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The biology of reactive intermediates in systemic lupus erythematosus

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Abstract

Systemic lupus erythematosus (SLE) is an autoimmune syndrome marked by autoantibody production. Innate immunity is essential to transform humoral autoimmunity into the clinical lupus phenotype. Nitric oxide (NO) is a membrane-permeable signaling molecule involved in a broad array of biologic processes through its ability to modify proteins, lipids, and DNA and alter their function and immunogenicity. The literature regarding mechanisms through which NO regulates inflammation and cell survival is filled with contradictory findings. However, the effects of NO on cellular processes depend on its concentration and its interaction with reactive oxygen. Understanding this interaction will be essential to determine mechanisms through which reactive intermediates induce cellular autoimmunity and contribute to a sustained innate immune response and organ damage in SLE.

Keywords

Nitric oxide; systemic lupus erythematosus; reactive oxygen species; free radicals; oxidative stress

Introduction

The broad phenotype of systemic lupus erythematosus (SLE) is unified by autoantibody production, a function of the acquired immune response. However, an inappropriately active and sustained innate immune response is implicated in both the initiation and pathogenic consequences of autoantibody production in SLE. An important arm of the innate immune response is the production of reactive intermediates (RIs). The role of these RIs and subsequent oxidative products in the initiation and exacerbation of the lupus phenotype is discussed below.

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Reactive intermediates

RI's are short-lived molecules formed by chemical reactions that are capable of rapidly modifying other molecules. Through these modifications, they act as signaling molecules for a broad array of cellular functions. RI's important in biology include the reactive oxygen intermediates (ROIs such as super-oxide (SO) and hydrogen peroxide (H_2O_2)), reactive nitrogen intermediates (RNIs such as nitric oxide (NO) and peroxynitrite (ONOO^-)), and hypochlorous acid (HOCl). The pathogenic potential of NO is partly dependent upon its concentration and whether its production occurs in proximity to the formation of ROI such as SO . Catalysis of RI's to less reactive molecules occurs through enzymes such as SO dismutase (which catalyzes the reaction of SO to H_2O_2). H_2O_2 , not a free radical, is further catalyzed in some cell types to HOCl (bleach) by myeloperoxidase with antimicrobial effect.

H_2O_2 can also be converted to hydroxyl radicals (OH) in the presence of Fe(II) via the Fenton reaction or to water and oxygen by catalase [1]. NO is produced by one of the three known isoforms of NO synthase (NOS). NOS dimerizes to form the active enzymes. Uncoupled monomers or low arginine substrate conditions result in NOS producing SO [2]. NO and SO rapidly combine to form ONOO^- . Thus, RI production depends on catalytic enzyme activity and substrate availability as well as the amount and activity of detoxifying enzymes (Figure 1) [3].

NO biology

NO is a membrane-permeable free radical that is formed by one of the three NOS using arginine and oxygen as substrates (Figure 1). NO has the potential to induce both physiologic and pathologic effects, a seemingly contradictory notion that complicates interpretation of the literature. The effect of NO production on cellular processes is largely dependent on its concentration and the local presence of other free radicals. Lower concentrations of NO have a direct effect on processes such as proliferation and cell survival, while higher concentrations have an indirect effect through both oxidative stress (via a first-order, diffusion-limited reaction with SO to form ONOO^-) and nitrosative stress (through a second-order reaction with NO to form N_2O_3). Because NO is freely diffusible across cell membranes and SO is not, the reaction of SO and NO occurs within cells/organelles producing SO [activated leukocytes, endothelial cells, and mitochondria] in proximity to diffusible NO from the target cell or a neighboring cell [4].

NO itself can reversibly nitrosylate thiol groups on proteins in a fashion much akin to phosphorylation. This process may be indirectly mediated by carrier thiol peptides or proteins such as thioredoxin [5]. NO also modifies iron centers in proteins such as p450 enzymes and soluble guanylate cyclase, the latter being responsible for the vasodilating effect of NO . N_2O_3 can modify zinc finger domains and nitrosylate thiol groups. ONOO^- can oxidize lipids such as those found in LDL or arachidonic acid [6]. ONOO^- can also act as a peroxide substrate for peroxidases such as those found in cyclooxygenase (COX) [7]. Finally, ONOO^- can nitrate DNA (Figure 2) [8].

