Chapter

Nitric Oxide and Oxidative Stress-Mediated Cardiovascular Functionality: From Molecular Mechanism to Cardiovascular Disease

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Abstract

The underlying pathology of most cardiovascular diseases (CVDs) such as coronary artery disease, high blood pressure, and stroke involves decreased cardiovascular contractility and anatomic alterations in cardiovascular structures. Nitric oxide (NO) regulates vascular tone and contractile function of myocardium and maintains blood vessel homeostasis. Interestingly, the effect of NO is like a double-edged sword in the body. Insufficient NO causes hypertension and atherosclerosis, while an overproduction of NO may foster inflammation and cause heart infarction and shock. In addition, growing evidences have shown that oxidative stress plays pivotal roles in the initiation and progression of CVDs. This chapter will discuss in detail the roles NO plays in the cardiovascular system under both physiological and pathological conditions. We will focus on: (1) the molecular mechanism of cardiovascular contraction, (2) NO/Ca^{2+}-induced muscle relaxation, (3) NO-related structural change in blood vessels, and (4) redox balance in the cardiovascular system. The relationships between these molecular mechanisms and the characteristics of CVDs will be highlighted.

Keywords: cardiovascular diseases, muscle contraction and relaxation, cytoskeleton, nitric oxide, nitroso-redox balance

1. Introduction

Cardiovascular diseases (CVDs), i.e., ischemic heart disease and stroke, remain the leading cause of death in the past decades around the world, especially in the developed countries [1]. CVDs can start from risk factors that may cause local vascular lesion and end up with systematic complications, which lead to organ failure and death. Thus, understanding the biochemistry of events involved in the whole process of CVD progression is crucial to prevent and treat the disease.

Epidemiological data show that various factors are associated with the increase of cardiovascular morbidity and mortality, including hypertension, smoking,

hypercholesterolemia, diabetes mellitus, obesity, stress, low fruit and vegetable dietary, lack of regular exercise, and abnormal sleep [2]. Current therapeutic strategies mainly focus on reducing patients' blood pressure, restoring redox balance, controlling cholesterol, and implementing physical activity programs [3]. In this chapter, we explore the physiological and pathological events in the cardiovascular system from the molecular biology's perspectives. Molecular mechanism of muscle contraction and relaxation in the cardiovascular system will be discussed first. Then, we will delve into the biological effects of Nobel Prize molecule nitric oxide (NO), the most important vasodilator in the body. In addition, due to the inspiring clinical outcomes of using isosorbide dinitrate (an NO stimulus) and hydralazine (an antioxidant) to treat patients with symptomatic congestive heart failure [4], we will also discuss how nitroso-redox balance mediates cardiovascular functions.

2. Muscle contraction and relaxation

2.1 Sliding filament theory

Skeletal, cardiac, and smooth muscles have different structures and regulatory mechanisms, but they share the same molecular mechanism of contraction and relaxation, i.e., the relative sliding between myofilaments [5]. To understand how the heart beats and how blood vessels regulate their tones, it is important to look into the subcellular structure of these tissues (**Figure 1**).

Heartbeat relies on myofibrils, a fiber bundle structure that abounds in cardiomyocyte (**Figure 1c**). When a number of myofibrils are highly aligned, sarcomere, a repeat unit in the myofibril, can be observed under the microscope. Sarcomere is the basic unit for motion. Two most important proteins in the sarcomere are: myosin and actin. A myosin contains the N-terminal globular head domain, the short neck domain, and the long C-terminal coiled-coil tail domain. The globular head works as a specialized adenosine triphosphatase (ATPase), responsible for adenosine triphosphate (ATP) binding, actin binding, and generating force from ATP hydrolysis. The neck domain transduces force generated by the head. And the fibrous tails are bundled together to form the thick filament (**Figure 1g**). Actin, together with troponin and tropomyosin, forms the thin filament. When the two fibers slide toward each other, the overlapped region increases, which is the mechanism of muscle contraction. Similarly, when the fibers slide away from each other, muscles relax (**Figure 1e**).

2.2 Cross-bridge cycling

The filament sliding depends on cross-bridge cycling [6]. A cross-bridge refers to the two globular heads of myosin, which take turns to bind, pull, and detach from the actin fiber to achieve relative movement between the filaments. An analogy is that a person alternately uses two hands to pull a rope. One alteration of the hand is considered to be one cycle. There are four basic states [7] (sometimes detailed to six states [8]) in cross-bridge cycling. Each state corresponds to one behavior of ATP and one response of myosin. State 1: activation of myosin head, when ATP binds to myosin, it is hydrolyzed to ADP and Pi (inorganic phosphate); myosin becomes the "cocked position." State 2: cross-bridge formation, the activated myosin binds to actin; Pi is released to stabilize the binding. State 3: power stroke, ADP is released; myosin generates force to pull actin filament. State 4: detachment of cross bridge, another ATP binds to myosin; myosin disengages from actin; then State 1 is repeated. The continuous cross-bridge cycling allows myosin to pull actin to its tail side, resulting in

