Atherosclerosis: Open Access

Wang et al., Atheroscler Open Access 2018, 3:1

Review Article Open Access

# Roles of Nitric Oxide Signaling Pathway in Atherosclerosis

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**Received date:** January 12, 2018; **Accepted date:** February 28, 2018; **Published date:** March 05, 2018



**Abstract**

Nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) plays a critical role in the vasoprotective function of the endothelium under physiological conditions. However, under pathological conditions, eNOS and nNOS become dysfunctional, and inducible nitric oxide synthase (iNOS) is stimulated to produce excessive NO, which induce endothelial dysfunction. NO signaling pathways mainly include phosphatidylinositol 3-kinase (PI3K)/serine threonine protein kinase B(AKT)/eNOS pathway, nuclear factor-kappa B(NF-κB)/iNOS pathway, extracellular regulated protein(ERK)1/2/nNOS pathway, etc. which are involved in the development and progression of atherosclerosis through affecting NO synthesis and its functions. Meanwhile, NO-soluble guanylyl cyclase (sGC)-cyclic guanosine monophosphate (cGMP) pathway is closely related to atherosclerosis, due to the ability of regulating the degree of vasodilatation. Elucidating NO signaling pathway associated with atherosclerosis is necessary for the improvement and treatment of atherosclerosis. This review summarized the relationships between the different NO signaling pathways and atherosclerosis in the present relative studies.

**Keywords:** Nitric oxide; Atherosclerosis; Signaling; Pathway

## Introduction

Atherosclerosis is the underlying cause of most cardiovascular diseases, such as coronary artery disease (CAD), heart failure, stroke and so on. These diseases are the main cause of human mortality and disability in the world today [1]. NO was firstly proposed as an endothelium-derived relaxing factor by Furchgott and Ignarro in 1986, and it was also the first gaseous molecule accepted to be a signaling mediator in the organism [2]. Many studies have shown that NO produced under different conditions has an inhibitory or promoting effect on atherosclerosis. The role of NO in the pathogenesis of atherosclerosis has not yet been fully elucidated. In this paper, the progress in relationships between NO signaling pathways and atherosclerosis was reviewed.

## Pathological Characteristics of Atherosclerosis and its Presumptive Pathogenesis

Atherosclerosis is an inflammatory disease that results in the development of atherosclerotic plaques and progressive stenosis of the coronary arteries [3]. Atherosclerosis starts at the arterial intima [4] where endothelial cells become activated, resulting in recruitment and accumulation of monocytes, which differentiate into macrophages [5]. Macrophages become foam cells through the uptake of oxidized low density lipoprotein (oxLDL). In addition, it has been reported that foam cells also derive from smooth muscle cells (SMCs) [6]. Mature SMCs that are present in the media of the nonatherosclerotic vessel wall possess the potential to become activated during plaque development and transdifferentiate to macrophage-like cells with a Mox phenotype that reside within the lesion. The SMCs derived plaque macrophages have a clonal origin in the vascular media and can make up a major fraction of the intimal cells. Foam cells produce high levels of pro-inflammatory cytokines, chemokines and growth factors that induce SMCs proliferation and migration to the intima, leading to plaque growth. Due to intimal hyperplasia with macrophages and SMCs infiltration, vascular lumen becomes narrowed, causing the ischemic changes of the organ [7].

A variety of hypotheses have been developed to explain atherosclerosis, in which cholesterol deposition in arterial wall, viral or bacterial infection, response-to-injury, local arterial inflammation and autoimmune response were involved [8]. Although some progress has been made, the exact pathogenesis still remains to be clarified [9].

The current views on the pathogenesis of atherosclerosis are as follows: (1) Atherosclerosis has been considered as an inflammatory/autoimmune disease, and its attack is initially caused by proinflammatory Th1 cells and cytokine immune responses. Now, the Th17/Th1 axis is thought to be shared by most sterile inflammatory diseases. The anti-inflammatory Th2 cells and cytokine immune response are initiated concomitantly with the former, the latter dampening their harmful reactions which culminate in full-blown atherosclerosis. Interleukin-33 (IL-33), as a novel member of the IL-1 cytokine superfamily, is a multifaceted mediator with both pro- and anti-inflammatory activities, and it participates in antiatherogenic response through mediating the Th1-to-Th2 switch of immune response [10]. The IL-1β induces the procoagulant activity, promotes monocyte and leukocyte adhesion to vascular endothelial cells, and accelerates the growth of SMCs in the development of

atherothrombotic plaque [11]. Meanwhile, IL-1β drives the IL-6 signaling pathway, which promotes the development of inflammation. In CANTOS [11], anti-inflammatory therapy targeting the IL-1β innate immunity pathway with canakinumab led to a significantly lower rate of recurrent cardiovascular events than placebo, independent of lipid-level lowering. (2) DNA methylation in the genome plays an important role in the development of atherosclerosis. DNA methyltransferases is crucial in maintaining endothelial cell integrity, promoting SMCs proliferation, and inducing atherosclerosis formation in animal models. Some studies have shown that oxidative stress affected DNA methylation in the progression of atherosclerosis [12]. (3) Mitochondrial dysfunction and mitochondrial DNA (mtDNA) damage may be directly involved in atherosclerosis formation [13]. At least four mitochondrial genomic mutations, namely, A1555G in MT-RNR1 gene, C3256T in MT-TL1 gene, G12315A in MT-TL2 gene and G15059A in MT-CYB gene are related to lipofibrous plaques in human aortic intima [8]. (4) Endothelial cell metabolism is perturbed to promote atherogenesis when endothelial cells become dysfunctional [5]. Peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) is a major regulator of atherosclerosis-related cellular energy metabolism. It has been reported that PGC-1 was modulated at its transcriptional level by miR-19-3p, miR-221-3p and miR-222-3p targeting PGC1α in atherosclerosis, which contribute to endothelial cell dysfunction [14].

