

Nitrosative modifications of protein and lipid signaling molecules by reactive nitrogen species

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White PJ, Charbonneau A, Cooney GJ, Marette A. Nitrosative modifications of protein and lipid signaling molecules by reactive nitrogen species. *Am J Physiol Endocrinol Metab* 299: E868–E878, 2010. First published September 28, 2010; doi:10.1152/ajpendo.00510.2010.—This review is the last of four review articles addressing covalent modifications of proteins and lipids. Two of the reviews in this series were previously published (15, 28) and dealt with modifications of signaling proteins by GlcNAcylation and serine phosphorylation. In the current issue of the *Journal*, we complete this series with two reviews, one by Riahi et al. (102a) on the signaling and cellular functions of 4-hydroxyalkenals, key products of lipid peroxidation processes, and our present review on the effects of nitrosative modifications of protein and lipid signaling molecules by reactive nitrogen species. The aim of this Perspectives review is to highlight the significant role that reactive nitrogen species may play in the regulation of cellular metabolism through this important class of posttranslational modification. The potential role of nitrosative modifications in the regulation of insulin signal transduction, mitochondrial energy metabolism, mRNA transcription, stress signaling, and endoplasmic reticulum function will each be discussed. Since nitrosative modifications are not restricted to proteins, the current understanding of a recently described genus of “nitro-fatty acids” will also be addressed.

insulin signaling; metabolism; transcription; bioactive lipids; endoplasmic reticulum stress

FOLLOWING THE NOBEL PRIZE-WINNING DISCOVERY that endogenously produced nitric oxide (\bullet NO) possesses important bioactivity in vivo, targeted nitrosative modifications of proteins and lipids have emerged as a key signaling mechanism in cell physiology. With a clear role in pathophysiology, elevated formation of nitrosative adducts has been implicated in the etiology of multiple disease states, including insulin resistance (33, 135), atherosclerosis (95), and Alzheimer's disease (126). The aim of this Perspectives review is to highlight the significant role that reactive nitrogen species (RNS) may play in the regulation of cellular metabolism through this important class of posttranslational modification. The potential role of nitrosative modifications in the regulation of insulin signal transduction, mitochondrial energy metabolism, mRNA transcription, stress signaling, and endoplasmic reticulum function will each be discussed. Since nitrosative modifications are not restricted to proteins, the current understanding of a recently described genus of “nitro-fatty acids” will also be addressed.

Biochemistry of Nitrosative Modifications

Nitrosative modifications are selective processes that target precise molecular sites in proteins or lipids for gain or loss of function in a manner somewhat analogous to the better-known phosphorylation or acetylation signal transduction mechanisms (49, 52, 115). In proteins, with the exception of heme iron binding, these modifications manifest in two main forms, either as *S*-nitrosylation¹ of cysteine thiols or as nitration of tyrosine residues. *S*-nitrosylation occurs through the covalent attachment of a diatomic nitroso group to a reactive thiol sulfhydryl in a redox-dependent fashion (49). Tyrosine nitration on the other hand, results from the covalent addition of a triatomic nitro group (NO₂) to the phenolic ring of tyrosine residues (52). Both *S*-nitrosylation and tyrosine nitration may arise from protein interactions with \bullet NO or secondary intermediates of \bullet NO, otherwise termed reactive nitrogen species (RNS). Higher nitrogen oxides such as N₂O₃, formed through previous reaction of \bullet NO with O₂, are thought to be a major *S*-nitrosylating species (50, 132). Protein *S*-nitrosylation may also occur as a result of transnitrosylation, which involves

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¹ The covalent attachment of the NO group to sulfhydryl residues in proteins is defined as *S*-nitrosylation. General NO attachment to nucleophilic centers is referred to as nitrosation (110a).

NO⁺ transfer from abundant low-weight *S*-nitrosothiols such as *S*-nitrosoglutathione (GSNO) (50). On the other hand, peroxynitrite (ONOO⁻), produced from the reaction of •NO with superoxide anion (O₂⁻), is regarded as one of the major cellular nitrating agents (50, 53). Other notable nitrating agents include myeloperoxidase (MPO)-derived nitrosonium cation (•NO₂), produced from the reaction of nitrite (NO₂⁻) with hydrogen peroxide (H₂O₂) (34, 107), and nitroso-peroxocarbonate (ONO-OCO₂⁻) which is formed via the reaction of carbon dioxide (CO₂) with ONOO⁻ (32, 66). Interestingly, lipid peroxy radicals (LOO•) have also been recently shown to promote tyrosine nitration by inducing tyrosine oxidation and also by reacting with NO₂⁻ to produce •NO₂ (9).

