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Mitochondrial nitric oxide in the signaling of cell integrated responses

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Carreras MC, Poderoso JJ. Mitochondrial nitric oxide in the signaling of cell-integrated responses. *Am J Physiol Cell Physiol* 292: C1569–C1580, 2007; doi:10.1152/ajpcell.00248.2006.—Mitochondria are the specialized organelles for energy metabolism, but, as a typical example of system biology, they also activate a multiplicity of pathways that modulate cell proliferation and mitochondrial biogenesis or oppositely promote cell arrest and programmed cell death by a limited number of oxidative or nitrosative reactions. These reactions are influenced by matrix nitric oxide (NO) steady-state concentration, either from local production or by gas diffusion to mitochondria from the canonical sources. Likewise, in a range of ~30–200 nM, NO turns mitochondrial O₂ utilization down by binding to cytochrome oxidase and elicits a burst of superoxide anion and hydrogen peroxide that diffuses outside mitochondria. Depending on NO levels and antioxidant defenses, more or less H₂O₂ accumulates in cytosol and nucleus, and the resulting redox grading contributes to dual activation of proliferating and proapoptotic cascades, like ERK1/2 or p38 MAPK. Moreover, these sequential activating pathways participate in rat liver and brain development and in thyroid modulation of mitochondrial metabolism and contribute to hypothyroid phenotype through complex I nitration. On the contrary, lack of NO disrupts pathways like S-nitrosylation or H₂O₂ production and likewise is a gateway to disease in amyotrophic lateral sclerosis with superoxide dismutase 1 mutations or to cancer proliferation.

peroxynitrite; hydrogen peroxide; mitochondrial nitric oxide synthase; mitogen-activated protein kinase

MITOCHONDRIA PLAY A PIVOTAL role in cell physiology, producing the cellular energy and acting as signaling organelles. A mitochondria-based network elicits a multiplicity of effects and responses, depending on the course of cell life and on the environmental stimuli. This organization resulted from the selective pressure applied on eukaryotes to evolve the original endosymbiotic process between bacteria, i.e., *Cyanobacteria* sp., and the primordial prokaryote structure. Accordingly, contribution of mitochondria includes several cohorts of integrated biochemical pathways shared with nucleus and mainly directed to execute the different cell programs or with the plasma membrane to elicit the responses to environmental challenges. Interestingly, mitochondrial pathways involve a limited number of reactions; depending on the steady-state concentrations of substrates and products and on the rate of oxidative reactions, there exist dual or even opposite effects on cell responses.

NITRIC OXIDE IN MITOCHONDRIA

Mitochondria are the central organelles in cell bioenergetics. Most available oxygen is consumed in the electron transfer chain, placed in the inner membrane of the two membranes that limit the differentiated mitochondrial compartment. Electron transfer through mitochondrial complexes I, III, and IV is joined to proton pumping across the inner membrane, creating a proton electrochemical gradient between the intermembrane

space and the matrix. The gradient is dissipated by the reentry of protons through F₁F₀ ATP synthase channels (complex V), which couple ATP synthesis to the electron transfer activity. From a classic perspective, it is accepted that the rate of this process is regulated by O₂ and substrate availabilities as well as by the ADP-to-ATP ratio, likely the main mitochondrial modulator in response to cell demands.

During the past years, significant regulatory effects of nitric oxide (NO) on mitochondrial respiration became evident as resulting from its high-affinity binding to cytochrome oxidase (COX), the final electron acceptor (19). The synthesis of NO from L-arginine and O₂ is catalyzed by nitric oxide synthases (NOS) (92). There exist three canonical isoforms [neuronal (NOS I or nNOS), inducible (NOS II), and endothelial (NOS III)] and a significant number of spliced and posttranslationally modified variants. In addition, new isoforms or mitochondrial variants of NOS (mtNOS) were recently described in rat liver (60, 63), thymus (29), and brain (119); mtNOS is bound to mitochondrial PDZ domains of COX (57, 103) and to complex I (57), thus favoring a steric relationship between the released NO, vectorially directed to the matrix and inner membrane, and the control of respiration. In pathological conditions such as extreme hypoxia, mitochondrial NO could come either from stimulated NOS (116, 142) or from the reduction of nitrite by COX (39).

NOS I and III (45, 82) are constitutively expressed in different tissues, although their expression is tightly regulated. These enzymes are bound to anchoring proteins in cell membrane (caveolin) and in synaptic vesicles (PSD95) in skeletal and cardiac muscles (dystrophin) and to heat shock protein

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(HSP) 90 or 70 chaperones (79). The interaction with these proteins may involve different effects: 1) increased (HSP90-NOS) or decreased (caveolin-NOS) enzyme activity, 2) modified subcellular traffic (dystrophin impedes NOS I traffic to mitochondria) (76), and 3) participation in ubiquitination and degradation (54). Constitutive NOS are activated by Ca^{2+} pulses after activation of cell surface receptors by effectors like bradykinin or acetylcholine (endothelial NOS III) or excitatory amino acids like glutamate (neuronal synaptic NOS I) (45). NOS I and III are characterized by fast and transient responses to facilitate neural transmission or to induce a prompt vasodilation in response to changes in blood flow. In contrast, NOS II is not constitutive and does not depend on Ca^{2+} concentration; NOS II gene expression is modulated by inflammatory mediators, like cytokines TNF- α , IFN- γ , and LPS that activate transcription factors like NF- κ B or AP-1 (59). The mtNOS is nNOS- α translocated to mitochondria with specific posttranslational modifications such as myristoylation and phosphorylation of Ser-1412 in an Akt-dependent motif (RXXRXXS/T); it is constitutively expressed and Ca^{2+} dependent, and it is subjected to modulation by drugs or hormones or during development (21, 32, 34, 119).