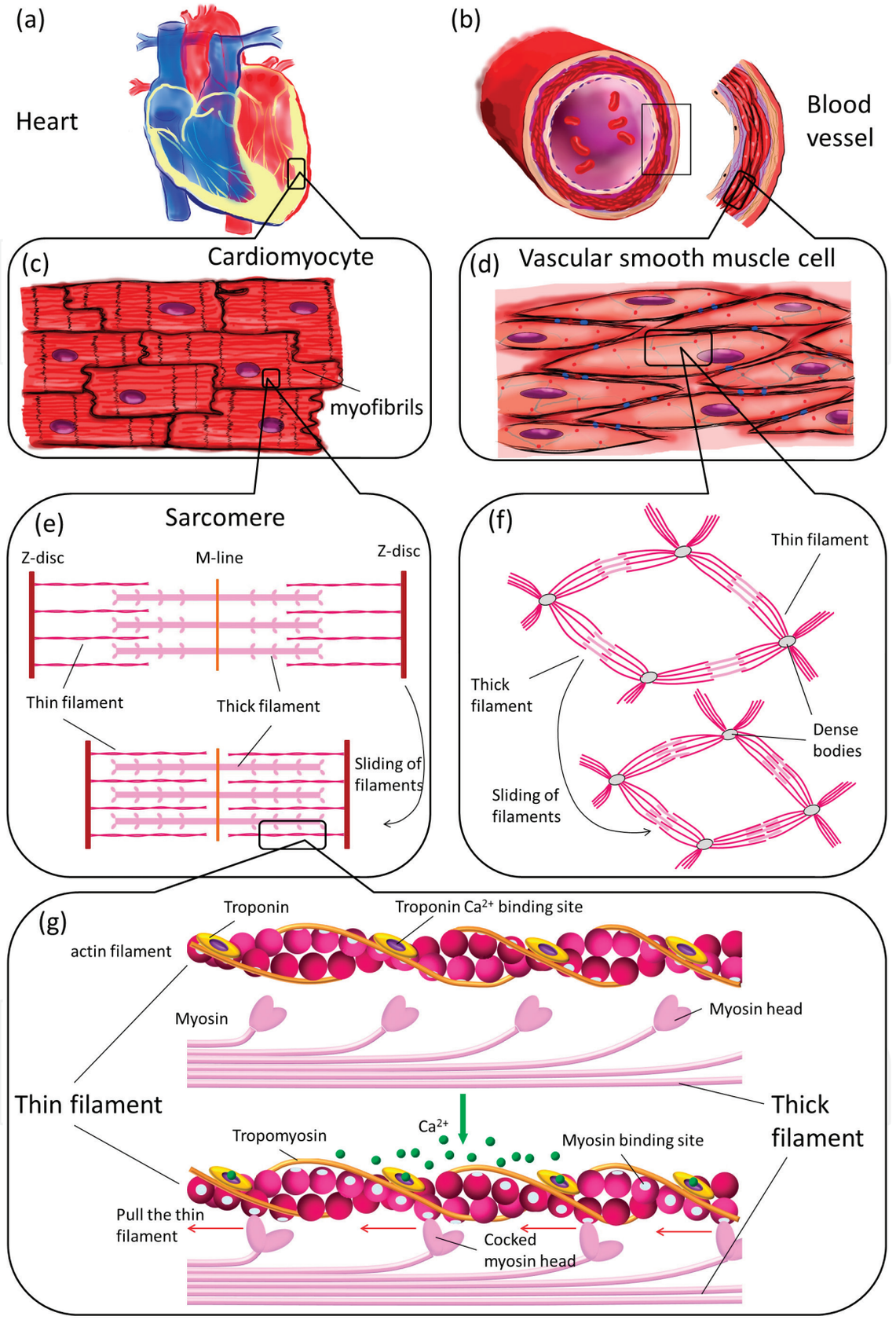

Figure 1.
Muscle contraction illustrated on different structural levels in the cardiovascular system: tissue level—heart (a) and blood vessel (b); cell level—cardiomyocyte (c) and vascular smooth muscle cells (SMCs) (d); subcellular level—filament sliding in cardiomyocyte (e) and SMCs (f); and molecular level—thin and thick filament for cross-bridge cycling (g).

filament sliding and muscle contraction. At the resting state, actin's myosin-binding site is blocked by troponin and tropomyosin [9] (**Figure 1g**). A switch mechanism is needed to expose and mask the myosin-binding site to regulate muscle contraction.

Intracellular Ca^{2+} works as a secondary messenger that quickly bonds to troponin, causing a quick conformational change of troponin and tropomyosin [10]. Thus, the myosin-binding site on actin filaments is exposed, and cross-bridge cycling proceeds. Intracellular Ca^{2+} concentration, or $[Ca^{2+}]_i$, can return to a very low level to cease the contraction and cause relaxation by different mechanisms, such as extruding Ca^{2+} out of cells or storing cytosolic Ca^{2+} into sarcoplasmic reticulum (SR) which functions as the Ca^{2+} reservoir in the cardiomyocyte. Similar mechanisms exist in the vascular tissue. SMC layer lies in between the endothelium layer and adventitia. There is no organized contractile protein fibril or sarcomere structure in SMCs [11]. Instead, the contractile fibrous proteins along with other intermediate filaments form bundles that are immobilized by anchoring proteins onto cell cytoskeletons. These filaments distribute all over the cytoplasm and connect each other through anchoring proteins (dense bodies) to form a three-dimensional network (**Figure 1d** and **f**). Unlike in cardiac muscles, actin filament in smooth muscles is associated with caldesmon, tropomyosin, and calmodulin (CaM) [12]. CaM is an important Ca^{2+} sensing protein, which binds and mediates many enzymes' activities upon Ca^{2+} signaling. Caldesmon binds to actin, which inhibits the activity and motility of actin-myosin ATPase, and this binding is greatly strengthened by tropomyosin [13]. Ca^{2+} binds and activates CaM to uncouple the interaction between caldesmon and actin. Thus, actin's myosin-binding sites are exposed to myosins. Different from skeleton or cardiac tissues, the contraction in smooth muscles also depends on phosphorylation level of myosin light chain, which is adjusted by the enzyme activity of CaM-dependent myosin light chain kinase (CaM-dependent MLCK) and myosin light chain phosphatase (MLCP) [14]. MLCK adds the phosphoryl group to the myosin light chain, while MLCP removes it. Thus, increase of $[Ca^{2+}]_i$ also facilitates muscle contraction through enhancing myosin phosphorylation [15].

Cardiovascular contractility is crucial for blood pressure homeostasis, thermal exchanging, mass transfer, immune responses, and organ functions [16]. Impaired coronary artery contractility (caused by the block of blood vessel or poor dilation) incurs ischemia heart disease [17]. To maintain cardiomyocyte viability and vascular tones, enhancing vasodilation and restricting oxidative stress are critical.

3. NO-related cardiovascular physiology

3.1 Biosynthesis of NO

The biochemical history of NO dates back to 1860s when the therapeutic use of nitroglycerin became a prevalent treatment for angina and hypertension [18]. Consequent studies showed that relaxation of blood vessels depended on a molecule present in an intact endothelium lining, which was named as endothelium-derived relaxing factor (EDRF). In 1977, it was demonstrated that NO was the chemical released from nitroglycerin metabolism [19], and EDRF was later identified to be NO [20]. Since then, NO's cardiovascular effect and medical applications have drawn great research interest.

NO biosynthesis primarily relies on the enzyme nitric oxide synthase (NOS). The reaction uses substrate L-arginine and oxygen to generate L-citrulline and NO in the presence of the cofactors, including Ca^{2+}/CaM, reduced nicotinamide-adenine-dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and tetrahydrobiopterin (BH_4) [21].

NO is an excellent messenger molecule in the human body because it is a highly reactive free radical and a highly diffusible molecule [22]. NO is able to react with

various species, such as oxygen, superoxide, lipid oxygen radicals, thiols, and metals [23], which is the biochemical base for NO signaling. The ultimate biological effect of NO depends on its concentration, duration of release, and physiological environment [24]. NO concentration varies from subnanomolar (cell survival signaling) to micromolar (cytotoxic effect and apoptotic signaling) in the body [25]. Its small size and lipophilic characteristic allows it to move rapidly across cell membranes. However, NO's effective distance is limited by its extremely high reactivity with species like oxygen, superoxide, and hemoglobin [23]. NO concentration decreases rapidly around the NO source, which makes NO's effect extremely localized (within hundreds μm).