Despite being nonenzymatic in nature, both protein *S*-nitrosylation and tyrosine nitration appear to be remarkably selective processes. Indeed, only a relatively small number of tyrosine and cysteine residues appear to be the target of nitrosative adducts (3, 115). It has been established that the local environment of the residue plays a major role in whether it will react with RNS. In the case of tyrosine nitration, Souza et al. (114) revealed that proximity to turn-inducing amino acids, such as proline or glycine, and a nearby negative charge contribute to this selectivity. In contrast, the presence of an acid-base consensus motif has been shown to confer reactivity for *S*-nitrosylation to cysteine thiols (116). Interestingly, hydrophobic pockets in proteins are thought to promote *S*-nitrosylation of internal cysteines independently of the identified consensus motif by concentrating local •NO and O₂, which are hydrophobic in nature (88).

Endogenous •NO is produced enzymatically by a family of •NO synthase (NOS) isozymes, which convert O₂ and L-arginine to •NO and L-citrulline (42, 86). The NOS family comprises two constitutively expressed isoforms, endothelial NOS (eNOS) and neuronal NOS (nNOS), as well as one transcriptionally regulated isoform, inducible NOS (iNOS), which plays an important role in host defense (42). The enzymatic source of •NO synthesis is a key determinant of the cellular nitrosative milieu. Indeed, the NOS activity of the constitutively expressed isoforms is transient in nature and depends on activation by intracellular cues such as calcium or phosphorylation through protein-protein interactions with heat shock protein (HSP)90 and Akt (63, 79, 121). In contrast, iNOS activity is regulated at the biosynthetic level, and induction of this enzyme leads to higher •NO output that is sustainable even for days (48, 87). These differences imply discrete nitrosative signaling by constitutive NOS-derived •NO vs. widespread accumulation of nitrosative modifications resulting from iNOS induction.

In addition to the quantity of •NO produced, the spatial localization of NOS enzymes is also an important determinant of nitrosative outcomes. On this note, the constitutive, nNOS, and eNOS isozymes have been shown to display differential colocalization with intracellular calcium sources that is believed to underlie part of the disparity in their biological functions (8). Furthermore, NOS enzymes are also known to complex with their intracellular nitrosative targets either directly or via adaptor proteins (37, 62, 77, 104). One well-recognized example of this being the regulation of cyclooxygenase-2 activity in inflammatory settings by iNOS (62).

Nitrosative protein adducts are not only governed by the enzymes involved in •NO generation, they also appear to be subject to negative regulation by cellular denitrosylating and

denitrating enzymes (1, 13). *S*-nitrosoglutathione reductase (GSNOR) has been identified as a physiologically relevant “denitrosylase”, which acts by reducing the low-weight nitrosothiol GSNO (73, 74). Other candidates with potential denitrosylase activity include thioredoxin (89), protein disulfide isomerase (111), and xanthine oxidase (124). Compared with the abundance of potential denitrosylases, there is very little information available concerning candidate denitrating enzymes. However, exposure of tyrosine-nitrated proteins to various components of whole blood (46, 68, 106, 112) as well as different tissue lysates and crude extracts (46, 57, 65) has been shown to reduce nitrotyrosine abundance independently of any proteolytic activity. This evidence strongly supports the existence of tyrosine nitratase activity *in vivo*. As a whole, the identification of counterregulatory enzymatic processes is a relatively important advance in nitrosative cell biology that certainly warrants further investigation. Future findings in this field will likely be reminiscent of those that revealed the vital functions of protein phosphatases. Importantly, the regulation of these enzymes could confer an essential degree of specificity to nitrosative signaling that is yet to be fully understood.

Nitrosative Modifications in Insulin Signaling

Our group was the first to demonstrate the link between nitrosative stress and the etiology of obesity-linked insulin resistance. We observed that iNOS expression is induced in dietary (high-fat feeding) and genetic (*ob/ob* mice, Zucker diabetic rat) models of obesity (99). We then provided genetic evidence for the deleterious role of iNOS in obesity by showing that high-fat (HF)-fed obese mice lacking iNOS (iNOS KO) are protected from the development of skeletal muscle insulin resistance, as revealed by the normalization of insulin-dependent tyrosine phosphorylation of the insulin receptor, IRS-1, and Akt/PKB (99). Further studies by three independent groups confirmed that iNOS is a mediator of insulin resistance. Indeed, Kearney's group confirmed that iNOS disruption protects against insulin resistance for glucose metabolism in HF-fed mice (91). iNOS disruption was also found to partially prevent muscle insulin resistance in genetically obese *ob/ob* mice (117), whereas downregulation of iNOS by selective inhibitors or antisense oligonucleotides improved hepatic and muscle insulin action, respectively, in these animals (21, 40). Importantly, iNOS is also induced in skeletal muscle and adipose tissues of type 2 diabetic subjects (36, 123), where its expression correlates with the occurrence of insulin resistance and obesity (36). Furthermore, the iNOS gene has been shown to be genetically modulated by a 4-bp insertion/deletion (±) polymorphism in patients with coronary artery disease, and those patients homozygous for the + allele showed higher glycemia and elevated waist/hip ratio as well as a greater risk for unstable angina (82). The + allele of the iNOS promoter variant was later found to confer higher iNOS expression and may also be implicated in the risk for developing diabetic complications (e.g., microalbuminuria, nephropathy, neuropathy, retinopathy) in carriers of this variant (83). Taken together, these studies indicate that iNOS may also play a key role in the pathogenesis of human insulin resistance, diabetes, and associated debilitating complications.

