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Targeting nitric oxide as a key modulator of sepsis, arthritis and pain

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ABSTRACT

Nitric oxide (NO) is produced by enzymatic activity of neuronal (nNOS), endothelial (eNOS), and inducible nitric oxide synthase (iNOS) and modulates a broad spectrum of physiological and pathophysiological conditions. The iNOS isoform is positively regulated at transcriptional level and produces high levels of NO in response to inflammatory mediators and/or to pattern recognition receptor signaling, such as Toll-like receptors. In this review, we compiled the main contributions of our group for understanding of the role of NO in sepsis and arthritis outcome and the peripheral contributions of NO to inflammatory pain development. Although neutrophil iNOS-derived NO is necessary for bacterial killing, systemic production of high levels of NO impairs neutrophil migration to infections through inhibiting neutrophil adhesion on microcirculation and their locomotion. Moreover, neutrophil-derived NO contributes to multiple organ dysfunction in sepsis. In arthritis, NO is chief for bacterial clearance in staphylococcal-induced arthritis; however, it contributes to articular damage and bone mass degradation. NO produced in inflammatory sites also downmodulates pain. The mechanism involved in analgesic effect and inhibition of neutrophil migration is dependent on the activation of the classical sGC/cGMP/PKG pathway. Despite the increasing number of studies performed after the identification of NO as an endothelium-derived relaxing factor, the underlying mechanisms of NO in inflammatory diseases remain unclear.

1. Introduction

Nitric oxide (NO) is an ancient molecule present in the Earth's primitive atmosphere [1]. It is therefore not surprising that NO is broadly distributed in several species, including mammals, invertebrates, low eukaryotic organisms and plants [2]. The conservation of NO during evolution illustrates its fundamental role in biological systems. Since the development of primitive microorganisms, NO has been involved in defense mechanisms. During early days of life on Earth, the photolysis of oxygen (O_2) by cyanobacteria increased the atmospheric levels of ozone (O_3) with consequent oxidative destruction of microorganisms [3]. The production of NO most likely provided a strong evolutionary advantage by neutralizing the toxic O_3. Currently, NO acts as a scavenger for reactive oxygen species (ROS) due to its high reactivity with superoxide (O_2^-) [4]. The free diffusion of NO throughout tissues and cell membranes helped this molecule reach intracellular targets and gain new functions during evolution. Since the identification of NO as the endothelium-derived relaxing factor (EDRF) produced by endothelium, numerous studies have demonstrated the various functions of this compound in physiological and pathological conditions [5].

NO is produced by mammalian cells through metabolism of L-arginine by the activity of nitric oxide synthases (NOS). Three different NOS isoforms encoded by distinct genes have been described in mammals. The neuronal (Nos1, NosI, nNOS) and the endothelial (Nos3, NosIII, eNOS) isoforms are constitutively expressed and their activities are calcium-dependent. Activities of eNOS and nNOS result in low levels of NO, which are mainly involved in several physiological processes. Other works also have described the role of eNOS- and nNOS-derived NO in pathological functions [5]. The third NOS isoform, the inducible NOS (Nos2, NosIII, iNos), produces high levels of NO in response to inflammatory mediators and/or to pathogen-associated molecular patterns (PAMPs). The iNOS isoform is regulated at transcriptional level and its activity is calcium independent [6]. In addition to inflammation-induced iNOS expression, this enzyme can be present constitutively in both mouse and human neutrophils as iNOS has been observed in the cytosol, elastase and gelatinase granules, and subcellular organelles in resting neutrophils [7]. NO has a central function in regulating inflammatory and immune responses [8] and our group has been

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attempting to understand the role of NO in these systems. In the present review, we will cover important contributions to understanding the mechanisms of NO in arthritis, pain and sepsis outcome.