## The Putative Role of NO in Atherosclerosis

NO is generated by a family of three nitric oxide synthase enzymes(NOS) isoforms, namely endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) [15]. Most of the NO in the vascular system is produced by eNOS [16]. Endothelium-derived NO (eNO) is a multifunctional signaling molecule that, as an effective endogenous vasodilator, inhibits the key process in vascular lesion formation. eNO reduces the production of reactive oxygen species(ROS) and lipid peroxidation [17]. In addition, eNO also has the effect of inhibiting platelet adhesion and aggregation, inhibiting adhesion molecule and chemokine expression, as well as reducing inflammatory cell infiltration and SMCs migration and proliferation [18].

The first evidence that nNOS played a role of vascular protection in atherosclerosis was from the work of Wilcox et al., which showed the correlation between the progression of plaque formation and nNOS mRNA [19]. And it is compatible with the studies showing accelerated neointimal formation and constrictive vascular remodeling in nNOS-knockout (nNOS-KO) mice in a carotid artery ligation model [20].

Vascular cells express iNOS, which produces large amounts of NO leading to vascular dysfunction in inflammatory conditions [16]. Zhao et al. proved that the high levels of NO produced by iNOS may interfere with the efficacy of the liverX receptor α(LXRα)-ATP-binding cassette transporter A1(ABCA1)–dependent cholesterol efflux and thereby promote oxLDL-mediated cholesterol accumulation in foam cells, thus leading to atherosclerosis progression [21]. The experimental results indicated that the anti-atherosclerosis effect of adiponectin is to reduce oxidative/nitrative stress by inhibiting iNOS, superoxide and peroxynitrite production [22]. Superoxide usually functions as a reducing agent, and the fate and effect of this radical depend on surrounding conditions. Under normal physiological conditions, cells generate small but significant amounts of superoxide. The superoxide dismutase catalyzed dismutation is the most important antioxidant systems in terms of superoxide anions [23]. Superoxide formation increases dramatically, and at the same time iNOS produces high concentrations of NO in atherosclerosis. Both radicals react in a diffusion-controlled fashion to yield the toxic and highly reactive nitrogen species peroxynitrite, which might account for numerous deleterious effects associated with nitro-oxidative stress [24]. In human atherosclerosis and in a mouse model of vascular remodeling (carotid artery ligation), Reventun et al. [25] found a significant correlation between increased levels of inducible nitric oxide and decreased integrin-linked kinase levels (ILK, plays a key role in controlling vasomotor tone and is decreased in atherosclerosis) comparing with iNOS knockout mice, and NO/iNOS promoted the dissociation of the complex ILK/heat shock protein 90/eNOS, leading to eNOS uncoupling. They provided the first molecular evidence about the negative effect of NO/iNOS on ILK stability by inducing internalization and molecular degradation of ILK in lysosomes. Inflammation of the vascular wall during atherosclerosis and vascular remodeling induces iNOS to release vast amounts of NO, promoting the development of atherosclerosis.

It can be seen that the NO/eNOS and NO/nNOS produced in physiological conditions have the effect of anti-atherosclerosis, and the NO/iNOS generated under pathological conditions will trigger or accelerate atherosclerosis.

## NO Synthesis Pathway and Atherosclerosis

The main signaling cascades associated with NO in atherosclerosis are as follows (Figure 1): (1) Under pathological conditions, the activated nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase) induces endothelial cells to produce a large amount of ROS, which blocks PI3K-mediated and adenosine monophosphate activated protein kinase (AMPK)-mediated phosphorylation of AKT, and then lead to a decrease in NO produced by eNOS. (2) Oxidative stress activates nuclear factor erythroid 2-related factor 2 (Nrf2), which can induce eNOS phosphorylation and enter the nucleus to trigger Heme oxygenase-1 (HO-1) gene expression, and then inhibits the development of atherosclerosis. (3) eNOS is a Ca (2+)-dependent enzyme, and atherosclerosis is associated with the Ca(2+)/calmodulin-dependent kinase II(CaMKII) or calmodulin-dependent protein kinase kinase(CaMKK)/AMPK/AKT/eNOS/NO pathway abnormality. (4) Under inflammatory conditions, NF-κB p50 and p65 transfer to the nucleus, and induce iNOS gene expression, then accelerate the process of atherosclerosis. Meanwhile, ROS promotes the above cascade reaction through activating p38 mitogen-activated protein kinase (MAPK). What's more, the active MAPK (ERK1/2, c-Jun N-terminal kinase (JNK)1/2 and p38) can increase the concentration of NO produced by iNOS directly.