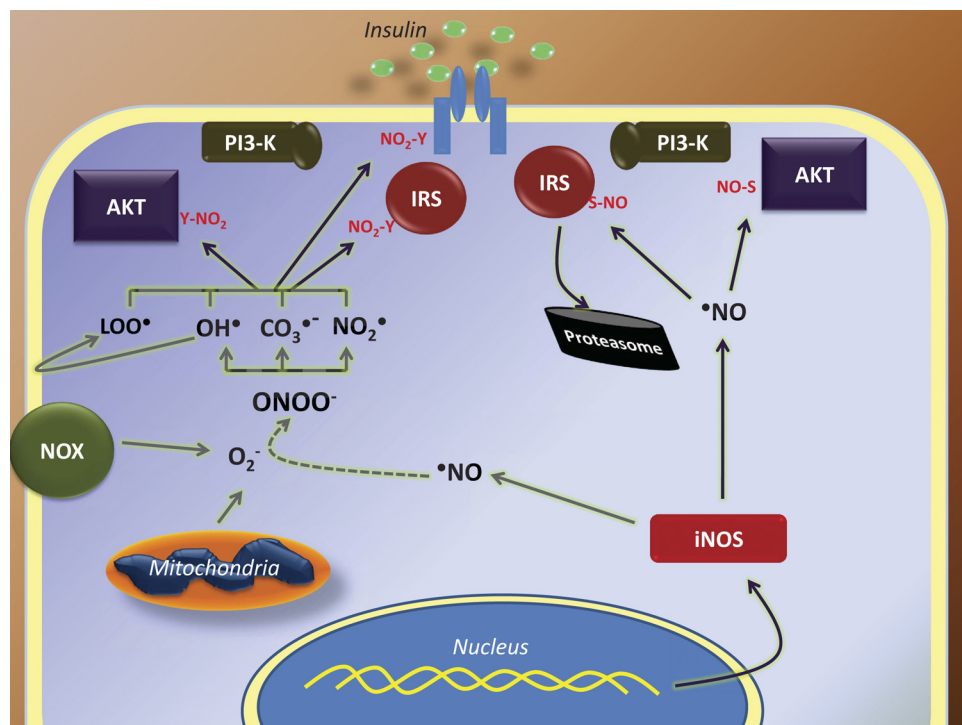
In light of the importance that tyrosine phosphorylation holds for insulin signal transduction, it is logical to assume that interfering tyrosine nitration underlies iNOS-induced insulin resistance. However, studies providing important information on the role of this posttranslational protein modification in obesity and lipid- and inflammation-induced insulin resistance are still scarce. In support of the interference theory, *in vitro* work has shown that exposure to ONOO⁻ can induce tyrosine nitration of IRS-1, consequently impeding tyrosine phosphorylation and activation of downstream insulin-signaling intermediates (90). Recent *in vivo* data also suggest that tyrosine nitration of insulin receptor (IR), IRS, and Akt in skeletal muscle and liver of HF-fed and lipid-infused animals could be responsible for impaired insulin signal transduction (23, 33, 139). Genetic evidence for a key role of tyrosine nitration in the control of insulin signal transduction was provided by the observation that lipid infusion induced tyrosine nitration of the IR, IRS-1, IRS-2, and Akt in wild-type mice but not in iNOS KO animals (23). Furthermore, *in vitro* tyrosine nitration of hepatic Akt by ONOO⁻ blunted insulin-induced Akt tyrosine phosphorylation and kinase activity. Using the ONOO⁻ decomposition catalyst FeTPPS [5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron (III)] was also found to effectively prevent tyrosine nitration of IR and IRS-1 in skeletal muscle of HF-fed animals and alleviated the concomitant defects in insulin-dependent tyrosine phosphorylation (139). Together, these data provide support for tyrosine nitration as a molecular mechanism by which iNOS induction reduces insulin action in obesity and after lipid overload (Fig. 1).

S-nitrosylation of key insulin-signaling intermediaries has also been hypothesized as a potential mechanism of insulin resistance in altered metabolic states (Fig. 1). In skeletal

muscle, S-nitrosylation of IRS-1 and Akt/PKB has been associated with impaired insulin signal transduction in multiple models of insulin resistance (19–21, 135). Interestingly, it appears that S-nitrosylation is key for proteasomal downregulation of IRS-1 in cultured skeletal muscle cells (117) as well as in liver (4). Carvalho-Filho and colleagues (19–21) also demonstrated that S-nitrosylation of insulin signaling intermediates was tightly linked to iNOS induction and that reducing iNOS expression by gene disruption and antisense oligonucleotide treatment preserved insulin action in all models of obesity. Importantly, Yasukawa et al. (135) showed that Akt/PKB is negatively regulated by S-nitrosylation. Using site-directed mutagenesis to replace the cysteine at position 224 in Akt/PKB with a serine, they were able to show that this site was vital to the inhibitory effect of S-nitrosylation, since mutated forms of Akt/PKB were resistant to •NO donor-induced S-nitrosylation and inactivation. These studies suggest an important regulatory role for this posttranslational protein modification in insulin signal transduction and insulin resistance.