1.1. Paradoxical role of nitric oxide in acute sepsis physiopathology

By generating iNOS-deficient mice, two independent groups confirmed previous pharmacological demonstrations of the central role of iNOS-derived NO against infections. MacMicking et al. showed that iNOS-deficient mice are more susceptible to *Listeria monocytogenes* infection [9] while Wei and colleagues showed that these animals are highly susceptible to a *Leishmania major* challenge [10]. Next, several studies demonstrated the major role of iNOS-derived NO against bacterial, virus and parasite infections [8]. Otherwise, systemic NO production inhibits several steps of neutrophil migration, compromising the migration of these cells to the focus of infection, and consequently for the bacterial clearance [11]. In this context, our group has been investigating the role of neutrophil-derived NO to sepsis outcome. Neutrophils are essential for eradicating a bacterial infection and avoiding bacterial spread [12]. Consistent with this concept, a reduction on the number of neutrophils was observed in the focus of infection during experimental severe sepsis induced by cecum ligation and the puncture model, as well as by intraperitoneal administration of Gram-positive, Gram-negative or cecal bacteria [13–15]. The low numbers of neutrophils in the infection focus are preceded by a reduction on rolling and adhesion of neutrophils to the mesenteric endothelium, denoting a breakdown in the early steps of migration to the infection focus [13–16]. Of note, the neutrophil migration failure is not due to a deficiency in production of chemotactic factors, since the massive bacterial load is followed by increased levels of chemotactic cytokines and chemokines at the focus of infection [13–16]. Recovering the neutrophil migration to the focus of infection in severe sepsis is associated with bacterial clearance and low mortality rates [17]. In mice subjected to nonsevere sepsis, the high numbers of emigrated neutrophils to the infection focus are associated with a reduction of bacterial load [13–16]. These data suggest that during experimental severe sepsis there is a failure of neutrophil migration to the focus of infection, which is correlated to a poor bacterial clearance and high mortality rate [13–16].

Once neutrophils reach the focus of infection during nonsevere sepsis, they eliminate microorganisms by phagocytosis and the production of microbicidal mediators. Among these mediators, reactive oxygen (ROS) and nitrogen species (RNS) are produced in the phagosome compartment, with consequent formation of peroxynitrite (a by-product of NO and superoxide interaction), and subsequently bacteria nitrosylation [18]. Nitric oxide also may lead to bacterial killing independent of ROS production [19]. Using pharmacological tools and iNOS-deficient mice, we and others demonstrated that neutrophil-derived NO is necessary at the focus of infection for effective bacterial clearance [13–16]. Nitric oxide production during neutrophil microbicidal activity involves interaction of iNOS with Ras-related C3 botulinum toxin substrate 2 (Rac2) protein, a Rac small GTPase [7]. Patients with an inhibitory mutation in Rac2 have several leukocyte disorders, including decreased bactericidal activity and recurrent infections [20,21]. In accordance, Rac2-deficient mice have defects in microbicidal activity and increased susceptibility to fungal infection [22]. Extrusion of neutrophil extracellular traps (NETs) is also an important mechanism of bacterial killing during sepsis, a process that appears to involve Rac2 and NO production [23–26].

There are evidences in the literature that systemic inflammatory response is a chief mechanism of neutrophil migration failure in severe sepsis [13,17,27]. Indeed, the concentrations of circulating cytokines and chemokines, as well as the byproducts of NO such as nitrite and nitrate, are increased in animals subjected to lethal sepsis [28,29]. Consistently, the systemic inflammatory response was reduced in TLR4-, TLR2- or TLR9-deficient mice subjected to severe polymicrobial sepsis compared to wild-type mice. These TLR-deficient mice exhibited efficient neutrophil migration to the focus of infection, low bacterial load and a reduced mortality rate [13,17,30]. Impairment of tumor necrosis factor (TNF) receptor signaling also decreased the systemic inflammatory response concomitant with an efficient neutrophil recruitment and bacterial clearance. As a consequence, these mice present a lower mortality rate during severe experimental sepsis [31].

Serum cytokines and TLR intracellular signaling induce expression of acute phase proteins in the liver and leukocytes, which in turn contribute to dampening the neutrophil response [29,32,33]. We observed that intravenous administration of human alpha-1-acid glycoprotein (AGP) inhibited carrageenan-induced rolling, adhesion and neutrophil migration. The inhibitory activity of AGP was blocked by aminoguanidine, a selective iNOS inhibitor and was not observed in iNOS knockout mice. The AGP intravenous pretreatment of rats with nonsevere sepsis inhibited neutrophil migration and reduced 7-day survival from approximately 80%–20%. *In vitro*, AGP significantly increased nitrite content in the supernatant of neutrophils isolated from healthy donors and inhibited IL-8-induced neutrophil chemotaxis by an iNOS-sGC-dependent mechanism [28].