The activities of classic NOS isoforms are able to sustain NO cytosolic concentrations large enough to reach mitochondria. The diffusion coefficient of NO in aqueous solutions is similar to that of O_2 , in $\sim 4 \times 10^{-6} \text{ cm}^2/\text{s}$ (143); activation of endothelium is compatible with a concentration in the arterial wall of 2 μM NO. Most NO binds to cytosolic compounds, like myoglobin (35, 111). Therefore, mitochondrial NO coming from classic cytosolic NOS results in a considerably lower concentration, ~ 20 – 100 nM (18, 111); however, it may increase by fivefold after induction of inducible NOS in endotoxemia (14). In this condition, both increased NO and NO-derived superoxide anion (O_2^-) favor the formation of intramitochondrial peroxynitrite (13).

In addition, new evidence supports the notion that mitochondrial NO steady-state concentration is also sustained by the constitutive activity of mtNOS (64). It is noteworthy that changes in the expression and activities of NOS isoforms, particularly of intra-mtNOS, will be followed by significant variations in NO steady-state levels in the relatively small and well-differentiated mitochondrial compartment.

OXYGEN AND NO UTILIZATION

In mitochondria, NO acts at different levels. As previously reported by us and others, in rat heart mitochondria (108), skeletal muscle, and liver mitochondria (48, 110) and synaptosomes (25), NO reversibly binds to Cu^{2+} B center of COX and consequently inhibits electron transfer to O_2 and mitochondrial O_2 uptake. The same effects were described by our group in the isolated rat heart (111) and by others in different organs (78) and in the whole animal, evaluated as systemic oxygen uptake (130). It is noteworthy that NO-dependent inhibition of O_2 utilization is achieved at very low physiological NO concentrations; 50–100 nM NO inhibits by half the activity of COX (108, 110).

An elegant computational study on mitochondrial NO effects was done by Antunes et al. (5). The authors simulated typical NO effects in experiments of inhibition of mitochondrial respiration, whereas the rate of O_2 utilization was evalu-

ated in a variety of conditions. At steady state and in the absence of NO, the rate of oxygen consumption predicted by the utilized model resulted in a hyperbolic function of O_2 concentration. The $K_{0.5}$ value is the O_2 concentration ($[\text{O}_2]$) that provides the half-maximal rate of O_2 uptake (also referred as K_{MO_2} and $[\text{O}_2]_{0.5}$) and indicates apparent affinity of respiration for O_2 . In this study, $K_{0.5}$ values for O_2 consumption in states 3 and 4 were 0.22 and 0.022 μM , respectively, in reasonable agreement with the experimental values (0.35–1.7 μM in state 3 and 0.08–0.6 μM in state 4) (20–65). In addition, the model predicted that, at the micromolar levels of oxygen found in tissues, the rate of respiration would be nearly saturated with O_2 (Eq. 1) (for details, see supporting text in Ref. 5)

$$-\frac{d[\text{O}_2]}{dt} = \frac{k_{\text{IV}}[\text{COX}]\text{tot}[\text{O}_2]}{k_{\text{IV}}/k_{\text{O}_2\text{app}} + [\text{O}_2]} = \frac{k_{\text{IV}}[\text{COX}]\text{tot}[\text{O}_2]}{K_{0.5} + [\text{O}_2]} \quad (1)$$

where brackets indicate concentration, $k_{\text{O}_2\text{app}}$ is apparent rate of O_2 uptake, tot is total, and k_{IV} is the rate of O_2 consumption in state 4. With respect to NO inhibitory effects, the main results obtained by the authors reflected that, at the same NO concentration, the degree of inhibition increased when the oxygen concentration decreased and that the degree of inhibition caused by the same concentrations of NO and O_2 was much more pronounced in state 3 than in state 4 (5). NO inhibited mitochondrial respiration at high ratios ($>1,000$) of O_2 to NO, and the onset of inhibition was extremely fast, occurring virtually immediately after NO addition. Therefore, mathematical calculation results were identical to those observed experimentally (16, 20, 24, 97, 137).

To evaluate whether the fast inhibition of mitochondrial respiration by NO represents a simple competition between NO and O_2 for COX, the formation of the complex between COX and NO was followed by the authors after addition of NO. As observed experimentally (62, 139), the formation of COX-NO occurred within a few seconds after NO addition. The time scale for the inhibition of COX by NO is given by $k_{\text{NOon}} \times [\text{NO}]$; at typical $[\text{NO}] = 10^{-8} \text{ M}$, a time scale of $\sim 1 \text{ s}$ was obtained, with higher NO concentrations (10^{-7} to 10^{-6} M) giving faster inhibitions (110). Therefore, the fast inhibition of COX by NO is completely compatible with a direct competition between NO and O_2 for COX; at the steady state and in the presence of NO, the rate of respiration is given by Eq. 2, which describes a simple linear competitive inhibition of COX by NO with an inhibition constant (K_i) given by $k_{\text{NOoff}}/k_{\text{NOon}}$ (k_{NOon} and k_{NOoff} are defined in supporting material of Ref. 5). Thus NO acts by increasing the apparent $K_{0.5}$, as observed experimentally (16, 20).

$$-\frac{d[\text{O}_2]}{dt} = \frac{k_{\text{IV}}[\text{COX}]\text{tot}[\text{O}_2]}{\frac{k_{\text{IV}}}{k_{\text{O}_2\text{app}}} \left(1 + \frac{k_{\text{NOon}}}{k_{\text{NOoff}}} [\text{NO}] \right) + [\text{O}_2]} = \frac{k_{\text{IV}}[\text{COX}]\text{tot}[\text{O}_2]}{K_{0.5} \left(1 + \frac{[\text{NO}]}{K_i} \right) + [\text{O}_2]} \quad (2)$$