The use of mass spectrometry and site-directed mutagenesis will be vital to the future identification of tyrosine and cysteine residues in insulin-signaling proteins that are targeted by nitration and S-nitrosylation and thus responsible for impaired insulin signal transduction in dietary and genetic models of insulin resistance. It will be interesting to see whether the contribution of these nitrosative modifications to insulin resistance varies among insulin target tissues. In future studies, it will also be important to define the precise radical species that are responsible for these nitrosative adducts *in vivo* and whether altered denitrosylation/denitration events contribute to the pathogenesis of insulin resistance.

Fig. 1. Nitrosative modifications in insulin signaling. The figure shows identified targets of tyrosine nitration (NO₂-Y, on the left) and S-nitrosylation (S-NO, on the right) in the insulin-signaling cascade. Left: inducible NO synthase (iNOS) expression leads to •NO production, which, upon interaction with mitochondrial or NADPH oxidase (NOX)-derived superoxide (O₂⁻), forms peroxynitrite (ONOO⁻). Peroxynitrite may then react to form hydroxyl radicals (OH•), carbonate radicals (CO₃^{-•}), and nitrogen dioxide (NO₂•). OH• may also interact with unsaturated fatty acids in membranes to form lipid peroxyl radicals (LOO•). These reactive intermediates may then work in concert to promote tyrosine nitration of the insulin receptor, insulin receptor substrate (IRS), and Akt. Each of these modifications is believed to impede insulin signal transduction, leading to insulin resistance. Right: iNOS induction leads to •NO production, which results in S-nitrosylation of IRS and Akt. S-nitrosylation of IRS promotes its degradation in the proteasome. S-nitrosylation of these key insulin-signaling intermediates is also believed to promote insulin resistance.



