Nitric Oxide and Oxidative Stress in Cardiovascular Aging

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The long-standing free radical theory of aging, which attributes cellular pathology to the relentless accumulation of reactive oxygen species (ROS), remains attractive but controversial. Emerging insights into the molecular interactions between ROS and reactive nitrogen species such as nitric oxide suggest that, in biological systems, one effect of increased ROS is the disruption of protein S-nitrosylation, a ubiquitous posttranslational modification system. In this way, ROS may not only damage cells but also disrupt widespread signaling pathways. Here, we discuss this phenomenon in the context of the cardiovascular system and propose that ideas regarding oxidative stress and aging need to be reevaluated to take account of the balance between oxidative and nitrosative stress.

Introduction

One of the most intriguing theories concerning the biochemical mechanisms underlying aging is the view that endogenously produced reactive oxygen species (ROS)—highly reactive molecules with unpaired electrons—increase in abundance with age, produce oxidative stress (OS), and in turn lead to cellular toxicity or impaired second messenger signaling (see "The Two Faces of Oxygen" at http://sageke.sciencemag.org/cgi/content/ full/2001/1/oa5) (*1*, *2*). Implicit in this theory is the idea that the more free radicals, of which superoxide (O_2^-) is prototypic, the greater the toxicity. However, our understanding of free radical signaling has evolved considerably with the more recent discovery of another important free radical molecule, nitric oxide (NO). The existence of interactions between O_2^- and NO calls for a change in our thinking regarding the effect of free radicals on aging. O_2^- (*3*) and NO (*4*–*7*) both contribute, alone and in combination, to OS and aging, but it is critical to appreciate that the relation between the degree of OS and the pathological consequences of aging is not linear, but rather that it is a disruption of the physiological balance between NO and O_2^- that leads to pathology. We illustrate this here in the context of cardiovascular disease, a pervasive corollary of advancing age.

Oxidative Stress and Aging

Free radicals were first described by Moses Gomberg (*8*) a little over a century ago, but it was not until the late 1960s, when McCord and Fridovich discovered the antioxidant enzyme superoxide dismutase (SOD) (http://sageke.sciencemag.org/cgi/genedata/sageke GdbGene;141) (*9*), that the importance of free radicals in biological systems gained credence. According to Harman's free radical theory of aging, published in 1956 (see Harman Classic Paper at http://sageke.sciencemag.org/cgi/content/ abstract/sageke;2002/37/cp14) (*1*), and the mitochondrial free radical theory of aging put forward in 1972 (*10*), accumulated damage to mitochondrial DNA wrought by free radicals in aging cells causes defective protein synthesis, progressive deterioration of cellular bioenergetic pathways, decreased oxidative phosphorylation, and reduced ATP formation. Ironically, as bioenergetic efficiency declines owing to free radical damage, increased electron leakage from the electron transport chain results in further free radical production (see Kristal Perspective at http://sageke.sciencemag.org/cgi/content/full/2003/5/pe3). Thus, damage to mitochondrial DNA caused by OS results in a self-perpetuating "vicious cycle" (Fig. 1) (*11*).

Based on earlier studies, it was hypothesized that O_2^- and other ROS, produced both by mitochondria and cellular enzymatic reactions, were responsible for OS-induced cellular damage (*12*, *13*). Observations indicating that reduced OS leads to increased longevity in laboratory animals support this contention, although the underlying mechanisms remain controversial in some cases [see Finkel (*14*) and Junqueira *et al.* (*15*) for recent reviews]. For

