Physiological Effects of Nitric Oxide on Cartilage

Nitric oxide (NO) is a small gaseous molecule that is widely distributed in the human body, and its synthesis is dependent on NO synthase (NOS). NO plays an important role in various physiological processes such as the regulation of blood volume and nerve conduction.

1. NO and NO Synthase (NOS)

Nitric oxide (NO) is an unstable, uncharged free radical, and the N atom in NO with free electrons gets rapidly oxidized. Hence, its biological lifetime is only several seconds; subsequently, NO is active only in the immediate proximity of NO-producing cells. Meanwhile, NO has a small size and favorable lipid solubility, thereby exhibiting high membrane permeability [1]. Therefore, NO can rapidly diffuse out of cells and cross through more than several microns into target cells, acting as a paracrine-signaling molecule.

NOS produces NO as a byproduct while oxidizing L-arginine to L-citrulline, using oxygen and nicotinamide adenine dinucleotide phosphate (NADPH) as substrates (**Figure 1**). In addition, this reaction requires flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), and (6R-)5,6,7,8-tetrahydro-L-biopterin (BH_4) as cofactors. Among these cofactors, FAD and FMN are responsible for the transfer of electrons from NADPH to heme, and BH_4 can bind to the oxygenase domain of NOS as an essential cofactor for substrates. Notably, this reaction is stereospecific; NOS can metabolize L-arginine, but not D-arginine [2]. There are three subtypes of NOS present in the body: neuronal NOS (nNOS; NOS1), inducible NOS (iNOS; NOS2), and endothelial NOS (eNOS; NOS3). They have the same NO production process; however, their structures and functions are tailored to the different body parts. nNOS and eNOS are constitutively expressed (thus also called constitutive NOS, cNOS) and can rapidly produce NO based on an increase in cytoplasmatic calcium. In some specific cases, such as oxidation of BH_4 or depletion of L-arginine, eNOS can transform from a NO-producing enzyme into an enzyme that generates superoxide anion (O^{2-}), which is called NOS uncoupling. iNOS is only induced when macrophages are stimulated by cytokines including tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) and other factors such as bacterial toxoids. iNOS produces far more NO as compared to that produced by nNOS or eNOS [3]; this increased production might have various pathological effects.
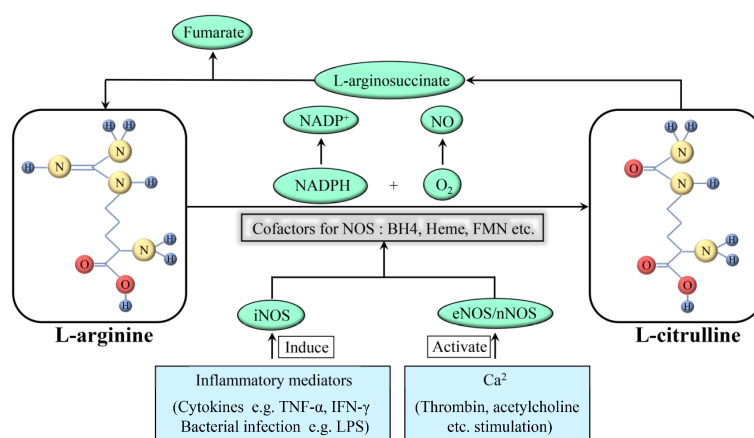

Figure 1. Formation and synthesis of NO. NAPDH: nicotinamide adenine dinucleotide phosphate; $NAPD^+$: oxidation form of NAPDH; NO: nitric oxide; O_2: oxygen; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase; nNOS: neuronal nitric oxide synthase; TNF: tumor necrosis factor; IFN: interferon; LPS: lipopolysaccharide.

NO is a reactive molecule that can act via numerous pathways, depending on the relative concentrations of NO and the surrounding environment in which NO is produced. The effects of NO can be direct or indirect, depending on the NO

concentration—NO can show direct effects (<1 μM NO) as well as indirect effects (>1 μM NO). The indirect effects are induced by the reactive nitrogen species, produced by the interaction of NO with superoxide or oxygen [4].

In inflammatory diseases, NO acts as a double-edged sword. It can produce anti-inflammatory effects under physiological conditions. The agents, such as cytokines, promote iNOS activity, resulting in the synthesis of NO in large amounts by monocytes, macrophages, and granulocytes, among many other cells. NO then scavenges free radicals or kills microbes, thereby preventing cell injury.

NO can also act as a pro-inflammatory mediator. Its overproduction can be cytotoxic [5]. NO can react with superoxide and generate peroxynitrite. The subsequent secondary chain reaction leads to the production of NO_2 and hydroxide, which can be even more toxic. This results in damaging the normal tissues, thereby increasing inflammation.