As stated by Antunes et al. (5), the ratio of the respiration rate in the absence of NO to the respiration rate in the presence of NO, deduced by combining Eqs. 1 and 2, is

$$\frac{\text{Noninhibited respiratory rate}}{[\text{NO}] - \text{inhibited respiratory rate}} = 1 + \frac{K_{\text{NOon}} [\text{NO}]}{k_{\text{NOoff}} (1 + [\text{O}_2] k_{\text{O}_{2\text{app}}}/k_{\text{IV}})} \quad (3)$$

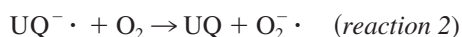
and the concentration of NO that inhibits mitochondrial respiration by 50% (IC₅₀) is given by Eq. 4:

$$\text{IC}_{50} = \frac{k_{\text{NOon}}}{k_{\text{NOoff}}} \left(1 + \frac{k_{\text{O}_{2\text{app}}}}{k_{\text{IV}}} [\text{O}_2] \right) = K_i \left(1 + \frac{[\text{O}_2]}{K_{0.5}} \right) \quad (4)$$

These calculations are useful for isolated COX, although they should be modified to include additional NO effects on other respiratory components, as occurs in vivo. Accordingly, NO inhibits COX and increases the reduction levels of the components of the electron transfer chain, including ubiquinol and ubisemiquinone, on the substrate side of COX and reacts directly with ubiquinol to produce nitroxyl anion (NO⁻) and ubisemiquinone (109, 110). A decade ago, we found that mitochondrial utilization of NO involves a similar inhibition of the *b-c1* region at complex III that increases the production rate of O₂⁻ and H₂O₂ (108, 109). Hence, the reactions of NO with components or products of the mitochondrial respiratory chain are, as in bacteria, reductive and yield NO⁻ or they are oxidative and yield ONOO⁻ (18).

NO UTILIZATION IS OBLIGATORILY LINKED TO THE MITOCHONDRIAL PRODUCTION OF OXYGEN ACTIVE SPECIES

Almost 20 yr ago, Boveris et al. (22) reported that mitochondria produce oxygen active species by auto-oxidation of intermediary ubisemiquinone (UQ), a transitional redox state of membrane ubiquinol. About 2–3% of utilized O₂ undergoes one-electron reduction by ubisemiquinone forming O₂⁻. In the presence of mitochondrial manganese superoxide dismutase (MnSOD), most of O₂⁻ is dismutated to H₂O₂, which is freely diffusible to cytosol.

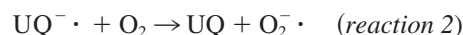
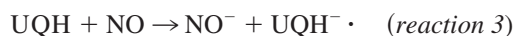


To sustain this notion, the ubisemiquinone pool and O₂⁻ production rate can be experimentally increased by the utilization of specific compounds like antimycin (complex III inhibitor), which blocks electron flow between cytochromes *b* and *c* (22).

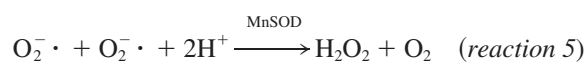
Considering that mitochondrial NO metabolism involves regulatory aspects on O₂ consumption and O₂⁻ and H₂O₂ production and the resultant effects of freely diffusible H₂O₂ outside mitochondria on gene expression and cell signaling, it is surmised that these activities markedly contribute to the mitochondrial modulation of life processes.

It is remarkable that the enzymes of the electron transfer chain show a different sensitivity to NO (108). Accordingly, at 0.3–0.5 μM, NO inhibits electron transfer between cytochromes *b* and *c1* in the respiratory chain (108, 111), whereas relatively prolonged 0.5–1 μM NO exposure selectively inhibits the NADH dehydrogenase activity at mitochondrial complex I in intact cells (50) and isolated mitochondria (120), a hallmark of neurodegenerative experimental or clinical entities like Parkinson disease. Finally, albeit controversial, some au-

thors believe NO is able to inhibit complex II activity, which probably depends on the time of exposure and the type of utilized mitochondria in the experimental setting (38, 133). As a consequence of the above-mentioned NO effects, it results in an increase in the reduction level of the mitochondrial components, which, in turn, favors the reaction of NO with ubiquinol and with complex I components. NO oxidizes ubiquinol with a second-order reaction rate constant of $2.3 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ (109, 110) to the respective semiquinone (reaction 3), which decays by auto-oxidation to ubiquinone, forming O₂⁻ (reaction 3 followed by reaction 2).



Finally, O₂⁻ reacts with NO to form peroxyntirite anion or dismutates to H₂O₂, a reaction catalyzed by MnSOD (reactions 4 and 5):



Considering the reaction rate constants of reactions 4 and 5 [2×10^{10} and $2.3 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$, respectively (56 and 80)], its relative participation in mitochondrial metabolism will depend on NO concentration and on MnSOD level. In agreement, supplementation of submitochondrial particles with complex I or complex II substrates increases by 10- to 20-fold NO utilization; in contrast, addition of superoxide dismutase (SOD) decreases NO utilization and prolongs its mean life and increases H₂O₂ (110). From these observations, it is noteworthy that 1) in mitochondria, most NO decays by reaction 4 and 2) depending on NO and MnSOD concentrations, mitochondrial utilization of NO involves the formation of H₂O₂, a species freely diffusible outside of mitochondria (30).