Primary and secondary effects of NO on cellular processes

Different cellular proteins have different concentration thresholds for modification by NO or its oxidative/nitrosative products. Below 30 nM, NO can have a protective and proliferative effect by activating c-GMP-mediated processes, the most recognized of this is c-GMP-mediated vasodilatation from endothelium-derived NO . In the 30–100nM concentration range, the Akt pathway is activated, leading to phosphorylation of BAD and caspase-6, both of which lead to cell survival [9]. S-nitrosylation of caspases by NO in the cytoplasm has an anti-apoptotic effect. On the other hand, mitochondrial NO can combine with SO to form

ONOO⁻, which nitrates cytochrome *c*, leading to mitochondria-mediated apoptosis [10]. Another example lies in the interaction between RNI and eicosanoid synthesis. NO produced in the presence of SO, as is often seen in inflamed tissues or in the presence of antiphospholipid antibodies [11], results in the rapid formation of ONOO⁻, which in turn nitrates and inhibits the activity of prostacyclin synthase with an IC₅₀ of about 50 nM [12]. ONOO⁻ also acts as a peroxide substrate to COX2 and increases its activity [7]. This results in increased availability of prostaglandin H₂ (PGH₂) substrate for thromboxane and PGE₂ synthases in the vasculature and glomerulus.

The inhibition of prostacyclin synthase and activation of COX2 can reverse the vasodilatory and antithrombotic effects of PGI₂ and increase the formation and, thus, inflammatory effects of TXA₂ and PGE₂. At 100 nM, hypoxia-induced factor 1α is stabilized, increasing proliferation and protection against cellular injury. Above 400 nM, p53 is phosphorylated and acetylated, leading to cell cycle arrest and apoptosis. Above 800 nM, nitrosative stress (with the formation of N₂O₃) occurs, which tends to result in nitrosation of thiol, lysine, and zinc fingers [8]. Specific to apoptotic signaling, N₂O₃ can lead to nitrosation of PARP and caspases [9].

NOS isoforms

The two isoforms [eNOS and neuronal (nNOS)] are generally constitutively expressed and are calcium dependent. In the vascular system, NO produced by eNOS acts as a vasodilator through activation of soluble guanylate cyclase.

The inducible NOS gene (NOS2) transcribes iNOS that is primarily expressed in immune cells including macrophages and macrophage-like cells such as mesangial cells. iNOS expression is stimulated in murine cells by several cytokines and toll-like receptor ligands such as lipopolysaccharide (LPS), interleukin-6 (IL6), interferon-γ (IFNγ), IL1β, and tumor necrosis factor-α (TNFα). In human monocytes and macrophages, induction *in vitro* can be attained with IFN γ and LPS, but the levels of NO produced are log-fold lower than those seen in murine macrophages.

In most cells, signaling pathways for iNOS induction converge on the janus kinase/signal transducer and activator of transcription and/or the nuclear factor kappaB (NF-κB) pathways. iNOS is expressed during pathological states in human eNOS cells, synovial fibroblasts, polymorphonuclear cells, lymphocytes, and natural killer cells. In normal human tissue, expression is strong in myocytes, skeletal muscle, and Purkinje cells.

iNOS produces log-fold higher amounts of NO than the constitutively expressed isoforms. When iNOS is uncoupled or in a low arginine environment, SO is produced with NO, resulting in ONOO⁻ formation. ONOO⁻ produced by immune cells is capable of killing intracellular pathogens and tumor cells. Glutathione peroxidase, catalase, SO dismutase, heme oxygenase, and antioxidants serve to protect host cells during inflammatory states by reducing the total ROI burden that can contribute to ONOO⁻ production [3].

Increased markers of NO production with lupus disease in humans

Given that no selective inhibitors of iNOS have been approved or successfully completed the early development for human use, it is difficult to directly study the pathogenic potential of iNOS in human lupus. However, several independent studies have demonstrated a significant correlation between markers of systemic NO production and global lupus disease activity [3]. One of these studies demonstrated more prominent increases in markers of NO production among African-Americans with lupus disease activity [13].

This predisposition to produce increased NO in response to disease activity may be inherited, as two NOS2 polymorphisms were significantly more prevalent in female African-American SLE subjects than matched controls. Supporting a functional role for the polymorphisms in combating infection are studies reporting increased markers of systemic NO production and improved malaria survival in some African populations with these polymorphisms [14].

