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The chemical biology of nitric oxide – an outsider's reflections about its role in osteoarthritis

Martin Feelisch, Ph.D.*

Professor of Experimental Medicine & Integrative Biology, Clinical Sciences Research Institute, Warwick Medical School, The University of Warwick, Coventry, UK

"If we knew what it was we were doing, it would not be called research, would it?"
Albert Einstein, 1930

Abstract

Excess formation of nitric oxide (NO) has been invoked in the development of osteoarthritis and blamed for triggering chondrocyte apoptosis and matrix destruction. Much of the evidence for a deleterious role of NO in disease progression has been obtained indirectly and inferred from the measurement of nitrite/nitrate and nitrotyrosine concentrations as well as iNOS expression in biopsy specimen, cartilage explants and cytokine-stimulated cells in culture. While these results clearly indicate an involvement of NO and suggest additional contributions from oxidative stress-related components they do not necessarily establish a cause/effect relationship. Many NO metabolites are not mere dosimeters of local NO production but elicit potent down-stream effects in their own right. Moreover, oxygen tension and other experimental conditions typical of many *in vitro* studies would seem to be at odds with the particular situation in the joint. Recent insight into the chemical biology of NO, in particular with regard to cellular redox-regulation, mitochondrial signaling and nitration reactions, attest to a much richer network of chemical transformations and interactions with biological targets than hitherto assumed. In conjunction with the emerging biology of nitrite and nitrate this information challenges the validity of the long-held view that "too much NO" is contributing to disease progression. Instead, it suggests that part of the problem is a shift from NO to superoxide-dominated chemistries triggering changes in thiol-dependent redox signaling, hypoxia-induced gene expression and mitochondrial function. This essay aims to provide a glimpse into research areas that may hold promise for future investigations into the underlying causes of osteoarthritis.

Key words: Oxidative stress, redox regulation, chemical biology, nitrite

Introduction and scope of the problem

Osteoarthritis (OA), the most common form of joint disease, is a major cause of discomfort and disability in the elderly population. It affects >2 million people in the UK, >20 million people in the United States and accounts for ~25% of all visits to primary care physicians in these countries. While the cause of the disease remains unknown, systemic and local mechanical factors seem to be involved with age, gender, heredity and life-style related factors such as body weight and physical activity all affecting disease risk one way or another. With obesity and sedentary life-styles on the rise the problem is expected to increase and become an even more significant public health issue in the near future. Destruction and loss of articular cartilage is a central feature of OA and chronic pain its cardinal symptom, compromising mobility and quality of life of those affected.

No curative therapies are available for OA, limiting current treatment goals to the management of symptoms and disease progression. OA alone accounts for roughly half of all non-steroidal anti-inflammatory drug prescriptions; besides analgesics other treatment options include intraarticular glucocorticoid and hyaluronic acid injections and, in severe cases, joint replacement surgery. It has become increasingly clear in recent years that multiple cell types and tissues that contribute to joint structure and function contribute to the disease and that an inflammatory component is involved in its progression.[1] Pro-inflammatory cytokines play a central role in the formation of reactive oxygen species (ROS) and nitric oxide (NO), and both have been hypothesized to contribute to the development of OA.

The focus of the current paper is on NO and its relationship to OA; it does neither attempt to provide a general overview about the chemistry and enzymology of NO nor summarize the wealth of information available on its generation in OA. For this purpose the reader is referred to a number of excellent reviews[2–6] and comprehensive monographs.[7,8] Instead, some aspects of NO biology that recently emerged in other research areas but do not seem to have been discussed much in the OA community will be highlighted in the hope of providing new "food for thought" and inform future research efforts on the role of NO in OA.

Nitric oxide – a jack-of-all-trades in cell regulation

NO is a pleiotropic signaling and effector molecule that has garnered a great deal of attention by both the basic and clinical research community. More than 80,000 papers related to the biochemistry as well as the cellular and molecular biology of NO have been published since its biological significance was discovered, rather serendipitously, as "endothelium-derived relaxing factor (EDRF)" in isolated blood vessels about a quarter of a century ago.[9] Within

*Address correspondence and reprint requests: Dr. Martin Feelisch, Ph.D., Clinical Sciences Research Institute, Warwick Medical School, The University of Warwick, Gibbet Hill Road, Coventry CV4 7AL, UK. Phone: +44 (0)24 7652 8372, Fax: +44 (0)24 7615 0589, e-mail mf@warwick.ac.uk

a relatively short period of time EDRF was identified as NO[10,11] and its enzymatic production from the amino acid, L-arginine shown to be a mammalian process. NO is produced by three distinct isoforms of the NO-synthase (NOS) enzyme family and their genes were mapped to three different chromosomes of the human genome. These discoveries triggered a flurry of research worldwide and it was soon established that NOS enzymes are not only constitutively expressed in discrete compartments of virtually every cell investigated but that the expression of one particular inducible isoform increases dramatically during inflammation.[a] NO emerged as a key regulator of blood vessel tone in the cardiovascular system, as a neurotransmitter in the central and peripheral nervous system, as a modulator of immune responses and as a cytotoxic effector molecule involved in host defense mechanisms.[12]

Within a couple of years NO had metamorphosed from an environmental pollutant and poisonous gas associated with 'chemical smog' to an endogenous master regulator of cell function.[13] In recognition of its growing significance NO was selected "Molecule of the Year" in 1992 by the journal *Science*, formed the basis for the 1996 Albert Lasker Basic Medical Research Award and led to the award of the 1998 Nobel Prize for Physiology or Medicine for the discovery of NO "as a signaling molecule in the cardiovascular system".