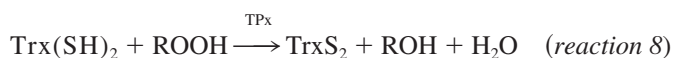
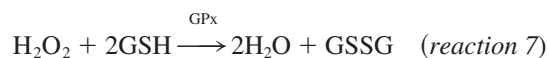
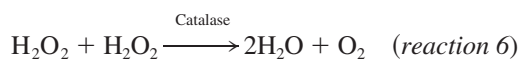
From this view, the modulation and activities of NO utilization pathways and mitochondrial NO, superoxide anion, H₂O₂, and peroxyntirite yield participate significantly in life processes. In the past decades, cumulative evidence has shown that H₂O₂ and the related oxidative stress level play significant roles in the activation of signaling pathways to control cell proliferation, differentiation, apoptosis, and senescence. Furthermore, redox status is clearly related to the activity of growth factors and to cell transformation and cancer. Our underlying proposal is that grading expression and activities of NOS isoforms and the concentration of matrix NO modulate H₂O₂ and oxidative stress. As described in cell transformation (71), concomitant changes in MnSOD have two effects: to increase cytosolic H₂O₂ and to prolong NO effects on mitochondria. These two effects are the principles of system biology that signal for different physiological or pathological responses.

PRODUCTION AND CELL STEADY STATE CONCENTRATION OF H₂O₂

The mitochondrial and cytosolic rates of formation and utilization of NO and derived oxidants and the calculation of their cell steady-state concentrations ([NO]_{ss} and [H₂O₂]_{ss}) are important because of their impact on cell processes. The

assessment of steady-state calculations implies the assumption that, in a determined period of time, the production and utilization of the compounds are equalized: $+d[\text{H}_2\text{O}_2]/dt = -d[\text{H}_2\text{O}_2]/dt$ and $+d[\text{NO}]/dt = -d[\text{NO}]/dt$.

The $d[\text{H}_2\text{O}_2]/dt$ is contributed by mitochondria and peroxisomes and to a lesser extent by tissue-specific reactions. Because peroxisomes contain the highest concentration of catabolizing catalase, mitochondria with very low levels of degrading enzymes are accepted as the main source of O_2 species (17, 33). The dismutation of O_2^- to H_2O_2 entails a two-electron reductive reaction catalyzed by compartmentalized enzyme isoforms. In microsomes, O_2^- is dismutated to H_2O_2 by Cu/ZnSOD, whereas in mitochondria the reaction is catalyzed by MnSOD. Otherwise, enzymes that catabolize H_2O_2 , catalase, glutathione peroxidase (GPx; reactions 6 and 7), and thioredoxin peroxidase (Trx; reaction 8) are preferentially distributed in cytosol and peroxisomes.



H_2O_2 is freely diffusible through cell membranes, and thus a similar mitochondrial and cytosolic $[\text{H}_2\text{O}_2]_{\text{ss}}$ should be expected. In agreement, $[\text{H}_2\text{O}_2]_{\text{ss}}$ has been calculated as $\sim 10^{-8}$ to 10^{-9} M in rat liver cytosol (40), rat liver mitochondria (40), and stimulated perfused liver (27), as well as after diffusion in hepatocytes (141). Thus cell or tissue $[\text{H}_2\text{O}_2]_{\text{ss}}$ could be calculated as the ratio between H_2O_2 mitochondrial yield and the catabolizing cytosolic enzyme activities.

In rat liver mitochondria, the physiological $[\text{NO}]_{\text{ss}}$ levels have been estimated to be $\sim 0.5\text{--}1 \times 10^{-7}$ M (18, 111). At physiological or even lower O_2 levels, it should be reasonable to expect less O_2^- production because O_2 uptake and electron transfer are proportionally lower. However, experimentally, the O_2^- production in living tissues at $5\text{--}10 \mu\text{M}$ O_2 is not far from that at the atmospheric level (12, 40).

At $0.1 \mu\text{M}$ NO, $+d[\text{H}_2\text{O}_2]/dt$ is $\sim 0.8 \times 10^{-6}$ M/s. Considering the reduction to H_2O by liver cytosolic GPx and catalase at the respective concentrations of 2.7×10^{-6} M and 1.2×10^{-6} M and the rate constants for reactions 7 and 8, the NO-dependent $[\text{H}_2\text{O}_2]_{\text{ss}}$ could be calculated as follows (17):

$$[\text{H}_2\text{O}_2]_{\text{ss}} = \frac{+d[\text{H}_2\text{O}_2]/dt}{k[\text{GPx}] + k[\text{catalase}]}$$

$$[\text{H}_2\text{O}_2]_{\text{ss}} = \frac{0.8 \times 10^{-6} \text{ M} \cdot \text{s}^{-1}}{\left[5 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} (2.7 \times 10^{-6} \text{ M}) + 4.6 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1} (1.2 \times 10^{-6} \text{ M}) \right]}$$

$$\text{NO-dependent } [\text{H}_2\text{O}_2]_{\text{ss}} = 0.4 \times 10^{-8} \text{ M}$$

Previous references indicated that, in liver, cytosolic $[\text{H}_2\text{O}_2]_{\text{ss}} = 0.8 \times 10^{-8}$ M (17); on the basis of experimental data and of the present calculations, H_2O_2 generated physio-

logically after NO mitochondrial utilization in liver accounts for one-half of its cytosolic concentration. These data emphasize the importance of mitochondrial NO metabolism in the generation of oxygen active species, particularly at near-physiological NO concentrations.

On these bases, the modulation of $[\text{H}_2\text{O}_2]_{\text{ss}}$ implies an effective regulation of the different pathways participating in the process, including the concentration and activities of mt-NOS, cytosolic classic NOS isoforms, MnSOD, catalase, and peroxidases. Thus most of the experimental evidence is based on manipulation of the related enzymes, either by disruption of specific genes, by transfection of encoding cDNA, or just by addition or subtraction of antioxidant enzymes, like catalase.