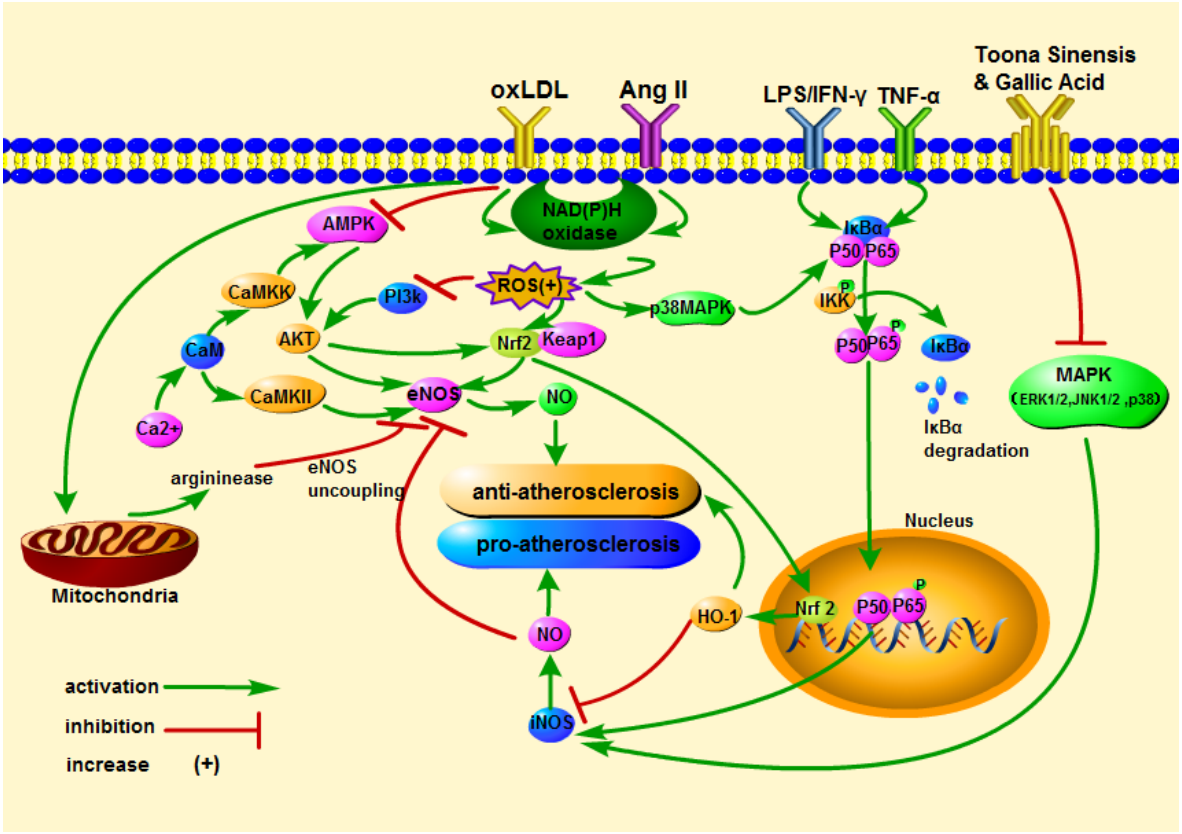


**Figure 1:** The main signaling pathways of eNOS/NO and iNOS/NO in atherosclerosis. OxLDL: Oxidized low density lipoprotein; Ang II: Angiotensin II; LPS: Lipopolysaccharide; IFN-γ: Interferon-γ; TNF-α: Tumor necrosis factor-α; NAD(P)H oxidase: Nicotinamide adenine dinucleotidephosphate-oxidase; ROS: Reactive oxygen species; PI3K: Phosphatidylinositol 3-kinase; AKT: Serine threonine protein kinase B; eNOS: Endothelial nitric oxide synthase; NO: Nitric oxide; AMPK: Adenosine monophosphate activated proteinkinase; CaMKII: Calmodulin-dependent kinase II; CaMKKII: Calmodulin-dependent protein kinase kinaseII; Nrf2: Nuclear factor erythroid 2-related factor 2; Keap1: Kelch-like ECH-associated protein 1; HO-1: Heme oxygenase-1; iNOS, Induciblenitric oxide synthase; IκB: Inhibitory subunit of NF-κB; IKK: IκB kinase complex; NF-κB: Nuclear factor-kappa B; MAPK: Mitogen-activated protein kinase; JNK: c-JunN-terminalkinase; ERK1/2: Extracellular regulated protein1/2.

## The eNOS/NO Signaling and Atherosclerosis

### The PI3K/AKT/eNOS/NO pathway and atherosclerosis

OxLDL binding to lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) triggers the activation of NADPH oxidase, which results in the production of ROS and lipid peroxidation (Table 1) [26]. In addition, the PI3K/AKT / eNOS/NO pathway disordering induced by oxLDL leads to the reduced NO production, and these abilities of NO scavenging superoxide anions, preventing superoxide anion from forming its dismutation product and hydrogen peroxide are impaired, eventually induces endothelial cell damage [27]. PI3K, which is a heterodimeric enzyme, plays a critical role in proliferation and apoptosis, while its downstream serine-threonine kinase, AKT, transmits survival signals from growth factors [28]. The activation of AKT mediated by PI3K involves its phosphorylation on threonine 308 and serine 473, and the activated AKT promotes eNOS to produce NO [27]. Phosphocreatine (PCr) [27] and Myricitrin [26] have been proved to inhibit the apoptosis of human umbilical vein endothelial cells (HUVECs) induced by ox-LDL and reduce the formation of atherosclerotic plaques by activating PI3K/AKT/eNOS/NO pathway. The vascular endothelial dysfunction of high fat diet induced atherosclerosis rat model [29] and mouse model [30] was eased with drugs which upregulated the mRNA and protein expression of PI3K, AKT and increased eNOS phosphorylation.