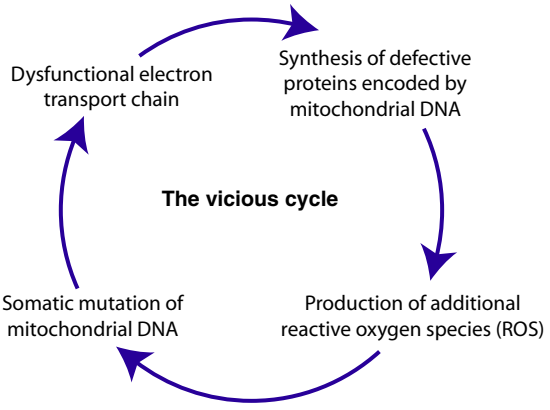

Fig. 1. The vicious cycle of oxidative stress. Free radical damage to mitochondrial DNA in aging cells causes defective protein synthesis, progressive deterioration of cellular bioenergetic pathways, and decreased oxidative phosphorylation, resulting in reduced ATP formation. Declining bioenergetic efficiency from free radical damage results in increased electron leakage from the electron transport chain, further increasing free radical production. Thus, damage to mitochondrial DNA by oxidative stress results in a self-perpetuating "vicious cycle" (*1*, *11*).

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example, reduction of O_2^- production by caloric restriction (see Masoro Review at http://sageke.sciencemag.org/cgi/content/full/sageke;2003/8/re2) in laboratory animals increases longevity and slows the characteristic changes associated with aging (*16*).

There is increasing appreciation of the general consequences of OS. For example, telomere shortening, associated with cessation of cell division and cellular senescence, is prevented by the enzyme telomerase (see "More Than a Sum of Our Cells" at http://sageke.sciencemag.org/cgi/content/full/sageke;2001/1/oa4 and Heist Perspective at http://sageke.sciencemag.org/cgi/content/full/ 2003/19/pe11). The catalytic reverse transcriptase subunit of telomerase, TERT (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;205), is inactivated by OS (*17*). OS also leads to DNA damage (see Skinner Review at http://sageke.sciencemag.org/cgi/content/full/2005/9/re3). Moreover, ROS trigger cellular apoptosis by releasing cytochrome c from mitochondria (*18*).

OS in the heart is associated with cardiac mechanoenergetic uncoupling, a divergence between energy use and the force of ventricular contraction (*19*–*21*). This is a primary mechanism by which both cardiac and skeletal muscle tissues fail, owing to an inability to convert energy supplies into mechanical work. This uncoupling is a characteristic of heart failure, a syndrome that now affects elderly individuals in epidemic proportions (*22*). A major source of OS causing mechanoenergetic uncoupling (*23*) is xanthine oxidase, a key enzyme involved in purine metabolism: Xanthine oxidase produces O_2^- as a by-product of the terminal two steps of the conversion of adenosine to uric acid (*24*). Up-regulation of this enzyme in the failing heart is associated with a progressive loss of cardiac contractility for any given level of oxygen consumption. These findings provide support for the concept that elevated ROS formation leads to cellular toxicity, thereby hastening cell senescence and death, and, conversely, the notion that strategies reducing OS may prolong life.

Nitric Oxide

In the myocardium, NO is produced by the enzyme nitric oxide synthase (NOS), which is present in three isoforms: neuronal NOS (nNOS or NOS1), inducible NOS (iNOS or NOS2), and endothelial NOS (eNOS or NOS3) (*25*). NOS1 and 3 are present constitutively and are calcium/calmodulin dependent, whereas NOS2 is induced in cells after stimulation by cytokines and other immunological agents (*25*) and is calcium independent by virtue of its increased affinity for calmodulin. Aging leads to increased expression of both NOS2 and NOS3 (*26*, *27*). There is also evidence of NOS1 up-regulation in aging rats following a myocardial infarction (heart attack) (*28*). NOS2 has been implicated as one of the main mediators of the hemodynamic and cardiovascular collapse that occurs with sepsis and heart failure (*29*), with deleterious effects on both β-adrenergic contractility, the increase in force of heart contraction stimulated by the sympathetic nervous system, and vascular tone.