2. Physiological Effects of NO on Cartilage

As a chronic evolutionary disease, OA causes structural changes in the normal articular cavity. In order to comprehensively understand the mechanism of OA, the regulation of osseous tissue under physiological conditions should be explored.

The musculoskeletal system is an essential part of the human body's functions and is affected by the biomechanical environment. The osseous tissue's shape and density alter with the changes in the biomechanical environment. Qualitatively, the bone mass and density increase in areas with higher loads and decrease in areas with lower loads; this process is called bone remodeling. Bone remodeling consists of two steps: the stimulation of resorption in response to bone formation and the generation of new bone after bone degradation. Studies have shown the complexity of the process, involving multi-tiered communication networks of osteoclasts, osteoblasts, and other cell types in bone [6][7][8]. In addition, the coordination among endocrine, autocrine, and paracrine signals harmonizes human bone remodeling based on the adjustment of these cells in different stages [9]. Therefore, the recruitment, differentiation, and function of cells in bone remodeling are governed by a series of systemic regulators of bone metabolism (parathyroid hormone, vitamin D, and estrogen), local mediators (receptor of activated nuclear factor kappa-B ligand [RANKL], its antagonist osteoprotegerin, and Wnts/sclerostin), free radicals (superoxide, hydrogen peroxide, and NO), and bone matrix components [10]. The subsequent sections focus on the effects of the NO/NOS system on bone processes (**Table 1** and **Figure 2**).
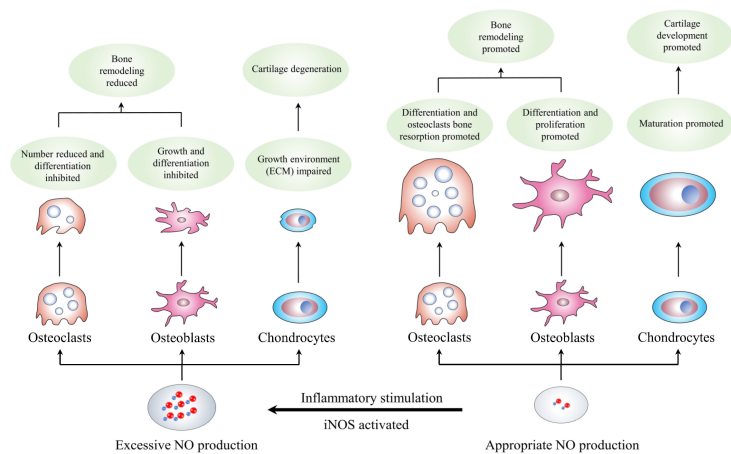

Figure 2. Formation and synthesis of NO. NO: nitric oxide; iNOS: inducible nitric oxide synthase; ECM: extracellular matrix.

Table 1. Possible effect of NO on different type of cells and its related mechanisms.

Targeted-Cell	Type of Study	NO Concentration	Related Mechanisms	Effect of NO	Reference
Osteoclasts	In vivo and in vitro	High	Reduces the number of osteoclasts and inhibits its spread	Inhibits mature osteoclasts bone resorption	Kalyanaraman et al., 2017 [11]
Osteoclasts	In vitro	High	Induced by inhibited cGMP-degrading activity of PDE	Inhibits precellular osteoclasts differentiation	Amano et al., 2019 [12]

Targeted-Cell	Type of Study	NO Concentration	Related Mechanisms	Effect of NO	Reference
Osteoclasts	In vitro	Low	Induced by RANKL and produces downstream molecular 8-nitro-cGMP	Promotes osteoclasts differentiation	Kaneko et al., 2018 [13]
Osteoclasts	In vitro	Low	Stimulated by cytokines and other mediators such as PG	Promotes osteoclasts bone resorption	Mentaverri et al., 2003 [14]
Osteoblasts	In vitro	Low	Induces osteoblast differentiation factor (Cbfa1) expression	Promotes osteoblasts differentiation	Gloria et al., 2020 [15]
Osteoblasts	In vivo and in vitro	Low	Activates Src, Erk-1/2 and Akt signaling pathway through sGC and PKG2	Promotes osteoblasts proliferation and anti-apoptotic effects	Cepeda et al., 2020 [16] Ramdani et al., 2018 [17]
Osteoblasts	In vivo and in vitro	Low	Stimulated by estrogen	Promotes osteoblasts growth and development	Gerbarg et al., 2016 [18] Crescitelli et al., 2019 [19]
Osteoblasts	In vitro	Low	Responses to mechanical stimulation	Promotes osteoblasts proliferation and survival	Wittkowske et al., 2016 [20] Maycas et al., 2017 [21]
Chondrocytes	In vitro	High	Induces caspase expression upregulation	Induces chondrocytes apoptosis	Poderoso et al., 2019 [22] Kamm et al., 2019 [23]
Chondrocytes	In vivo and in vitro	High	Stimulated by inflammatory mediators	Affects numerous biomolecular processes in chondrocytes	Wojdasiewicz et al., 2014 [24]
Chondrocytes	In vitro	Low	Stimulated chondrocytes hypertrophy and increased the expression of alkaline phosphatase and type X collagen	Promotes chondrocytes maturation	Teixeira et al., 2005 [25] Drissi et al., 2005 [26]