| Pretreatment | Animal | Cells | Signaling pathway | NO levels | Atherosclerosis | References |
|---|---|---|---|---|---|---|
| Myricitrin | atherosclerotic mouse model | ox-LDL-induced HUVECs | PI3K/AKT/eNOS(+); STAT3(+) | ↑ | ↓ | [26] |

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| Phosphocreatine | | ox-LDL-induced HUVECs | PI3K/AKT/eNOS (+) | ↑ | ↓ | [27] |
| Klotho | | ox-LDL-induced HUVECs | PI3K/AKT/eNOS (+) | ↑ | ↓ | [28] |
| Wen-Xin Decoction | Wistar rats models of atherosclerosis | | PI3K/AKT/eNOS (+) | ↑ | ↓ | [29] |
| Salidroside | apoE(-/-) male mice with high-fat diet | | AMPK/PI3K/AKT/ eNOS (+) | ↑ | ↓ | [30] |
| Ethanol extract of *Rumex acetosa* L. | phenylephrine-precontracted rat thoracic aorta | HUVECs | PI3K/AKT/eNOS (+) Ca(2+)-eNOS-NO (+) | ↑ | ↓ | [31] |
| Progranulin | | HUVECs | AKT/eNOS (+) | ↑ | ↓ | [32] |
| Osteocalcin | apoE (-/-) mice with high fat diet | HUVECs | PI3K/AKT/eNOS (+) | ↑ | ↓ | [33] |
| Olmesartan | carotid atherosclerosis patients | | PI3K/AKT/eNOS (+) | ↑ | ↓ | [34] |
| Irisin | apoE(-/-) streptozotocin-induced diabetic mice | HUVECs | AMPK-PI3K-AKT-eNOS (+) | ↑ | ↓ | [35] |
| TRAIL | diabetic rats | HUVECs | AKT/eNOS (+) | ↑ | ↓ | [36] |
| Vaspin | | Ratbonemarrowderived endothelial progenitor cell with glucose treatment | PI3K/AKT/eNOS (+) | ↑ | ↓ | [37] |
| EGCG | | homocysteine- induced HUVECs | PI3K/AKT/eNOS (+) | ↑ | ↓ | [38] |
| Interleukin-1β | | HUVECs | Ca(2 +) / CaMKII / eNOS (-) | ↓ | ↑ | [39] |
| β-carotene | | HUVECs | Ca (2 +) / CaMKKII / eNOS (+) | ↑ | ↓ | [39] |
| Betulinic acid | | HUVECs | Ca(2+)/CaMKII/ eNOS (+); Ca(2+)/CaMKK/ AMPK/eNOS (+) | ↑ | ↓ | [40] |
| CytochromeP450 enzymes(CYP) 2C8-derived epoxyeicosatrie-noic acids | | TNF-α-induced HUVECs | Nrf2/HO-1/eNOS (+) | ↑ | ↓ | [41] |
| Piceatannol | | palmitic acid-stimulated HUVECs | Nrf2/HO-1/eNOS (+) | ↑ | ↓ | [42] |
| Prunella vulgaris | | HUVECs with high glucose treatment | Nrf2/HO-1/eNOS (+) | ↑ | ↓ | [43] |
| Quercetin | | ox-LDL-induced HUVECs | AMPK/NADPH oxidase/AKT/eNOS (+) | ↑ | ↓ | [44] |
| Ginkgo biloba extract | | ox-LDL-induced HUVECs | AMPK / AKT /eNOS (+) | ↑ | ↓ | [45] |
| Vaspin | Sprague-Dawley rat | HAECs obtained from Lonza Inc. | STAT3/ DDAH / ADMA/ eNOS (+) | ↑ | ↓ | [46] |
| Korean red ginseng water extract | wild type miceatherogenic model mice with high cholesterol diet | | OxLDL/arginase/ eNOS-uncoupling(-) | ↑ | ↓ | [47] |

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|---|---|---|---|---|---|---|
| OxLDL | ApoE(-/-) mice | human aortic endothelial cells; murine aortic endothelial cells | OxLDL/arginase/ eNOS-uncoupling(+) | ↓ | ↑ | [48] |
| OxLDL | ApoE(-/-) mice with Arg2(-/-);lectin-like OxLDL receptor-1 knockout mice | | OxLDL/arginase/ eNOS-uncoupling(-) | ↑ | ↓ | [48] |
| Tongxinluo | male Wistar Kyoto rats | | ERK1/2-nNOS-NO(+) | ↑ | ↓ | [49] |

Abbreviations: NO: Nitric oxide; eNOS: Endothelial Nitric oxide Synthase; nNOS: Neuronal Nitric Oxide Synthase; ox-LDL: Oxidized Low Density Lipoprotein; HUVECs: Human Umbilical Vein Endothelial Cells; PI3K: Phosphatidylinositol 3-kinase; AKT: Serine threonine Protein Kinase B; STAT3: Signal Transducer and Activator of Transcription 3; AMPK: Adenosine Monophosphate Activated protein Kinase; TRAIL: Tumor Necrosis Factor-related apoptosis-inducing ligand; EGCG: Epigallocatechin Gallate; CaMKII: Calmodulin-dependent Kinase II; CaMKKII: Calmodulin-dependent Protein Kinase II; TNF-α: Tumor Necrosis Factor-α; Nrf2: Nuclear Factor Erythroid 2-related factor 2; HO-1: Heme oxygenase-1; NADPH Oxidase: Nicotinamide Adenine Dinucleotide Phosphate-oxidase; DDAH: Dimethyl Arginine Dimethyl Amino Hydrolase; ADMA: Asymmetric Dimethyl Arginine; ERK1/2: Extracellular Regulated Protein1/2.