NO, which is produced through oxidative deamination of L-arginine by NOS, exerts cellular effects through activation of soluble guanylyl cyclase to produce the second messenger cGMP and through direct posttranslational modification of proteins (*30*, *31*). S-nitrosylation, the transfer of NO to sulfhydryl moieties on proteins (*31*), is a ubiquitous mode of posttranslational modification akin to phosphorylation, with far-reaching effects in regulating protein function (*32*). S-nitrosylation is implicated in the regulation of more than 100 proteins and participates in the regulation of the cardiac ryanodine receptor (RyR) and possibly the L-type calcium channel. With regard to RyR, S-nitrosylation of a single cysteine residue increases the open probability of the channel: The nitrosylation/denitrosylation cycle is thought to occur rapidly so as to modulate the channel's activity within the time-frame of the cardiac cycle (*33*). The reaction of NO with sulfhydryl moieties to form RSNO, where R represents either a protein or a small molecule that can be nitrosylated (e.g., glutathione), is facilitated by the presence of electron acceptors (such as metals), which are abundant in biological systems.

NO and Oxidative Stress

The traditional view of the interplay between reactive nitrogen species (RNS) and ROS has been that the direct chemical interaction between these classes of molecule was the main determinant of downstream signaling and that the primary reaction depended mainly on relative rates of production and the ensuing concentrations of NO relative to O_2^-. Viewed by this rubric, NO would act either as an antioxidant or a pro-oxidant depending on its abundance, and conversely ROS would decrease NO bioactivity by chemically inactivating it (*4*, *34*–*37*).

However, the growing awareness of the critical role played by S-nitrosylation in modulating the activity of proteins participating in various signaling pathways offers new insight into the role of ROS in biology. Although it has long been appreciated that low concentrations of O_2^- facilitate RSNO formation (*37*), there is abundant new data to the effect that high concentrations may disrupt this signaling pathway either by directly oxidizing the relevant cysteine sites or by altering the permissiveness of the protein in question to SNO modification (*2*). Recent data suggest that RSNO formation can be regulated enzymatically by proteins such as ceruloplasmin (*38*), albumin (*39*), and even hemoglobin (*40*). A variety of electron acceptors in cell systems, such as iron nitrosyl complexes (*41*), can regulate S-nitrosylation as well.

In a series of recent studies, we have demonstrated that NO directly regulates an enzymatic source of ROS, namely xanthine oxidase. When the specific NOS inhibitor L-N^G-monomethyl-arginine (L-NMMA) is administered to animals with normal heart function, cardiac mechanoenergetic uncoupling results, owing to increased cardiac oxygen consumption relative to work performed. The production of ROS by xanthine oxidase is implicated in this phenomenon by the observation that mechanoenergetic uncoupling produced by L-NMMA can be reversed either by the xanthine oxidase inhibitor allopurinol or by the antioxidant ascorbate (*21*). Furthermore, in heart failure, where xanthine oxidase activity is increased and NO bioavailability is reduced, the restoration of myocardial efficiency observed in response to allopurinol was abolished by pretreatment with L-NMMA (*21*). Thus, heart failure represents a loss of balance between RNS and ROS formation.

New work from our laboratory further elucidates the mechanism for xanthine oxidase-NOS cross-talk (*36*). In this study, xanthine oxidase-mediated O_2^- production was substantially increased in the absence of NOS1. The resulting deleterious effect on myocardial excitation-contraction coupling in NOS1-deficient mice could be reversed with allopurinol. The converse of this situation has also been demonstrated: The NO /O_2^- donor 3-morpholinosydnonimine (SIN-1) (*42*) produces a positive inotropic effect (an increase in cardiac contractile activity) in whole hearts that is abolished by SOD, highlighting the importance of the physiological NO/O_2^- balance. There are other examples in which NO regulates ROS activity

through interactions with enzymes, including S-nitrosylation of thioredoxin, which augments its antioxidant properties (*43*), and the fact that several antioxidant enzymes—most notably copper/zinc SOD—can modify or be modified by NOS products (*44*).