How exactly does NO signal?

NO was long considered a highly diffusible 'gas' that freely moves about, crosses cell membranes with ease and can travel tens or hundreds of microns away from its site of production.[14] More recent concepts have raised doubt about this concept and suggested a very different picture where NO actions are much more localized and highly influenced by the local microenvironment. Early studies with NO and NO donors suggested that the binding of NO to the heme moiety of soluble guanylyl cyclase, an interaction that results in nitrosyl-heme formation and enzyme stimulation with increased production of cyclic guanosine monophosphate (cGMP), is the major if not sole pathway of NO signaling.[15] The second messenger cGMP interacts with specific binding sites on target proteins (including protein kinases, phosphodiesterases, and cyclic nucleotide-gated ion channels) to elicit its downstream effects. Other examples of NO binding to heme proteins include interactions with cytochrome P-450 enzymes and cytochrome c oxidase (complex IV of the mitochondrial respiratory chain), leading to inhibition of enzyme activity. These NO effects have the potential to affect drug metabolism and modulate cellular energetics and are cGMP-independent.

Later studies confirmed the existence of numerous other non-cGMP mediated effects in which NO either leads to post-translational modifications at sites other than hemes or contributes to the oxidation of biomolecules. The best characterized example of the former is represented by the S-nitros(yl)ation of low-molecular-weight thiols (e.g., cysteine and glutathione) and sulfhydryl groups of proteins. S-nitros(yl)ation has been shown to affect the activity of enzymes and transcription factors and coined the "prototypic redox-based signaling pathway".[16] Protein glutathiolation, i.e. the addition of glutathione to a sulfhydryl group to form a mixed disulfide, represents an alternative pathway of redox-regulation by NO.[17] In the majority of cases either type of reaction requires NO to be converted into secondary reaction products before it can interact with the sulfhydryl group.

An early recognition of the speed with which NO reacts with superoxide ($O_2^{\bullet -}$) has lead to the appreciation that the formation of peroxynitrite ($ONOO^-$) is intricately linked to NO biology. In the black-and-white images of the not-so-distant past NO was viewed as "good", superoxide as "bad" and peroxynitrite, a potent pro-oxidant with cell damaging effects, as "ugly"[18] (Figure 1). However, peroxynitrite is more versatile than originally presumed, and its formation can give rise to oxidation and nitration reactions. Protein nitration has received the most attention, in part due to the ease with which nitrotyrosine (NO_2Tyr) can be detected by immunostaining. The latter advanced to become a universal hallmark of inflammation and is frequently interpreted as "the footprint of peroxynitrite chemistry" as much as nitrite, nitrate and NO_2Tyr are used as biomarkers of NO formation. As discussed later, there are biological targets other than tyrosine residues that become nitrated, including tryptophan,[19] catecholamines, lipids, sugars[20] and even cGMP itself.[21,22] More importantly, the majority of NO_2Tyr may be derived from sources other than peroxynitrite which will be further discussed. Nevertheless, the original notion that NO_2Tyr originates from iNOS-derived peroxynitrite production stuck and NO_2Tyr somehow managed to become synonymous with "too much NO". The origin of this claim remains elusive, but is presumably based on the assumption that iNOS always produces much more NO than the constitutive NOS isoforms (in fact, this has more to do with the shear amount of inducible protein expressed under inflammatory conditions than its specific activity) and that high rates of NO production can lead to DNA damage.[23]

A quarter of a century after the discovery of EDRF and >80,000 publications later, the interactions of NO with their biological targets remain incompletely understood. How NO makes it through the sea of antioxidants and other free radicals within the cell to reach its biological targets is just one of several aspects that remain unanswered. Although great strides have been made in unraveling a surprisingly rich chemistry of NO in biological environments, implicating transport and storage forms of NO that have the potential to enhance its radius and mode of action, the study of NO biology remains a challenging area of research. Analytical problems are just one of several areas of concern. Casual yet inappropriate oversimplification of the biological chemistry of NO has led to an unfortunate level of confusion surrounding the state-of-the-art. This seems to be particularly true for the area of translational research, and the fact that clinicians rarely become enthused about papers abounding with chemical formulas and reaction pathways has not helped the case.

Why is NO involved in the regulation of so many different functions?

NO formation and inflammation may have evolved as the first-line defense mechanisms of the innate immune system to combat microbial infections.[24] A question that has puz-

[a]The different NOS isoforms were originally named after the cell/tissue type in which they were first discovered, later renamed according to the order of their cloning, as eNOS (endothelial; NOS-3), nNOS (neuronal; NOS-1) and iNOS (inflammatory or LPS/cytokine-inducible; NOS-2).[3] Adding to the confusion, we now know that the expression of the two "constitutive" enzymes, eNOS and nNOS can also be induced and that the "inducible" isoform is constitutively expressed in some tissues even in the absence of inflammation.

