas of old rats (Rajapakse et al. 2011). Likewise, short-term CR initiated in old age reverses age-associated vascular endothelial dysfunction by restoring NO bioavailability, reducing NADH oxidase-mediated O_2^- production, stimulating antioxidant enzyme activity, and upregulating SIRT1 (Rippe et al. 2010).

7.2 Low-Grade Inflammation as an Essential Consequence of Obesity?

In regard to obesity, there are many unsolved problems. Is obesity a disease? Is the fat deposited in the subcutaneous adipose tissue (SAT) benign, but the fat deposited in the visceral adipose tissue (VAT) malignant? Does the brown adipose tissue (BAT) or the white adipose tissue (WAT) distinguish the healthy or unhealthy obesity? Does chronic inflammation induce obesity and insulin resistance? Does the antihypoxic intervention reduce body weight and improve insulin sensitivity?

There has a definition of obese/lean not strictly based on the body mass index (BMI), but has no specific standards to distinguish the composition of an individual that is composed of the adipose and muscle tissues. However, some above mentioned terms such as SAT or VAT as well as BAT or WAT have been employed to describe the features of adiposity. SAT localizes under the skin, arms, breasts, buttocks, hips, and thighs, while VAT distributes around or within the liver, heart, muscles, and pancreas. BAT rather than WAT has an extraordinary number of mitochondria and numerous capillaries, allowing it to look dark and gland-like. BAT is more active than WAT in lipid degradation and energy expenditure.

Although the American Medical Association (AMA) declare obesity to be a disease, Katz (2014) recently argued that obesity is not a disease, but a risk factor of other chronic diseases. This is because not only can chronic diseases develop in the absence of obesity, but not every obese person develops any such conditions. A BMI-based thin person might have an increased fat depot in VAT. In similar,

those individuals with a normal BMI might possess a higher body fat proportion. Compared with people with a normal body fat composition, those persons with VAT have a nearly fourfold risk of prediabetes, and nearly twice as likely to have high blood pressure or heart disease (Levine and Levine 2011). As to BAT and WAT, while adults have much WAT, new born babies have much BAT. Obese individuals have more WAT than do lean individuals.

Is low-grade inflammation originated from obesity? There are much discrepant opinions regarding the origin and cause of obesity-induced inflammation. Some authors thought that inflammation might be derived from the activated macrophages within the adipose tissue (Weisberg et al. 2003), some authors suggested that increased adipocyte O_2 consumption would trigger HIF-1α, causing inflammation and insulin resistance in obesity (Lee et al. 2014), and other authors supposed that hypoxia-induced heme oxygenase-1 (HO-1) might drive the chronic inflammation and insulin resistance (Jais et al. 2014). However, it was also believed that macrophage activation induces proinflammatory cytokines, which can in turn activate iNOS to produce NO and drive hypoxia via HIF-1α.

A logical relationship is inflammation cause hypoxia, hypoxia results in reduced energy expenditure, and reduced energy expenditure leads to obesity. Indeed, BAT is usually "whitening" due to decreased mitochondria and compromised angiogenesis (Shimizu et al. 2014). So it can be anticipated that anti-inflammation and antihypoxia are generally effective for weight reduction. It was found that chronic blockade of iNOS by L-NMMA reduces adiposity and improves insulin resistance in HFD-induced obese mice (Tsuchiya et al. 2007). Through anti-inflammation, salisylate as a degraded product of aspirin was found to reduce circulating lipids in obese rats and to improve insulin sensitivity (Yuan et al. 2001). Like metformin, aspirin was shown to able to treat type II diabetes as an activator of AMPK (Hawley et al. 2012). Nitroaspirin was suggested to have therapeutic potential for NAFLD (Ibrahim et al. 2011). Overexpression of or supplementation with EPO induces reduced blood glucose levels and body mass in mice (Katz et al. 2010). Similarly, it was also noticed that EPO treatment can inhibit WAT inflammation, normalized insulin sensitivity, and reduced glucose intolerance in mice (Alnaali et al. 2014).