Tissue- and cell-specific NOS expression

Skin

The skin often reflects disease activity in SLE, and iNOS expression in this organ appears to parallel that activity. Immunostaining for iNOS protein and mRNA was elevated in 33% of epidermal tissue samples from cutaneous lupus subjects before exposure to ultraviolet B (UVB) irradiation, but expression was increased in all samples after UVB exposure [15]. Among subjects with systemic disease, skin biopsy specimens from the buttocks revealed higher iNOS expression in endothelial cells and keratinocytes than in controls. Endothelial expression correlated with lupus disease activity [16]. The presence of iNOS in unaffected skin eNOS cells suggests systemic expression, while its induction with UV exposure offers one mechanism for increased expression during disease activity.

eNOS cells

Lupus patients often display a phenotype of defective eNOS function, as subjects with SLE have reduced endothelium-dependent vasodilation [17]. The mechanism behind this defect is unclear, but the increased levels of circulating endothelial cells seen in lupus subjects may be a maker of damage to the endothelium. The level of circulating endothelial cells in lupus subjects correlated inversely with complement levels, and these cells stained for nitrotyrosine. This observation, combined with the observation that eNOS cells stain for iNOS even in non-lesional skin, suggests an immune complex-mediated production of ONOO⁻ by iNOS in endothelial cells [16,18].

Renal cortex

More recent longitudinal observational studies demonstrate increased markers of systemic NO production (serum NO_x) in lupus patients with proliferative lupus nephritis when compared with those with non-proliferative renal disease or those with lupus, but without nephritis. In the same nephritis patients, those who did not achieve renal response to therapy had significantly higher serum NO_x levels in the first 3 months of therapy than those who achieved a renal response [19]. This provides the rationale for the hypothesis that sustained RNI production leads to renal damage in lupus nephritis.

Several laboratories have described increased iNOS expression in the glomeruli of subjects with proliferative lupus nephritis [13,20,21]. In biopsies from those with class IV disease, citrulline staining increased with iNOS staining, suggesting that the iNOS was functionally active [22]. In one study, glomerular iNOS staining co-localized with markers of apoptosis and staining for p53, a proapoptotic signaling molecule [21]. This is consistent with the known effect of higher levels of NO on p53 phosphorylation [9]. In another study, in patients with class IV LN, iNOS expression was increased in glomerular, tubular, and interstitial cells. iNOS expression in the tubulointerstitium correlated significantly with total lesion index on biopsy and the extent of proteinuria and creatinine clearance at the time of biopsy. iNOS and NFκB co-localized with apoptotic cells in the glomerulus [23]. These data suggest that two mechanisms for iNOS-mediated glomerular damage in proliferative

nephritis are increased by signaling for apoptosis via increased p53 activity and through activation of NFκB.

Potential pathogenic mechanisms of increased RNI production suggested by studies of human SLE subjects

One mechanism through which NO can be pathogenic, in the setting of SLE, is through the creation of neoepitopes by ONOO⁻-mediated nitration of nucleophilic domains on self-antigens. In one study, serum from SLE patients bound more avidly to ONOO⁻ nitrated versus native poly-L-tyrosine. Binding of serum from patients with high dsDNA antibody titers was inhibited by nitrated poly-L-tyrosine, nitrated BSA, nitrated DNA, and nitrated chromatin much more effectively than native forms of these antigens. DNA, modified in this fashion, is also a better immunogen for inducing anti-dsDNA antibody production in experimental animals [24]. Similarly, peroxynitrite-treated DNA is more immunogenic than native DNA as the antigen for dsDNA antibody testing of serum from patients with SLE [25,26]. These combined studies suggest that ONOO⁻ modifications of self-antigens can create neoepitopes with increased binding affinity over native antigens. Whether the increased immunogenicity of nitrated DNA stems from cross-reactivity of these epitopes with native DNA or later leads to epitope spreading to unmodified epitopes has not been determined.