Three different isoforms of NOS have been identified in the human body. Endothelial NOS (eNOS) is mainly found in vascular endothelial cells (ECs) and is involved in regulating vascular tone and blood clotting. It is also present in cardiomyocytes where it mediates contractility of cardiac muscle [26]. Inducible NOS (iNOS) predominates in immune cells to produce large amounts of NO that aids in the host defense mechanisms. Examples include NO produced by macrophages and neutrophils when induced by lipopolysaccharide, interferon-γ, and tumor necrosis factor alpha [24]. Neuronal NOS (nNOS) is mainly identified in nerve cells where NO assists in nerve transmission. It has also been isolated from cardiomyocytes where NO regulates excitation contraction coupling of cardiac muscles [27]. NO generation by iNOS is mainly regulated at the transcription level, while eNOS and nNOS are constitutive NOS isoforms whose activities are Ca^{2+}- and CaM dependent. Shear forces during blood flow stimulate the opening of Ca^{2+} channels on ECs to increase $[Ca^{2+}]_i$, resulting in eNOS activation [28]. NO biosynthesis can be inhibited by various chemicals that selectively bind to NOS with high affinities, such as N^G-monomethyl L-arginine (L-NMMA) and asymmetric dimethyl L-arginine [9].

S-nitrosothiols and nitrite are alternative endogenous sources of NO. S-nitrosothiols decompose to release NO under physiological pH and are formed through the reaction of NO and thiol [29]. Nitrite can be reduced to NO in the body through pathways involving reducing agents and proteins, such as ascorbic acid, thiols, hemoglobin, and myoglobin [30]. These backup NO generation pathways are emphasized during hypoxia and acidosis when oxygen-dependent NOS-mediated NO production is limited.

3.2 NO-cGMP pathway

Endogenous NO has various physiological effects in the body including inhibiting platelet aggregation, regulating SMC proliferation, modulating immune response, participating in neuron signal transmission, and inducing vasodilation [25]. In the vascular system, NO is primarily generated by eNOS in ECs (**Figure 2**). Shear stresses induced by blood flow and chemical stimuli, known as agonists including acetylcholine, bradykinin, adenosine triphosphate, estrogen, and vascular growth factors, are able to activate eNOS [28, 31]. NO is a highly dynamic molecule that diffuses fast in both aqueous and lipid environment (with diffusion coefficient D = 2.07–3.30 × 10^{-5} cm^2/s in water and polymer matrices [32–34] at physiologically relevant temperatures). Thus, NO readily enters the SMCs and binds to the heme moiety of soluble guanylyl cyclase (sGC) to activate sGC, probably the most important protein target of NO. This binding causes the formation of a nitrosyl-heme complex, heme conformational change, and breaking-apart of sGC His105 from heme iron [35]. Then, an open central core is formed in porphyrin, which can accept guanosine triphosphate (GTP) to generate cyclic guanosine monophosphate (cGMP).

Cyclic-GMP binds and activates cGMP-dependent protein kinase (protein kinase G, or PKG), which can phosphorylate the downstream targets to trigger vasodilation

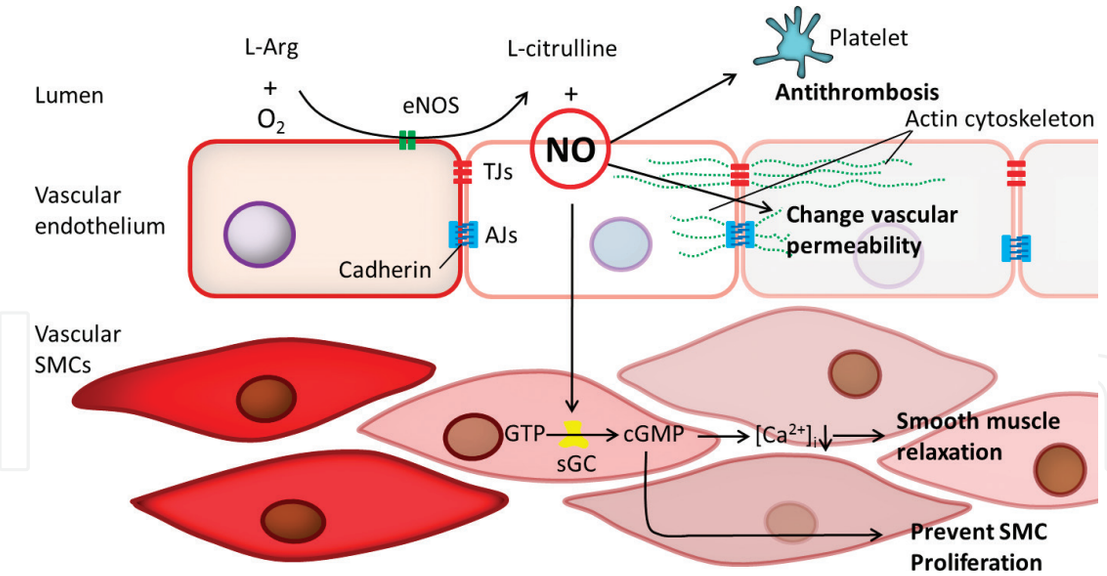

Figure 2.
Biosynthesis of NO by eNOS and the biological effects of NO in the vascular system.

(**Figure 2**). This signaling pathway can be terminated by removing cGMP through converting cGMP to GMP by various phosphodiesterases (PDE) [35]. There are many types of PDE in the human body, and they play critical roles in regulating cardiovascular function, adrenal steroidogenesis, and phototransduction [35].

Interestingly, protein kinase A (PKA, cAMP-dependent protein kinase) and PKG share very similar nucleotide binding domains. Many studies have shown that cGMP can activate PKA downstream pathways and cAMP can also cross-activate PKG [36]. This cross regulation between cGMP and cAMP pathways sometimes complicates NO signaling, which will be shown later.

Due to NO's vasodilation effect, NO releasing drugs such as organic nitrate, and nitro- and nitroso compounds have been used for treating angina pectoris, congestive heart failure damage from ischemia–reperfusion, and pulmonary hypertension [37, 38]. Potent drugs also include chemicals that target members involved in NO-cGMP pathway. For example, Sildenafil, known as Viagra, is a PDE5 inhibitor. It prohibits cGMP from being hydrolyzed by PDE5 and extends the activation time of vasodilation to widen the blood vessel and increases blood flow into the penis to treat erectile dysfunction [39].