What are the primary and cardinal reasons triggering adipose, muscular, and systemic inflammations? Such issues are debating in either noninfectious origin or infectious origin. Nevertheless, some scientists seem to insist a dual origin pattern, by which the free fatty acids (FFA) and LPS can equally activate proinflammatory cytokines and induce insulin resistance (Heinrichsdorff and Olefsky 2012). Although some authors supposed FFA-mediated inflammation in obesity, they are unable to reasonably explain how can FFA do this. While the interaction of LPS with Toll-like receptor 4 (TLR4) via CD14 has been confirmed, an interaction of FFA with TLR4 is illy elucidated for a long time. Eventually, the liver secretory protein Fetuin A (FetA) was found to function as an adapter between FFA and TLR4, by which the triple interaction of FFA with TLR4 via FetA has been established (Pal et al. 2012). Nevertheless, it remains awaiting for answering why short-chain fatty acids (SCFAs) prevent rather than promote obesity (Blaut 2014), and how can FetA distinguish and selectively bind to saturated FFA but not unsaturated FFA.

Interestingly, a previous finding delineated that the disruption of FatA expression renders animals more susceptible to endotoxemia, whereas the supplementation of FetA confers protection against lethal endotoxemia (Li et al. 2011). This result implies that elevation of FetA might be due to LPS accumulation in obesity because FetA can competitively bind to TLR4 with LPS. In fact, it seems that the LPS-originated inflammation theory has evoked much enthusiasms in recent years. Ding et al. (2010) indicated that intestinal inflammation precedes and correlates with HFD-induced obesity, adiposity and insulin resistance. They suggested that HFD interact with gut microbiota to trigger the expression of TNF-α and NF-κB. The absence of gut microbiota in germ-free mice blunts the upregulation of primary inflammatory indicators. A relevance of HFD to gut dysbiosis was revealed as that HFD increases the ratio of *Firmicutes* to *Bacteriodetes*, and induces the overgrowth of *Enterobecteriaceae*, which exacerbates inflammation and obesity in mice via the TLR4 signaling pathway (Kim et al. 2012). A recent work indicated that adipocyte inflammation is essential for healthy adipose tissue expansion and remodeling, in which visceral fat is deposited for effectively filtering the gut-derived LPS (Asterholm et al. 2014). This finding seems imply an association of gut bacterial dysbiosis with visceral adipose storage.

Recently, we investigated the effects of different dosages of LPS on the expression levels of proinflammatory cytokines in mice fed with HFD. Surprisingly, we found that low-dose (0.25 mg/kg) LPS enhances the low-grade inflammation in the visceral tissue, whereas high-dose (1.2 mg/kg) LPS does not induce or even suppresses inflammation in the peripheral tissue. For example, intramuscular injection of HFD-induced mice with 1.2 mg/kg LPS extremely downregulates proinflammatory cytokines in the skeletal muscle. In contrast, intraperitoneal injection of HFD-induced mice with 0.25 mg/kg LPS mildly upregulates proinflammatory cytokines for only 2–3 folds.

Accordingly, we also noticed that the serum NO and 3NT levels that represents the extent of iNOS activation by proinflammatory cytokines are gradually declined during high-dose LPS injection. In contrast, low-dose LPS can mimic the gastrointestinal infection by G^- bacteria that produce and leak LPS into the blood stream. It could be concluded that low-grade inflammation is originated from low-dose LPS, but we are currently unknown why high-dose LPS exerts an immunosuppressive effect. We found that ART, DNP, and NG exert weight-reducing effects through anti-inflammation and mitochondrial biogenesis. For example, ART, DNP, and NG were found to downregulate iNOS and downstream genes, and upregulate eNOS and downstream genes, suggesting activated eNOS/inactivated iNOS might be a pivotal switch for weight loss.

I propose here a novel hypothesis of healthy/unhealthy obesity based on the comparison of low-grade inflammation, insulin resistance, SAT/VAT, and BAT/WAT (Fig. 7.2).

