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IMPACT OF NITRIC OXIDE ON METABOLISM IN HEALTH AND AGE-RELATED DISEASE

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Abstract

Nitric oxide (NO) serves as a messenger molecule in a variety of physiological systems and also converts into toxic radical species that can damage cells through a process known as nitrosative stress. While the physiological role of NO in blood vessel dilation, the nervous system, and the immune system is well established, recent studies have begun to investigate the role of NO in metabolism and energy expenditure through modulation of mitochondria. NO appears to stimulate mitochondrial biogenesis in certain situations through activation of proteins such as peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC1-α). Because of this link between NO and mitochondrial biogenesis, the role of NO in certain aspects of metabolism, including exercise response, obesity, fat cell differentiation, and caloric restriction, are the subject of increasing investigation. In addition to its role in mitochondrial biogenesis, NO also stimulates mitochondrial fragmentation, which can be caused by too much mitochondrial fission or inhibition of mitochondrial fusion and can result in bioenergetic failure. While the contribution of NO-mediated mitochondrial fragmentation to neurodegenerative diseases seems clear, the mechanisms by which NO causes fragmentation are uncertain and controversial. In this review, we discuss the role of NO in manipulation of mitochondrial biogenesis and dynamics and how these events contribute to human health and age-related disease.

Keywords

mitochondrial biogenesis; mitochondrial fission and fusion; nitric oxide; PGC1-α; sirtuins

Introduction

Age-related diseases are collectively the greatest health problem plaguing the populations of industrialized countries. With readily available food supplies and vastly improved medicine and technology has come a shift in the types of diseases that are most prevalent and damaging in the developed world, from infectious and acute diseases in the past to age-related diseases such as diabetes, neurodegenerative diseases, and certain types of cancer that riddle our modern societies (1). From the very earliest days of molecular aging research, the role of mitochondria has been heavily suspected. While reactive oxygen species (ROS)

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produced during mitochondrial respiration was the initial focus, further research has identified many more pathways involving mitochondria that affect cellular function both positively and negatively. For example, caloric restriction has been shown to increase lifespan in all species tested including non-human primates (2, 3). Proteins such as sirtuins regulate the effects of caloric restriction, which notably include increased mitochondrial function (2, 3). Caloric restriction is just one example that illustrates the complexity of the aging process and age-related diseases, especially as it relates to mitochondrial function. In particular, the factors and events that initiate mitochondrial activities in normal physiology and mitochondrial dysfunction in diseases remain a mystery in most cases.

Nitric oxide (NO) is a messenger molecule with important roles in both physiology and pathophysiology. Here, we review the links between NO and age-related diseases and present the evidence for NO's role as an initiating factor in multiple mitochondrial pathways. In particular, we focus on the effect of NO on various aspects of mitochondrial function including biogenesis, fission, and fusion, and examine how these processes are involved in important concepts in health and age-related disease such as the physiological response to exercise and caloric restriction, differentiation of fat cells, and obesity-linked inflammation. In addition, we discuss the role of NO in mitochondrial dysfunction related to neurodegenerative diseases, which has been the topic of recent controversy and debate.

Nitric Oxide Synthases and the Physiological Role of NO

NO is a gas that has important physiological functions in different organ systems in addition to its pathophysiological roles in nitrosative stress, protein modification, and excitotoxicity. Nitric oxide synthases (NOSs) synthesize NO from L-arginine in mammals (4, 5). Studies have positively identified three distinct NOS isoforms – endothelial NOS (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) (6). In addition, more recent studies have identified a fourth NOS isoform, mitochondrial NOS (mtNOS) (7, 8).