ONOO⁻ can also modify lipids. Peroxidation of arachidonate by ONOO⁻ can lead to formation of isoprostanes [6] that stimulate monocyte adhesion to endothelial cells [27] and induce vasoconstriction in smooth muscles [28]. ONOO⁻ can oxidize arachidonic acid or lipids such as those found in LDL. Oxidized, but not native, LDL complexes with β2-glycoprotein I. Antibodies to this complex were elevated in subjects with SLE and antiphospholipid syndrome, and this antibody/antigen complex enhances influx of oxidized LDL into foamy macro-phages, providing a plausible mechanism for accelerated atherosclerosis in SLE [29]. Some phospholipids within oxidized LDL have platelet-activating factor-like activity and can stimulate growth of smooth muscle cells [30].

ONOO⁻ derived from myeloperoxidase and not iNOS can lead to nitration of tyrosine 166 in apolipoprotein A-I within HDL. Nitration of this amino acid leads to a loss of cholesterol efflux capacity of HDL [31], and this modification along with chlorination of this site confers a 6- to 16-fold risk for cardiovascular disease that is independent of Framingham risk factors [32]. The extent to which this phenomenon occurs in SLE subjects is unclear. Thus, the complete clinical effect of ONOO⁻ formation on lupus disease phenotype and cardiovascular disease associated with SLE is unknown.

Perl and colleagues identified that normal T cells express eNOS and nNOS (but not iNOS) and that expression of these NOS isoforms increased with CD3/CD28 costimulation. In addition, they demonstrated that NO induced an increase of mitochondrial hyperpolarization (MHP) in normal human T cells[33]. In contrast, they found that T lymphocytes of SLE patients exhibited persistent MHP and mitochondrial mass, accounting for increased production of ROI. These data suggest that mitochondrial dysfunction leading to ATP depletion is ultimately responsible for diminished activation-induced apoptosis and sensitizes lupus T cells to necrosis [34–36]. More recent studies have identified that NO-induced MHP in SLE T cells leads to activation of mTOR, a sensor of mitochondrial potential and target of the drug rapamycin [37].

Observational murine studies implicating iNOS activity in lupus disease progression

Although iNOS activity can suppress parasitemia or tumor growth, its over-expression in the setting of lupus disease activity is associated with not only organ damage but also more subtle changes in cellular phenotype and survival. Several studies involving murine models of lupus support this hypothesis. Both MRLMpJ-*Fas^{lpr}*/J (MRL/lpr) and (New Zealand Black $\times$ New Zealand White)_{F1} (NZB/W) mice develop spontaneous proliferative lupus nephritis. MRL/lpr mice developed increasing levels of urine NO metabolites (nitrate + nitrite or NO_x) in parallel with the onset of glomerulonephritis [38].

This increase in iNOS activity was associated with formation of nitrotyrosine (NTyr), a product of ONOO⁻-mediated nitration of tyrosine (Tyr). Such modifications reduced the activity of catalase in the MRL/lpr kidney. Because catalase removes SO, its inactivation may have exposed cells to increased oxidative stress and could, thus, lead to redox signaling and transcriptional events [39]. iNOS expression is increased in the brain tissue of NZB/W mice compared with BALB/c mice and is associated with an increase in p53. The pro-apoptotic role of p53 activated by iNOS may lead to brain injury in SLE [40].

Potential mechanisms of increased iNOS expression in MRL/lpr mice

Immune complex formation and tissue deposition are not dependent on iNOS activity in murine lupus, as iNOS inhibitor therapy had no effect on glomerular immune complex deposition in MRL/lpr mice [38]. However, nephritogenic autoantibodies, when injected into young, disease susceptible MRL/lpr mice, induced NOS2 gene transcription in both an Fc-receptor-dependent and an Fc-receptor-independent manner. Similarly, MRL/lpr mesangial cells exposed to nephritogenic anti-dsDNA antibodies had increased levels of NOS2 message compared with control antibodies [41]. The histone deacetylase inhibitor trichostatin A, which attenuated renal disease in MRL/lpr mice, also inhibited NO production in cultured MRL/lpr mesangial cells [42]. This suggests that epigenetic regulation has a direct and/or indirect effect on iNOS expression. LPS/IFN γ -induced iNOS expression in MRL/lpr mesangial cells can be abrogated through inactivation of the interferon regulatory factor-1 (IRF1) gene.