**Table 1:** The role of different eNOS or nNOS/NO signaling in atherosclerosis.

The NOS-NO signaling of HUVECs treated with ethanol extract of Rumex acetosa L (ERA) [31], granule protein [32] and osteocalcin (OCN) [33] have been evaluated. The results showed that the phosphorylation of eNOS was induced through PI3k / AKT signaling in HUVECs treated with the former three, and the level of NO was significantly up-regulated, while the above effects could be blocked by PI3K inhibitors and AKT inhibitors [31-33]. In animal experiments, the isolated thoracic aortic vasodilatation induced by ERA was eliminated by L-NAME (NOS inhibitor) [31].

Gong et al. [34] selected 40 patients with carotid atherosclerosis. Patients were administrated olmesartan 20 mg/day for 3 months. The results showed that olmesartan could increase the number of circulating endothelial progenitor cells and the serum levels of eNOS and NO. Spearman rank correlation analysis showed there was no relationship between the promoting effects of olmesartan on endothelial progenitor cell mobilization and the clinical characteristics (e.g, gender, age, blood pressure, etc.) ($P>0.05$). They concluded that olmesartan effectively promoted the mobilization of endothelial progenitor cells and improved their function in patients with carotid atherosclerosis relying on the PI3K/AKT/eNOS/NO pathway [34].

Irtrexate [35], tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [36], and Vaspin [37] have been proved to activate the PI3K/AKT/eNOS/NO pathway in HUVECs, increase NO production, inhibit apoptosis, oxidative stress, NADPH oxidase production induced by high glucose, increase the expression of antioxidant enzyme, and finally improve diabetes-related atherosclerosis [35-37].

## The Ca (2+)/CaMKII or CaMKK/AMPK/AKT/eNOS/NO pathway and atherosclerosis

eNOS is a Ca (2+) -dependent enzyme that is regulated by store-operated calcium entry (SOCE), and SOCE inhibitors impair the activity of eNOS [50]. Yamagata et al. [39] found that IL-1β enhanced the expression of adhesion molecules, reduced NO production and induced atherosclerosis. The results showed that IL-1β reduced the expression of Ca (2+)/CaMKII, PI3K and eNOS in cultured human endothelial cells. In contrast, it increased the expression of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1). It was found that IL-1β-induced atherosclerosis was associated with the Ca (2+)/CaMKII/eNOS/NO pathway abnormality.

Both AMPK and AKT are important regulators of eNOS activity, which promote NO secretion by increasing eNOS phosphorylation in human endothelial cells and vessels [44]. β-carotene and betulinic acid (BA) have the effect of preventing atherosclerosis, which is mediated by the Ca (2+)/CaMKII or CaMKK/AMPK/eNOS/NO pathway [39,40]. The research showed that the treatment of β-carotene [39] and BA [40] induced AMPK, eNOS phosphorylation and increased the level of NO in the cultivated human endothelial cells. They also increased the level of intracellular Ca (2+) and phosphorylation of Ca (2+)/CaMKIIα and Ca(2+)/CaMKKβ. At the same time, the treatment with AMPK inhibitor compound C, L-type Ca (2+) channel (LTCC) inhibitor, W7 (CaM antagonist), KN-93 (selective inhibitor of CaMKII) or STO 609 (selective inhibitor of CaMKK) reduced eNOS phosphorylation and the level of NO [39,40].

Studies have shown that oxLDL-induced endothelial oxidation involves the blockage of the AMPK/AKT/eNOS/NO pathway in atherosclerosis [44,45]. Hung et al. [44] pretreated HUVECs with or without quercetin and treated them with oxLDL. The results showed that quercetin protected against the decrease of AMPK activity and reduced oxLDL-activated NOX2 and NOX4. However, silent AMPK weakened quercetin's protective function of anti-oxidation. At the same time, oxLDL inhibited AKT/eNOS/NO pathway, impaired mitochondrial function and enhanced the formation of ROS. Ou et al. [45] pretreated HUVECs with or without Ginkgo biloba extract (GbE) and treated them with oxLDL. Western blot results showed that GbE inhibited membrane translocation of NADPH oxidase subunits p47 (phox) and Rac-1, and attenuated the AMPK dephosphorylation and the increase of subsequently activated protein kinase C (PKC)-induced membrane subunits gp91 and p22 (phox) protein expression. AMPK-α(1)-specific small interfering RNA-transfected cells that had been exposed to GbE followed by oxLDL revealed the increased levels of PKC and p47(phox). In addition, exposuring to oxLDL resulted in the reduced AMPK-mediated AKT/eNO signaling [45].

## The Nrf2/HO-1/eNOS/NO pathway and atherosclerosis

Nrf2 is a redox-sensitive master regulatory transcription factor which usually binds to Kelch-like ECH-associated protein (Keap)-1 in the cytoplasm. Nrf2 plays an important role in ensuring sustained release of NO and protecting endothelial cells from apoptosis [41]. HO-1 is an antioxidant and anti-inflammatory inducible enzyme. Nrf2 is activated by oxidative stress and then enters the nucleus, where it

binds to AU rich elements in the HO-1 promoter to trigger HO-1 gene expression [42]. Tumor necrosis factor-α (TNF-α) incubation significantly reduced Nrf2 expression in the nuclear fraction and inhibited Ser-1177 eNOS phosphorylation as well as HO-1 induction in HUVECs, causing endothelial cell apoptosis [41]. Meanwhile, other experiment showed that aqueous extract from Prunella vulgaris (APV) induced the activation of HO-1 and eNOS and enhanced the production of NO by activating the PI3K/AKT-mediated Nrf2 pathway and inhibiting Keap1 degradation, thus inhibited the diabetes-related atherosclerosis [43].