In addition to sepsis models, intravenous administration of LPS, TNF, interleukin-8 (IL-8), macrophage-derived neutrophil chemotactic factor (MNCF) or IL-2 inhibits the recruitment of neutrophils to the inflammatory site by a NO-dependent mechanism [34–36]. Interestingly, inhibition of LPS-derived NO prevents the reduction of neutrophil migration without decreasing the levels of serum cytokines. These results demonstrate that the excessive systemic inflammatory response-induced NO is the proxy of neutrophil migration failure toward the peritoneal cavity [37]. Investigating the molecular mechanisms involved in disruption of neutrophil migration during severe sepsis, we demonstrated that neutrophils from severely septic mice have a downregulation of chemokine receptor CXCR2, which compromised the rolling, adhesion and migration *in vivo* as well as the chemotactic response toward CXCR2 agonists *in vitro*. CXCR2 expression and neutrophil migration in severe sepsis were reestablished by pharmacological or genetic ablation of iNOS. Likewise, neutrophil incubation with SNAP (S-nitroso-N-acetyl-D,L-penicillamine), a NO donor, downregulated CXCR2 receptor and reduced CXCL-8-induced neutrophil chemotaxis [27]. Inhibition of iNOS constrains LPS- or IL-8-induced CXCR2 downregulation and the chemotactic response to CXCR2 agonists [27,38]. These results highlight NO as a major contributor for the impairment of neutrophil migration to the focus of infection in experimental sepsis. Despite the fact that ablation of iNOS improved neutrophil migration, the mortality rate of severe sepsis remains high. This apparent paradox could be explained by the key role of neutrophil-derived NO as a microbicide agent. These effects highlight the dual role of NO in sepsis as this reactive gas is important for restraining pathogens at the local infection but also inhibiting the neutrophil migration to the focus of infection. These results led us to ask whether the fine-tune regulation of NO production could improve survival in experimental sepsis. We observed that subcutaneous pretreatment of severe septic mice with aminoguanidine at 10, 30, and 90 mg/kg prevented failure of neutrophil migration, however only doses of 10 and 30 mg/kg improved survival rate [15]. It suggests that the modulation of NO production could be useful to improve the outcome of sepsis.

The improvement of cardiovascular functions and sepsis outcome by soluble guanylate cyclase (sGC) inhibitors suggests that NO achieves its systemic deleterious effects by classical signaling pathway involving L-arginine–NO–sGC-cyclic guanosine monophosphate (cGMP) [39–41]. To preserve the microbicidal mechanisms of NO, we evaluated the effect of ODQ (1H- [1,2,4]oxadiazolo[4,3-a] quinoxalin-1-one), an sGC inhibitor, on neutrophil migration and sepsis outcome. Pretreatment of septic mice with ODQ increased the survival rate by preventing the reduction of neutrophil migration concomitant with efficient neutrophil bacterial killing. Additionally, the beneficial effect of sGC inhibition was observed when mice were posttreated with ODQ. *In vitro*, inhibition

of iNOS, sGC or protein kinase G (PKG, a downstream target of cGMP) inhibited the LPS-induced downregulation of CXCR2 and the upregulation of GPCR kinases 2 (GRK2) expression in neutrophils, and, consequently, increased neutrophil chemotaxis response [38]. Heterologous and homologous desensitization of G-protein-coupled receptors (GPCRs) is a common mechanism involving receptor phosphorylation by GRKs [42]. Therefore, high serum levels of CXCR2 agonists, bacterial components or cytokines might account for the CXCR2 downregulation on neutrophils during severe sepsis. Using murine models of sepsis or *in vitro* and *in vivo* approaches, we demonstrated that activation of TLR2, TLR4, TLR9 or TNFRs induces neutrophil CXCR2 downregulation by GRK2 activation. The consequence of this response is reduced neutrophil migration to the focus of infection and a decreased neutrophil chemotactic response to CXCR2 agonists [13,17,30,31,43]. In agreement, neutrophils isolated from septic patients have a reduction in chemotaxis response, downregulation of CXCR2 and high expression of GRKs [44].

NO can react with superoxide anion (O_2^-) in aqueous solutions to yield peroxynitrite ($ONOO^-$). Peroxynitrite oxidization of multiple targets leads to significant changes in lipids, proteins, and nucleic acids functioning. In sepsis, peroxynitrite contributes to damage in several tissues, including cardiovascular system, kidney, liver, central nervous system and immune system [45,46]. We observed that pretreatment of septic mice with the $ONOO^-$ scavengers uric acid (UA) or Tetrakis (FeTPPs) enhanced neutrophil rolling, adhesion, and migration to the focus of infection, improving infection control and survival rates [47].

As noted previously, high levels of NO in sepsis lead to several changes in the cardiovascular system, such as hypotension, low peripheral vascular resistance, poor response to vasoconstrictors and heart dysfunction [48]. We observed that in murine experimental severe sepsis there is an increase in iNOS expression in heart tissues, which was associated with a downregulation of β-adrenergic receptor, an upregulation of GRK2 and a decreased cardiac *ex vivo* response to isoproterenol (a β-adrenergic agonist). Blockade of iNOS-derived NO *in vivo* inhibited the low density of the β-adrenergic receptor and prevented the upregulation of GRK2 and heart responses. Of note, pharmacological inhibition of GRK2 prevented the downregulation of β-adrenergic receptor, restored the *ex vivo* heart response to isoproterenol and increased the survival rate of septic mice. In cardiomyocytes isolated from neonatal mice, LPS/IFN-γ-induced NO production upregulated GRK2 levels by the canonical GC/cGMP/PKG pathway [49]. The presented pieces of evidence show that iNOS-derived NO from neutrophils is necessary for bacterial clearance in different experimental models of sepsis; however, systemic high levels of NO lead to impairment of neutrophil migration to the focus of infection as well as to cardiovascular hyporesponsiveness in severe sepsis (Fig. 1).