Nitrosative Stress

When NO is produced in excess, either alone or in combination with ROS, a situation of nitrosative stress ensues that is akin to oxidative stress. Excessive NO may contribute to pathology, either by uncoupling electrons (*45*), reacting with ROS (*46*), inactivating antioxidant enzymes (*47*, *48*), or initiating apoptosis (*49*). The formation of peroxynitrite is one example of a direct interaction between NO and O_2^-, although the biological importance of peroxynitrite is unclear at present.

Regulation of Cellular Function by NO and O_2^-

O_2^- itself can produce biological effects similar to those elicited by NO. Although ROS are implicated as physiological signaling molecules in some cases (*24*, *50*, *51*), the following example demonstrates a situation in which NO leads to reversible effects, whereas O_2^- acts irreversibly to produce pathological consequences. In the heart, NO modulates β-adrenergic signaling and sarcoplasmic reticulum Ca^{2+} cycling and is therefore an important mediator of contractility. Through reversible protein nitrosylation of RyR (also known as calcium release channels), NOS1 increases Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum, thus optimizing contractile regulation (*33*). However, under conditions of elevated OS, RyR is maximally activated with loss of feedback inhibition, dysregulation of sarcoplasmic reticulum Ca^{2+} release, and consequent sarcoplasmic reticulum Ca^{2+} leak (*33*, *52*, *53*). The net result is an impaired ability of the heart to contract and relax in its normal cycle of systole and diastole and a diminished ability to respond to physiological stimuli that typically lead to increased cardiac contractility. In essence, ROS disrupt cardiac function by preventing normal regulation of ion channels through posttranslational modification.

Mitochondria are another site for NO/O_2^- modulation of cellular functioning. A major source of O_2^- production is mitochondrial respiration, particularly involving complex I and complex III of the electron transport chain (see Fig. 1 in Nicholls Perspective at http://sageke.sciencemag.org/cgi/content/full/2002/31/pe12/F1) (*16*). NO regulates mitochondrial respiration by reversibly inhibiting complex IV (cytochrome c oxidase), thereby reducing the consumption of oxygen and consequent O_2^- generation (*4*, *54*). This process is a good example of a situation in which small amounts of free radicals participate in physiological signaling. When there is a breakdown of this regulation, resulting in excessive NO or O_2^- production, the physiological balance is upset and pathological changes ensue (*4*).

These examples argue that it is the loss of balance between NO and O_2^- that is responsible for disrupted cell and organ function, not just a simple accumulation of free radicals (Fig. 2). Accordingly, we propose that redox balance in biological systems may be more appropriately considered in terms of nitroso-redox balance.

NO and Oxidative Stress in Cardiovascular Pathology

NO and O_2^- act within precise subcellular compartments, and disruption of this spatial confinement may lead to OS and disease. We and others have shown this to be critical in determining the precise regulatory effect that NO has within the cardiac myocyte.