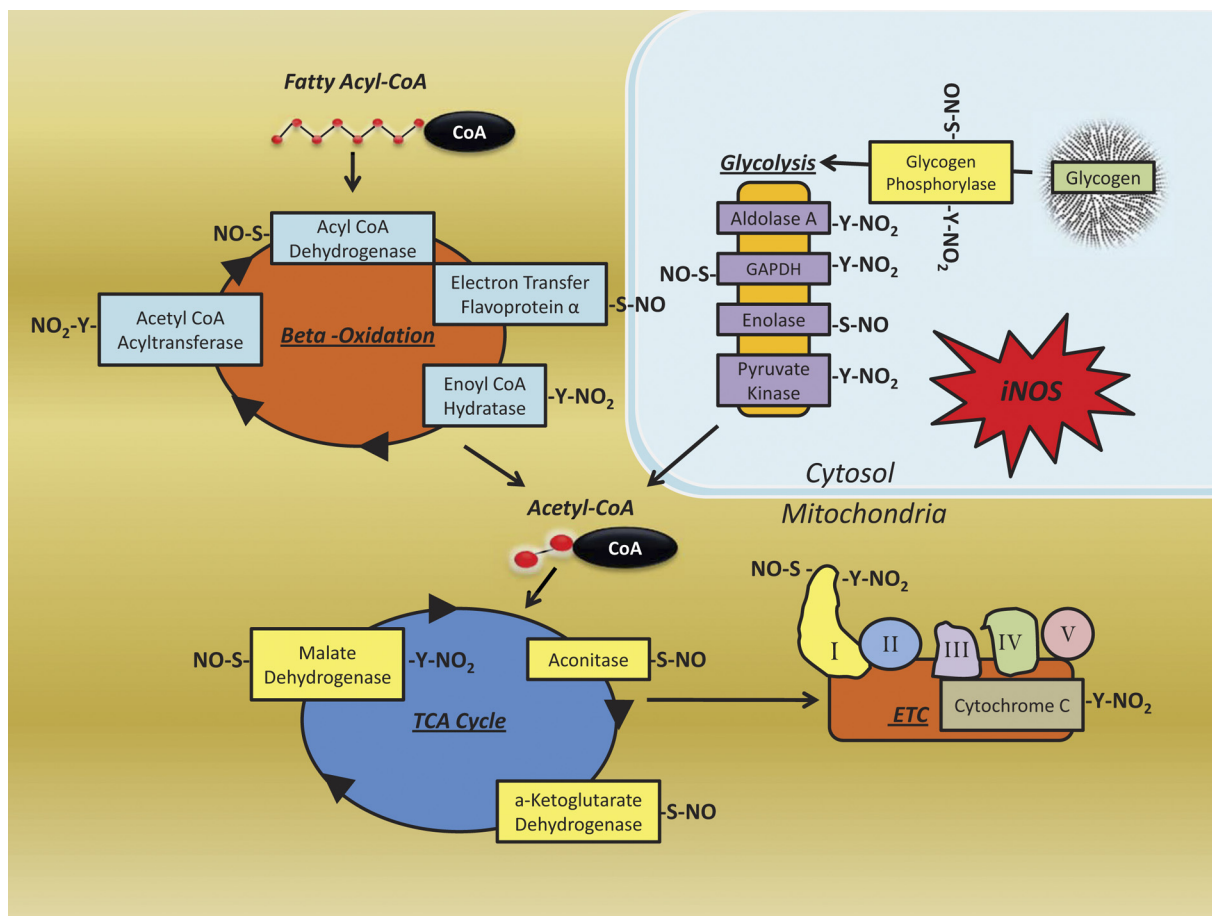


Fig. 2. Identified targets of nitrosative modifications in energy metabolism. The figure shows key enzymes involved in glycolysis, β -oxidation, the TCA cycle, and electron transport chain (ETC) that have been identified as targets of tyrosine nitration ($\text{NO}_2\text{-Y}$) or *S*-nitrosylation (*S*-NO).

Nitrosative Signaling and Mitochondrial Energy Metabolism

Careful investigation of the *S*-nitrosylation and tyrosine nitration proteomes has revealed that many enzymes involved in cellular energy metabolism, including glycolysis and fatty acid oxidation, are susceptible to nitrosative modifications on both cysteine and tyrosine residues, with some proteins in these pathways being susceptible to both (38, 59, 64, 76, 102, 118) (Fig. 2). Importantly, these analyses highlight the mitochondria as a primary hub of nitrosative signaling in the cell. Of note, key enzymes involved in β -oxidation and the TCA cycle as well as mitochondrial complex I and cytochrome *c* of the electron transport chain were shown to be subject to nitrosative modifications (summarized in Fig. 1). Mitochondrial proteins not related to energy metabolism are also targets of nitrosative signaling. An elegant example of this comes from the work of Cho et al. (24), who found that *S*-nitrosylation of Drp1 in neurons leads to altered mitochondrial fission and the development of Alzheimer's disease.

The finding that the mitochondria are a natural focal point for nitrosative protein modifications is likely due in part to the elevated potential for $\bullet\text{NO}$ interaction with O_2 species within this organelle. Indeed, $\bullet\text{NO}$ may be produced within the mitochondria by a splice variant of nNOS or may be a product of cytosolic NOS activity (43, 101). Importantly, the recent discovery of mitochon-

drial denitrosylation and denitration pathways suggests that nitrosative modifications within this organelle may be highly coordinated to ensure that mitochondrial activity matches changes in environmental demands (11, 64). On this point, Koeck et al. (64) have shown that protein tyrosine nitration in the mitochondria is rapidly and selectively reversed with changing O_2 tension. It will be interesting to see in future studies whether nitrosative modifications regulate mitochondrial substrate utilization and if obesity-linked iNOS induction contributes to the known pathogenic perturbations in mitochondrial energy metabolism in the obese state. Recent data have confirmed that nitrosation of mitochondrial proteins using mitochondrial targeted *S*-nitrosothiol alters the activity of key TCA cycle enzymes in a reversible fashion (25). These studies suggested that one role for the reversible inhibition of aconitase, α -ketoglutarate dehydrogenase, succinate dehydrogenase, and complex I could be to protect tissues such as heart from free radical bursts during ischemia/reperfusion. However, modifications that reduce the activity or function of important TCA cycle and electron transport proteins also have the potential to slow substrate oxidation and potentially lead to the buildup of metabolic intermediates (particularly lipids) that could impinge on signaling pathways to reduce insulin action. In addition to identifying the targets of nitrosative modifications within the mitochondria, future studies should define the functional impact that each nitrosative modification has on enzyme function

and mitochondrial energy metabolism as a whole. Forthcoming works should also be aimed at delineating the influence that altered substrate supply in the context of metabolic challenges such as exercise and obesity have on nitrosative signaling within the mitochondria.