## The STAT3/DDAH/ADMA/eNOS/NO pathway and atherosclerosis

Signal transducer and activator of transcription 3(STAT3) is a key transcription factor that regulates cell survival, inflammation and angiogenesis. ApoE-/- mouse atherosclerosis model [26], ox-LDL-induced HUVECs [26] showed that drug therapy activated the STAT3 signaling to relieve endothelial cell apoptosis and the early atherosclerotic plaque formation.

Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of eNOS. And ADMA derives from methylation of arginine residues in proteins and is metabolized to citrulline and dimethylamine by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) [46]. Jung et al. [46] proposed that vaspin affected the activity of DDAH II promoter, activated DDAH II gene expression and reduced the level of ADMA by mediating STAT3 signaling in the aorta model isolated from Sprague-Dawley (SD), finally increased STAT3 phosphorylation and eNOS activity in STAT3-dependent way and played the role of anti-atherosclerosis.

### The OxLDL/arginase/eNOS-uncoupling/NO pathway and atherosclerosis

Hypercholesterolemia reduces the bioavailability of NO by affecting the function and activity of eNOS. OxLDL stimulates NADPH oxidase in the vessel wall, which mediates the oxidation of eNOS cofactor tetrahydrobiopterin (BH4), resulting in uncoupling of eNOS [51]. Arginase (a key enzyme in the urea cycle) directly competes with eNOS for the common substrate L-arginine, inhibits the activity of eNOS and affects the production of NO, leading to endothelial dysfunction. Recent studies have supported that arginase (including enzyme I and enzyme II) causes eNOS uncoupling, and the expression and activity of arginase are upregulated in atherosclerosis [47,52,53]. Pandey et al. [48] found that oxLDL triggered arginase II transfering from mitochondria to cytoplasm in human aortic endothelial cells and rat aortic intima, accompanied by an increase in arginase activity and eNOS uncoupling. Mitochondrial processing peptidase inhibitors or LOX-1-knockdown reduced the decrease in endothelial-specific NO production and the increase in superoxide production induced by ox-LDL [48].

## The iNOS/NO Signaling and Atherosclerosis

### The NF-κB-iNOS-NO pathway and atherosclerosis

NF-κB represents the transcription factor family, including p50 and p65 that are important in the regulation of inflammatory responses. NF-κB p50 and p65 transfer to the nucleus, and bring out a variety of gene transcription participating in diverse cellular processes (including inflammation, proliferation, apoptosis and cellular senescence) (Table 2) [54]. Activation of the NF-κB signaling has been found in macrophages, SMCs and endothelial cells in human atherosclerotic lesions, but has not been found in healthy blood vessels [32]. NF-κB regulates iNOS gene: iNOS catalyzes NO generation, and NO is a molecule that plays a role in inflammation and immune response [55]. Vascular inflammation induced by pathogens or cytokines is accompanied by the production of peroxynitrite, which is an effective vasotoxic molecule formed by the reaction of NO and superoxide [56]. The activation of NF-κB-iNOS-NO pathway accelerates the development of atherosclerosis.

| Pretreatment | Cells | Signaling pathways | NO levels | Atherosclerosis | References |
|---|---|---|---|---|---|
| Tormentic acid | H2O2-induced RVSMCs | IKK/IκBα/NF-κB/iNOS(-) | ↓ | ↓ | [54] |
| Progranulin | LPS-induced HUVECs | IKK/IκBα/NF-κB/iNOS(-) | ↓ | ↓ | [32] |
| Artemisinin | RVSMCs co-incubated with TNF-α | IKK/IκBα/NF-κB/iNOS(-) | ↓ | ↓ | [55] |
| Andrographolide | TNF-α-stimulated HUVECs | JNK-AKT-NF-κBp65-iNOS(-) | ↓ | ↓ | [56] |
| hydrophilic α-tocopherol derivative(PMC) | LPS/IFN-γ-stimulated VSMCs | ROS-PP2A-NF-κBp65-iNOS(-) | ↓ | ↓ | [57] |
| Cyanidin-3-Glucoside (C3G) | Ang II-stimulated human endothelial cells | IKK/IκBα/NF-κB/iNOS(-) | ↓ | ↓ | [58] |
| atorvastatin and C3G | Human aortic smooth muscle cells exposed to Ang II | IKK/IκBα/NF-κB/iNOS(-) | ↓ | ↓ | [59] |
| GPx1 knockout | primary aortic endothelial cells isolated from GPx1 KO mice | IKK/IκBα/NF-κB/iNOS(+); MAPK(P38, ERK and JNK)-iNOS(+) | ↑ | ↑ | [60] |
| Honokiol | palmitic acid-stimulated HUVECs | IKK/IκBα/NF-κB/iNOS(-) | ↓ | ↓ | [61] |

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| Resokaempferol | LPS-stimulated RAW264.7 macrophages | JAK2 / STAT3-iNOS(-); NF-κB-iNOS(-); JNK/p38 MAPK-iNOS(-) | ↓ | ↓ | [62] |
| Prunella vulgaris ethanolextract | TNF-α-stimulated human aortic smooth muscle cells | p38MAPK/ERK-iNOS(-) | ↓ | ↓ | [63] |
| Toona sinensis and gallic acid | LPS-treated rat aortic smooth muscle cells | NF-κB/iNOS(-); MAPK (ERK1/2, JNK1/2, and p38)-iNOS(-) | ↓ | ↓ | [64] |