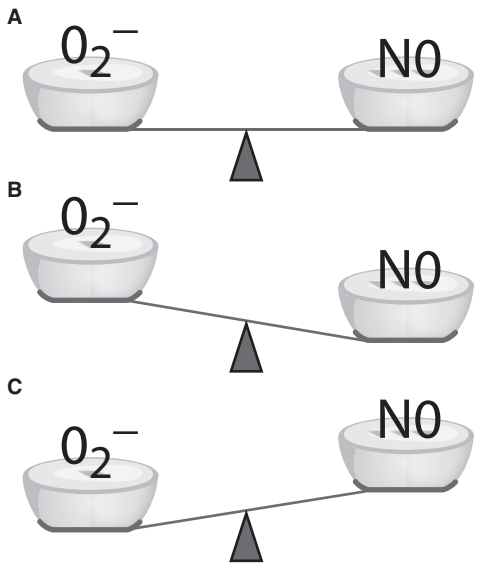
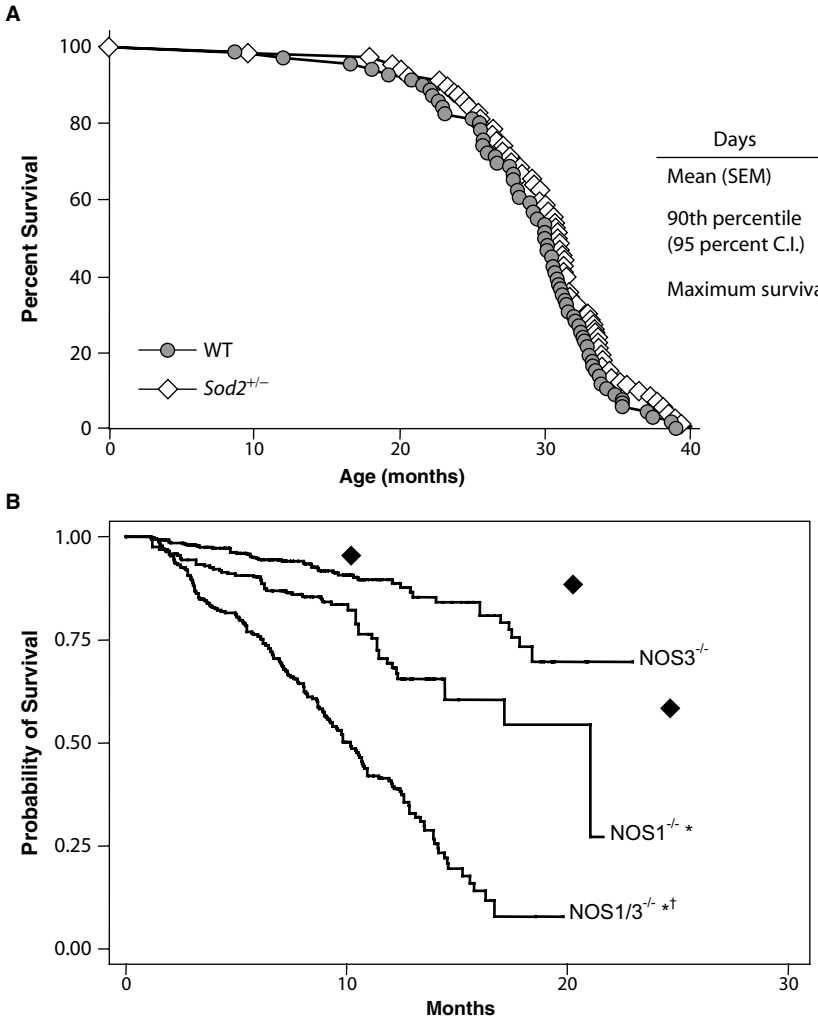


Fig. 2. A modified theory of aging. (**A**) Physiological balance between NO and O_2^- contributes to the maintenance of normal cellular signaling and function. (**B**) Excess O_2^- or depressed NO produces pathological effects owing to OS. Low concentrations of NO augment O_2^- production, exacerbating OS. (**C**) Nitrosative stress resulting from excess NO has deleterious effects similar to OS. The interaction between NO and O_2^- can result in the formation of even more reactive and damaging peroxynitrites. Additionally, increased NO contributes to OS by directly uncoupling electrons or by reacting with other ROS.