The different NOS isoforms exhibit tissue and cell-type specific distributions and activities, which reflect their specific physiological roles. eNOS is active primarily in the endothelial tissue of blood vessels, where NO mediates vasodilation and relaxation of soft tissue (9). eNOS is a constitutively active isoform that produces low levels of NO at a steady rate over long periods to achieve its functional roles (9). iNOS is active primarily in immune cells and glial cells and is activated by pathogen recognition and cytokine release (9, 10). The primary function of iNOS is to mediate cell death in response to pathogens by generating NO at toxic levels. Thus, iNOS produces high concentrations of NO over short periods (6). nNOS is active primarily in central and peripheral neurons where NO serves as an important neurotransmitter in cell-to-cell communication and neuronal plasticity (6). Similar to eNOS, nNOS is constitutively active and produces low levels of NO over long periods. Finally, mtNOS is the most recently identified member of the NOS family (7, 8). mtNOS localizes to the mitochondrial inner membrane and plays a role in the regulation of bioenergetics and Ca^{2+} buffering (8). The relationship between mtNOS and the other NOS isoforms remains unclear (6).

While NO contributes to various pathologies through formation of reactive nitrogen species and modification of proteins, NO also plays important physiological roles in blood vessel dilation, neurotransmission, and immune cell response. NO was first identified as the endothelium-derived relaxing factor (EDRF) that mediates blood vessel dilation (11). In addition, NO is involved in multiple nervous system activities including nerve-mediated relaxation of the gut during digestion (12), innervation of neural blood vessels in cerebral and penile arteries (13–15), and prevention of excitotoxicity by S-nitrosylation of NMDA (*N*-methyl-*D*-aspartate) glutamate receptors (16, 17). Furthermore, immune cells and microglia rely on the toxic ability of NO to perform their pathogen fighting activities (18, 19). Finally, recent studies have identified NO as an important mediator of mitochondrial biogenesis. Here, we will discuss this physiological role of NO in greater detail.

Pathophysiological roles of NO

In addition to its roles in normal physiology, it is important to appreciate that NO also has pathophysiological actions. NO reacts with various oxygen species in the cell to form highly reactive molecules that damage cellular components through various mechanisms. First, NO reacts with superoxide anions (O_2^-) to form peroxynitrite ($ONOO^-$). $ONOO^-$ formation in mitochondria induces cytochrome *c* release (20), which is an indicator of mitochondrial stress and inducer of cell death, and blocks complexes I and IV of the mitochondrial respiratory chain in many cell types (6). In addition, NO reacts with cysteine residues in interacting proteins to form nitrosothiols in a process known as S-nitrosylation (21). S-nitrosylation is a form of protein regulation that is important for normal physiology, but also can cause protein dysfunction. For example, S-nitrosylation of various proteins has been associated with neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD), and stroke (6). Finally, NO also plays a role in glutamate excitotoxicity in neurons, which is caused by overstimulation of glutamate receptors and excessive influx of calcium ions. While under certain conditions NO can block NMDA receptors (a subtype of glutamate receptor) by S-nitrosylation and protect against excitotoxicity (16), under different conditions NO can enhance neuronal injury caused by excitotoxicity (22). In sum, the pathophysiological actions of NO are also well-established and both the positive and negative actions of NO are relevant to our discussion of NO's role in mitochondrial function, metabolism, and age-related diseases.

NO regulation of mitochondrial biogenesis

The term mitochondrial biogenesis refers to the formation of new mitochondria in cells, which involves the coordinated transcriptional regulation of respiratory protein complexes consisting of both nuclear and mtDNA-encoded components, enzyme production, and replication of mitochondrial DNA (mtDNA) (23). Because mitochondria are crucial to cellular health, a high level of mitochondrial biogenesis is considered to be indicative of intact metabolic and bioenergetic functionality and cellular well-being. Interestingly, recent studies suggest that chronic, small to moderate increases in NO stimulate mitochondrial biogenesis (23). For example, treatment of cultured mouse brown adipocyte precursors and white fat cells with NO donors increased mtDNA content, MitoTracker fluorescence (an indicator of mitochondrial membrane potential), expression of cytochrome *c* oxidase subunit