3.3 NO-induced muscle relaxation through Ca^{2+} signaling

To fully understand how NO causes vasodilation, it is necessary to perceive the relationship between NO-cGMP pathway and $[Ca^{2+}]_i$. At rest, extracellular Ca^{2+} concentration is high (1–2 mM), while the cytosolic Ca^{2+} is over 1000 times lower (>1 μM) [40, 41]. In the endoplasmic reticulum (ER, or SR in cardiac muscles), Ca^{2+} concentration is also high (about 400 μM) [40]. NO modulates $[Ca^{2+}]_i$ by controlling Ca^{2+} exchange mechanisms on both cell and SR membranes.

3.3.1 Ca^{2+} exchange through plasma membrane

Voltage-gated Ca^{2+} channels regulate $[Ca^{2+}]_i$ through sensing electrical signals to allow Ca^{2+} entering the cell. High voltage-activated L-type channels are broadly found in the cardiovascular system. L-type Ca^{2+} channel inhibitors, such as dihydropyridines, phenylalkylamines, and benzothiazepines, are a major class of drugs for treating CVDs [42]. The opening probability of L-type Ca^{2+} channel can be

lowered by PKG indirectly [43]. PKG phosphorylates the K^+ channel and increases its opening probability to hyperpolarize the cell membrane [44, 45]. The hyperpolarized cell membrane can no longer send electrical signals to activate the Ca^{2+} channel, and therefore, Ca^{2+} influx is inhibited. Besides PKG, high NO level also directly activates K^+ channels to achieve aorta relaxation in a cGMP-independent fashion [46].

Ca^{2+} pumping ATPase located on the cell membrane also extrudes Ca^{2+} from the cytosol. It binds Ca^{2+} with a high affinity and forces Ca^{2+} out of the cell even when $[Ca^{2+}]_i$ is very low to maintain the low $[Ca^{2+}]_i$ level at rest [47]. PKG can stimulate the Ca^{2+} pump, initiating the expulsion of cytosolic Ca^{2+} [48].

Unlike Ca^{2+} pump, Na^+/Ca^{2+} exchanger is more effective in quickly removing cytosolic Ca^{2+}, but its Ca^{2+} binding affinity is low [47]. This mechanism is crucial for preventing cells from the cytotoxicity of an acute high Ca^{2+} concentration. The driving force for Na^+/Ca^{2+} exchanger is the stored sodium electrochemical gradient created by Na^+/K^+ channels. PKG can activate the Na^+/K^+ channel to cause more Na^+ accumulated to indirectly facilitate Ca^{2+} removal [49, 50].

3.3.2 Ca^{2+} exchange through ER

Ca^{2+} pump ATPase also resides on the ER responsible for the uptake of cytosolic Ca^{2+} into the ER. NO pathway regulates ER Ca^{2+} pumping through phosphorylation of phospholamban by PKG [51]. Mainly identified in cardiac tissues, phospholamban is an inhibitor of SR Ca^{2+} pump. Phospholamban is normally phosphorylated by PKA, which diminishes its inhibitory effect to Ca^{2+} pump [52]. Interestingly, in neonatal cardiomyocytes and vascular SMCs, NO pathway also demonstrated relaxation effect through differentially phosphorylating phospholamban [53, 54].

Inosital 1,4,5-trisphosphate (IP_3) is a critical messenger molecule that can induce Ca^{2+} release from the ER reservoir. IP_3 receptor resides on the ER and works as a chemical-activated Ca^{2+} channel. NO-cGMP pathway can reduce IP_3 generation [55], and PKG can phosphorylate and inactivate IP_3 receptor in vascular SMCs to inhibit ER Ca^{2+} release [35, 56].

3.3.3 Ca^{2+}-independent muscle relaxation regulated by NO

Independent from NO-Ca^{2+} pathway, in SMCs NO also increases MLCP activity and limits MLCK activity, resulting in a dephosphorylation shift of myosin light chain phosphorylation balance [15]. Thus, myosin cross-bridge cycling is inhibited, causing smooth muscle relaxation.

4. Vascular structural integrity mediated by NO

Anatomic alterations in the cardiovascular structure directly deteriorate cardiovascular functions. NO is a multifunctional regulator for homeostasis in the cardiovascular system. An intact endothelial layer is the hub for NO generation. Pathological changes in NO generation can trigger various local flaws that may progress to be systematic cardiovascular issues with time.

4.1 NO-induced alterations in endothelial permeability

Deviant NO level causes change of endothelial permeability, a key characteristic for mass transfer and extravasation. Interestingly, increase, decrease, and no change of vascular permeability due to the presence of NO have been reported. Using

high concentration (millimolar level) of exogenous NO donor spermine NONOate decreased endothelial permeability in the *in vitro* human umbilical vein endothelial cell (HUVEC) model [57]. And this effect was amplified by vitamin C, a chemical that increases the apparent half-life of NO. However, in the frog mesenteric capillary model, inhibition of NO synthesis by L-NMMA decreased capillary permeability [58]. Moreover, although NO effectively regulated basal vascular tone in the blood-brain barrier, it demonstrated no effect on its basal permeability [59]. Again, these results demonstrate that NO's biological effect is sensitive to NO concentration, duration, and environment.

Vascular permeability is mainly determined by tightness of cell-cell junctions [60]. Tight junctions (TJs) and adherens junctions (AJs) are the most abundant interendothelial junctions. And both junctions are closely related to actin cytoskeleton dynamics [61] (**Figure 2**). TJs are composed of series of transmembrane proteins that anchor to the actin cytoskeleton to hold cells together. They seal the cells to maintain cell polarity and prevent the molecules from traveling through the space between cells. AJs consist of clusters of transmembrane protein cadherin, which is connected to actin cytoskeleton on its cytoplasmic side and binds strongly with cadherins residing on the neighboring cell membrane. These junctions are important for transmitting mechanical force between cells and reinforcing tissues. Since both junctions directly connect cytoskeletons, the cytoskeleton's behavior will influence cell-cell junctions and thus control vascular permeability. When actin and myosin filaments undergo relative sliding to cause cell contraction, the cytoskeleton-associated membrane proteins will be pulled into the cells, and cell-cell junctions are disrupted. NO mediates cell contraction by adjusting $[Ca^{2+}]_i$. Therefore, deviated NO level may cause the change of cell-cell junctions [60].

Another important downstream molecule of NO is vascular endothelial growth factor (VEGF) which has been extensively studied in cancer research due to its angiogenic effect. VEGF was initially considered as a vascular permeability factor, because it caused the formation of leaky capillaries [62], which is an important characteristic in tumor and retinopathy. Low NO level induces VEGF synthesis under normoxia through the transcription factor hypoxia-inducible factor 1 (HIF-1) [25, 63]. VEGF activates Src kinases, which further phosphorylate cadherin and elicit its internalization [64]. Once cells lose cadherin interactions, gaps between cells form and endothelial permeability is increased.