Abbreviations: iNOS: Induciblenitric Oxide Synthase; $H_2O_2$: Hydrogen Peroxide; RVSMCs: Rat Vascular Smooth Muscle Cells; IκB: Inhibitory Subunit of NF-κB; IKK: IκB Kinase Complex; NF-κB: Nuclear Factor-kappa B; LPS: Lipopolysaccharide; JNK: c-JunN-terminal Kinase; IFN-γ: Interferon-γ; VSMCs: Vascular Smooth Muscle cells; ROS: Reactive Oxygen Species; PP2A: Protein Phosphatase 2A; Ang II: Angiotensin II; MAPK: Mitogen-activated Protein Kinase; JAK2: Janus Kinase 2.

**Table 2:** The roles of different iNOS/NO signaling in atherosclerosis.

## The IKK/IκBα/NF-κB/iNOS/NO pathway

The most common form of NF-κB is the p65/p50 heterodimer in most cell types, and the NF-κB signaling is controlled by the IκB(inhibitory subunit of NF-κB) kinase(IKK) complex, which is composed of IKKα, IKKβ, IKKγ and the downstream substrate IκBα (the main type of IκB) [55]. Activated IKK phosphorylates IκBα due to stimulation, resulting in IκBα degradation, enhancing NF-κB nuclear translocation and subsequent transcriptional activation [55].

Angiotensin II(Ang II) [56], hydrogen peroxide($H_2O_2$) [52], TNF-α [53] activate NADH/NADPH oxidase, and induce the ROS-activated NF-kB signaling. ROS has been assumed to be the second messenger of NF-kB activation [56]. The increased production of ROS activated the NF-kB signaling in Ang II-induced human endothelial cells [56,57] $H_2O_2$-induced rat vascular smooth muscle cells (RVSMCs) [52] and TNF-α-induced RVSMCs [53]. Pretreating the above models with drugs could attenuate the formation of NO by iNOS and atherosclerotic plaques by inhibiting the degradation of IkBα inhibitors and down-regulating the expression of NF-κB subfamily (P50 and p65) [52,56,57]. In diabetic-related atherosclerosis, glutathione peroxidase-1 (GPx1) is a key antioxidant enzyme [58]. The IkBα degradation was prolonged in the primary aortic endothelial cells (PAECs) isolated from GPx1-KO mice, and the activation of NF-κB signaling promoted the development of atherosclerosis [58].

In the model of HUVECs damage induced by palmitic acid (PA), Honokiol reduced iNOS expression and NO production by inhibiting IκB phosphorylation and NF-κB activation in IKK/IκB/NF-κB pathway, thereby reduced the PA-induced inflammatory response and repaired endothelial dysfunction [59].

## The IKK-IκBα-independent-NF-κB-iNOS-NO pathway

The combination of lipopolysaccharide (LPS) with interferon (IFN)-γ [55] and TNF-α [54] induced VSMCs to produce inflammatory responses that promoted the progression of atherosclerosis. The pathway involved in above reaction is the IKK-IκBα-independent-NF-κB-iNOS-NO pathway.

The treatment with 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) [55] suppressed the action of LPS and IFN-γ in VSMCs. PMC significantly inhibited the translocation and phosphorylation of p65, protein phosphatase 2A(PP2A) inactivation and ROS formation, and decreased the expression of iNOS, but it had no effect on IκBα, IKK. PP2A-selective inhibitor okadaic acid and PP2A siRNA transfection significantly reversed the role of PMC on inhibiting iNOS expression, NF-κB-promoter activity and p65 phosphorylation. Immunoprecipitation analysis of LPS/IFN-γ-stimulated VSMCs extracts showed that p65 colocalized with PP2A, and PP2A induced p65 phosphorylation. It indicated that LPS/IFN-γ stimulated activation of ROS-PP2A-NF-κB p65-iNOS-NO pathway to promote atherosclerosis [55].

Andrographolide [54] inhibited the action of TNF-α in VSMCs. Andrographolide reduced the TNF-α-induced phosphorylation of JNK, AKT and p65, thereby inhibited iNOS from producing NO. But Andrographolide affected neither IκBα degradation nor p38MAPK or extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. The activity of NF-κB in the VSMCs inhibited by Andrographolide was mediated by JNK-AKT-p65 pathway [54].

## The JAK2/STAT3-iNOS-NO pathway and atherosclerosis

The STAT3 activation in endothelial cell and immune cell may exacerbate inflammation in the advanced stage of atherosclerosis [26]. Excessive or prolonged production of inflammatory mediators can lead to atherosclerosis. The productions of iNOS, NO, cyclooxygenase-2 (COX-2) were increased in the LPS stimulated RAW264.7 macrophages, and the medication inhibited nuclear translocation of signal transducer and STAT3 activation, reduced LPS-mediated phosphorylation of Janus kinase (JAK) 2 and STAT3 at serine 727 and tyrosine 705 sites, thereby reduced LPS-mediated inflammatory responses of macrophage [60].

### The MAPK (ERK1/2, JNK1/2 and p38) iNOS-NO pathway and atherosclerosis

Inhibiting the production of NO could reduce TNF-α-induced inflammation of human aortic smooth muscle cells (HASMC) through inhibiting p38 MAPK / ERK signaling [61].