IV (COX IV) of the mitochondrial respiratory chain, cytochrome *c*, and mitochondrial mass (24). The observed changes stimulated by NO were brought about by guanosine 3′,5′-monophosphate (cGMP)-dependent increased expression of peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC1-α), nuclear respiratory factor 1 (NRF-1), and mitochondrial transcription factor A (Tfam), all of which are important mediators of gene expression for mitochondrial biogenesis (24, 25). In addition, HeLa cells transfected with eNOS exhibited the same increases in mitochondrial biogenesis and inhibition of NOS abolished this effect (24). Furthermore, eNOS deficient mice exhibited decreased levels of mtDNA, COX IV, and cytochrome c in the brain, liver, and heart tissue, indicating that deletion of eNOS was sufficient to reduce mitochondrial biogenesis despite the fact that the animals still expressed other NOS isoforms such as nNOS and iNOS (24). Finally, NO-dependent mitochondrial biogenesis increased ATP production and oxygen consumption (26).

Role of NO in Metabolism and Energy Expenditure

Because of NO's role in mitochondrial biogenesis, it is important to consider the potential effects of NO on metabolism and energy expenditure. Metabolism and energy expenditure are important processes in overall health maintenance in humans as evidenced by the correlation between obesity, which results from insufficient energy expenditure in relation to caloric intake, and a wide spectrum of age-related disorders including diabetes, cancer, heart disease, and neurodegeneration. In addition, caloric restriction diets and moderate exercise, both of which increase mitochondrial function, fat and glucose metabolism, and energy expenditure, correlate with increased longevity and decreased risk of age-related disorders. As one might expect, NO plays a role in multiple aspects of metabolism and energy expenditure including the differentiation of fat cells, obesity, and caloric restriction, while its role in the physiological response to exercise is less clear.

Exercise and NO

The beneficial effects of physical exercise have long been appreciated. Interestingly, PGC1-α levels in muscle increase following exercise (27). Because of the link between NO and PGC1-α discussed earlier, it is tempting to presume that NO might mediate this physiological response. To examine this possibility, a recent study measured the effect of pharmacological inhibition of NOS by N^G-nitro-L-arginine methyl esther (L-NAME) and found that L-NAME did not attenuate exercise-induced upregulation of PGC1-α (28). However, further studies utilizing NOS knockout models will be necessary to confirm that NO does not play a substantive role in exercise-induced PGC1-α response (28). Of note, several independent sources have indicated that technical issues with NOS null mice persist as different strains of mice continue to produce variable results (29–31).

Composition and Differentiation of Fat Tissue and NO

Another important factor in mammalian metabolism and energy expenditure that is becoming the focus of increasing research attention is the composition profile of body fat and the differentiation of fat cells. The majority of body fat in adult humans is white fat, which is found mostly in the abdomen and subcutaneous areas and functions as energy

storage reservoirs (32). The other, less common type of fat is brown adipose tissue (BAT), which is confined to smaller pockets and was until recently thought to be relatively sparse in adult humans compared to newborns (32). Typically, smaller mammals exhibit higher levels of BAT because of its role in heat production. However, recent studies are beginning to suggest that BAT is more common in adult humans than previously recognized, especially in response to cold temperatures (33). In contrast to white fat, BAT is highly vascularized tissue that contains abundant mitochondria, which operate in heat generation to maintain body temperature as mediated by activation of uncoupling protein 1 (UCP1) (32). UCP1 is a mitochondrial transmembrane protein that allows BAT to perform its primary role of heat generation by creating proton leaks across the mitochondrial inner membrane and thus uncoupling oxidative phosphorylation from ATP production in favor of heat generation (32). Intriguingly, the presence of BAT correlates with reduced propensity for obesity in animals, hence the interest in the mechanism of fat cell differentiation (32).

The role, if any, of NO in BAT formation remains unclear (34). PPARγ plays a central role in the differentiation of fat cells, but PGC1-α is merely a regulator of adaptive thermogenesis in BAT and does not seem to play a direct role in brown fat cell differentiation (33). NO (particularly NO produced by iNOS) regulates BAT thermogenesis by increasing BAT mass and UCP1 levels (35). Interestingly, a recent study reported that protein kinase G (PKG) is required for brown fat cell differentiation and that PKG mediated the induction of UCP1 expression and mitochondrial biogenesis by NO and cGMP (36). This finding suggests a potential indirect role for NO (and cGMP) in stimulating expression of genes necessary for brown fat cell differentiation and the formation of BAT.