Healthy obesity was assumed to be germ-free (without LPS leakage) or G^+ bacteria-induced adiposity with a high BMI but without VAT and insulin resistance, whereas unhealthy obesity was supposed to be G^- bacteria-induced adiposity with high BMI, VAT and low-grade inflammation, probably exhibiting insulin

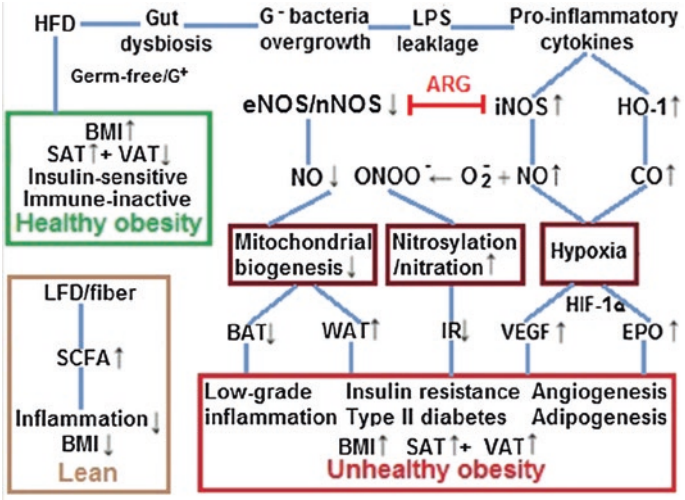

Fig. 7.2 A hypothesized mechanism of adipogenesis/obesity. *ARG* L-arginine; *BAT* brown adipose tissues; *BMI* body mass index; *CO* carbon monoxide; *eNOS* endothelial nitric oxide synthase; *EPO* erythropoietin; G^- Gram negative bacteria; G^+ Gram positive bacteria; *HIF-1α* hypoxia inducible factor α; *HO-1* heme oxygenase 1; *iNOS* inducible nitric oxide synthase; *IR* insulin receptor; *LFD* low-fat diets; *LPS* lipopolysaccharide; *nNOS* neuronal nitric oxide synthase; *NO* nitric oxide; *SAT* subcutaneous adipose tissues; *SCFA* short-chain fatty acids; *VEGF* vascular endothelial growth factor; *VAT* visceral adipose tissues; *WAT* white adipose tissues

resistance or type II diabetes. Chronic inflammation is most likely derived from an interaction of HFD with gut dysbiosis, leading to the overgrowth of G^- bacteria and the leakage of bacterial LPS. Upon activation of proinflammatory cytokines, LPS can upregulate the expression of iNOS and HO-1, which directly lead to the overproduction of NO and CO. Both gas molecules can competitively bind to hemoglobin and myoglobin with O_2, leading to metabolic hypoxia. The hypoxic condition can subsequently induce HIF-1α, VEGF, and EPO, thereby driving angiogenesis and adipogenesis. The commonly occurred hyperinsulinemia/hyperlepti nemia in obesity might be explained by the $ONOO^-$-mediated nitrosylation/nitration of insulin/leptin receptors. For BAT whitening to WAT in obesity, it might be explained by the dramatic decline of NO that is derived from eNOS/nNOS and the suppression of mitochondrial biogenesis.

By comparison of the gut microbiota between the healthy adults and the patients with type II diabets, Qin et al. (2012) found the hypoproliferation of butyrate-producing bacteria and the overgrowth of sulfate-reducing bacteria among other abnormalities of gut microbiota. The butyrate-producing bacteria are fiber-digested bacteria, whereas the sulfate-reducing bacteria are meat-addicted bacteria. For example, the δ-proteobacteria, *Desulfovibrio piger*, without any forms of sulfatases can only survive in the gut depending on the sulfate released from *Bacteroides thetaiotaomicron*. It was found that chondroitin

sulfate and mucin are the main sources of sulfate uptaken by *B. thetaiotaomicron*. Interestingly, enhanced mucus degradation was considered a couse of reduced gut lining integrity (Rey et al. 2013).

The gastrointestinal tracts of centenarians were recently found to have less abundant sulfate-reducing bacteria such as *Desulfovibrio*, but have much plentiful butyrate-producing bacteria such as *Clostradia* (Wang et al. 2015). Nevertheless, it is currently inconclusive whether obesity is caused by the dysbiosis between sulfate-reducing bacteria and butyrate-producing bacteria. However, we can image that the trace amount of hydrogen sulfide that is released by less abundant sulfate-reducing bacteria might be beneficial to extend lifespan (Qabazard et al. 2014), but overproduction of hydrogen sulfide by the overgrown sulfate-reducing bacteria might induce the cancerous alteration (Attene-Ramos et al. 2007) and initiate an adipogenetic process (Tsai et al. 2015) directly or indirectly through conversion of nitrate/nitrite to large-amount NO (Vermeiren et al. 2012).