It is difficult to conclude whether this mechanism directly reduces the iNOS expression seen in MRL/lprIRF1^{-/-} mice, as this genetic manipulation also reduces anti-dsDNA antibody production and glomerular immune complex deposition in these mice[43]. In NZB/W mesangial cells, IL20 and IL20 receptors are upregulated, and addition of IL20 induces expression of iNOS along with IL6, RANTES, IP 10, and MCP-1 [44]. Some interventions that do not directly inhibit iNOS enzyme also reduce expression of iNOS. For instance, chemical induction of heme oxygenase-1 and oral administration of mycophenolate mofetil both effectively treated glomerulonephritis in MRL/lpr mice while concurrently reducing iNOS expression in the kidney[45–47]. These data suggest that glomerular iNOS expression in murine models can be induced by nephritogenic autoantibodies and innate immune factors such as IL20 through several independent mechanisms.

Effects of manipulation of iNOS in murine lupus

Several studies utilizing competitive inhibitors of iNOS suggest that iNOS activity is pathogenic in murine lupus. Inhibiting iNOS activity in MRL/lpr mice before disease onset with the non-specific arginine analog L^N^G-monomethyl-L-arginine (L-NMMA) reduced 3NTyr formation in the kidney, partially restored renal catalase activity, and inhibited cellular proliferation and necrosis within the glomerulus[38,39,48].

This effect occurred without a change in immunoglobulin or complement deposition in the glomerulus, suggesting that increased iNOS expression occurred distal to immune complex deposition and complement activation [38]. The partially selective iNOS inhibitor L-N6-(1-iminoethyl)lysine (L-NIL) had a similar effect when used to treat these mice prior to disease onset. L-NIL-treated mice had lower glomerular pathology scores compared with controls [48]. L-NMMA used to treat NZB/W mice with nephritis had a similar but less profound effect on proteinuria and renal histopathology than did L-NMMA given before disease onset. L-NMMA monotherapy was less effective in treating the more aggressive, rapidly progressive nephritis seen in MRL/lpr mice [49].

In contrast to the effectiveness of pharmacologic iNOS inhibition in murine lupus was the observation that iNOS $-/-$ MRL/lpr mice, while having reduced signs of vasculitis and IgG rheumatoid factor production, had similar glomerular pathology to their MRL/lpr wild-type litter-mates [50]. To address whether the beneficial effect of L-NMMA in lupus was unrelated to iNOS, MRL/lpr NOS2 $-/-$ mice were administered an iNOS-selective inhibitor prior to and throughout the progression of disease. NOS2 $-/-$ mice had elevated anti-dsDNA antibody levels and had, as observed in the past, no significant reductions in glomerular pathology or proteinuria. However, iNOS inhibitor therapy significantly reduced proteinuria and podocyte flattening/eNOS cell swelling by electron microscopy [51]. This suggests that iNOS inhibitor therapy reduces pathologic changes in podocyte and endothelial cell pathology in this model in an iNOS-independent (and possibly eNOS-dependent) fashion.

Potential mechanisms for pathogenicity of RNI suggested by studies in murine models of lupus

The mechanisms through which iNOS activity may be pathogenic in SLE have been studied in animal models and *in vitro* (Table I). As mentioned above, ONOO $^-$, a by-product of iNOS activity, can nitrate protein amino acids and change the catalytic activity of enzymes. One such enzyme, catalase, serves to protect host tissues from free radical attack [39]. In vascular tissue, prostacyclin synthase [52] and eNOS [53] are inactivated by ONOO $^-$, leading to vasoconstriction. These observations suggest that one mechanism through which iNOS activity is pathogenic is via deactivation of tissue protective enzymes.

Because nuclear antigens are presented in late apoptotic blebs [54], regulation of apoptosis and clearance of apoptotic cells is an important area of investigation. NO and ONOO $^-$ are both integral in regulating non-receptor-mediated apoptosis in many cellular systems [55]. To investigate the role of iNOS activity in apoptosis, MRL/lpr mice with active disease were treated with L-NMMA, an iNOS inhibitor. Compared with controls, treated mice exhibited reduced levels of splenocyte apoptosis. Treatment of cultured splenocytes isolated from mice with active disease with an NO donor resulted in increased levels of apoptosis [56]. NO or other RNI appeared to increase non-receptor-mediated apoptosis despite the well-described defect in receptor-mediated apoptosis in this murine model of lupus [57].

iNOS activity can lead to ONOO $^-$ production only if iNOS activity is accompanied by equimolar levels of SO. One mechanism for simultaneous production of SO and NO is through the parallel production of SO by the reductase domain of iNOS itself. Support for this mechanism in lupus comes from experiments involving pharmacological inhibition of iNOS in the MRL/lpr and NZB/W models. Mice given iNOS-specific and non-specific inhibitors demonstrated significant reductions in markers of systemic oxidant stress (urine F2-isoprostanes) compared with mice treated with distilled water [58]. This observation raises the possibility that some of the pathogenic effects of iNOS activity in SLE arise from its ability to produce ROI in proximity to NO.