Preclinical and clinical data showing benefits of NO inhibition supported the development of a randomized controlled trial that included 797 patients. This study was suspended after showing increased mortality in patients that received high doses of L-N-monomethyl arginine (L-NMMA), a nonselective NOS inhibitor [50]. The high doses of this nonselective NOS inhibitor could be accounted for the unsuccessful results of this trial. It argues that preclinical data should be carefully analyzed and taken into account during design of clinical trials since dose titration of a selective iNOS inhibitor could yield a different outcome in patients.

Despite the enormous progress achieved in the last decades to understand the pathophysiological mechanisms of sepsis and the improvement of the survival rates showed by preclinical models, including NO as a target, septic patients remain without an effective treatment. Many factors might account for inefficient translation of data from bench to bedside in this complex disease, some of them are difficult to control, but others may be considered in experimental studies. As examples, the majority of experimental studies in sepsis uses young animals, while human sepsis is often observed either in patients over 60 years old or in premature babies. Septic patients have different diseases and comorbidities, including diabetes, cancer, immune-deficiencies, whereas experimental animals are completely healthy. Moreover, different from experimental models, these patients are frequently under pharmacological treatment for their comorbidities, which might also contribute to sepsis worsening. Taking this into account and other particularities during the design of preclinical and clinical studies, it will improve the success of clinical translation. Notwithstanding these mentioned limitations of preclinical models, several findings obtained in murine models in our and other groups have counterparts in human condition [17,38,44,51,52].

1.2. Role of NO to arthritis outcome

Rheumatoid arthritis (RA) is a progressive systemic autoimmune disease characterized by joint inflammation [53]. RA is a multifactorial complex condition where genetic inheritance and environmental factors, such as infections and smoking, are the main contributors to disease development [54]. This chronic disease is most common in women and elderly people and may lead to joint damage, disability, cardiovascular comorbidities and a decreased quality of life [53,54]. Many drugs have been used to reduce synovial, systemic inflammation and to improve joint function. These agents are known as disease-modifying antirheumatic drugs (DMARDs). First choice of drug for RA is methotrexate, which can be administered alone or associated with other DMARDs [55]. Biologics, including TNF, IL-1, or IL-6 inhibitors, anti-B and anti-T drugs, and small molecules designed to target intracellular kinases are available to treat RA patients [56]. Patients treated with DMARDs presented a marked decrease in reactive oxygen and nitrogen species concomitant with increased antioxidant defenses, suggesting the success of RA treatment-involved reduction of oxidative and nitrosative stress [57].

Several works, some of them conducted by our research group, have provided solid results about NO as a remarkable protagonist during the development and progression of RA [58–68]. NO also has a predominant role in osteoarthritis (OA) [69]. Using a quantitative method of OA associated to weight bearing in the anterior cruciate ligament transection (ACLT) we observed that NO production is linked to pain development and poor OA outcome in this model [62]. As mentioned, high concentrations of iNOS-derived NO under inflammatory conditions can be toxic, leading to tissue injury [70]. Both serum and synovial fluid of patients with RA or OA present high levels of nitrite, express iNOS in synovial fluid and show evidence of protein nitration, highlighting the involvement of NO in distinct types of arthritis [66,71]. During RA, the prevalent source of NO seems to be the inflamed joint [65,66]. Constitutive and inducible isoforms of NOS have been linked to the development of inflamed joint erosion in RA, implying that damage mediated by NO is independent of the NOS isoform [72,73]. While the regulation of nNOS and eNOS is intracellular Ca^{2+}-dependent, iNOS is regulated by nuclear transcription factors, including NFkB [74], allowing for a variety of different cell types to produce and secrete NO into inflamed joints. These cells include fibroblasts, osteoclasts, osteoblasts, endothelial cells, and immune cells, such as macrophages and neutrophils [75,76]. NO leads to tissue injury by releasing proteolytic enzymes, inducing dysregulation of osteoblasts/osteoclasts balance, and by a combination with superoxide to generate peroxynitrite, which contributes to destructive events in cartilage, including apoptosis induction [77,78]. Osteoblasts and osteoclasts are responsible for bone mass formation and resorption, respectively. In RA, chronic inflammation triggers irreversible structural damage seen by the unbalance of those bone modeling and remodeling processes. T helper cells are reportedly osteoclastogenic once they provide osteoclast differentiation by activating RANK in monocyte-macrophage lineage [79,80]. In response to inflammatory mediators, macrophages and $CD4^+$ T lymphocytes infiltrate and release cytokines that induce fibroblast-like synoviocytes (FLS) to express RANK ligand (RANKL). Additionally, infiltrated T cells induce osteoclasts to release cathepsin K