MITOCHONDRIAL NO AND REDOX METABOLISM ARE DETERMINANTS OF CELL DECISION CONCERNING PROLIFERATION OR CELL CYCLE ARREST

Although high levels of reactive oxygen species (ROS) are frequently associated with cytotoxicity, H_2O_2 mediates the transmission of many intracellular signals, including those related to growth and transformation. Hence, proliferating mammalian cells have different responses to oxidative stress, likewise depending on the stress level (52). Those grading effects of H_2O_2 have been reported by Antunes and Cadenas (4) in Jurkat T cells. Although the authors did not explore the proliferative phase, in these cells, very low ($0.7 \mu\text{M}$) H_2O_2 steady-state concentration represented the edge between survival and apoptosis, a level completely in line with that measured in different cell lines and tissues (17). It is noteworthy that after "in vitro" supplementation of exogenous H_2O_2 to cell cultures, antioxidant enzymes will set the $[\text{H}_2\text{O}_2]_{\text{ss}}$ to a considerably lower value in the intracellular milieu. In agreement and at a similar H_2O_2 concentration ($1 \mu\text{M}$), we observed an increase of the proliferation rate of PO7 lung tumor cells (58). In the same way and considering oncogenic transformation as a state of uncontrolled proliferation with no differentiation, H_2O_2 and oxidative stress have been accepted to have a major role (74, 135, 136). In an elegant study, Arnold et al. (7) showed that transfection of oxidase Nox1 to NIH/3T3 fibroblasts, which increases cell H_2O_2 levels by fivefold, resulted in a transformed phenotype with aggressive tumor formation. In this study, cells were reverted to a normal phenotype by cotransfection with catalase, and the authors concluded that a major role of H_2O_2 is to activate genes related to the proliferating cascade.

On the other hand, high H_2O_2 concentration ($100\text{--}200 \mu\text{M}$) induces temporary cell cycle arrest followed by overexpression of genes encoding antioxidant and protective enzymes in fibroblasts (113, 114). Even at higher concentrations ($250\text{--}400 \mu\text{M}$), H_2O_2 induces an almost permanent growth-arrested state but without cell damage.

Therefore, the progression of NO and H_2O_2 effects from proliferation to cell cycle arrest and differentiation arises in differential effects elicited by low and high cellular concentrations to related effects, such as 1) kinase activation or inhibition (112), 2) effects on cyclins (37), and 3) Ca^{2+} release (90).

It is noteworthy that effects of NO and H_2O_2 are confluent on modulation of MAPKs and cyclin D₁. MAPKs, including SAPK/JNK, p38 MAPK, and ERK, are believed to be redox-dependent biomolecules that modulate cell proliferation, sur-

vival, and apoptosis (41). ERKs stimulate cell proliferation and induction of active cyclin D₁ by different mechanisms, including the enhancement of AP-1 activity. In the same way, NO determines a gradual, moderate elevation of intracellular [Ca²⁺] and leads to activation of ERKs and potentiates cell division; functionally blocking Ca²⁺ or inhibiting calmodulin or MAPK activities prevents ERK activation and antagonizes the mitogenic effect of NO (90). On the other hand, p38 SAPK transcriptionally downregulates cyclin D₁. Casanovas et al. (37) reported that oxidative and osmotic stresses decrease cyclin D₁ activity and expression, the former by activating p38 pathway (37). This effect is due to phosphorylation of cyclin D₁ at Thr-286, which leads to the ubiquitination of the enzyme. The same effects were observed by Awad and Grappuso (8) who reported a temporal inverse correlation between activation of p38 MAPK and cyclin D₁ content during liver development or liver regeneration in the rat. Similarly, NO activates p38 MAPK and suppresses proliferation through the activation of JAK2-STAT5 and cyclin D₁/cdk4 (70). In accordance, the responses to shear stress of cultured microvascular endothelial cells are mediated by signal pathways involving both production of NO and activation of p38 MAPK pathway (93).

Considering the occurrence of reactions 2–5 in mitochondria, previously, our group (32, 57) attempted to connect oxidative stress and NO levels to MAPKs, D cyclins, and cell proliferation. Our studies confirmed that NO yield and the oxidative stress level modulate the different cell responses.

Both H₂O₂ and NO react with thiol groups of cysteine, methionine, or another amino acids that may modify the activity of tyrosine kinases, tyrosine phosphatases, and other proteins (10, 140). Another mechanism is the activation of specific phosphorylation cascades that participate in the progression of the cell cycle; for instance, H₂O₂ acts as a messenger in growth factor-induced p70(S6k) signaling pathway in mouse epidermal cells, which plays an important role in the transition from G₀/G₁ to S phase of the cell cycle (9). Finally, our group (2) observed ERK1/2 activation in brain mitochondria by very low concentrations of H₂O₂ (0.1 μM) but kinase inhibition when cells or isolated organelles were exposed to higher H₂O₂ concentrations (1–10 μM). It is not clear whether it implies a direct redox effect on the protein or actions on ERK-MEK (MAPK kinase) interactions. In this way, it was proposed that duration of MAPK activation determines whether a stimulus produces proliferation or differentiation (69). Activation of a myeloid leukemia cell line (TF-1a) by granulocyte/macrophage colony-stimulating factor induced only a transient activation of MEK and ERK1/2 and 50% increase in cell proliferation, whereas prolonged stimulation with 10⁻⁸ to 10⁻⁶ M PMA underwent 91–98% cell differentiation without proliferation. The findings gain significance considering that ERK1/2 are activated by ROS (134).