Further indicating a link between NO and BAT function are recent observations in eNOS null mice. eNOS null mice exhibit similar characteristics as genetic models of obesity (24). Specifically, these mice, when compared to wild-type mice, have similar food consumption but greater weight due to increased feed efficiency resulting from decreased energy expenditure (24). Reduced energy expenditure and increased feed efficiency suggests there are fewer uncoupled mitochondria, which might imply decreased BAT-dependent thermogenesis. Accordingly, levels of UCP1 and PPARγ mRNAs were lower in eNOS null mice (24). Finally, excess weight gain in mice early after birth can reprogram BAT, make it less thermogenically active, and reduce BAT levels later in life (37). This observation is potentially of great importance due to the increasing concern over rising obesity levels in children (38). Whether these observations in mice will apply to humans, considering the differences in fat profiles between mice and humans, will be of great interest.

Obesity-Linked Inflammation and NO

The prevalence of obesity continues to rise throughout the world and is a growing threat to human health (39). Studies have linked obesity to a number of health problems including cardiovascular disease, neurodegenerative disease, diabetes, and some cancers (40). It is becoming increasingly clear that inflammation is a key feature of obesity and diabetes (40). One of the first studies to identify a role for inflammation in obesity reported that tumor necrosis factor-α (TNF-α), a cytokine that promotes inflammation, is overexpressed in obese mice (41). In this context, TNF-α seems to inhibit insulin sensitivity. Since this

seminal finding, other studies have reported overexpression of other inflammatory factors and activation of various inflammatory pathways in adipose tissue (40). Whether NO participates in the inflammatory response in obesity and diabetes remains unclear, but recent studies have provided some clues.

An emerging concept in the mechanism of obesity-induced inflammation is the potential role of endoplasmic reticulum (ER) stress. The ER is responsible for protein quality control in the cell and the accumulation of unfolded proteins in the ER can trigger ER stress and a cascade of signal transduction pathways such as the unfolded protein response (UPR) (39). There are many potential links between ER stress, the UPR, and inflammation. Of note, NO is one common link between ER stress and inflammation (42). Specifically, NO S-nitrosylates protein-disulfide isomerase (PDI) in the brain and can cause ER stress and initiate the UPR (43).

In relation to inflammation, iNOS, which mediates inflammation, has been implicated in insulin resistance, a common complication of obesity that leads to diabetes (44). Increased iNOS expression has been associated with obesity in insulin-sensitive tissues and protein S-nitrosylation is elevated in diabetes patients and obese mice (44). Finally, disrupting iNOS can protect against obesity-induced insulin resistance (45). Hence, there is ample evidence suggesting that NO plays a role in obesity-linked inflammation. Whether NO works through ER stress and the UPR to cause inflammation is an intriguing possibility and is an area for future research.

Caloric Restriction, Sirtuins, and NO

Caloric restriction (CR), the reduction of dietary caloric intake without malnutrition, correlates with lifespan extension and prevention of disease in all species examined so far including non-human primates (2, 3). There is strong evidence that sirtuins, a family of class III histone deacetylases, help mediate the positive effects of CR (46, 47). While the exact mechanisms by which CR and sirtuins promote longevity and health remain under investigation, there is a strong link between CR, sirtuins, and metabolic activities. For example, sirtuins 3, 4, and 5 (SIRT3, SIRT4, SIRT5) are localized to mitochondria and appear to orchestrate a coordinated program of metabolic regulation (3). In addition, SIRT3 upregulates fatty-acid oxidation in response to fasting (48). There is also convincing evidence that sirtuin 1 (SIRT1), a nuclear sirtuin, deacetylates and activates PGC1-α in some situations (49–51). Activation of SIRT1 and PGC1-α by CR in these studies was associated with increased mitochondrial biogenesis (49–51). However, there is at least one study that contradicts these findings, so the relationship between CR, SIRT1, and PGC1-α remains somewhat controversial (52).