2.1. NO and Osteoclasts

Osteoclasts resorb bone and originate from somatic cells related to the single-core macrophage lineage [27]. Although they have unique bone resorption capabilities, they have various characteristics in common with macrophages, some of which are selectively expressed and regulated. This might be due to the adaptation of osteoclasts to osteoclast physiology, thus developing their specific functions. The similarities and nuances that distinguish osteoclasts from non-bone-resorbing macrophages reflect some specific mechanisms in bone remodeling. The NO/NOS system might be one such mechanism.

Thirty years ago, NO was confirmed to regulate the osteoclasts' roles. MacIntyre reported that in isolated rat osteoclasts, NO could inhibit the spreading of the cells and bone resorption [28]. Moreover, nitrosyl-cobinamide has been recently reported as an immediate NO release agent, which reduces the number of osteoclasts in intact mice and inhibits the increase in osteoclast numbers in ovariectomized mice [11]. Amano found that during osteoclast formation in mouse bone marrow cells, the osteogenic helioxanthin derivative could inhibit the cyclic guanosine monophosphate (cGMP) degradation activity of phosphodiesterase, promote NO production, and inhibit the differentiation of osteoclasts dose-dependently [12]. Other experiments, such as the mouse skull assay and the rat long bone assay, also demonstrated similar results [29][30]. Taken together, these results indicated the inhibitory effects of high NO concentrations on bone resorption via two types of effects: an immediate inhibitory effect on mature osteoclast bone resorption and a reasonable inhibitory effect on precellular osteoclast differentiation. In this pathway, the effects of NO are cGMP-independent and different from the common pathway in most other systems. Notably, NO can induce mature osteoclasts to get rid of bone and reduce their acid secretion, thereby inhibiting bone resorption [31]. This process appears to be mediated by endogenous NO production and requires cGMP and protein kinase G (PKG).

In addition, lower NO concentrations can also promote osteoclast differentiation and survival. Interleukin (IL)-1β and TNF-α are powerful stimulators of bone resorption; they could appropriately enhance NO formation in the organ cultures of

bone as well as bone marrow cultures. The addition of NOS inhibitors resulted in inhibiting the induced bone resorption, demonstrating that the NO/NOS system was stimulated by cytokines and other cytokine-induced mediators, such as prostacyclin (PG), to enhance bone resorption [30][32].

Kaneko et al. found that 8-nitro-cGMP, which is a NO derivate that is formed when NO reacts with cGMP in the presence of reactive oxygen species (ROS), could promote RANKL-induced osteoclast differentiation [13]. Moreover, a study assessed the levels of all three NOS modes in bones as well as isolated osteoblasts and osteoclasts using reverse transcription-polymerase chain reaction (RT-PCR) and immunohistochemistry [33]. Knocking out the nNOS gene increased the relative density of trabecular and cortical bone minerals in mice; the accurate measurement of the bone structure indicated a decrease in the total number of osteoclasts and osteoblasts, with worsened bone remodeling, which was reflected by the low mineral accumulation and bone formation rate [34][35][36]. These major manifestations indicated that nNOS might be essential for the differentiation and/or survival of all normal osteoclasts in vivo.

Moreover, nNOS-deficient bone marrow monocytes could produce poorly functional osteoclasts in vitro. Correspondingly, the iNOS-deficient mice showed no significant bone abnormalities, and their femoral length, trabecular bone volume fraction, bone formation rate, and osteoclast surface were normal [37]; this confirmed that the NO production by iNOS was much larger than that produced by nNOS, resulting in the loss of the promotion function. Therefore, in bone, iNOS might limit osteoclast activity by increasing NO levels, thus preventing excessive bone resorption in inflammatory diseases.

In other words, the high NO concentration can reduce the number of osteoclasts and inhibit their differentiation, demonstrating inhibitory effects on osteoclast bone resorption. On the other hand, the low NO concentration, mainly produced by cNOS, can promote the differentiation and survival of osteoclasts. Thus, the osteoclasts produced in nNOS-deficient individuals can lead to poor function. However, these conclusions are based on only in vivo and in vitro studies and lack the support of clinical data.