4.2 Inhibition of SMCs proliferation by NO

One distinctive characteristic of vascular SMCs is the phenotypic plasticity. Two most important phenotypes are contractile and synthetic. Contractile SMCs guarantee the good performance of muscle contraction/relaxation, while synthetic SMCs are highly proliferative and migratory, crucial for vascular remodeling during pregnancy and injury healing. Dysregulation of the phenotype transition causes neointima formation [65]. NO plays important roles in suppressing SMCs' contractile to synthetic transition.

NO donors and 8-Br-cGMP showed similar effect in inhibiting SMC migration and proliferation, indicating NO's inhibitory effect might be through the cGMP-dependent pathway [66]. SMCs overgrow when stimulated by serum and epidermal growth factor (EGF). Many studies were based on these models, though divergent results were reported. EGF induces SMC proliferation through mitogen-activated protein kinases (MAPK) pathway, also called extracellular signal-regulated kinases (ERK) pathway. Ras (a small GTPase) and Raf (kinase of MAPK kinase, or MAPKKK) are the critical upstream protein kinases in this pathway. NO blocks MAPK pathway by prohibiting Raf from being activated by Ras-GTP in rat aortic SMCs. It is believed that PKG

phosphorylates Raf, resulting in the conformation change. Thus, Ras-GTP cannot recognize Raf, causing the block of MAPK pathway and the accumulation of Ras-GTP [67]. Meanwhile, elevation of cGMP induced by IL-1β is correlated with the activation of PKA, and it can be prevented by blocking NO and cGMP pathways. Interestingly, this effect is cAMP independent, but PKA inhibitor, not PKG inhibitor, can prevent the inhibition of the proliferation, indicating that cGMP-PKA cross talk plays important roles in suppressing rat aortic SMCs' proliferation [68].

NO-cGMP pathway may inhibit SMC growth by impairing cytoskeleton reorganization. Vasodilator-stimulated phosphoprotein (VASP) is characterized as a substrate of both PKG and PKA [69]. It targets focal adhesions and is involved in actin filament formation. Cell morphology change during proliferation relies on VASP, and its activation relies on the phosphorylation of Ser157 primarily mediated by PKA [70]. However, PKG can phosphorylate Ser239 and Thr278 to impair VASP's activity and inhibit actin cytoskeleton reorganization [70, 71].

NO also directly mediates proteins associated with cell cycle and cell metabolism by cGMP-independent mechanisms. Cyclin A and cyclin-dependent kinase 2 expression levels can be blunted by exogenous NO donor DETA NONOate in an *in vitro* vascular SMC model [72]. Ornithine decarboxylase (ODC) catalyzes the ornithine decarboxylation to form polyamines, which are necessary for cell growth and proliferation. ODC's active center can be masked by nitrosylation. And NO biosynthesis's intermediate product N(omega)-hydroxyarginine can inhibit ODC enzyme activity [73] to disrupt cell proliferation.

4.3 Prevention of thrombogenesis by NO

Thrombus formation is critical for hemostasis during injury. However, thrombus in blood vessels can cause stroke and heart attack. Stable thrombus reduces lumen size and stiffens blood vessels. Unstable thrombus may rupture with blood flow and block the vessel. Activation of platelet is a critical step for thrombus formation, which involves exocytosis processes to expose P-selectin on the platelet surface and activate glycoprotein IIb/IIIa. Both processes depend on the elevation of $[Ca^{2+}]_i$ controlled by IP_3 pathway. NO suppresses platelet activation through NO-cGMP pathway [74]. Although, the inhibition pathway has not been fully characterized, evidences have shown that cGMP-PKG blocks agonist-induced IP_3 formation in platelet [75], and PKG can phosphorylate IP_3 receptor to inhibit Ca^{2+} release from the ER [35].

When the endothelium loses its integrity, there will be a local shortage of thromboregulators such as NO, prostacyclin, and ectonucleotidase CD 39, resulting in thrombogenesis [76]. Collagen and tissue factors also trigger the coagulation reactions [76]. The use of blood contact implant is another common source of thrombus. Note that, all materials are thrombogenic to some degrees. To enhance implant biocompatibility, an efficient method is to use NO releasing polymers to fabricate or surface coat the blood contacting devices (such as vascular graft/stent, intravascular catheter, and sensor implants). Common strategies include: physically incorporating NO releasing chemicals into polymer matrices, chemically linking NO releasing agent to polymer backbones, and developing materials that can trigger NO generation using endogenous NO donors circulating in the blood. By using the first two strategies, successful trials have been reported to achieve long-term (over few weeks to months) NO releasing and antithrombotic applications [77–79]. Good NO donors include N-diazeniumdiolate and S-nitrosothiols. Both hydrophilic and hydrophobic polymers that are commonly used in medical device fabrication have been successfully modified for NO release including poly(vinyl chloride), polymethacrylates, various hydrogels, polyethylene terephthalate, polyurethane, and silicone rubbers [77]. The third strategy directly uses endogenous NO donors as

the NO reservoir to catalyze NO generation from S-nitrosoglutathione or nitrite in the body. Currently, its main challenge is to adjust the NO releasing rate to be more biologically relevant.

5. Nitroso-redox balance in the cardiovascular system

Oxidative stress is always associated with ischemia reperfusion injury, dilated cardiomyopathy, and heart failure [80]. It is crucial to restore redox balance in the cardiovascular system when treating these diseases. Redox balance is governed by changes in the oxidative state in tissues, where addition and loss of electrons result in reduction and oxidation of molecules, respectively [80]. Oxygen can accept an electron to become reactive oxygen species (ROS). ROS are highly reactive chemical species that contain oxygen atoms, mostly free radicals with one or more unpaired electrons [81]. NO is a free radical signaling molecule. Under pathological conditions, it reacts with superoxide to generate reactive nitrogen species (RNS) that have detrimental consequences to cells. Herein, we highlight the causes of redox imbalance, their functions in the cardiovascular system, and the roles they play in the progression of CVDs.