The two constitutive isoforms of NOS are compartmentalized within different organelles: NOS1 in the sarcoplasmic reticulum and NOS3 in caveolae, which are invaginations of cell membranes that serve to compartmentalize receptors involved in signal transduction. The NO produced by each isoform has opposite effects on excitation-contraction coupling and inotropy, determined primarily by its subcellular localization (*25*). Damy and colleagues demonstrated the importance of this spatial localization in heart failure (*55*), showing not only that increased NO production is due to up-regulation of NOS1 in the failing heart but also that NOS1 is translocated from its usual subcellular location in the sarcoplasmic reticulum to caveolae. The consequences of this translocation are highlighted by our observation that NOS1 deficiency in the sarcoplasmic reticulum leads to decreased reserves of Ca^{2+} in the sarcoplasmic reticulum, which in turn directly impairs the ability of the heart to contract (*56*). As previously mentioned, a likely mechanism for the loss of sarcoplasmic reticulum Ca^{2+} reserves is that diminished sarcoplasmic reticulum NOS1 not only reduces NO production but directly increases O_2^- formation via xanthine oxidase, which oxidizes the RyR and causes a leak of Ca^{2+} through that channel (*36*). Increased NO production at the sarcolemma may further depress contractility by increasing the inhibition of L-type Ca^{2+} channels, membrane ion channels that initiate the cardiac cycle by allowing extracellular Ca^{2+} to enter the cell and initiate sarcoplasmic reticulum Ca^{2+} release. In addition, NOS activation has been shown to result in compartmentalized RSNO formation (*57*).

Although NOS1 and NOS3 cause opposite effects on excitation-contraction coupling and hemodynamic parameters, deficiency of



Days	WT (n = 69)	*Sod2*$^{+/-}$ (n = 70)
Mean (SEM)	894 (19)	918 (19)
90th percentile (95 percent C.I.)	1034 (1002–1099)	1088 (1027–1154)
Maximum survival	1189	1239

Fig. 3. (**A**) Survival and life-span characteristics of mice lacking one copy of the gene encoding manganese superoxide dismutase (Sod2$^{+/-}$), a model of increased oxidative stress, as compared with wild-type mice. Figure and legend adapted from Van Remmen *et al.* (*79*) with permission. (**B**) Mortality in mice lacking NOS enzymes. In NOS1$^{-/-}$ mice, mortality exceeded that of NOS3$^{-/-}$ mice (RR 2.5, 95% C.I. 1.7-3.6). NOS1/3$^{-/-}$ animals fared even worse, with greater mortality than either NOS3$^{-/-}$ (RR 7.3, 95% C.I. 5.2-10.0) or NOS1$^{-/-}$ mice (RR 3.0, 95% C.I. 2.2-3.9). Survival of wild-type mice is shown by the solid diamonds, obtained from published data (*83*). (*, $P < 0.001$ versus NOS3$^{-/-}$; †, $P < 0.001$ versus NOS1$^{-/-}$). RR, relative risk; C.I., confidence interval. [Figure and legend adapted from Barouch *et al.* (*58*).]

either NOS1 (http://sageke.sciencemag.org/cgi/content/full/2003/29/tg5) or NOS3 (http://sageke.sciencemag.org/ cgi/content/full/2003/29/tg6) leads to cardiac hypertrophy in mice. Furthermore, loss of both isoforms produces a classical cardiovascular phenotype of aging with concentric left ventricular (LV) remodeling, in which markedly increased wall thickness is accompanied by reduced cavity size (*25*, *58*). This mouse phenotype is very similar to the hypertensive hypertrophic cardiomyopathy observed in elderly humans (*59*–*62*), which is an independent clinical predictor of mortality. This observation provides further evidence for the importance of NOS isoforms and NO in the pathophysiology of aging. Thus, changes in the net concentration or functionality of either the parent enzyme (NOS in this case) or free radical product (NO) can result in adverse events that appear ultimately attributable to the loss of the physiological NO/O_2^- balance.

NO and Apoptosis

Apoptosis, or "programmed cell death," is characterized by a set of distinctive cellular changes beginning with cell shrinkage and progressing to widespread nuclear and cellular fragmentation and hastened phagocytosis due to the exposure of novel cell surface molecules (*63*). Apoptosis is a recognized participant in the pathogenesis of both myocardial aging and heart failure (*64*–*66*). High levels of apoptotic activity have been observed in animal models of heart failure (*67*, *68*) and in the explanted hearts of patients undergoing cardiac transplantation (*69*). Indeed, even low levels of myocyte apoptosis are thought to contribute to the worsening of heart failure (*70*).