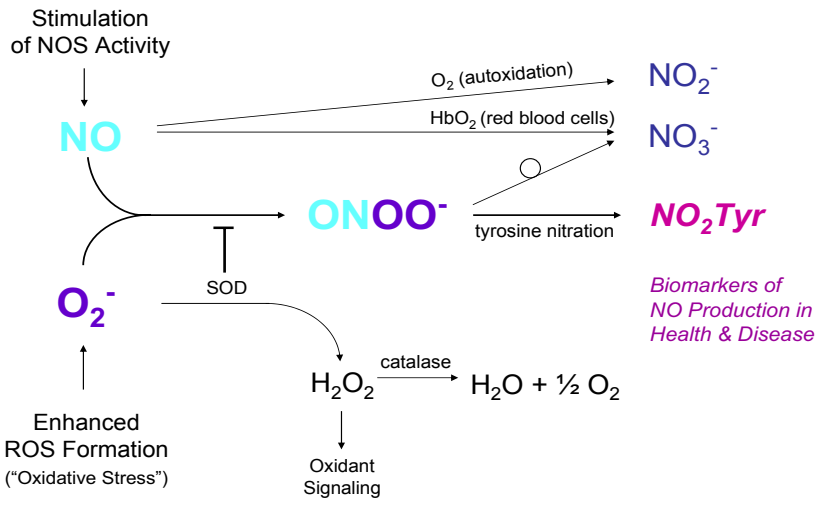

Fig. 1. NO Metabolism as Conceptualized a Decade Ago – The Good (NO), the Bad (O_2^-), and the Ugly ($ONOO^-$). Abbr.: H_2O_2 = hydrogen peroxide, HbO_2 = oxyhemoglobin, NO = nitric oxide, NO_2Tyr = nitrotyrosine, NO_2^- = nitrite, NO_3^- = nitrate, NOS = nitric oxide synthase, O_2^- = superoxide, $ONOO^-$ = peroxynitrite, ROS = reactive oxygen species, SOD = superoxide dismutase.

zled the research community for some time is how such a seemingly simple molecule as NO can fulfill so many different cell regulatory functions from bacteria to man and across the plant to the animal kingdom. More than a decade ago, Feelisch and Martin hypothesized that this may be so because the production of NO and its interaction with biological targets was one of the first and most primitive of signaling systems that has survived to current use in more complex organisms because it provided, at a crucial phase of the development of life on earth, an evolutionary advantage.[25] If this is true, it would explain also why NO tends to interfere with signaling at multiple levels – from gene expression over modulation of enzyme activities by direct binding and post-translational protein modification to antioxidative effects and redox regulation of cellular function. Curiously, one of the biggest problems we faced during the writing of this hypothesis article then was to find an explanation for the transition from a relatively simple pathway of NO generation (e.g., denitrification) to the current pathway of NO formation via NOS. Clearly, the oxidation of L-arginine with its sophisticated substrate transporter and cofactor requirements looks way too complicated to have evolved from scratch. Much to our surprise, a decade later research suggested there was no need to look for an explanation as both pathways appear to happily coexist still in contemporary mammalian cells.[26]

It is now known that NO can be produced not only from L-arginine by NOS, but also via reduction of nitrite and, due to reduction by the commensal bacterial flora in our mouth and gut, even nitrate.[27] The latter is likely a vestige of the evolutionary older pathway of NO production with contributions from multiple enzyme systems and subcellular compartments. Both pathways appear to regulate the expression, function and activity of proteins in similar ways, except that NO formation from L-arginine requires the presence of oxygen whereas that from nitrite is inhibited by its presence. While nitrite has been demonstrated to protect tissues against ischemia/reperfusion-related damage in several organs,[27] it is not yet clear what role, if any, physiological levels of nitrite, might play. Given the inverse dependence on oxygen, nitrite reduction to NO may serve to prevent a drop in NO concentration when oxygen levels fall below a critical threshold and NO production from NOS becomes insufficient.

Nitrite and nitrate – not just markers of NO

Nitrite and nitrate have long been considered biologically inert and traditionally been used as markers of NO formation. In inflammation, increased levels are observed due to NOS upregulation and increased NO production. However, as discussed above, it is clear that these simple oxyanions of nitrogen are not merely decomposition products of NO but have significant biological effects in their own right. Whatever function nitrite may have in physiology and inflammation, hypoxic NO formation from nitrite is likely to be of particular relevance to OA because i) oxygen availability in the joint is rather low compared to other tissues; ii) its concentration is further reduced in inflammation; and iii) physiological oxygen supply of joint constituents is intermittent, reminiscent of recurring ischemia and reperfusion[28] – conditions under which nitrite has been shown to exert protective effects. In addition to being reduced in hypoxia, nitrite was found to undergo rapid conversion to an array of metabolites (including S-nitrosothiols and NO-heme species) indistinguishable from those produced by NO under aerobic conditions.[26] Thus, nitrite and nitrate can no longer be regarded as simple dosimeters of enzymatic NO formation from NOS alone, but have to be considered precursors of NO production in tissues with limited oxygen availability.

In addition to the nitrite/nitrate originating from endogenous NOS activity, the nutritional intake of nitrate (largely in the form of vegetables) may play an important disease modulatory role. Attempts to influence the course of arthritis by dietary means are not new, but little systematic research has been undertaken. In contrast, large well-controlled studies have demonstrated beneficial effects of a diet rich in fruits and green leafy vegetables (e.g., Mediterranean diet) in chronic degenerative diseases, and nitrate has recently been proposed to account for part of this protective effect.[29] Whether a similar degree of protection by dietary modulation of nitrate intake can be achieved in OA is unknown. Similarly

unclear is which of the functional joint components would be most effective in generating NO from nitrite and which ones are likely targets of the NO produced. Since cartilage is an avascular tissue it would seem to be difficult supplying NOS with all its necessary cofactors for prolonged periods of time. Although it has been demonstrated that cartilage explants can generate NO for days after removal from the joint[30] the source of this NO remains unclear. Besides from NOS, it may originate from the decomposition of preformed NO storage forms or reduction of nitrite.