Conclusions

The effects of NO production *in vivo* are dependent on focal cellular levels of NO production and its proximity to other reactive species. This makes it impossible to declare NO as either a pathogenic or protective molecule in lupus. It also complicates attempts to study the effect of RI on pathogenic lupus cellular processes *in vitro*. Future work investigating the effect of NO and reactive oxygen production in lupus will require determination of the concentration and cellular location of NO and reactive oxygen production in affected tissues *in vivo*. Newer live imaging and *ex vivo* and *in vivo* spin trap techniques are potential tools for this inquiry. This knowledge will provide a rational basis for mechanistic studies *in vitro* and the development of targeted therapies. Drugs that modulate the effects of reactive intermediate production in lupus hold promise but have yet to be tested in human SLE.

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Figure 1.

The fate of NO and SO is dependent on the production of other RI and detoxifying pathways. NO is produced by NOS, which also produces SO when arginine is in short supply or if NOS is uncoupled. SO is also produced by inflammatory cells or cells under stress. NO reacts rapidly and preferentially with SO to form ONOO⁻. SO can be catalyzed to hydrogen peroxide (H₂O₂) by SO dismutase (SOD). H₂O₂ can undergo the Fenton reaction with Fe(II) to form OH which is converted into water by catalase. Myeloperoxidase found in neutrophils and other cells converts H₂O₂ to HOCl.

Figure 2.

The actions of NO on biologically relevant molecules. NO can nitrosylate thiol amino acids in proteins or peptides to form nitrosothiols. It can nitrosylate iron in p450 enzymes and guanylate cyclase to alter enzyme activity. NO also acts as a radical scavenger of SO (SO or O_2^-). When this latter reaction occurs, oxidative stress occurs through the action of $ONOO^-$, which itself can nitrate protein tyrosines and DNA, a process that can alter enzyme function and increase immunogenicity of DNA and proteins/peptides. $ONOO^-$ and NO_2 can oxidize lipids to form mediators of inflammation. Finally, $ONOO^-$ can act as a peroxide substrate to increase the activity of enzymes such as COX.

Table I

Mechanisms through which reactive nitrogen species can affect cell cycle/apoptosis and inflammation.

Reactive Species	Target	Process	Effect	Reference(s)
NO _x	Caspases	Nitrosation	Reduced enzyme activity	[9,10]
NO _x	p53	NO-induced DNA strand breaks?	Phosphorylation of p53	[9,59]
NO _x	HIF1 α	Disruption of Hsp90–client protein interactions	HIF1 α stabilized, half-life increased	[9,59]
NO _x	Nitrosylation	Cytochrome <i>c</i> oxidase complex IV S2	Inhibition of cytochrome <i>c</i> and mitochondrial membrane hyperpolarization	[60]
ONOO [−]	eNOS	Thiol oxidation	eNOS uncoupling/SO production	[52]
ONOO [−]	PGI ₂ synthase	Nitration	Reduced enzyme activity	[52]
ONOO [−]	Catalase	Nitration	Reduced enzyme activity	[39]
ONOO [−]	Cytochrome <i>c</i>	Nitration	Mitochondria-induced apoptosis	[10]
ONOO [−]	Protein Tyr	Nitration	Neopeptide formation/increased antigenicity	[24]
ONOO [−]	DNA	Nitration	Neopeptide formation/increased antigenicity	[26]
ONOO [−]	Arachidonic acid	Peroxidation	Formation of isoprostanes	[6]
ONOO [−]	LDL	Oxidation	Increased uptake by macrophages, increased binding of β 2-glycoprotein I	[29]
ONOO [−]	COX2	Peroxide substrate	Increases enzyme activity	[7]

NO_x = NO or N₂O₃