As discussed, NO stimulates PGC1-α activity and mitochondrial biogenesis, thus whether NO regulates the cellular response to CR is an important question to consider. Interestingly, CR induces eNOS expression (but not iNOS or nNOS expression) and cGMP formation in mice and stimulates mitochondrial biogenesis and SIRT1 expression (53). In addition, other mitochondrial proteins with increased expression following CR in the presence of eNOS include, NRF-1, Tfam, mitofusin 1 (MFN1), mitofusin 2 (MFN2), COX-IV, and cytochrome c. eNOS is required for CR mitochondrial benefits and SIRT1 induction as eNOS null mice

do not show the same effects (53). eNOS null mice have decreased longevity, which is consistent with an inability to generate new mitochondrial proteins and activate stress responses such as sirtuins (53). These findings are of great significance and further clarify the potential relationship between CR and mitochondrial biogenesis.

Mitochondrial fission and fusion

The synthesis of new mitochondrial proteins by PGC1-α mediated mitochondrial biogenesis is not the only mechanism by which cells maintain bio-energetic functionality. In addition, mitochondrial biogenesis is coordinated with mitochondrial division (fission) and mtDNA replication. What mediates the cross-talk between these cellular processes and how they are synchronized is poorly understood, but changes in cellular energy levels and expenditures likely play a role. After division, mitochondria can re-fuse to form elongated filaments or mitochondrial networks. Mitochondrial fusion may facilitate mixing of metabolites and compensate for mtDNA mutations that are lined to aging, thereby improving cellular metabolism and energy production. Three conserved large GTPases regulate mitochondrial fission and fusion with dynamin-related protein 1 (DRP1) triggering mitochondrial fission (54, 55) and Mitofusin (MFN1) (56, 57), and optic atrophy 1 (OPA1) regulating fusion (58–60). In healthy cells, mitochondria undergo cycles of fission and fusion. Mitochondrial fission occurs when cells divide, but can also occur in post-mitotic cells such as neurons. Mutations in fusion proteins MFN2 and OPA1 cause Charcot-Marie-Tooth subtype 2A (a human peripheral neuropathy affecting motor and sensory neurons) and autosomal dominant optic atrophy (the most common form of optic atrophy affecting retinal ganglion cells), respectively (61–64). Thus, loss of mitochondrial fusion and subsequent accumulation of divided mitochondria causes neurodegenerative disease, suggesting that neurons are particularly vulnerable to a shift of the mitochondrial fission/fusion balance towards fission.

Nitrosative stress triggers mitochondrial fragmentation and neuronal injury

While the discovery of rare disease-causing mutations in mitochondrial fusion GTPases suggested an important role for fission and fusion in human health, more recent studies have focused on investigating whether a disrupted mitochondrial fission/fusion balance is also of significance to sporadic neurodegenerative disease. In the nervous system, NO functions as an important neurotransmitter and is generated by neurons and glia. In common neurodegenerative conditions, such as stroke, AD, PD, and amyotrophic lateral sclerosis (ALS), NO accumulates, forming highly neurotoxic reactive nitrogen species (RNS) such as $ONOO^-$, which in turn can covalently modify tyrosine or cysteine residues in proteins and alter their structure and function. Numerous studies support the idea that this form of nitrosative stress plays a causal role in these disorders (6). Mechanisms that might account for an increase in RNS include inflammation, microglia activation, and cytokine-mediated iNOS activation. Another possible mechanism is accumulation of excitatory amino acids such as glutamate at synapses due to defective re-uptake by glutamate transporters in neighboring astrocytes. Overstimulation of neuronal glutamate receptors then results in Ca^{2+}-mediated NOS activation and $NO/ONOO^-$ increase.