Nitrosative Modifications at the Hub of Stress Signaling

RNS may be produced as a result of the activation of multiple well-known cellular stress signaling pathways that initiate iNOS transcription. Importantly, these pathways also appear to be tightly regulated by nitrosative modifications, which offer a complex form of feedback control. One relevant example of this is the nuclear factor- κ light-chain enhancer of activated B cells (NF- κ B) signaling pathway. NF- κ B activation is dependent on proteasome-mediated degradation of inhibitory proteins termed I κ Bs, which bind NF- κ B and mask its nuclear translocation signal. I κ B kinase (IKK)-mediated phosphorylation of I κ B targets I κ B for degradation in the proteasome leading to NF- κ B nuclear translocation. It has been reported that RNS may negatively regulate NF- κ B via *S*-nitrosylation of a critical Cys residue (Cys²⁹) within IKK β , which inhibits I κ B phosphorylation and therefore prevents NF- κ B translocation to the nucleus (49). On the other hand, there is also evidence of nitrosative stress-dependent activation of the NF- κ B pathway. In L6 and L8 myotubes, RNS activated NF- κ B via tyrosine nitration of I κ B α (7). Taken together, these data suggest that nitrosative stress may have both stimulatory and inhibitory effects on the NF- κ B signaling pathway depending on the nature of the prevailing posttranslational protein modifications. It is thus conceivable that, in acute inflammatory settings, *S*-nitrosylation of the Cys residue on IKK β could serve as a negative feedback control on the NF- κ B-dependent inflammatory response, whereas in a more persistent state of inflammation tyrosine nitration could supersede the effect of *S*-nitrosylation and exacerbate the inflammatory response. Very recent studies from our group suggest that iNOS-generated ONOO⁻ in response to both inflammatory and lipid challenges is necessary for the maximal activation of the NF- κ B pathway in the liver (23). Studies in mice lacking iNOS further indicated that iNOS-derived ONOO⁻ can nitrate tyrosine residues in both IKK β and I κ B α resulting in maximal NF- κ B activation. Yasukawa et al. independently observed similar results in INS-1 β -cells, where iNOS functions not only as a downstream effector of cytokine-mediated β -cell damage but also appears to act as an upstream enhancer of sustained NF- κ B activation in β -cells (135). These results suggest the existence of an iNOS-dependent feed-forward mechanism on the NF- κ B signaling pathway. Given that the NF- κ B signaling pathway has been implicated in the induction of inflammation in obesity-induced insulin resistance (61, 137), further studies are warranted to determine how nitrosative modifications of elements of the NF- κ B pathway contributes to the initiation and propagation of inflammation in tissues of obese animal models and subjects.

The activity of mitogen-activated protein kinases (MAPKs) might also be regulated by nitrosative modifications. Studies in cardiomyocytes (70, 100), fibroblasts (138), enterocytes (47), human neutrophils (140), astrocytes (136), and murine

neural cells (56) have clearly demonstrated that ONOO⁻ is a potent activator of both p38 MAPK and extracellular signal-regulated kinase (ERK). Furthermore, c-Jun NH₂-terminal kinases (JNKs), have also been shown to be regulated by various forms of RNS (16, 69). Indeed, JNK activation occurs in a myriad of cells ranging from rat cardiomyocytes to human cancer cells and human bronchial epithelial cells when treated with exogenous •NO donors (2, 55, 84). JNK activation was also observed in cells with enhanced levels of endogenously produced •NO from iNOS (26, 98). Interestingly, similar findings in shear-stressed cells were attributed to elevated levels of endogenous ONOO⁻ (44).

Despite the overwhelming evidence showing that RNS might stimulate JNK activity, contradictory reports demonstrate that nitrosative stress may also inhibit JNK activation. Studies by Park and colleagues (96, 97) and So et al. (113) suggest that such inhibition, like in NF- κ B signaling, is due to *S*-nitrosylation of JNK, which leads to suppression of its DNA binding activity. Importantly, in cardiomyoblasts, •NO was found to block H₂O₂-induced JNK activation, further demonstrating an inhibitory role of RNS on JNK signaling (22).

Collectively, these studies suggest that nitrosative signaling plays a complex dichotomic role in the regulation of stress signaling pathways. On the one hand, *S*-nitrosylation may prevent exaggerated responses to extracellular stimuli, while on the other, tyrosine nitration may promote the exacerbation of stress responses. These observations suggest that the functional impact of nitrosative signaling depends on various factors: the concentration of actual RNS, the cell type, and the presence of other stressing factors such as reactive oxygen species. The importance of cellular denitrosylating and denitrating activity in determining the outcome of nitrosative signaling is also of interest. In the perspective of inflammation and/or obesity-induced insulin resistance, the specific role of these posttranslational protein modifications on stress signaling pathways in metabolic tissues is yet to be completely resolved. As for insulin signaling, the use of mass spectrometry combined with site-directed mutagenesis in upcoming studies will be vital to our comprehension of the role that specific nitrosative modifications play in regulating stress signaling. Future studies should also be directed toward identifying the specific radical species and radical-generating enzymes that contribute to each nitrosative modification.

Transcriptional Regulation by Nitrosative Modifications

In addition to modulating canonical signaling pathways, RNS also critically regulate gene transcription through direct nitrosative modifications of key transcription factors and associated nuclear binding proteins. *S*-nitrosylation has been shown to effectively limit the DNA binding activity of a long list of important nuclear receptors and transcription factors, including NF- κ B, activation protein-1, MyB, hepatocyte nuclear factor 4, and the estrogen receptor (17, 41, 78, 120, 130). This regulatory role of *S*-nitrosylation on DNA binding activity appears to depend on the presence of susceptible reactive cysteine thiols in the critical DNA binding domains of transcription factors. In line with this, we recently showed that iNOS-derived •NO desensitizes

obese mice to the antidiabetic actions of the thiazolidinedione rosiglitazone by reducing the DNA binding and transcriptional activity of their target nuclear receptor peroxisome proliferator-activated receptor- γ (PPAR γ) (29).