In LPS-induced rat aortic smooth muscle cell inflammatory model, Yang et al. [64] found that pretreatment with non-cytotoxic concentrations of Toona sinensis (TS) and gallic acid (GA) (anti-atherosclerosis effect) inhibited inflammatory NO production by down regulating iNOS expression, and further investigation showed that the inhibition of iNOS/NO signaling was associated with the decreased phosphorylation of MAPK (ERK1/2, JNK1/2 and p38). Yu et al. [62] found that Resokaempferol (RES) (anti-inflammatory effect) reduced NO, iNOS production in LPS-induced RAW264.7 macrophages and inhibited LPS-induced activation of JNK/p38 MAPK signaling. The study from Sharma et al. [60] confirmed the enhancement of TNF-α-induced phosphorylation of P38, ERK and JNK in PAECs isolated

from GPx1 KO mice. It is concluded that the MAPK signaling associated with inflammatory response promotes the development of atherosclerosis.

### The Nrf2/HO-1/iNOS/NO pathway and atherosclerosis

Some studies have shown that Ang II-treated human endothelial cells [56] and HASMCs [57] had the increased oxidative stress. Enhancing the production of superoxide dismutase (SOD) and the expression of HO-1 through Nrf2 signaling decreased ROS production induced by Ang II, thereby inhibited the ROS-NF-κB-iNOS-NO pathway and relieved Ang II-induced vascular endothelial dysfunction [56,57]. The effective Nrf2/anti-oxidative response element (ARE) activation inhibits LPS-induced NF-kB nuclear translocation and DNA binding activity, and further prevents iNOS expression and NO production in liver [65]. Nrf2-KO mice showed the significantly elevated iNOS expression [66]. To sum up, Nrf2 signaling reduces the expression of iNOS and the excessive production of NO to relieve the development of atherosclerosis.

## The nNOS/NO Signaling and Atherosclerosis

### The ERK1 / 2-nNOS-NO pathway and atherosclerosis

NO synthesis by vascular nNOS can regulate vascular tone and have the effect of anti-atherosclerosis. Guan et al. [49] divided 24 male Wistar Kyoto rats into two treatment groups (n=12): vehicle and Tongxinluo (TXL) (400 mg·kg·d). After 2 weeks of treatment, adventitia injury was induced by placing the silicone collar around the left carotid artery for 2 weeks, which leaded to chronic vasoconstriction and vascular hypersensitivity to serotonin, and the above reaction was attenuated by TXL treatment. TXL improved carotid blood flow and normalized the vascular hypersensitivity to serotonin. The expression of nNOS and ERK 1/2 phosphorylation was increased in both collared carotid and VSMCs with the TXL treament, and the above effect was abolished by ERK kinase inhibitor PD98059, indicating that TXL increased the expression of nNOS mediated by ERK 1/2 phosphorylation (Table 1) [64].

## The NO-sGC-cGMP Pathway and Atherosclerosis

NO regulates the degree of VSMCs contraction by stimulating soluble guanylate cyclase (sGC) to produce cyclic guanosine monophosphate (cGMP). In addition, NO also mediates the augmentation of coronary artery contraction in hypoxia state, which depends on sGC but is independent of cGMP production [2]. Sun et al. [31] have demonstrated that ethanol extract *Rumex acetosa* L. (ERA)-induced vasorelaxation was removed by ODQ (as GC inhibitor) in the isolated phenylephrine pre-contracted rat thoracic aorta, and concluded that the vasodilatation medicated by NO was regulated by the muscle NO-sGC-cGMP pathway [31]. In addition, ROS promotes the development of atherosclerosis by oxidizing NO receptor sGC to impair NO function [65]. GUCY1A3 was identified as a risk gene for coronary artery disease and myocardial infarction. It promotes the formation of atherosclerosis by promoting the transformation of SMCs phenotype from the contracted state to the synthetic state through encoding the α1 subtype of sGC. Preventing this conversion by inhibiting sGC activity may provide a new therapeutic goal for atherosclerosis [67].

## Summary

The appropriate amount of NO synthesized by eNOS and nNOS plays the anti-atherosclerosis role, but the excessive amount of NO synthesized by iNOS promotes the occurrence and development of atherosclerosis. Under the pathological conditions, such as lipid infiltration, oxidative stress and inflammatory reaction, the activity of eNOS and nNOS decreased, whereas the activity of iNOS increased, which involved a series of signaling pathways to promote the formation of atherosclerosis, and among them, the main signaling pathways are the PI3K/AKT/eNOS/NO pathway, the Ca(2+)/ CaMKII or CaMKK/ AMPK/ AKT/ eNOS/ NO pathway, the Nrf2/HO-1/ eNOS/NO pathway, and the IKK/IκBα/NF-κB/iNOS/NO pathway. The enhancers or inhibitors of signaling molecules in the above NO pathway and avoiding harmful stimulation on the NO pathways will contribute to the treatment of atherosclerosis. However, the mechanism of NO signaling pathways associated with atherosclerosis is not yet fully elucidated. Further research is helpful to better understand and interfere atherosclerosis with NO signaling pathway related anti-atherosclerosis drugs.

## Acknowledgements

This work was supported by National Natural Science Foundation of China (№ 81660751, 81660151, 81260504); Key Research and Development Program of Jiangxi Province of China (No 20161BBG70067and Jiangxi Provincial Natural Science Foundation of China (No 20171BAB205085).

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