Given that gut bacteria, especially G^-, are responsible of LPS leakage and inflammation induction, how to prevent such an adverse outcome? The most important consideration should be prevent the dysbiosis of gut microbiota. For this purpose, you should maintain a balanced daily diet that contains low fat and high fiber. If you are an obese person or a patient with type II diabetes, you should restrict your calorie uptake, and recover your microbiota homeostasis. For example, it is beneficial to eat more fiber-rich vegetables and fruits to nourish the butyrate-producing bacteria, but eat less meats or chondroitin sulfate-rich foods to avoid the overgrowth of sulfate-reducing bacteria.

Interestingly, dietary yeast has been reported to reduce hepatic steatosis, obesity, and type II diabetes in mice (Everard et al. 2014) as well as neuroinflammation in mice (Takata et al. 2015), suggesting that yeast might narrow the occupying space by bacteria, and compromise LPS-induced inflammatory diseases. If this situation is also confirmed in human, supplementation with dietary yeast as an probiotic alternative to bacterial prebiotics might be helpful to decrease the risk of obese, fatty liver, and type II diabetes.

7.3 The Origin of CSC: Next Breakthrough on Tumorigenesis/Carcinogenesis?

Regardless of debating on the concept of CSC, tumorigenesis/carcinogenesis must experience the extensive genetic alteration and chromosome instability. It is known that the chronic inflammation is linked to cancer by rehearing the scenarios of tumor initiation, promotion, malignant conversion, invasion, and metastasis (Grivennikov et al. 2010). How inflammation causes tumor/cancer remains largely unknown, but many types of tumor/cancer are known to be originated from a long-term immune activation that upregulates proinflammatory cytokines and triggers NO burst. Given that NO is implicated in inflammation-originated tumor/cancer,

how it acts on, directly or indirectly, maturated cells or stem sells remains obscure. Nevertheless, we could still anticipated that the origin of tumor/cancer should at least engage the following effectors: (1) NO-driven hypoxia. Sustained NO burst due to chronic inflammation may cause hypoxia, angiogenesis and hyperplasia that are seen in the benign tumor. (2) $ONOO^-$-mediated mutagenesis and modification. The concomitant occurrence of high-level NO and O_2^- promotes the formation of $ONOO^-$ and enables the denaturation of proteins and DNA, which are frequently seen in either benign or malignant tumor/cancer. (3) NO-confered cytoprotection. Tumor/cancer cells are characterized by extreme longevity, and there must be an association of NO with antiapoptosis, antiautophagy, antisenescence, and anticytotoxicity in tumor/cancer cells.

Hypoxia promotes tumor development through multiple mechanisms including initiating tumor angiogenesis (Choi et al. 2003), causing genome mutagenesis (Papp-Szabo et al. 2005), maintaining anaerobic metabolism (Gillies and Gatenby 2007), and modifying acidic microenvironment (Fang et al. 2008; Chen et al. 2010; Hjelmeland et al. 2011). Early in 1998, Wink et al. had reviewed that chronic inflammation can lead to the de novo production of many kinds of chemical intermediates, among which $ONOO^-$ that mediates nitrogen stress is able to make DNA damage. Previously, it was detected that purine and pyrimidines deamination, strand breaks and purine modification, and nitroguanine adduction can occur upon exposure of DNA to $ONOO^-$ (Yermilov et al. 1995; Zingarelli et al. 1996). Usually, DNA damage necessarily leads to DNA repair by tumor suppressors, thereby uneasily leading to genetic mutagenesis. Surprisingly, why are genetic mutations not rare in tumor/cancer? The most possibility is that $ONOO^-$ inactivates DNA repair responsible enzymes such as tumor suppressors, p53 and BRCA1, via nitrosylation/nitration. Therefore, genes are highly prone to be mutated when DNA repair responsible tumor suppressor genes have been mutated. Indeed, evidence for $ONOO^-$-mediated p53 modification in human gliomas had been declared (Cobbs et al. 2001).