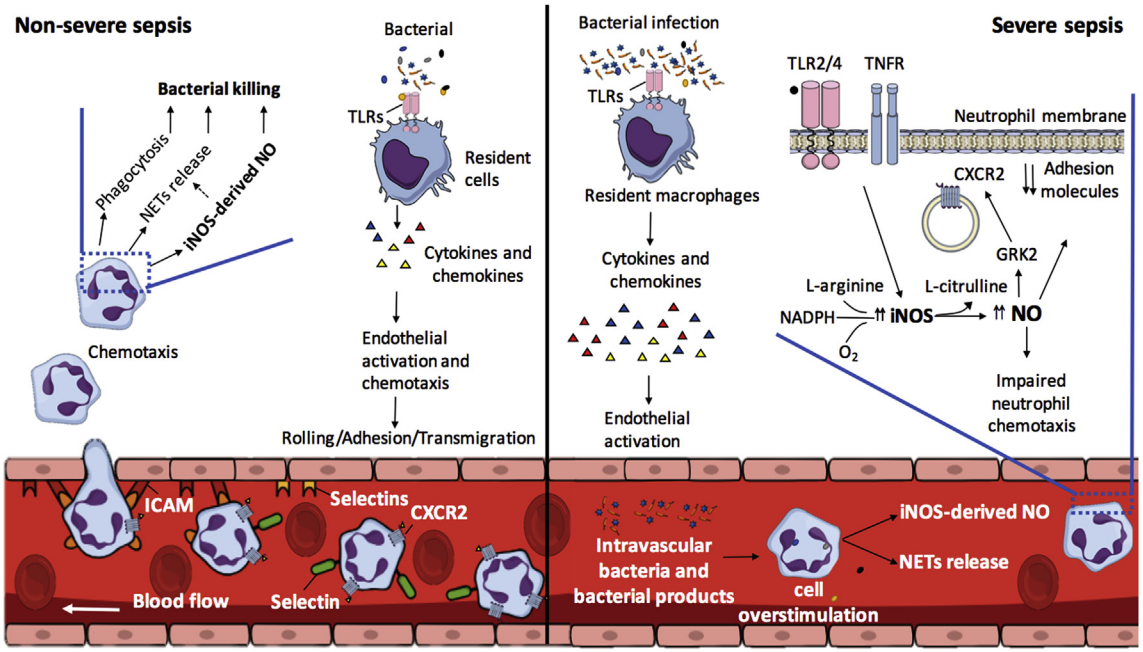

Fig. 1. Role of nitric oxide in sepsis outcome. Nitric oxide in nonsevere sepsis. Polymicrobial infection induced Toll-like receptor 2 and 4 activations in resident cells such as macrophages, results in the release of proinflammatory cytokines and chemokines. These mediators create a chemoattractant gradient in the extracellular matrix and induce endothelial cell activation. P- and E-selectin expressed on endothelial cells and L-selectin on leukocytes engage carbohydrate ligands and facilitate the initial capture and rolling of leukocytes under shear flow. Chemokines bound to the luminal surface of the endothelium activate seven transmembrane–spanning G protein–coupled receptors on leukocytes (i.e., CXCR2), resulting in integrin activation and firm adhesion of rolling cells. Leukocytes traverse the endothelium into the underlying tissue, where they chemotaxis toward the focus of infection to initiate the control of infection. Phagocytosis, neutrophil extracellular traps (NETs) release and NO production are key events for bacterial killing. Nitric oxide in severe sepsis. Spreading bacteria leads to systemic TLR activation. In endothelial cells, TLR activation induces cytokine/chemokine release (i.e., TNF, IL-8) and loss of adhesion molecules in these cells. The overstimulation of neutrophils by bacterial products and/or cytokines/chemokines induces the expression of iNOS, resulting in upregulation of GRK2, downregulation of CXCR2 and shedding of L-selectin. NO also reduces neutrophil chemotaxis. These events lead to neutrophil migration failure. iNOS = inducible nitric oxide synthase; CXCR2 = CXC chemokine receptor 2; TLR2 = Toll-like receptor 2; TLR4 = Toll-like receptor 4; TNFR = tumor necrosis factor receptor.