5.1 Biochemistry and physiology of ROS and RNS

5.1.1 ROS and oxidative stress

The electron transport chain (ETC) located in the inner membrane of mitochondria is crucial for energy and ROS generation (**Figure 3**). Normally, the final electron acceptor oxygen is reduced to water. However, in pathological conditions, electrons uncouple from the chain and react with oxygen without passing *cytochrome c* oxidase to form superoxide. Other ROS sources include NADPH oxidase, xanthine oxidase (XO), eNOS, and cytochrome P450s (CYP). NADPH oxidases belong to NOX family proteins, which transfer electrons across intracellular membranes. NADPH oxidases transfer electron from NADPH to oxygen to form superoxide for immune responses [82, 83]. Three NOX enzymes have been found in the vascular wall, NOX 1, 2, and 4. NOX 1 and 2 result in the formation of superoxide and NOX 4 produces hydrogen peroxide (H_2O_2) [83]. NOX 4 is also present in the mitochondria and SR in cardiomyocytes [80]. XO is found in the heart and ECs. It catalyzes purine metabolism with superoxide and H_2O_2 produced. The activity of XO is enhanced under ischemic reperfusion injury and oscillatory shear stress [83, 84]. Another important source of ROS is the uncoupling of eNOS, which causes eNOS to produce superoxide, instead of NO. One of the reasons for eNOS uncoupling is the deficiency in the substrates (L-arginine and oxygen) and co-factor BH_4 [80, 83]. Uncoupling of eNOS may explain why diabetic patients are susceptible to CVDs. High glucose increases arginase levels in ECs, which competes with eNOS for the substrate L-arginine [85]. In addition, overload of ROS in diabetes mellitus limits BH_4 biosynthesis to further facilitate eNOS uncoupling [86]. Cytochrome p450s (CYP) are a group of hemoproteins similar in structure and function to eNOS under oxidative stresses. The catalytic activity of CYP requires oxygen and two electrons to form a ferrous-dioxy complex [87]. CYP is involved in the metabolism of cholesterol, vitamins, and arachidonic acid [88]. When there is excessive oxygen consumed, the enzyme uncouples, and the ferrous-dioxy complex diverts back to the ferric state to produce superoxide [89].

Superoxide is an anion-free radical that can produce other ROS including H_2O_2, hydroxyl radicals ($^{\cdot}OH$), and hypochlorous acid (HClO) [80, 82, 90, 91].

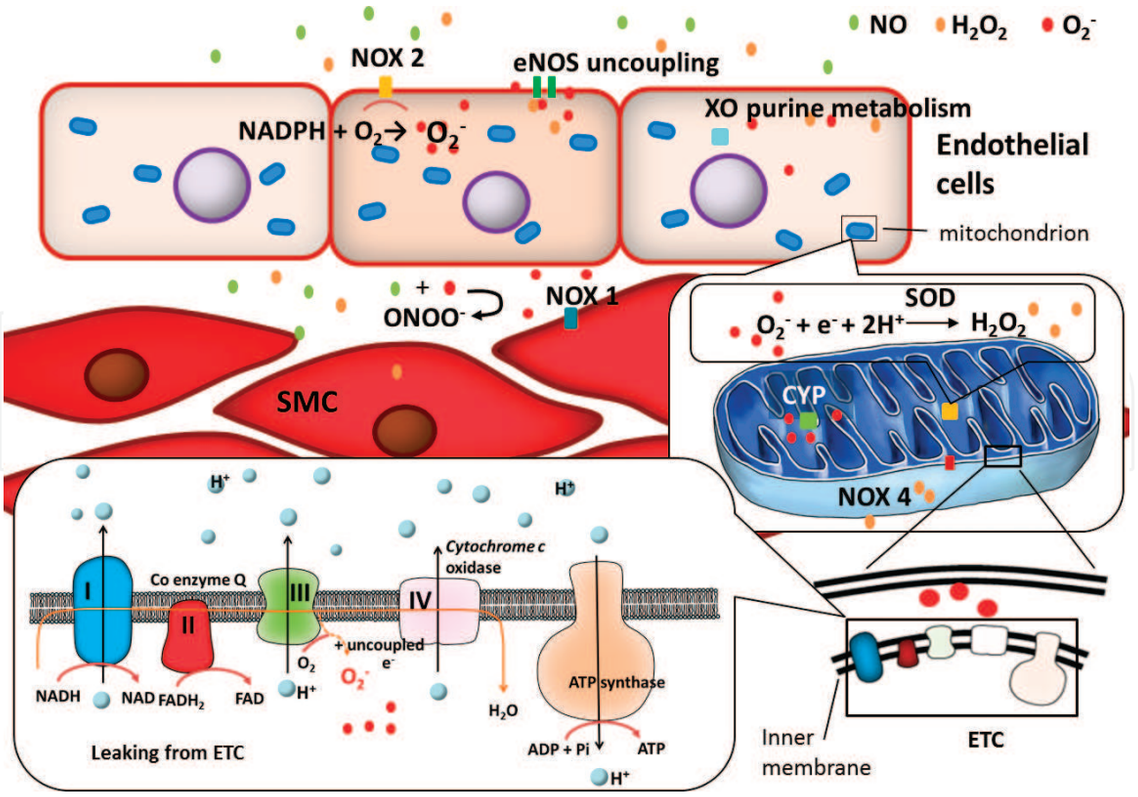

Figure 3.
The major sources of ROS and RNS in the cardiovascular system.

The spontaneous transfer of an electron to superoxide at low pH or by an enzyme reaction (superoxide dismutase, SOD) produces H_2O_2 [82, 91]. Low levels of H_2O_2 (1–10 nM) induce more antioxidant molecules that protect the cells, and high levels (>100 nM) are likely to generate more prooxidants [91]. For example, high level of H_2O_2 was generated in neutrophils for antimicrobial effects [92, 93]. Hydroxyl radical can be formed from the reaction between H_2O_2 and superoxide (Haber Weiss reaction) or the breakdown of H_2O_2 by metal ions, Fe^{2+} or Cu^{2+} (Fenton reaction) [94]. Hydroxyl radical is highly reactive. It alters DNA structure by attacking purine and pyrimidine bases, leading to mutations and cell damages [95]. In the pathological myocardial tissue, it is associated with decreased contractile function, increased membrane phospholipid peroxidation, and heart failure [96, 97]. HClO is mainly produced by leukocytes when H_2O_2 reacts with chloride anions. It facilitates the removal of foreign particles and is also implicated in the progression of atherosclerosis and ischemic reperfusion injury [81].

5.1.2 NO and nitrosative stress

NO acts in a diffusion- and concentration-dependent manner. Low concentrations of NO (nanomolar range) have a protective role, while high NO levels (micromolar range) can be detrimental [98]. The majority of NO's biological effect is attributed to sGC/cGMP pathway [21]. Additionally, NO acts as a signaling mediator through S-nitrosylation. NO can inhibit cardiac hypertrophy through nitrosylation of histone deacetylase 2 (HDAC2) released from chromatin [99]. HDAC2 regulates anti-hypertrophic genes. In ischemic preconditioning (the body's defense mechanism against myocardial necrosis), the S-nitrosylation of mitochondrial proteins protects the mitochondria from oxidative stress [100]. S-nitrosylation initiates excitation contraction coupling by increasing Ca^{2+} uptake, and the contraction may be sustained through releasing of Ca^{2+} via SR membrane ryanodine receptors

(RyRs) [26]. Quantitatively, when three thiols per subunit of RyR channels are nitrosylated, the process is reversible. However, if six or more thiols per subunit are nitrosylated, irreversible Ca^{2+} ion release occurs and can be detrimental to the cardiac muscle [26]. In addition, when too much NO is generated during inflammation or sepsis, NO may cause hypovolemia due to its excessive vasodilation effect [83]. Furthermore, upregulation of iNOS in ECs reduces the availability of BH_4 to eNOS, intensifying eNOS uncoupling and superoxide generation [83]. Thus, the physiological role of NO can be attenuated by ROS, because NO is quickly consumed by superoxide before it initiates any cell response [101].