An investigation on the genetic disease Laron's syndrome (dwarfism) that is resulted from the deficiency of growth hormone receptors has revealed a very low risk of suffering cancer and diabetes among patients. It is believed that the synchronously generated growth hormone (GH) and insulin-like growth factor (IGF) might be the enhancers promoting tumor proliferation (Leslie 2011). Furthermore, it has been documented that insulin and IGF facilitate the transport of glucose into cells through activating PI3K, which is antagonisted by the tumor suppressor PTEN. When PI3K is overactivated due to PTEN mutation, cells prone to uptake a large quantity of glucose for anaerobic respiration (Warburg effects), with which the metabolic activity increases for 10–20 folds. As the consequence, more ROS is released and more DNA damaged, hence leading to a higher frequency of tumorigenesis/carcinogenesis (Taubes 2012a). Interestingly, the diabetes pill metformin can decrease the cancer rate for 25–40 % via activating AMPK and decreasing blood insulin levels, thereby supporting the notion that high-level insulin increases the risk of cancer (Taubes 2012b).

Why does tumor cell prefer to glycolysis-based substrate phosphorylation (with the generation of only one mole of ATP) rather than citrate cycle-coupled oxidative phosphorylation (38 moles of ATP) even in an aerobic condition? This might be inflammation activates iNOS but inactivates eNOS, which should block NO-driven mitochondrial biogenesis. So the deficiency of functional mitochondria of tumor cells are forced to utilize glucose and other carbohydrates depending on the ineffective metabolic pattern, i.e. in aerobic respiration. If those possibilities could be confirmed, the unsolved issue on the origin of CSC should be readily solved, and a next breakthrough on the pathogenesis of cancer might be anticipated.

More enthusiastically, there have been most recent achievements to support such anticipation: (a) inflammasome-mediated dysbiosis regulates the progression of nonalcoholic fatty liver disease (NALFD) and obesity (Henao-Meijia et al. 2012); (b) hyperresponsibility to low-does endotoxin LPS during progression to nonalcoholic steatohepatitis (NASH) is regulated by leptin-mediated signaling (Imajo et al. 2012); (c) obesity-induced gut microbial metabolite promotes liver cancer through senescence secretome (Yoshimoto et al. 2013); and (d) obesity, rather than diet, drives epigenomic alterations in colonic epithelium resembling cancer progression (Li et al. 2014).

There seems a plausible association of HFD with NASH, during which the obesity-induced leptin plays a crucial role through inducing the hepatic expression of CD14 and increasing the cellular hyper-reactivity to low-dose LPS (Imajo et al. 2012). A large entity of literature has indicated that type II diabetes might be associated with an increased incidence of pancreatic cancer, hepatic cancer, colon cancer, bladder cancer, and breast cancer, suggesting a mitogenic effect of hyperinsulinemia (Joost 2014). Obesity has been identified as a cause for oesophageal, colon, uterine, kidney and postmenopausal breast cancers, and also as a significant risk factor for prostate cancer, pancreatic cancer, and non-Hodgkin lymphoma (Gong et al. 2014).

A hyperglycemic environment has been supposed to contributes to tumor progression through multiple pathways (Ryu et al. 2014). Cancer cell proliferation is promoted by the upregulation of glucose transporters (GLUT1 and GLUT3), growth factors and receptors, and other growth promoting signals. The levels of HIF-1α, prolyl hydroxylase, and cytochrome *c* regulated by hyperglycemia are associated with antiapoptotic activity of cancer cells. As a possible mechanism underlying insulin-driven cancer, it has been summarized that an increase of insulin levels is correlated with an increase of the bioavailable IGF, which promotes cellular proliferation and inhibits apoptosis in many tissue types (Suh and Kim 2011). Previously, it was found that many cancer cells have an increased content of insulin receptor (IR) (Papa et al. 1990) and an predominant expression level of the isoform A of IR (IR-A), whose activation elicits mitogenic effects (Frasca et al. 1999). By binding to the overexpressed IR-A, insulin may favor cancer progression and facilitate tumor growth.