Antiproliferative effects are related to suppression of growth signals or potentiation of inhibitory signals and to resulting changes in cell cycle regulators such as p21, p27, cyclins, and retinoblastoma protein. The proapoptotic effects depend on translocation of Bax to mitochondria and generation of ROS and ultimately on the release of cytochrome *c* but in a caspase-independent manner. For instance, activation of p38 MAPK and cell cycle arrest may finally progress to apoptosis in the presence of NO, which may activate the p38 MAPK pathway. In accord, the p38 inhibitor SB-203580 blocks proapoptotic

effects of NO in SH-SY5Y neurons (94). The activating NO effects and p38 MAPK signaling probably result in Bax translocation to mitochondria, a well-known constituent of programmed cell death (61). Kurata (81) reported that low oxidative stress (20 μM H₂O₂) causes a rapid activation of p38 MAPK cascade, with phosphorylation of MKK3/6 and p38 MAPK and activating transcription factors (ATF)-1 (cAMP response element-binding protein) and ATF-2. These effects were associated with mitotic arrest and inhibition of cell division in phase M of the cell cycle during 24 h and were cancelled by *N*-acetylcysteine or SB-203580, a specific p38 MAPK inhibitor. Similar effects were described by Sahl et al. (125) who observed that H₂O₂-induced cellular injury depends on JNK activation. In this case, also p38 and ERK were activated by H₂O₂; however, only JNK-related injuring effects were inhibited by the MEK inhibitor PD-98059. Moreover, JNK phosphorylation of p53 is important for the stabilization of proapoptotic p53 protein (28). Early activation of p38 MAPK and ERK does not seem to be dependent on cytotoxic factors like intracellular Ca²⁺ movements. These results suggest that physiological H₂O₂-dependent activation of p38 MAPK may proceed many steps before a significant Ca²⁺ release from intracellular and mitochondrial stores and that Ca²⁺-dependent tyrosine kinase-induced activation of SAPK/JNK reflects the progression of cell injury (73).

NITRATION AND NITROSYLATION OF MITOCHONDRIAL COMPONENTS: THE THYROID MODEL

Depending on the antioxidant levels, the consequence of high matrix [NO]_{ss} is that nitrosative and nitrative (and oxidative) reactions are magnified in mitochondria. The main mitochondrial antioxidant defense is MnSOD. At the same SOD level, the production of ONOO⁻ (and concurrent nitration) at 0.02 or 0.2 μM NO will be completely different. Dismutation of O₂⁻ by SOD proceeds with an order of magnitude lower than formation of peroxynitrite (10⁹ vs. 10¹⁰ M⁻¹·s⁻¹) (80); thus it may hardly compete with ONOO⁻ formation at high NO levels.

NO has both pro- and antiapoptotic effects. Some antiapoptotic effects include *S*-nitrosylation and inhibition of caspases, increase of HSPs and Bcl-2, and activation of Akt/PKB, which induces cytoprotective gene expression through NF-κB activation (85). *S*-nitrosylation is the modification of critical cysteine residues on proteins; dysregulation of this process by increased NO production has been linked to pathophysiology (86). Accordingly, endogenous NO production inhibits apoptosis of pulmonary epithelial cells in culture (75) and the apoptosis induced by bleomycin (53, 124); inhaled NO protects against apoptosis after hyperoxia (68) or ischemia-reperfusion injury (145). On the other hand, proapoptotic effects of NO include inhibition of NF-κB, decreased Bcl-2 expression (87–89, 118), and increased p53 expression, both by NO-mediated inhibition of proteasome degradation and by direct DNA damage.

Recent reported data suggest that excessive NO production and protein *S*-nitrosylation may contribute to disease. For instance, the E3 ligase parkin is aberrantly *S*-nitrosylated because of increased NO production in Parkinson disease (47, 146). *S*-nitrosylation inhibits parkin activity, leading to the accumulation of misfolded proteins and to abnormal aggrega-

tion as Lewy bodies, which contribute to apoptosis of dopaminergic neurons.

On the other hand, as referred in *reaction 4*, nitration on tyrosine, cysteine, or tryptophan follows the formation of peroxynitrite or protein exposure to NO_2^- ; ONOO^- -dependent tyrosine nitration is a radical-radical reaction between tyrosyl and caged distributed NO_2^- and OH^- (129).