Our group recently showed that nitrosative stress triggers persistent mitochondrial fragmentation (due to activation of fission or inactivation of fusion) prior to neuronal cell death in isolated neurons *in vitro* and in an experimental mouse model of ischemic stroke injury *in vivo* (65). NO-induced fragmentation results in ultrastructural damage of mitochondria, increased reactive oxygen species (ROS), and reduced ATP (65). Thus, persistent mitochondrial fission is accompanied by bio-energetic compromise. Free radical scavengers such as reduced glutathione prevent NO-mediated mitochondrial fragmentation and cell death. Using time-lapse imaging, we found that NO-induced mitochondrial fragmentation can be reversible in young neurons and neurons expressing survival genes such as Bcl-xL [(65) and unpublished observation]. In addition, we observed that mitochondrial fragmentation was accompanied by autophagosomes containing mitochondria. Thus, mitochondrial fission alone does not trigger neuronal cell death and may in fact represent a stress response to remove damaged organelles by autophagy, increasing survival. By contrast, when BAX, a pro-apoptotic BCL2 family member, moves into the fission sites mitochondria do not re-fuse and cell death occurs (66). In sum, short-term mitochondrial fragmentation increases cell survival and persistent fragmentation, marked by activation of downstream signal transduction pathways such as BAX relocation to mitochondria, triggers cell death.

SNO-DRP1 is not functionally implicated in Alzheimer's disease

The role of NO in AD has been a topic of recent interest. We and others have reported that amyloid-β (Aβ), the primary component of senile plaques found in the AD brain, induces mitochondrial fragmentation in cortical neurons (65, 67). Building on this finding, a recent study claimed that DRP1 S-nitrosylation at Cysteine 644 leads to its dimerization and enzyme activation and is the central mechanism of NO-induced mitochondrial fission, bioenergetic compromise, and neuronal injury in AD, but not in PD (68). However, this study remains controversial and is in disagreement with current literature of human DRP1.

First, the authors did not clearly determine DRP1 GTPase activity, but merely equated optical density at a single time point with enzyme activity using a malachite green-based assay (68). In addition, they reported an unexpected absorbance for the GTPase-defective $DRP1^{K38A}$ mutant. Based on this assay, the authors proposed a two-fold increase in DRP1 GTPase activity upon S-nitrosylation of cysteine 644. Second, the authors claim that formation of the DRP1 dimer is a reflection of increased enzymatic activity (68). We are aware of no other studies that have suggested dimer-stimulated DRP1 activation. Instead, protein cross-linking and gel filtration experiments have demonstrated that DRP1 is a tetramer under physiological conditions and self-assembly into higher order oligomers, rings, and spirals stimulates GTP hydrolysis (69–71). Further, oxidation does not seem to mediate oligomerization. On the contrary, reducing agents seem to be required for oligomerization of related proteins such as dynamin and OPA1 (72, 73). Third, the authors claim that all (seventeen out of seventeen) AD patient samples in their study, but none of the control and PD samples, exhibit increased SNO-DRP1 levels (68). This is counter intuitive given that AD is a late onset sporadic disease and it is puzzling that this event appears to occur only in AD, but not in PD, since NO stress is also implicated in PD pathogenesis.

To resolve the controversy and to reconcile the conflicting reports, we studied the relationship between NO and DRP1 in AD (74). Contrary to the report by Cho *et al.* we found that S-nitrosylation of DRP1 does not increase DRP1 GTPase activity or dimerization. We show that DRP1 is neither a monomer nor a dimer under physiological conditions, but rather a tetramer in the human brain *in vivo*, capable of forming spiral- or ring-like oligomers *in vitro*. This observation is consistent with the DRP1 literature (70, 71). Further, S-nitrosylation did not increase DRP1 oligomerization (74). We know that our negative results were not due to artifactual over-oxidation of the DRP1 protein, because we readily detected SNO-DRP1. Thus, the cysteine residues were not blocked by oxidation and were accessible to S-nitrosylation by NO. Most importantly, we found no significant difference in SNO-DRP1 levels in post-mortem human brains of normal, AD, or PD patients. All human samples contained detectable SNO-DRP1 at similar levels (74). Last, OPA1, a mitochondrial fusion GTPase, was also S-nitrosylated in the same patient sample set (74). Thus, S-nitrosylation is not specific to DRP1. Taken together, our results provide compelling evidence that the mechanism underlying nitrosative stress-induced mitochondrial fragmentation in AD and likely other neurodegenerative disorders is not DRP1 S-nitrosylation and the true mechanisms remain to be discovered.