According to above results, we suggested here a mechanistic model involving the origin of tumorigenesis and summarizing the conversion of benign tumor to malignant tumor, as depicted in Fig. 7.3, in which the relevant overweight/obesity, insulin tolerance/type II diabetes, and RA were also illustrated.

As described above, activation of proinflammation cytokines is derived from endotoxinemia, which is an essential consequence of HFD-induced gut dysbiosis, leading to the overgrowth of G^- bacteria and the leakage of bacterial LPS into the blood. Upon systemic, chronic and low-grade inflammation, both iNOS and HO-1 are upregulated for overproducing NO and CO and leading to hypoxia. Next, hypoxia-sensed HIF-1α, VEGF, and EPO are induced, thereby driving angiogenesis, cell proliferation, and inflammatory infiltration (benign tumor formation). Upon the activation of iNOS, both eNOS and nNOS should be inactivated due to the deprival of the NO precursor ARG by super activated iNOS. The lack of eNOS-derived NO would impede mitochondrial biogenesis and lead to adipose tissue whitening, i.e. BAT conversion to WAT, which remarkably decrease energy expenditure, alters lipid storage/usage homeostasis, and eventually allows overweight and obesity.

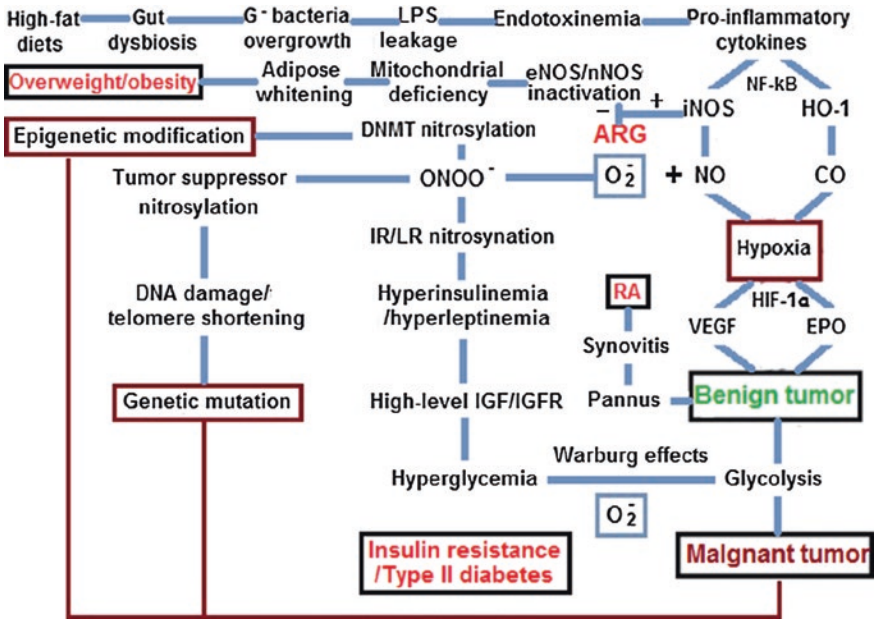

Fig. 7.3 A mechanistic model of the origin of a malignant tumor from a benign tumor. *ARG* L-arginine; *CO* carbon monoxide; *DNMT* DNA methyltransferase; *eNOS* endothenial nitric oxide synthase; *EPO* erythropoietin; *G*$^-$ Gram negative bacteria; *HIF-1α* hypoxia inducible factor α; *HO-1* heme oxygenase 1; *IGF* insulin-like growth factor; *IGFR* IGF receptor; *iNOS* inducible nitric oxide synthase; *IR* insulin receptor; *LPS* lipopolysaccharide; *LR* leptin receptor; *NF-κB* nuclear factor κB; *nNOS* neuronal nitric oxide synthase; *NO* nitric oxide; *RA* rheumatoid arthritis; *ROS* reactive oxygen species; *VEGF* vascular endothelial growth factor. The *plus symbol* (+) represents activation, and the *minus symbol* (−) represents inactivation

If hyperinsulinemia occurs due to $ONOO^-$-mediated nitrosylation of IR/LR, insulin resistance and even type II diabetes would take place. The synovial pannus, an earliest symptom of RA, should be a tumor-like hyperplasia in the articular synovium. Hyperglycemia can initiate Warburg effects for enhanced glycolysis, ROS burst, and nitrosylation, accompanying with the inactivation of tumor suppressors responsible for DNA repair and DNMT responsible for DNA methylation. Eventually, genetic mutation and epigenetic modification could occur (malignant tumor conversion).