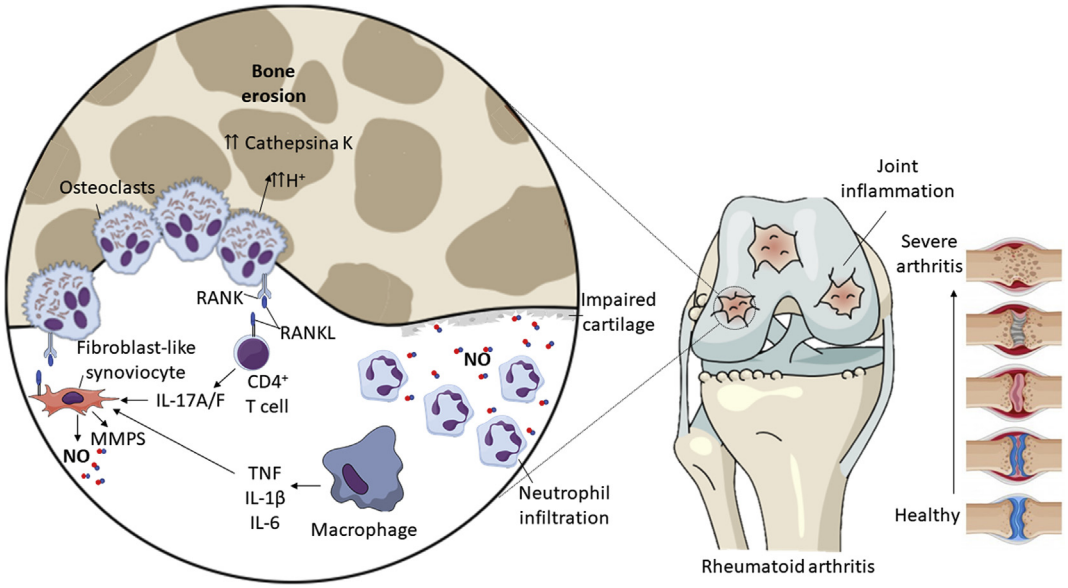

Fig. 2. Role of nitric oxide in inflammatory arthritis. Bone homeostasis maintains balanced osteoblast and osteoclast activity. Under severe inflammatory arthritis, infiltrated $CD4^+$ T and macrophages release proinflammatory cytokines, such as TNF-α, IL-1B, IL-6 and IL-17, which promote NO and MMP release by FLS. Infiltrated T cells and FLS also induce osteoclast proliferation via RANK-RANKL. The release of NO by neutrophils and cathepsin K and protons (H^+) by T cell-activated osteoclasts contributes to joint damage. FLS, fibroblast-like synoviocytes; IL, interleukin; MMPs, matrix metalloproteinases; NO, nitric oxide; RANKL, receptor activator of nuclear factor B ligand; TNF, tumor necrosis factor.

and protons (H^+). These events, together with matrix metalloproteinases and NO released by FLS and neutrophils, result in unbalanced bone resorption/formation and joint damage (Fig. 2) [80].

Several experimental models have confirmed the participation of NO in RA development. These studies have shown a high expression of iNOS in the joint of arthritic animals and regression of disease in

animals treated with selective and nonselective inhibitors of iNOS [81–84]. During early stage of sterile inflammation or during recrudescence of autoimmune inflammation, tissue damage seems to be related to neutrophil recruitment to an inflammatory focus and release of several cytotoxic mediators, such as metalloproteinases, proteolytic lysosomal enzymes, and reactive oxygen/nitrogen-derived species. Therefore, neutrophils do collaborate in the development of inflammatory process during RA. The high numbers of neutrophils in inflammatory synovial fluid suggest a multimediator process favoring the recruitment of these cells in arthritis. This process is mediated by many different chemoattractant factors, including leukotriene B_4 (LTB_4) and C5a [85,86]. In a physiological inflammatory response, after clearance of the proinflammatory stimulus, the resolution of inflammatory program changes to repair the damaged tissue. Apoptotic neutrophils are engulfed by macrophages to clean up the inflamed area, leading to release of lipoxins and resolvins to block the influx of additional neutrophils [87]. However, in the chronic inflammatory response of RA, resolution of inflammation does not occur precisely and neutrophils are constantly present in inflamed joints. The large numbers of emigrate neutrophils contribute to the high levels of NO and reactive oxygen species into inflamed joints. Those radicals can directly or indirectly compromise several cellular elements in cartilage and extracellular matrix [88,89]. Oxygen and nitrogen radicals promote the inhibition of the synthesis of proteoglycans, growth factors, and type II collagen by chondrocytes [90,91]. NO also decreases the number of chondrocytes by promoting apoptosis of these cells [90,92].