Potential mechanisms of NO-mediated mitochondrial fragmentation in neurodegeneration

Nitrosative stress impinges on a wide range of cellular and metabolic pathways, which can affect mitochondrial morphology and dynamics. For instance, nitrosative stress can directly inhibit mitochondrial respiratory chain complexes I and IV, causing bio-energetic failure (75–78). Thus, NO-induced respiratory inhibition might cause an instant decline in ATP synthesis and subsequent mitochondrial fragmentation, similar to other mitochondrial respiratory chain complex inhibitors such as rotenone (complex I) and 3-NP (complex II) (65, 79).

Another possibility is that nitrosative stress alters the microtubule organization derailing molecular motor proteins and resulting in a change in mitochondrial morphology, arrest in axonal trafficking of mitochondria, and subsequent loss of synapses due to depletion of mitochondria in nerve terminals. A loss of synapses and axonal organelle and vesicular trafficking has been well documented for AD and other neurodegenerative disorders (80).

Yet another potential mechanism of NO-mediated mitochondrial fragmentation in AD might be by tyrosine nitration of PPARγ, which impairs its translocation to the nucleus and thus prevents expression of NRF-1 and Tfam, and downstream mitochondrial proteins. Interestingly, PPARγ seems to play a role in activation of PGC1-α (81), which is in turn a key regulator of MFN2 (82). Thus NO-mediated inhibition of PPARγ may result in reduced MFN2 expression.

Nitrosative stress can activate signal transduction molecules such as kinases, which phosphorylate mitochondrial fission and fusion GTPases and thereby regulate their function. For example, Cdk1/cyclinB phosphorylates DRP1 at serine 616 and thereby promotes its translocation to mitochondria and fission (83). Importantly, we recently reported that NO

triggers phosphorylation of DRP1 at this serine, triggering its relocation to mitochondria (74). Thus, NO-mediated kinase activation is a plausible mechanism for activation of mitochondrial fission in AD and other neurodegenerative disorders, since cell cycle kinases are often abnormally activated in AD (84).

As mentioned above, nitrosative stress can result directly in inhibition of mitochondrial respiration and ATP synthesis. Low ATP levels can decrease the activity of the Na^+/K^+ ATPase pump resulting in membrane depolarization and activation of voltage-dependent Ca^{2+} channels (VDCC) (85). An increase in intracellular Ca^{2+} can then activate CaMKIα kinase or Ca^{2+}-dependent phosphatase calcineurin, causing increased mitochondrial fission (86, 87). Elevated Ca^{2+} may also activate proteases such as calpain, which could cleave mitochondrial fusion GTPases and inactivate their function.

These are but a few of the potential mechanisms that may account for the NO-mediated mitochondrial fragmentation. The range of possibilities underscores the tremendous complexity of the cellular response to nitrosative stress. It is conceivable that these pathways occur in parallel, vary in combinations dependent on cell type, and may mediate different cellular outcomes.

Conclusion

The roles of NO in human health and disease are many and our understanding of the physiological and pathophysiological importance of this molecule continues to grow. However, with increased knowledge comes an increased appreciation of the complexity of NO activity, which is dependent on many factors including cell type, relative abundance, identity of enzymatic synthase, and nature of reaction with target proteins. For example, studies have recently identified a new physiological role for NO in mitochondrial biogenesis. This finding represents yet another link between NO and metabolic function of the cell. In addition, there is compelling evidence that NO plays an important role in inflammation and obesity, fat cell metabolism, the body's response to caloric restriction, and mitochondrial dynamics, all of which are key factors in age-related disease. Because the toll of age-related diseases continues to increase in the developed world, it is critical that we continue to expand our understanding of the mechanisms underlying the pathophysiological activities of NO in these diseases. It is likely, due to the complex nature of NO we have highlighted here, that the pathogenic mechanism of NO in any disease is multi-dimensional rather than restricted to a single protein target. Thus, the path forward will require both continued target identification and integration of new findings into the growing network of NO physiological and pathophysiological activity in the human body.

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