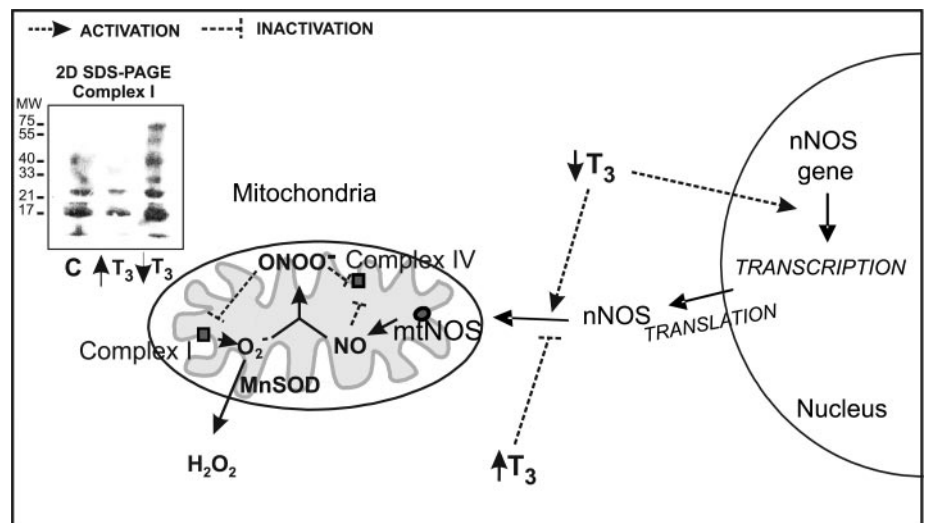
In mitochondria, most of O_2^- formation occurs in complexes III and I; however, other mitochondrial enzymes, like mono-amino oxidase, could contribute to ROS production (3). Thus, at high NO, ONOO^- formation is expected to specially proceed in those localizations. Clementi et al. (49) reported complex I inhibition in intact macrophages exposed to an NO donor, and we subsequently confirmed complex I inhibition and protein nitration in intact brain and liver mitochondria exposed to NO in a prolonged fashion. The inhibition of complex I limits the flow of electrons from NADH to the ubiquinol pool and markedly increases the O_2^- yield (36). However, it has been reported that, in intact mitochondria, rotenone inhibition of complex I did not increase ROS production with complex I substrates in myocardial ischemia (43). Similar to the NADH dehydrogenase site of electron leak forming complex I being directed into the matrix side of the inner mitochondrial membrane, in mitochondria, O_2^- can be detoxified by matrix antioxidant defense systems. Therefore, ROS production may differ substantially in mitochondria and corresponding submitochondrial particles that do not contain antioxidant enzymes.

To clearly exemplify mitochondrial effects of NO and the subsequent cohort of effects, we studied the hypothyroid condition. Thyroid status is crucial for energy homeostasis, and its physiological roles in growth and in organ and cell differentiation have been extensively studied. Thyroid hormone effects are exerted on mitochondria of specific target tissues like liver and skeletal muscle, whereas oxidative metabolism of other organs like brain is not affected (117). The action of thyroid hormone on respiratory functions has been considered to be operating at two levels: by altering mitochondrial inner membrane composition and permeability and by influencing respiratory gene expression. Thyroid status affects simultaneously the mitochondrial cytochrome content; in particular, cyto-

chrome aa_3 is strongly affected because it decreases by 80% in hypothyroidism, whereas it increases by 130% in hyperthyroidism (99). Changes in properties and composition of mitochondrial membranes, particularly in cardiolipin content, lead to variations of redox enzyme activities (72, 126) and in proton leak (23). However, although direct or transcriptional effects have considerable impact on oxidative metabolism, it is not defined how thyroid hormones set body metabolic rate. Considering NO effects on O_2 uptake, we analyzed the effects of thyroid status on mtNOS content (34, 57). At low levels of 3,3',5-triiodothyronine (T_3) in hypothyroidism, nNOS mRNA increased by threefold and nNOS translocation to mitochondria was favored with concomitant increase of mtNOS expression and activity. Two effects emerged from nNOS confinement. First, decreased O_2 consumption was more sensitive to L-arginine and to the NOS inhibitor N^G -monomethyl-L-arginine, indicating the modulation of O_2 uptake by mtNOS. Second, high matrix NO resulted in high O_2^- and H_2O_2 yields and to formation of peroxynitrite. Mitochondrial redox contribution to the activation of MAPK cascades was also confirmed in the hypothyroid model. Low T_3 -dependent mtNOS activation resulted in high oxidant production (H_2O_2 and peroxynitrite), with the concomitant activation of p38 MAPK and the inactivation of ERK1/2. As shown before, this MAPK pattern is consistent with cell cycle arrest and inhibition of cell proliferation, a hallmark of hypothyroidism. A similar effect of a NOS inhibitor [N^{ω} -nitro-L-arginine methyl ester (L-NAME)] or T_3 replacement in tuning hypothyroid cell signaling back to control status indicates that differential MAPK activation and cyclin D_1 expression should not depend on thyroid hormone themselves but on the relative production of mitochondrial oxidants, NO and peroxynitrite, at the different T_3 levels.

In addition, an extensive nitration of mitochondrial complex I proteins in this model (36, 57) was associated with a markedly reduced rate of electron transfer to ubiquinol acceptor, contributing to perpetuation of the process (Fig. 1) (57). Complex I derangement and decreased basal metabolic rate were reverted by previous administration of L-NAME to the hypothyroid rats. These findings suggest that most of O_2 consumption inhibition in hypothyroidism is the consequence of complex I inhibition by NO- ONOO^- overproduced by increased

Fig. 1. Transcriptional increase of neuronal nitric oxide synthase (nNOS) determines extensive binding to complex I and IV and contributes to hypothyroid phenotype. At low 3,3',5-triiodothyronine (T_3) levels, there is increased nNOS transcription, translation, and translocation into mitochondria, resulting in high mitochondrial NOS (mtNOS) activity and increased nitric oxide (NO), ONOO^- , and H_2O_2 levels, nitration of mitochondrial proteins, and inhibition of complex I and IV. *Top left*: nitration of components of mitochondrial complex I from control, hyper-, and hypothyroid rat livers. MW, molecular weight; MnSOD, manganese superoxide dismutase; 2D, two dimensional.



translocated nNOS (mtNOS). It is interesting that lack of T₃ stimulates nNOS gene expression, suggesting the existence of a tonic gene inhibition relying on effects of T₃ receptor dimers on the transcriptional machinery.