NO is involved in the progression of osteoarthritis – fact or fiction?

The versatility of NO's biological actions, in particular its ability to present as a physiological regulator of cellular processes and as a cytotoxic molecule that kills bacteria and cancerous cells has puzzled researchers for years. We have become accustomed to phrases like "yin and yang", "friend or foe", "janus-faced" and "double-edged sword" in the context of NO biology and medicine, and the "NO paradox" has become commonplace in discussions about inflammatory processes and degenerative diseases, regardless of discipline. While knowing you are not alone can provide comfort, it does not really help in science – all it tells us is that we do not yet understand how NO works.

There is no shortage of reports documenting cytotoxic and matrix destructive effects of NO in the OA literature[4] and countless papers reiterate the importance of NO in OA disease progression. This is probably due to the fact that the original discovery of elevated nitrite concentrations in synovial fluid and serum of patients suffering from rheumatic diseases,[31] later confirmed by other groups,[32,33] was made at a time when insight into the *in vivo* metabolism of NO and its diversity of actions at the subcellular level was limited. Moreover, NO_2Tyr was also detected in synovial fluid in patients with rheumatoid arthritis[34] and its presence is considered a hallmark of oxidative damage even today. NO_2Tyr will remain indicative of nitration reactions and may give rise to neoepitope and auto-antibody formation in joint synovial fluid,[35] but researchers can no longer be so sure about its origin and function.[36]

Experimentally induced OA tends to differ substantially from the human form and mechanisms vary between animal models depending on insult, age and species.[37] Even the use of NOS knockout animals has its limitations since the lack of one NOS isoform can lead to compensatory upregulation of one of the other isoforms, which may explain some of the controversy regarding the role of iNOS in OA.[38,39] Later studies linked NO production to chondrocyte apoptosis and inhibition of matrix synthesis, but revealed conflicting results concerning the role of NO.[40,41] The finding that NO-mediated cell death requires the generation of additional ROS[42,43] points to a cross-talk between NO and ROS signaling. While these studies clearly demonstrate an involvement of NO in OA and the differential effects of ROS and NO produced by different NOS isoforms in joint inflammation are increasingly appreciated,[44] these associations do not establish a cause/effect relationship. Thus, the question as to whether NO is of benefit or detriment in OA[45] remains open.

A closer look at the methods section of some papers and the context in which the results are discussed often reveals erroneous assumptions and misconceptions about the biochemistry (to the extent that NO is at times confused with ROS), questioning the validity of at least some of the conclusions drawn. Given the central role NO plays in many cell regulatory processes it is of obvious importance to define whether NO is "good" or "bad", whether there is "too much" or "not enough", and at what stage of the disease the patient is most likely to benefit from a pharmacological intervention if NO was the target. This would seem to be especially important for OA because of the current lack of true disease-modifying drugs, validated biomarkers and other diagnostic tools and the knowledge gap concerning disease mechanism and pathogenesis, in particular as to the relative contribution of bone, synovium, cartilage, inflammatory cells and T-cells because NO is involved in the functional regulation of each one of these cell types at multiple levels.

Too much or not enough NO in osteoarthritis and does it really matter?

Although the correct answer to the above question has an obvious impact on treatment options and choice of pharmacological intervention, it is unlikely that the absolute amounts of NO produced will tell us much about the direction in which the disease is progressing. Without understanding the relative contribution of local versus systemic factors, knowing what the concentration of nitrite/nitrate is in synovial fluid or blood is of limited value. We will need to know how much is produced over time in what particular cell/joint compartment and what effect it has on individual signaling pathways to make sense of this information. Currently available tools are too crude to address these issues. In addition to spatial and quantitative information about NO production we need to know in what form it arrives at its biological target(s). This requires better knowledge about the microenvironmental conditions, e.g. the redox poise in different compartments, as this has a major impact on the fate of NO. If NO as such is required to trigger a specific response and this pathway is important for cell integrity and proper joint function, any factor or event that has the potential to increase local ROS production will reduce the concentration of NO available for interaction with its biological target. The magnitude of the problem should encourage industry, academia and funding bodies alike to invest in the development of methodology capable of monitoring origin and fate of NO to obtain a "higher resolution picture of NO biochemistry" in complex biological systems such as the joint.

The ability to address the role of NO in OA is also hampered by the lack of a straightforward functional assay to assess NO availability in the joint. In the cardiovascular field impaired NO availability translates into readily measurable changes in vascular reactivity. Those changes can be assessed non-invasively by quantifying the increase in forearm blood flow in response to a brief period of ischemia using venous occlusion plethysmography or Doppler techniques. If the endothelium is intact and healthy (and NO availability adequate), a brief occlusion of arterial inflow results in blood vessel dilatation. Atherosclerosis, hypertension, obesity and other diseases known to be associated with enhanced oxidative stress are characterized by endothelial dysfunction, which presents as impaired dilatation or even vasoconstriction. Redox status and superoxide production are the major determinants of NO availability in the vasculature[46,47] and elsewhere. Thus, without any additional biochemical measurement this relatively simple functional test of systemic vascular reactivity allows identification of patients at risk for vascular complications in cardiometabolic disorders, and the forearm vasculature is often

used as a proxy for NO formation in other vascular beds.[48] Although there are indications for a link between endothelial dysfunction and OA,[49] which together with increased circulating nitrite/nitrate levels would suggest involvement of a systemic inflammatory component, there has been little research on the subject to date.