1.3. Role of NO in peripheral pain development

All of the mechanisms of tissue injury considered above lead to pain (nociception in animals) development. Therefore, relieving pain is a major concern during treatment of arthritis and other inflammatory diseases. Despite the arsenal of analgesic drugs available, they are still limited and, in some cases, inefficient. In this context, it is accepted that understanding the mechanism underlying the physiopathology of pain might contribute to the development of more effective treatments of pain observed in different inflammatory diseases.

Pain is defined by IASP as “an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage” [93–95]. The noxious stimuli are sensed and conducted to brain by nociceptors along the periphery of tissues, which are highly concentrated in primary sensory nerve fibers (Aδ and C) [96]. Peripheral nociception sensitization is triggered by inflammatory mediators released by injured tissues, such as prostaglandin E_2 (PGE_2), bradykinin, nerve growth factor (NGF) and others [97–99].

The role of NO for the onset and maintenance of pain during inflammatory diseases has been extensively investigated, at both peripheral and central levels and the results are still controversial. Taking into account the experience of our group, in the present review, we will focus mainly on the peripheral nociceptive role of NO. A local injection of NO produces pain behavior, suggesting that NO should activate the surrounding nociceptors [100]. Therefore, intra-articular injection of the nonspecific NOS inhibitor, L-NAME (N(ω)-nitro-L-arginine methyl ester), or injection of the selective nNOS inhibitor, 7-nitro-indazole (7-NINA), inhibited thermal hyperalgesia applied to a rat's paw in a model of carrageenan-induced acute joint inflammation [101]. Investigating the NO participation in zymosan-induced arthritis, we observed that systemic or intra-articular treatment with the nonselective inhibitors of NOS, L-N^G-nitroarginine methyl ester (L-NAME), or with the selective inhibitors of iNOS, aminoguanidine (AG) and N-(3-(aminomethyl) benzyl) acetamide (1400 W), prevented articular incapacitation and nociception [67,102]. As NO is involved in joint lesions in the models described above, it is possible that analgesic effect of the NOS inhibitors may reflect the reduction of joint lesion [87–90]. A quantitative approach was also applied by us to measure and analyze joint pain in experimental osteoarthritis (OA), which was induced by anterior cruciate ligament transection (ACLT) in rat knee joint [62,103]. In this model, a close relation between the increased concentration of NO and the development of joint pain was observed, as nociception was suppressed by the prophylactic administration of either 1400 W or L-NAME [62]. Analyzing animals subjected to ACLT, an increase of iNOS activity in the superficial synovial cells was detected, suggesting that these cells and chondrocytes are the main source of higher levels of NO [62]. These results point to an association between NO and joint tissue lesions with consequent joint pain. Alternatively, in bacterial septic arthritis induced by *Staphylococcus aureus*, the severity of arthritis is higher, followed by a poor infection control in iNOS-deficient mice when compared to wild-type mice. These results confirm the key role of NO in articular host defense and demonstrate that iNOS-derived NO is not necessary for articular injury in this particular model [104]. Altogether, the data presented here showed a deleterious involvement of NO to tissue damage in noninfectious arthritis. However, in infection-induced arthritis, NO has a protective role through its key function as a microbicide agent and does not play an important role in the joint lesion.

In inflammatory pain, NO is derived from resident cells or from new emigrate cells, such as neutrophils and monocytes [105–109]. In this context, Castro and Ferreira (1979) suggested for the first time the possible link between nociception and neutrophils. They observed that LPS administration into dog joints induced intense neutrophil accumulation followed by articular incapacity [110]. Subsequently, other studies demonstrated the dependence of neutrophil migration in hyperalgesia development [111–113]. Cunha et al. (2008) also showed that during an inflammatory process, migrating neutrophils participate in the cascade of events leading to mechanical hyperalgesia by releasing hypernociceptive mediators such as prostaglandin E_2. Pretreatment of rats with fucoidin, a leukocyte adhesion inhibitor, inhibited carrageenan-induced neutrophil migration and hyperalgesia but did not change the levels of cytokines and chemokines at the focus of inflammation. Additionally, hyperalgesia and neutrophil migration induced by TNF, IL1, or CINC-1/CXCL1 were inhibited by fucoidin, suggesting that neutrophils are involved on the production of hypernociceptive mediators [114].

Because activated neutrophils produce pronociceptive mediators, such as cytokines, PGE_2 [115–117] and NO [117], it is conceivable to suggest that neutrophils are the source of those molecules during pain development. Studies have previously demonstrated that large amounts of iNOS-derived NO are generated by neutrophils from acute myocardial infarction patients [118]. We observed that, in zymosan-induced arthritis, nociception and leukocyte influx were inhibited by prophylactic treatment with L-NAME or aminoguanidine [119]. Subsequently, Chaves et al. (2011) showed that zymosan-induced temporomandibular arthritis in rats was frequently associated with high levels of pain-related disability, neutrophil infiltration, increase of iNOS expression and mechanical hypernociception, which were inhibited by L-NAME [120]. These results suggest a potential participation of neutrophil-derived NO in the induction of inflammatory pain and tissue injury.