When NO collides with superoxide, peroxynitrite ($ONOO^-$) is formed, causing nitrosative stress. The chemical reaction is very fast and deleterious [98]. $ONOO^-$ is a very strong oxidant. It reacts with proteins through tyrosine and tryptophan residues to form nitrotyrosine and nitrotryptophan, respectively [80, 98]. In diabetic mice, tyrosine nitration of the voltage-gated K^+ channels in the vascular SMCs altered its dilation function, a possible mechanism of the progression of coronary artery disease [102]. Tyrosine nitration was also observed in cardiac myocytes desmin, myosin heavy chain, α-actin, and microtubules. These proteins play pivotal roles in maintaining cell morphology and cardiac contractility [98]. When free nitrotyrosine was incorporated into the carboxyl terminus of α-tubulin in microtubules, altered microtubule organization and redistribution of the motor cytoplasmic protein dynein were observed [103]. Protein activity can also be impaired by oxidation of thiols to disulfide bond by $ONOO^-$ [98]. In addition, $ONOO^-$ also reacts with lipids to yield nitrated lipids to promote atherosclerosis, and with nucleic acids via guanine and the sugar phosphate backbone to damage DNA [98].

On the other hand, low concentrations of $ONOO^-$ (10–200 uM) is associated with tyrosine kinase-dependent signaling. $ONOO^-$ has been shown to activate tyrosine phosphorylation and trigger glycolysis [98]. Another example involves MAPK pathway, where $ONOO^-$ activates Raf-1 kinase. The MAPK pathway is closely associated with anti-apoptosis and cardiac hypertrophy in the cultured cardiomyocyte model [104].

5.2 Atherosclerosis

5.2.1 Inflammatory mechanism of atherosclerosis

Atherosclerosis is characterized by the formation of plaques that reduce the lumen of arteries and consequently interfere with blood flow and tissue perfusion. The plaque consists of the lipid core and fibrous cap. In patients with hypercholesterolemia, ROS and RNS oxidize low-density lipoprotein (LDL) [105]. Oxidized LDL (Ox LDL) initiates a cascade of events that alters the endothelial permeability and leads to insudation of the lipoprotein in the arterial wall. Stimulated by atheroprone signals, ECs express selectins and vascular cell adhesion molecule (VCAM-1) to attract circulating blood monocytes. Monocytes penetrate the endothelial layer; i.e., diapedesis occurs, and become macrophages [106]. Macrophages target Ox LDL for phagocytosis and become foam cells, the accumulation of which causes the formation of fatty streaks. The foam cells initiate the production of transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) in the vascular system [107, 108]. These growth factors promote the change of vascular SMCs from a contractile to a synthetic phenotype. SMCs migrate from the media layer to the intima, where they secrete a complex extracellular matrix to form a fibrous cap around the lipid core to stabilize the plaque [109]. The proliferation of SMCs leads to neointima hyperplasia. Thus, the vessel becomes narrowed and the blood flow profile alters, further aggravating endothelial dysfunction (**Figure 4**).

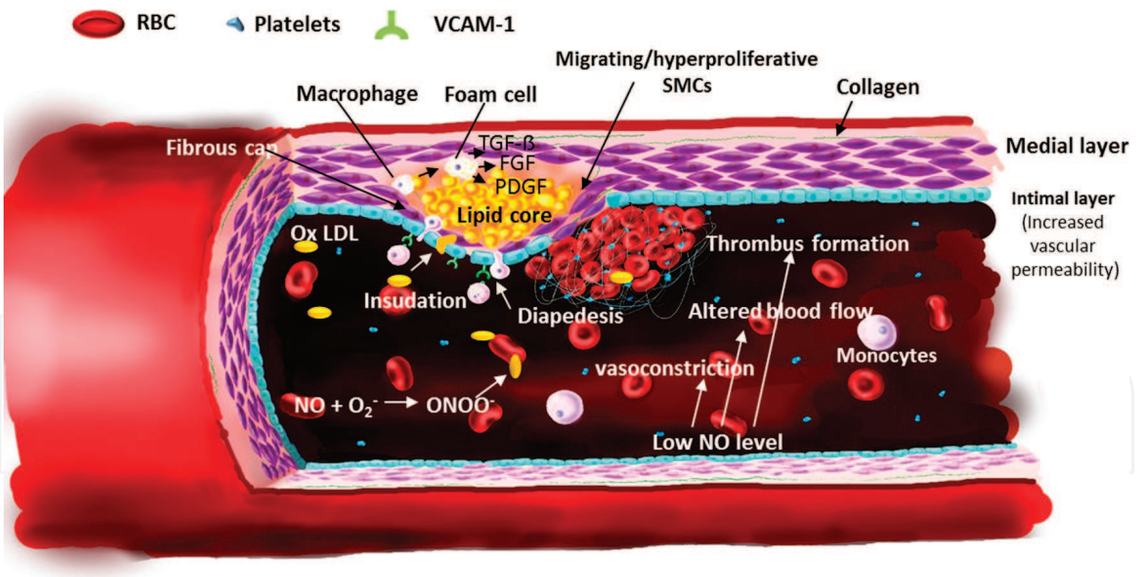

Figure 4.
NO's role in the initiation and progression of atherosclerosis.

5.2.2 Hemodynamics and atherosclerosis

In fact, disturbed blood flow at arches, branches, or bifurcations is always associated with the early appearance and fast development of atherosclerotic lesions. Blood flow influences ECs' gene expression through "shear-stress response elements" in the promoters of atherosclerosis relevant genes and "mechanotransducers" that can sense the force and transduce mechanical signal into biochemical events within the cell. Overall, in steady laminar flow, ECs express more antithrombotic, anti-inflammatory, and antioxidant proteins, such as eNOS, cyclooxygenase-2 (COX-2), and manganese-dependent superoxide dismutase (SOD) [110], while in turbulent flow, ECs show atheroprone phenotypes, which activate NF-κB pathways to promote the expression of cytokines and cell adhesion molecules [107].