Relatively little is known about the cell types and tissues that produce NO under physiological conditions in the joint and what particular isoforms are involved.[3] The latter may be important because fine-regulation by post-translational modification, cofactor requirements and specific activities differ between isoforms. Moreover, NO production is not only determined by the expression of specific NOS isoforms and local substrate/cofactor availabilities but, also by their interaction with other proteins. Nothing is known about changes in NOS trafficking and translocation processes in the different cell types that make up a functional joint.

Chemical biology of NO – the bare essentials

The realization that the chemistry of NO is a major determinant of the outcome of its interaction with biological targets has been crucial for understanding why the same molecule can be protective/regulatory under one condition and deleterious/cytotoxic under another. Wink and colleagues have been instrumental in developing a framework according to which the chemical biology of NO is divided into direct and indirect effects (see Figure 2; for a more comprehensive treatment of this subject see Refs. 50–52). Briefly, direct effects are those mediated by NO itself and include the interaction with metals and metalloproteins as well as the scavenging of other free radicals. Examples of the former would be the binding of NO to the heme group of e.g., soluble guanylyl cyclase, cytochrome P-450, cytochrome c oxidase, cyclooxygenase, peroxidases and others metalloproteins; examples of the latter are exemplified by the trapping of superoxide (forming peroxynitrite) and hydroxyl radicals (forming nitrous acid), lipid peroxides (a chain-breaking event that prevents lipid peroxidation) and other free radicals, reactions that contribute to the antioxidative effects of NO.[53] Direct effects dominate at low NO fluxes and probably account for a significant part of its regulatory effects in physiology. Under conditions of enhanced oxidative stress and higher rates of NO production the likelihood of interaction with oxygen and ROS increases, leading to enhanced formation of reactive nitrogen oxide species (RNOS; also called 'reactive nitrogen species'; these include $ONOO^-$, NO_2, N_2O_3 and other species). These secondary reaction products of NO are responsible for the so-called 'indirect effects of NO' and lead to nitrosation, oxidation, and nitration of biomolecules, chemistries NO itself does not entertain. The nature of the reaction products is dictated by the availability of target molecules in the vicinity of the RNOS and the prevalence of other competing and scavenging mechanisms. In a biological environment, a complex mixture of low-molecular weight compounds and post-translational protein modifications is expected to occur near 'hotspots' of RNOS formation. While some of the reaction products are stationary (e.g. nitrated tyrosine residues of proteins) and only become detectable in free form in the circulation following proteolysis, others (e.g. S-nitrosoalbumin) can travel significant distances to reach targets remote from the site of the actual nitrosative event. The half-lifes of NO-related metabolites vary substantially (from seconds to hours), depending on the chemical nature of the product and the availability of other cell constituents (e.g. reduced thiols, ascorbate, metals) triggering their degradation. This scenario helps understanding why predictions about the *in vivo* fate of NO are difficult to make and illustrates the complexity of NO metabolism from the angle of its production. Regrettably, the situation does not get much easier when it comes to NO breakdown. Contrary to the major inactivation mechanism for NO in blood, the conversion to nitrate by oxyhemoglobin in red blood cells (see Figure 1), the fate of NO in other cells and tissues is less well characterized. The reaction of NO with oxygen, yielding largely nitrite, is rather slow compared with other competing mechanisms and unlikely to play a significant role in regulating local NO availability. Mitochondrial consumption processes may occur via direct oxidation at the level of complex IV[54] or by reaction with ROS and may be involved in the regulation or fine-tuning of mitochondrial activity by NO. The latter is a crucial element of NO biology[55] and mitochondrial respiratory activity is altered

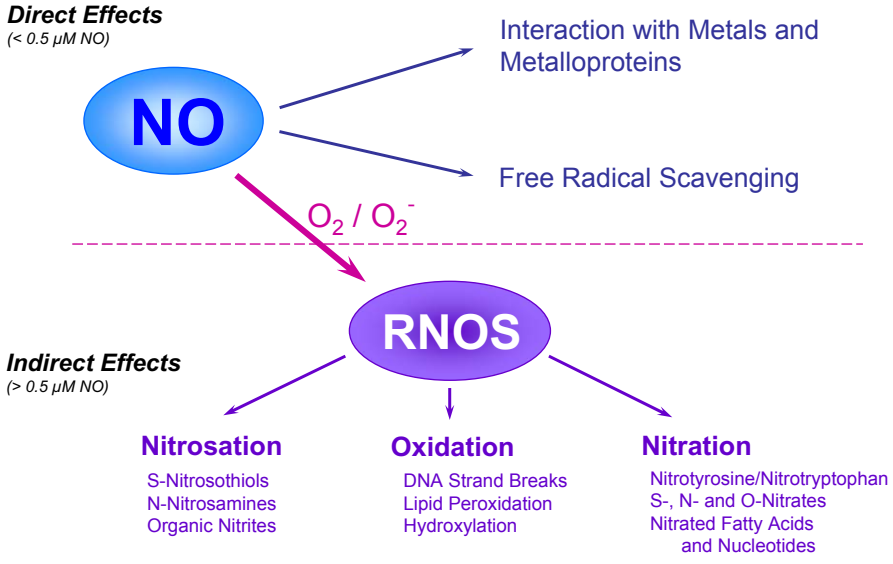

Fig. 2. Direct and Indirect Effects of NO. Abbr.: NO = nitric oxide, O_2 = oxygen, O_2^- = superoxide, RNOS = reactive nitrogen oxide species.