Despite of the abovementioned studies describing the nociceptive role of NO in different experimental models, there is fundamental literature describing that NO has antinociceptive effects. Intraplantar treatment with sodium nitroprusside, a NO donor, inhibited PGE_2-induced hyperalgesia in rat paws. Moreover, analgesic effect of acetylcholine was blocked by intraplantar administration of L-NMMA and methylene blue, an sGC inhibitor, and enhanced by cGMP phosphodiesterase inhibition [121]. Additionally, peripheral antinociception effects of morphine in rats subjected to PGE2-induced hyperalgesia were mediated by NO release via cGMP activation, supporting the hypothesis that peripheral analgesia of morphine and acetylcholine is subsequent to NO output [122]. Our group demonstrated that the antinociceptive effects are mediated by sGC/cGMP/PKG pathway, leading

to ATP sensitive K^+ channel (K_{ATP}) activation. The intracellular K^+ extrusion results in hyperpolarization and direct blockade of acute and persistent hyperalgesia [123]. In this context, we observed that activation of peripheral *kappa* opioid receptors (KORs) directly blocks inflammatory hyperalgesia by triggering the activation of the PI3Kγ/AKT intracellular signaling route, which is responsible for sGC/cGMP/PKG pathway activation [124]. Moreover, analgesic effects of dipyrone, another classical drug used to treat pain, are also mediated by activation of the arginine/NO/cGMP/PKG/K_{ATP} pathway [123].

Pharmacologic control of inflammatory pain is centered on the use of classical nonsteroidal anti-inflammatory drugs, such as aspirin and aspirin-like drugs, which inhibit cyclooxygenase-derived prostaglandin production and, consequently, reduce nociceptor sensitization. Additionally, other drugs are capable of directly blocking ongoing nociceptor sensitization through peripheral actions by stimulating NO production, such as opioids and dipyrone [123,125], as mentioned above. In this context, the development of NO donor-releasing NSAIDs has generated more potent anti-inflammatory drugs with increased safety profiles and presents new perspectives in pain therapy. A hybrid molecule containing both NO and H_2S donors in aspirin (NOSH-aspirin) was more potent than aspirin in inhibiting inflammatory hyperalgesia in a dose-dependent manner, due to reduction in IL-1β levels and activation of K_{ATP} channels [126].

Explanations for the apparent antagonistic studies showing nociceptive and antinociceptive roles of NO have been debated in the literature, and the possible reasons include different experimental models, different range of doses, administration routes, and local conditions associated with primary disorders that follow tissue injuries. Different pain models produce different pain states, sensitizing different sets of nociceptors and, therefore, may reveal different effects for drugs that interfere with NO production [127,128]. As an example, the administration of NO donors via intradermic route sensitizes the nociceptors, whereas a subcutaneous route causes anti-nociception [129]. Furthermore, in experimental models in which there is a massive recruitment of neutrophils, the antinociceptive action of NO could be explained by downmodulation of the migration of these cells, with a reduction of tissue lesions and consequently nociception [130]. Together, our studies suggest that during ongoing inflammatory nociception, the direct effect of NO at a nociceptor will promote antinociception due the hyperpolarization of these receptors mediated by activation of the NO/cGMP/PKG/K_{ATP} pathway.

2. Conclusions

We have reviewed some circumstances in which NO modulates sepsis, arthritis and pain and the ways it might perform these regulations. While low levels of NO have beneficial effects under physiological conditions, the increased NO production is linked to poor outcomes in pathological states. Neutrophil iNOS-derived NO is necessary for bacterial killing, however systemic high levels of NO impairs neutrophil migration to the focus of infection by a miscellaneous of mechanisms including dampening of adhesion, rolling, and chemotaxis, decreasing expression of selectins and chemotactic receptors. Furthermore, neutrophil-derived NO contributes to multiple organ dysfunction in sepsis. NO is also important for bacterial clearance in staphylococcal-induced arthritis. However, it contributes to articular damage and bone mass degradation. NO produced in inflammatory sites down-modulates pain by inhibition of neutrophil migration and by increase the classical sGC/cGMP/PKG analgesic pathway in nociceptive neurons. The data assembled here showed that the effects of NO are complex, diverse, and sometimes antagonistic. The underlining mechanisms of NO are not fully understood, and it remains a rich field to be explored.

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