Two highly differentially expressed transcription factors, zinc finger transcription factor Kruppel-like factor 2 (KLF2) and nuclear factor erythroid-2-related factor-2 (Nrf2), were identified by comparing endothelial gene expressions under different hemodynamic patterns [111]. KLF-2 maintains endothelial homeostasis at least in part by inhibiting local inflammation and restoring NO levels. Overexpression of KLF-2 blocks IL-1β-induced inflammation through inhibiting VCAM-1 and E-selectin expression to disturb the adhesion of immune cells [112]. In addition, it upregulates eNOS expression to improve vascular tones. Nrf2 is responsible for regulating redox-related genes (heme oxygenase 1, ferritin heavy chain, NADPH dehydrogenase quinone 1, and thioredoxin reductase) to maintain vascular redox balance in laminar flow [111]. Remarkably, it has been shown that KLF2 and Nrf2 work synergistically to integrate atheroprotective signals and active antioxidant responses, which may be a promising therapeutic strategy for CVDs.

5.3 Antioxidant mechanisms in nitroso-redox balance

To counteract the effect of excessive ROS and control CVD symptoms, introducing antioxidative mechanisms is an effective method (**Figure 5**). Increasing enzymes that can eliminate ROS is a commonly used strategy. For example, superoxide can be eliminated by dismutation of two superoxide molecules by SOD to O_2 and H_2O_2 [113]. H_2O_2 can undergo decomposition under the regulation of catalase and peroxiredoxin to oxygen and water [80, 114]. The thiol group in peroxiredoxins

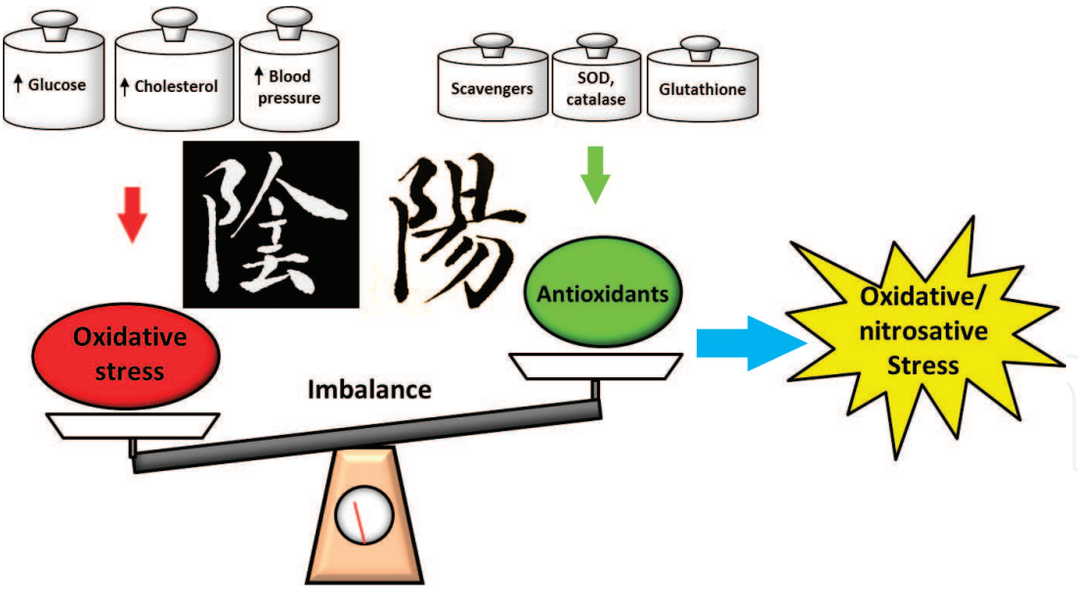

Figure 5.
Maintenance of redox balance in the cardiovascular system.

consumes H_2O_2 to form sulfenic acid, then subsequently disulfide bond [115]. Glutathione (GSH) peroxidase 1 uses the similar mechanism to inactivate H_2O_2, superoxide, and $ONOO^-$ in the presence of the tripeptide compound GSH. A prospective cohort study showed that reduced levels of GSH peroxidase 1 were associated with increased mortality in coronary disease patients [116].

Another effective antioxidative method is to protect redox-sensitive molecules from being oxidized. In the body, the thiol group on GSH can form reversible mixed disulfide bonds with cellular proteins under oxidative stress conditions. These disulfide bonds can be broken by the enzyme glutaredoxin when the surrounding cell environment reverts back to its normal state [80, 117]. The addition of scavengers to directly remove ROS/RNS can also restore the nitroso-redox balance. An example is the elimination of superoxide by ascorbic acid (vitamin C) [113]. By limiting superoxide, other reactive species can also be repressed, such as $^{\bullet}OH$ and $ONOO^-$. This may explain the success of the clinical trial of combining nitrate drug isosorbide dinitrate with hydralazine, a NADPH oxidase inhibitor, where heart failure was reduced by 45% [118]. By inhibiting superoxide generation from NADPH oxidase, $ONOO^-$ level may be reduced and NO function preserved.

The high concentrations of NO can be controlled through scavenging NO via oxyhemoglobin in red blood cells and myoglobin in the skeletal and heart muscle. These two proteins react with NO to form nitrate, which is considered as the primary method for inactivating NO in the cardiovascular system [119]. Hemoglobin and myoglobin can also scavenge $ONOO^-$ by their metal centers, generating nitrate from the reactions [120].

6. Conclusions and future outlooks

We briefly reviewed the molecular mechanisms of muscle contraction and relaxation in the cardiovascular system and highlighted the importance of physiological and pathological effects of NO and oxidative stress. NO and ROS both determine the structural integrity and functionality of the cardiovascular system. The cardiovascular system not only nourishes cells, but also provides paths for immune response and systematic signaling. Drugs are transported by this system to the correct site for metabolic reactions. Tissue regeneration also relies on a healthy

cardiovascular system. Therefore, to maintain, the homeostasis of the cardiovascular system is essential for overall health. Unfortunately, with aging, both cardiac function and cardiomyocyte number decline [121], and blood vessels undergo structural alterations [122]. Moreover, CVDs are also associated with other serious complications, such as diabetes, cancer, kidney failure, and inflammatory processes. Thus, multiple therapeutic strategies are needed to treat CVDs. According to 2011's American Heart Association's guidelines for preventing CVDs, therapeutic strategies include smoking cessation, blood pressure control, lipid management, physical activity programs, diabetes management, anticoagulation, dilation management, and depression prevention [3]. Besides traditional pharmaceutical management and surgeries, new perspectives to study, diagnose, and treat CVDs have also shown promising results, including development of biocompatible stents [123], stem cells therapies [124, 125], novel devices for mechanical thrombectomy [126], and inflammation management [127]. Although challenges still exist, the implementations of research findings from different disciplines in clinical trials will allow us to better understand and control CVDs in the future.

Conflict of interest

The authors have declared that no conflict of interest exists.

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