OS is known to be a trigger for cardiomyocyte apoptosis (see Kaminker Perspective at http://sageke.sciencemag.org/cgi/content/full/2004/8/pe8). Cesselli *et al.* examined the associa-

tion between OS and apoptosis in dogs with pacing-induced dilated cardiomyopathy: Events commonly associated with apoptosis, such as the expression of pro-apoptotic p66Shc (http://sageke.sciencemag.org/cgi/genedata/sagekeGdbGene;105) adaptor protein (see Friedman Perspective at http://sageke.sciencemag.org/cgi/content/full/2004/32/pe32) (*71*), the release of cytochrome c, and the activation of caspases, all occurred in response to OS (*66*). These changes preceded the induction of LV dysfunction, suggesting that apoptosis in response to OS contributes to the development of cardiac dysfunction and heart failure (*66*).

Excessive NO formation has also been shown to be a trigger for apoptosis (*72*). Conversely, however, protection against apoptosis can be achieved through NO-mediated gene transcription and translation (*73*), reiterating the idea that NO signaling pathways can be either protective or destructive depending on the context. Apoptosis can be prevented by up-regulation of heat shock proteins, cyclo-oxygenase-2, or heme-oxygenase-1, or by S-nitrosylation of caspases (*74*), which reduces their activity (*75*, *76*). The role of NO in apoptotic cell death is thus determined by the balance between two opposing NO-mediated effects and their relative relationship to OS. Furthermore, mitochondrial stress and Fas-associated apoptosis (an extrinsic pathway mediated by ligand binding to death receptors) appear to be mediated, at least in part, by release of S-nitrosylated caspases (3 and 9) from the mitochondrial intermembrane space (where S-nitrosylation protects them from degradation) to the cytosol, where denitrosylation leads to activation (*77*).

Oxidative Stress, NO, and Longevity

One of the major difficulties in providing definitive proof of the classical OS theory of aging has been the existence of confounding observations, as several mouse models of OS—animals deficient in various antioxidant pathways leading to increased sensitivity to OS—are not associated with reduced longevity (Fig. 3A) (*78*, *79*). It is important to note that NO signaling was not considered in these experiments. On the other hand, NOS1-deficient mice, in which the absence of NOS1 leads to up-regulation of ROS-producing enzymes and disruption of NO production both in terms of abundance and cellular localization (*36*), have substantially increased mortality (Fig. 3B) (*58*). Moreover, cardiac hypertrophy, which is observed in both NOS1- and NOS1/3-deficient mice, is a good predictor of frailty (http://sageke.sciencemag.org/cgi/content/full/2004/4/pe4) and early mortality (*80*–*82*). In this context, the observation of increased mortality in NOS1- and NOS1/3-deficient mice lends further support to the notion that loss of balance between ROS and RNS could be a key determinant in the aging process. The prevailing OS theory of aging cannot fully account for these findings, and we believe that further work is needed to determine the precise mechanisms by which this complex system is regulated.

Conclusion

Achieving a physiological balance between NO and O_2^- (nitroso-redox balance) is critical to the regulation of a variety of biological functions, including cardiac excitation-contraction coupling, mitochondrial respiration, and apoptotic cell death. Under physiological conditions, NO can reversibly modulate these diverse processes, and pathology results when the balance between O_2^- and NO is disrupted, resulting in oxidative and/or nitrosative stress. Here, we have used disorders of the aging cardiovascular system to illustrate the potential intricacies of this balance in general. Establishing the precise mechanisms that regulate the balance between OS and NO will be central to gaining better insight into cardiovascular pathology and ultimately into the aging process as a whole. These considerations offer new mechanistic insights into the manner by which ROS and RNS interact in biological systems and emphasize that spatial localization of the enzymes producing these species together with their target proteins is central. Both ROS and RNS participate in regulatory phenomena in which protein S-nitrosylation plays a primary role; excessive ROS production (which can be directly caused by NO deficiency) disrupts NO signaling not by chemical degradation but by disrupting the actions of NO at a target site on a regulated protein.

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