in OA.[56] ATP depletion leads to spontaneous knee OA in guinea pigs[57] and mitochondrial dysfunction is thought to be involved in OA.[58,59] While several reports have discussed the role of NO in modulating mitochondrial activity in the context of chondrocyte metabolism and matrix production,[60,61] this promising field seems to be in its infancy.

Oxidative stress and the role of NO as a cellular antioxidant

It has become increasingly clear over the years that "oxidative stress" is not simply an inevitable consequence of aerobic life and the formation of ROS (including $O_2^{\bullet-}$, $^{\bullet}OH$, and H_2O_2 among others) an unwanted side-effect, but that distinct ROS are purposefully generated to fulfill cell signaling and redox-regulatory functions.[62,63] ROS formation is increased in inflammation, and enhanced oxidative damage has been linked to OA and cartilage destruction. Oxidative stress markers are typically increased and antioxidant concentrations reduced in this setting.[64–66] There are a number of interesting parallels between ROS and NO formation and the "friend or foe" question surfaces more than once in the OA/ROS literature.[67] Of note, the low oxygen tension in the joint favors oxidative stress already under physiological conditions since ROS production is paradoxically enhanced in hypoxia.[68]

There are numerous sources of ROS within the cell including components of the mitochondrial respiratory chain, cytochrome P-450, xanthine oxidoreductase, NADPH oxidase and others. Superoxide, a major determinant of NO availability in the vasculature, has also been shown to limit NO signaling.[69] The classical definition of oxidative stress envisioned an imbalance between oxidant production and antioxidant capacity,[70] with free metals (iron, copper) determining the likelihood of hydroxyl radical formation and oxidative damage. The reaction of NO with superoxide is extremely fast, outcompeting the fastest enzymatic process currently known, that of superoxide dismutation. Oxidative stress may therefore be better understood as an imbalance in NO/superoxide availability with a shift towards a peroxynitrite/nitrogen dioxide (NO_2) dominated chemistry. This situation is synonymous with a state of low NO availability,[71] suggesting that associated perturbations of cell function under these conditions might benefit from an enhanced NO availability, provided those processes are still reversible.

NO availability can be increased by several means: i) scavenging of superoxide by either local injection of superoxide dismutase (SOD) – a therapeutic approach with limited effectiveness in OA – SOD overexpression or application of SOD mimetics; ii) enhancement of NOS substrate (L-arginine) or cofactor (tetrahydrobiopterin) availability; iii) inhalation of NO gas; and iv) application of NO donors.[b] Conceivably, nitrite may also be able to serve as a source of NO under hypoxic conditions (see above).

NO can abate the oxidative chemistry mediated by ROS, reduce lipid peroxidation and protect cells from metal and peroxide-induced oxidative damage.[73] Moreover, NO spares α-tocopherol and other antioxidants from oxidation,[74] a process shown to prevent cartilage matrix protein degradation.[75] In fact, NO is a key mechanism that limits oxidative injury to mammalian cells[53] and surprisingly small concentrations are required to effectively inhibit lipid peroxidation.[76] In addition to these antioxidative actions, NO has potent anti-inflammatory effects by down-regulation of the activity of activated neutrophils and macrophages, preventing neutrophil adhesion and modulating T-cell function.[77] Moreover, it modulates both production and release of pro-inflammatory cytokines and the activity of transcription factors such as NF-κB, a master-regulator of the inflammatory signaling cascade. Its effects on prostaglandin production are more difficult to assess given the complexity of the cross-talk between NOS and cyclooxygenase expression and activities.[78] Another potential anti-inflammatory mechanism transduced by NO is via nitration of unsaturated fatty acids (including prostaglandins) and the action of these reaction products on PPAR-γ receptors.[79]

Taken together, NO has potent antioxidative and anti-inflammatory effects. As much as the reciprocal regulation of ROS and NO offers opportunities for fine-regulation of a multitude of cell biological processes, it appears to become a source of concern when the balance shifts from an NO-dominated to a superoxide-dominated chemistry. Can this happen by mechanisms other than a reduced antioxidant availability or increases in ROS production? The following section will address the cellular NO-generating machinery.

Peroxynitrite, nitrotyrosine and NOS uncoupling

While peroxynitrite is a powerful oxidant and nitrating agent endowed with apoptosis-inducing, cytotoxic and pro-inflammatory effects, its formation is strictly limited to sites of almost equimolar fluxes of NO and superoxide.[80] This peculiar behavior implies that peroxynitrite formation (and consecutive oxidation/nitration chemistry) is less than that at a roughly 1:1 molar ratio of NO and O_2^- whenever NO production exceeds superoxide formation or *vice versa*. This would restrict NO_2Tyr formation to sites with comparable NO/O_2^- production rates, if it were not for another mechanism of nitration in cells.