Multiple receptors/enzymes have been implicated as the mediators linking hyperglycemia to cancer. An in vitro study suggested that glucose transporters, including GLUT1 and GLUT3, are regulated by hyperglycemia in choriocarcinoma cells (Hahn et al. 1998). In a hyperglycemic medium supplemented with 25 mM D-glucose, GLUT1 and GLUT3 are upregulated, and glucose uptake is enhanced. It was observed that the levels of epidermal growth factor (EGF) and its corresponding receptor (EGFR) that promote oncogenesis are augmented by high-level glucose in pancreatic cancer cells (Han et al. 2011). Similarly, hyperglycemic conditions was found to induce peroxisome proliferator-activated receptors (PPARs) in breast cancer cells (Okumura et al. 2002), and high levels of PPAR-α and PPAR-γ that influence lipid metabolic pathways accelerate cell proliferation (Mueller et al. 1998). Additionally, high glucose promotes the cell cycle through cyclin-dependent kinase 2 (CDK2), E2F, cyclin A, and cyclin E, resulting in abnormal proliferation (Masur et al. 2011).

Furthermore, hyperglycemic conditions were also noticed to augment the levels of glial cell line derived neurotophic factor (GDNF) in human pancreatic cancer cells (Liu et al. 2011). GDNF, a cytokine related to the transforming growth factor-β family, can enhance the survival, growth, and differentiation of midbrain dopaminergic neurons, which promote cell proliferation and invasion in pancreatic cancer (Lin et al. 1993; Veit et al. 2004). Taken together, these data highlighted that hyperglycemia serves as a contributing factor to enhanced cell proliferation and cancer.

In breast cancer, the family-transmitted *BRCA1* mutation is linked to estrogen receptor α negative ($ER\alpha^-$) with unknown reasons. It was suggested that 17β-estradiol or estrogen-$ER\alpha^-$ influences BRCA1 expression, and BRCA1 inhibits its ERα activity through a monoubiquitination mechanism, implying the existence of a negative feedback mechanism that regulates functional interaction between ERα and BRCA1 in breast cancer cells (Manavathi et al. 2013). However, this suggested model is unable to explain why *BRCA1* mutation is often accompanied with triple negative, i.e. $ER\alpha^-$, progesterone receptor negative (PR^-), and human epidermal growth factor receptor 2 negative ($HER2^-$).

It has been noted that loss of BRCA1 results in impaired DNA double-strand break repair and activated TP53-mediated apoptosis (Shukla et al. 2011; Pao et al. 2014). So I assumed that breast cells carrying *BRCA1* mutation might be destroyed by TP53-mediated apoptosis, which should lead to the undetection of abovementioned receptors. In contrast, if *TP53* is also mutated, the newly proliferated breast cancer cells with *Brca1* mutation should be survived, and all receptors could be detectable, i.e. receptor positive.

Because of *BRCA1* mutation-coupled ERα^{-}, the cellular level of estradiol must be raised in breast cancer cells. Such an extremely higher estradiol level can derive the generation of many hydoxylated products that are able to induce high frequent genetic mutations (Savage et al. 2014). Naturally, BRCA1 is responsible for repairing the damage of double-strand breaks on DNA. In normal conditions, estrogen can exert an anti-inflammatory role via binding to ERα (Morselli et al. 2014). So it is logically reasonable that the dual alteration of *Brca1* mutation with ERα^{-} could predispose the tumorigenesis/carcinogenesis due to high-level estrogen-mediated DNA damage, and the lack of anti-inflammatory effects that are conferred by the estrogen-ERα signaling should exacerbate the inflammatory lesions from metabolic hypoxia and Warburg effects.

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