In addition to limiting the DNA binding activity of multiple transcription factors, nitrosative modifications may also stimulate gene transcription. Indeed, \bullet NO has been shown to control cAMP response element-binding protein (CREB)-mediated DNA binding and gene expression through *S*-nitrosylation of nuclear proteins that associate with CREB target genes (92, 103). In this work, the authors noticed that \bullet NO signaling modulates histone H3 and H4 acetylation and decided to investigate whether \bullet NO directly influences histone deacetylase (HDAC) activity (103). It was found that HDAC2 can be *S*-nitrosylated, triggering its release from chromatin and thereby facilitating histone acetylation and CREB binding to the CRE-containing promoter sequences (92, 103). In addition to its role in CREB DNA binding, *S*-nitrosylation has also been shown to promote hypoxia-inducible factor (HIF)-1 α DNA binding by preventing the oxygen-dependent degradation of one of its two subunits (71). This \bullet NO-dependent HIF-1 α stabilization occurs as the result of *S*-nitrosylation on Cys⁵³³ in the oxygen-dependent degradation domain. Together, these data suggest that RNS may exert wide control on cell function through coordinated regulation of an array of transcription factors and nuclear binding proteins. Future studies aimed at understanding how nitrosative signaling integrates with alternative signal transduction pathways in the nucleus will be key to predicting the outcomes of RNS generation on transcription events. An effort should also be made to determine whether NOS or denitrosylating enzymes interact or colocalize with transcription factors or other key nuclear factors.

Nitrosative Signaling in the Endoplasmic Reticulum

The endoplasmic reticulum (ER) is responsible for the synthesis and folding of proteins and also represents a major cellular calcium store, playing an important role in calcium homeostasis. The ER is capable of importing calcium via the sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase (SERCA) and exporting calcium via the inositol triphosphate (IP₃) receptor and/or ryanodine receptor (RyR), respectively (45). High concentrations of calcium in the ER are essential for folding and disulfide bond formation in newly synthesized proteins, because the activities of several ER chaperones, such as calreticulin, calnexin, and protein disulfide isomerase (PDI) are all calcium dependent. These molecular chaperones are central in the maturation and transport of unfolded secretory proteins; therefore, any disruption of calcium homeostasis in the ER may lead to the accumulation of unfolded or misfolded proteins and the development of ER stress.

RNS have been proposed as important inducers of ER stress (94), since increased \bullet NO production has been shown to negatively impact ER calcium content in β -cells (18, 30, 35, 94), neurons (126, 127), endothelial cells, and foam cells (131). Nitrosative modifications appear to underlie the mechanism by which RNS exert this effect. Indeed, it has been reported that \bullet NO inhibits calcium-ATPase activity of cardiac SERCA by nitrating tyrosine residue as well as by *S*-nitrosylating functional cysteine residues within channel-like domains, thereby reducing ER calcium import and concomitantly prompting ER stress (128, 129, 134). *S*-nitrosylation has also been proposed

to explain the mechanism by which \bullet NO could pathologically accentuate calcium export from the calcium release channels known as RyRs. Reports demonstrated that the activity of skeletal muscle RyR1 is significantly increased by \bullet NO through *S*-nitrosylation (119, 134). Therefore, it seems that \bullet NO-mediated ER calcium depletion could be regulated by both tyrosine nitration of SERCA and *S*-nitrosylation of SERCA and RyR. Interestingly, it has been shown that RNS also contribute to ER stress-induced neurodegeneration in a calcium-independent manner by directly *S*-nitrosylating the ER chaperone PDI (12, 85, 126, 127). Together, these data suggest an important role for nitrosative modifications in the negative regulation of ER function. It is tempting to speculate that these nitrosative targets represent a mechanism by which cellular stress signaling can regulate protein synthesis and apoptosis. Since a growing body of evidence suggests that ER stress plays an integral role in the development of insulin resistance and diabetes (51), further studies are needed to determine whether RNS exert regulatory control on this process.

Nitrosative Modification of Lipid Species

It is becoming increasingly evident that RNS also play a major role in lipid biology. Not only do they influence bioactive lipid synthesis by targeting key enzymes such as COX-2 (62) and cytochrome *P*-450 (67) they may also directly interact with unsaturated fatty acids to form novel nitro-fatty acids that have distinct bioactivities from their precursor lipids (6, 39). Lipid reactions with RNS can result in the formation of *cis* or *trans* nitro-alkanes, where the NO₂ group is present at the site of the double bond, as well as nitro-hydroxy and nitro-hydroperoxy lipids (6). Multiple unsaturated fatty acids including oleate, linoleate and arachidonate have been shown to have nitro-derivatives (54, 125, 133). Importantly, the high number of nitro-fatty acid species identified to date likely represents the elevated formation of RNS in hydrophobic environments, such as the lipid bilayer, which inherently makes lipids excellent candidates for nitrosative signal transduction (31, 75, 80, 81, 122).