2.2. NO and Osteoblasts

Similar to their effects on osteoclasts, they might promote the differentiation and survival of osteoblasts and vice versa. The low NO concentration has been confirmed both in vivo and in vitro. Recent studies on human somatic cells and mouse models showed that the osteoblasts lacked argininosuccinate lyase, an enzyme participating in the synthesis of arginine and contributing to NO production, which resulted in low NO production and failure to differentiate [38]. Another study found that the vascular smooth muscle cells from the renal artery of male Wistar rats treated with aminoguanidine (AG; an iNOS inhibitor) showed a reduced expression of osteoblast differentiation factor (Cbfa1) [15]. Moreover, Wei et al. demonstrated that using the biochemical signaling molecules, such as PGs, NO, and insulin-like growth factor-1 (IGF-1) released by osteocytes, could increase osteogenesis, which showed a guiding significance in contemporary clinical treatment [39]. A study investigating the possible underlying mechanism of action showed that the proliferation of osteoblasts was simulated by cell-permeable cGMP analogs and prevented by pharmacological inhibition of soluble guanylyl cyclase (sGC) or protein kinase 2 (PKG2) or siRNA-mediated PKG2 knockdown; this suggests that the positive effects of low NO concentrations on osteoblast proliferation were mediated by sGC and PKG2 [16]. PKG2 exerts its antiapoptotic and proliferation-promoting effects in osteoblasts by activating Src, extracellularly regulated protein kinases-1/2 (Erk-1/2), and Akt [17]. The activated Akt phosphorylates and inactivates glycogen synthase kinase-3β, thereby stabilizing β-catenin and activating the Wnt pathway genes. The activation of the Wnt signaling pathway is a key factor in the differentiation, proliferation, and survival of (pre)osteoblasts and in driving bone formation [40][41]. In addition, a small dose of NO donors could activate the mRNA expression of osteoblast genes, such as alkaline phosphatase, osteocalcin, and collagen-1, and increase bone matrix synthesis and mineralization, thereby enhancing the osteogenic differentiation of (pre)osteoblasts in vitro [42][43][44].

In addition, based on estrogen stimulation, the moderate cNOS expression in osteoblasts can produce NO, which has a substantial role in the growth and development of osteoblasts as well as cytokine production [18][19].

The initiation of mechanical stimulation is highly important for the growth, development, and remodeling of bone [20][21]. When fluid flows through the bone canalicular system, the resulting shear stress stimulates osteoblasts and osteocytes to enhance their anabolism nonspecifically. With the increase in their proliferation and survival, the bone marrow stromal cells, osteoblasts, and osteocyte-like cells respond to fluid shear stress in vitro. This anabolic reaction requires moderate NO production from calcium-mediated eNOS.

The high NO concentrations resulting from NO donors or proinflammatory cytokines can effectively inhibit the growth and differentiation of osteoblasts [45][46]. These conditions often occur in inflammatory disorders and are related to the

inhibitory effects of pro-inflammatory cytokines on bone formation. Based on the animal model of inflammation-mediated osteopenia, the active cytokines are the reason for the reduced osteogenesis [47].

In summary, the high production of NO by iNOS in an inflammatory environment can effectively inhibit the growth and differentiation of osteoblasts. In most physiological conditions, the appropriate NO concentrations can promote osteoblast function, which might be mediated by sGC and PKG2. The initiation of mechanical stimulation can also contribute to the proliferation and survival of osteoblasts through moderate NO production.

2.3. NO and Chondrocytes

The effects of NO on chondrocytes in vivo under normal physiological conditions are hard to observe due to the dominance of iNOS in these bone cells. In other words, a necessary stimulation is required for the chondrocytes to produce NO.

A study using the chick cartilage model reported that the NO metabolites played a role in the maturation and differentiation of chondrocytes [25][26]. All three NOS isoforms are expressed and remain active in the growth plate. At least two NO-mediated functions are important in epiphyseal chondrocytes. After the maturation of chondrocytes, NO and related compounds stimulate chondrocyte hypertrophy via the cGMP-dependent pathway, thereby increasing the expression of alkaline phosphatase and type X collagen (maturation markers). In the late stages, inhibiting NO production can inhibit apoptosis, while exposure to NO donors increases apoptosis, suggesting that NO induces this process. This suggested that based on artificial stimulation, moderate NO production might contribute to chondrocyte proliferation in vitro; however, relevant in vivo data are still lacking.

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