Recent results suggest that a significant portion, if not the majority of NO_2Tyr originates from peroxidases/H_2O_2-mediated oxidation of nitrite to nitrogen dioxide (NO_2) rather than from peroxynitrite.[81] With the ubiquitous availability of nitrite and H_2O_2 (concentrations of which are increased in inflammation) peroxynitrite formation would seem to be no longer spatially limited to the sites of concomitant NO/O_2^- formation but rather dependent on the expression of peroxidases, which are abundant in inflammatory cells. While this may enlarge the activity radius of nitrative and oxidative chemistry and contribute to more widespread protein nitration, the consequences for enzymatic NO production from NOS are serious.

NOS requires several cofactors for NO generation, including oxygen, NADPH and tetrahydrobiopterin. The latter is required for the transfer of electrons from NADPH to its heme center, where L-arginine is oxidized to L-citrulline and NO. Increased oxidative stress (from whatever source) with enhanced formation of peroxynitrite and/or NO_2 formation from nitrite, leads to enhanced oxidation reactions. If this situation persists beyond the window of protection afforded by the endogenous antioxidant system the NO producing machinery itself can become a target of ROS/RNOS action. As tetrahydrobiopterin becomes gradually oxidized oxygen reduction uncouples from NO synthesis and transforms NOS into a superoxide-producing pro-inflammatory

[b] Of note, there are many types of NO donors used in experimental studies which may generate/donate nitrosonium (NO+), nitroxyl (NO-/HNO), peroxynitrite or cyanide instead of or in addition to the desired NO, complicating the interpretation of results and the assessment of what part of the effect was actually due to NO itself.[72]

enzyme.[82] Thus, not only is NOS no longer capable of generating NO, it now contributes to the perpetuation of oxidative stress itself. Given the large amounts of iNOS expressed in inflammation, the additional oxidative stress generated via this mechanism may be significant. No further enhancement of NOS expression or arginine availability will help correcting this situation; only local ROS scavenging (e.g., by application of combined SOD/catalase mimetics) or an increase in NO availability (e.g., NO donors) can abate this chemistry and stop the vicious cycle.

The NO/ROS balance and its significance for cellular redox signaling and hypoxia-induced gene expression

ROS and NO are generated by a multitude of mechanisms under physiological conditions and the production of both is enhanced in inflammation. Global and local changes in ROS production and redox poise have a major impact on NO metabolism and the balance between oxidative, nitrative and nitrosative chemistries (Figure 3), with vast differences in expression and activity of multiple enzymes despite little or no changes in overall NO production. Since both the NO source (L-arginine versus nitrite) and mechanisms of consumption (trapping by oxyhemoglobin or superoxide versus consumption by cytochrome c oxidase) may change as oxygen concentrations vary, the situation is complex and dynamic. What kind of metabolites are formed when and where will depend on the i) rates of formation of NO and $O_2^{\cdot-}$; ii) NO and ROS scavenging mechanisms; iii) local oxygen and carbon dioxide concentrations; iv) microenvironmental redox poise; v) chemical nature of the biological target; and – like in real estate – vi) location, location, location.

Since the pathways of NO and ROS generation are intimately connected it would seem that their contribution to OA cannot be studied in isolation. The particular situation of the joint places an emphasis on the availability of oxygen[28] and carbon dioxide[83] for modulation of the downstream chemistry of concomitant NO and ROS formation. The associated redox changes further suggest an involvement of specific hypoxic signalling events. Where does all this lead us to and what are the likely molecular targets?

The interaction of thiols with metals has shaped evolutionary biochemistry for eons.[84] Many mammalian proteins contain one or more sulfhydryl (SH) groups that render their activity subject to redox regulation. Thiols are "nanotransducers of redox chemistry" that define which ROS and RNOS acts as second messenger and in what direction.[85,86] Thiols present in various transcription factors, such as NFκB, AP-1, and p53 and transcription factors with antioxidative-response-element binding sites such as Nrf2 act as redox sensors and transcriptionally control the regulation of genes critical for cell homeostasis and redox status. It appears reasonable, therefore, to assume that the NO/ROS balance plays a crucial role in the development and progression of OA by virtue of its effects on redox signalling pathways.

The shift in redox poise towards more oxidation will not only affect general antioxidant status but also cause ascorbate depletion. This may have important implications for hypoxic signalling via hypoxia-inducible factor-α (HIF-1α) and matrix production. Besides its classical antioxidant function ascorbate serves as a cofactor for prolyl hydroxylases, enzymes important in the regulation of HIF-1α degradation and collagen production. Impairment of HIF-1α hydroxylation due to ascorbate deficiency would spare this transcription factor from ubiquitination and proteasomal