Contrary to the initial belief that nitro-fatty acids would be proinflammatory in nature, accumulating evidence suggests that the transfer of an NO₂ adduct to unsaturated fatty acids actually confers anti-inflammatory potential (14, 27, 58, 60, 72, 105, 133). Indeed, the biological activity of nitro-fatty acids resembles more closely that pertaining to recently identified families of proresolving hydroxy lipids, namely the lipoxins, resolvins, protectins, and maresins (108–110), rather than the well-known leukotriene or prostanoid classes of bioactive lipids. Notably, due to the important role of iNOS in host defense, the synthesis of nitro-fatty acids may also be invoked by an inflammatory signal. This suggests that these novel fatty acids likely form part of a growing lipid signaling network that ensures “catabasis”, or the timely return to homeostasis following an inflammatory stimulus (109).

Somewhat paradoxically, although nitrosative modifications directly impair DNA binding and transcriptional activity of PPAR γ (29), two separate nitro-alkanes have been shown to display physiologically significant PPAR γ agonist activity that is comparable to that of the synthetic agonist rosiglitazone (5, 14, 93). Since PPAR γ plays a major role as a transcriptional

switch for the resolution of inflammation (93) it will be interesting to see whether there is a timely progression from direct inhibitory RNS-PPAR γ interactions to stimulatory nitro-fatty acid-PPAR γ interactions in the course of self-resolving inflammation. Importantly, it is believed that a large part of nitro-fatty acid activity stems from their ability to form reversible covalent adducts on nucleophilic sites of proteins, such as cysteine residues, in a process termed nitroalkylation (10). Thus, nitro-fatty acids appear to be mediators of a third type of nitrosative protein modification that likely possesses differential regulation to that of *S*-nitrosylation and tyrosine nitration, further adding to the complexity of nitrosative signaling in vivo. Future studies are certainly warranted to determine whether nitroalkylation, like *S*-nitrosylation and tyrosine nitration, influences insulin signaling, mitochondrial energy metabolism, stress signaling, and gene transcription in vivo.

Current Limitations in Nitrosative Biology

As one might expect, due to the highly reactive nature and short half-lives of RNS and ROS, one of the major limitations in the field is to successfully measure and define the precise intermediates that are specifically responsible for inducing the nitrosative modifications of interest. This situation becomes increasingly difficult as we move from in vitro to in vivo studies. Currently, electron paramagnetic resonance (EPR) spin trapping is considered the gold standard for measuring free radicals, and advancements in this technology have greatly improved our capacity to study RNS and ROS; however, many limitations remain inherent to this technique. Notably, there is the requirement for spin traps or spin probes to be present at the site of interest. These may be either hydrophobic or hydrophilic; thus, delivering them to the site of interest may not be easy to accomplish. Spin trap stability in vivo is also a limiting factor in the efficacy of this technology. Furthermore, precise, considered controls are a necessity when EPR is used, since spin traps may also react with media to produce ex vivo artifacts. Finally, despite being the gold standard and the only analytical method to detect reactive molecules in living cells, the sensitivity of EPR spin trapping is still relatively low at around 10^{-6} M. Future developments in EPR methodologies will certainly help advance our understanding of nitrosative biology in vivo.

In addition to difficulties in measuring the reactive nitrogen and oxygen intermediates that are responsible for nitrosative modifications, the field is also restrained by the lack of efficient methodology for detecting nitrosative modifications per se. Although a few good antibodies exist for the detection of 3-nitrotyrosine, this is not the case for *S*-nitrosylation, and thus the detection of these adducts is somewhat more difficult. Indeed, the latter modification is highly labile; thus, to study *S*-nitrosylation in cells and tissues, it is necessary to perform a chemical reaction known as the "biotin switch", in which the cysteine-bound NO is replaced with a more stable biotin label that can then be detected by Western blotting or mass spectrometry. Since the sensitivity of this method is low, detection of *S*-nitrosylated proteins often requires the use of nitrosylating agents, which may give different or off-target results compared with physiological settings. Future technologies that improve our ability to detect or stabilize this labile adduct will advance the study of nitrosative biology.

Conclusions

This review has focused on the role of the RNS-generating systems such as the iNOS/ \bullet NO pathway in producing an array of coordinated nitrosative modifications on key signal transduction proteins, enzymes, and lipid messengers involved in metabolic control. We have summarized recent data that strongly suggest that tyrosine nitration and *S*-nitrosylation of insulin-signaling intermediates represent novel means to modulate metabolic functions in insulin target cells. The recent identification by proteomics of multiple mitochondrial enzymes that are susceptible to nitrosative modifications have also increased the interest to explore the potential impact of such modifications on metabolic control in states of increased RNS production. Future studies will be needed to fully unravel how these newly established nitrosative modifications of multiple proteins and lipid species exert a fine control of insulin action, metabolism, and inflammation in both physiological and altered metabolic states such as obesity, insulin resistance, and diabetes.

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DISCLOSURES

No conflicts of interest are reported by the authors.

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