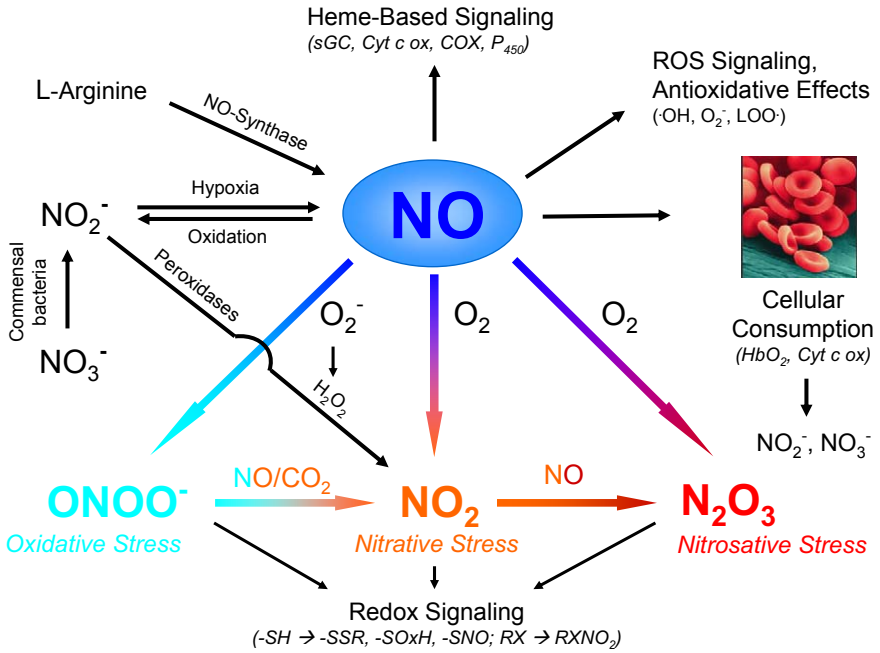

Fig. 3. Oxidative, Nitrative and Nitrasative Stress and Their Relationship to Cell Signaling? – A Balancing Act. Abbr.: COX = cyclooxygenase, Cyt C ox = cytochrome c oxidase, H_2O_2 = hydrogen peroxide, HbO_2 = oxyhemoglobin, $LOO^{\bullet}$ = lipid peroxyl radical, NO = nitric oxide, NO_2Tyr = nitrotyrosine, NO_2 = nitrogen dioxide, N_2O_3 = dinitrogen trioxide, NO_2^- = nitrite, NO_3^- = nitrate, O_2 = oxygen, $O_2^{\cdot-}$ = superoxide, $^{\bullet}OH$ = hydroxyl radical, $ONOO^-$ = peroxynitrite, P_{450} = cytochrome P_{450}, sGC = soluble guanylyl cyclase, SH = thiol, SSR = mixed disulfide, SOxH = oxidized thiol (sulfenic, sulfinic, sulfonic acids), SNO = nitrosothiol, RX = biomolecule, $RXNO_2$ = nitrated biomolecule.

degradation (its fate under normoxic conditions), leading to accumulation and "superinduction" of hypoxia-induced gene expression. In addition, redox changes are likely to affect HIF expression and activity in an ascorbate-independent manner. Together, these changes may induce local alteration in glycolysis, pH and energy regulation, apoptosis and other metabolic processes incompatible with the situation at hand and potentially detrimental for cell survival if uncoupled from other oxygen-dependent processes. The above scenario is consistent with the effectiveness of gold compounds and penicillamine in rheumatic disease;[87] the disappointing clinical results with ascorbate and other antioxidants in OA despite the fact that ascorbate deficiency in animal models is associated with ER stress and OA-like symptoms;[88,89] effects of ascorbate on cartilage metabolism and matrix production;[90] distinct threshold concentrations of NO regulating HIF-1α accumulation;[91] altered mitochondrial respiratory activity[56] and increased HIF-1α in osteoarthritic articular chondrocytes;[92] and peroxynitrite-mediated mitochondrial dysfunction with caspase-independent chondrocyte apoptosis.[93]

If some of the current investments in the search for possible genomic underpinnings of the disease would be used for integrative, systems-wide approaches using a combination of 'omics' techniques instead we might arrive earlier at the conclusion that there is nothing wrong with the genes in OA, but rather with the way their expression is regulated by NO and ROS. Insight into these processes might increase our chances for the development of true disease modifying drugs and preventive approaches to tackle this crippling disease.

Conclusions

NO is a pleiotropic signalling and effector molecule with a surprisingly rich chemistry, and many of its secondary metabolites (including nitroso and nitrosyl species as well as nitrite) have potent biological activities in their own right. The fate of NO *in vivo* is complex and differs profoundly between physiology and pathology. It is likely that local NO availability is impaired under inflammatory conditions, despite massive upregulation of NO production by iNOS in attempts to compensate for the accelerated breakdown secondary to increased oxidative stress. This creates a vicious cycle that leads to NOS uncoupling, further compromising NO availability. These changes lead to progressive nitrosation, nitration and oxidation of other proteins and biomolecules, exacerbating the shift in NO/ROS balance and the changes in downstream redox signalling and gene expression. Thus, the presence of increased concentrations of nitrosation and nitration products in the joint is not necessarily an indication for too much NO, but rather indicative of oxidative stress with consecutive alterations in NO metabolism. Nitrite and exogenous NO may conceivably protect tissues from the deleterious consequences of NOS uncoupling by virtue of its antioxidative and anti-inflammatory effects – a rescue mechanism that is independent of the classical cardiovascular effects of NO aimed at restoring normal physiological function. Given the prominent role NO plays in OA, modulation of NO availability and cellular NO/ROS-dependent redox poise appear to be attractive targets for future pharmacological intervention, but further research efforts are required to assess whether endogenous NO production needs to be inhibited or local NO concentrations enhanced and whether NO availability can be modulated differentially in different cell types.

Conflict of Interest statement

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