MITOCHONDRIA, CELL PROGRAMMED PROLIFERATION, AND NORMAL DEVELOPMENT

Cell proliferation is a complex programmed process responding to internal and periodical stimuli, to environmental ones, or both. Typically, growth factors elicit a cascade of events, including activation of kinases and transcription factors, that ultimately act on gene modulation by interacting with promoters or by increasing expression or activities of co- or transactivators and co- or transrepressors. To stimulate cell cycle transition from G₀ resting state to G₁ check point, cyclins are phosphorylated by cdk kinases (105, 131). Cyclin D₁ is implicated in the control of G₁ phase progression in hepatocytes and other proliferating cell types, and its expression is positively regulated by the ERK pathway and antagonized by stress-activated p38 MAPK cascade (84). During liver development, cyclin D₁ content is inversely related to p38 MAPK activity, which in turn may be regulated by ROS (81) and NO (104). NO may modulate per se the expression of cell cycle regulatory proteins (138); NO induces cytostasis by inhibition of cyclin D₁ or by inhibition of cdc2 (cyclin E and A pathways) (104). In accord, NO has antimetogenic effects on cultured hepatocytes (51).

During rat liver development, we observed that modulation of mtNOS and subsequent redox changes regulate MAPK cascades and cell cycle regulatory proteins in the sequence of proliferating to quiescent cell stages (Fig. 2) (32). Proliferating phenotypes were characterized by very low levels of mtNOS activity and expression, with a resulting NO-dependent [H₂O₂]_{ss} of ~10⁻¹¹ to 10⁻¹² M, and high cyclin D₁ expression associated with high ERK1/2 and low p38 MAPK activities. On the contrary, quiescent phenotypes presented an opposite

pattern with NO-dependent [H₂O₂]_{ss} of ~10⁻⁹ M. These differences are enhanced by the lower mitochondrial number per gram of liver tissue in proliferating tissues and paralleled the lower content of respiratory complexes and of antioxidant enzyme activities.

Similar effects of changes in [NO]_{ss} and [H₂O₂]_{ss} were observed in cell culture. Isolated neonatal hepatocytes supplemented with H₂O₂, catalase inhibitor 3-amino-1,2,4-triazole, or L-arginine invariably determined a dose-dependent negative modulation of cell proliferation. In contrast, decreasing cell H₂O₂ levels by controlled treatment with scavengers or NOS inhibitors, like N-acetylcysteine, glutathione, or L-NAME, increased proliferation rate up to 30%. Moreover, isolated hepatocyte proliferation rate may be modulated by MAPK inhibitors or stimulators like U-0126 (MEK inhibitor), SB-202190 (p38 inhibitor), or anisomycin (p38 activator), suggesting that hepatocyte proliferation signaling is related to a fine tuning of H₂O₂ level in the different developmental stages.

In this context, the synchronized increases of mitochondrial activities, mtNOS, and [H₂O₂]_{ss} operate on the balance of signaling pathways to drive the transition from proliferation to quiescence in rat liver development. In the same way, we recently reported that a critical reduction of mtNOS activity and [H₂O₂]_{ss} contribute to tumor-persistent (“embryonic”) behavior (58).

On the other side, an increase of mtNOS and H₂O₂ follows rat brain and cerebellum development, at the phase of synaptic plasticity (119). High mitochondrial NO and H₂O₂ yields are detectable over the last days of pregnancy and persist for ~10–12 days after delivery and for up to 20 days in cerebellum, with prolonged plasticity. This mitochondrial response is consistent with the arrest of neuroblast proliferation and with apoptosis, the two components of structural synaptic plasticity. Neurite growth and connection are the bases for the integration and differentiation of the neural anatomical network; however,

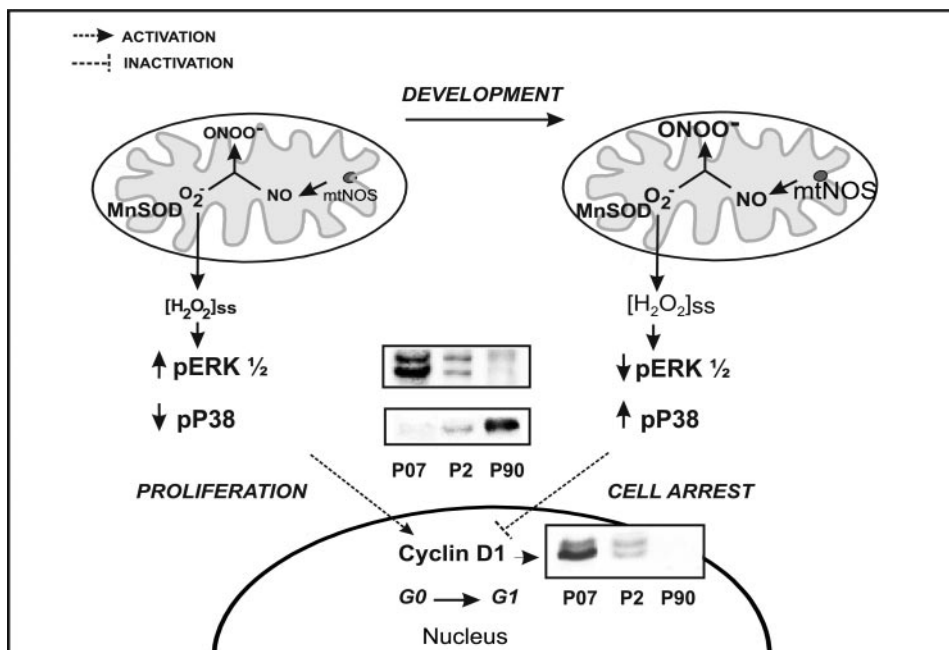


Fig. 2. Scheme of mitochondrial signals at two NO levels in the modulation of cell responses during development. In the proliferative stage, low NO and H₂O₂ levels activate ERK1/2, resulting in cyclin D₁-increased expression and proliferation. In the quiescent stage, higher NO, ONOO⁻, and H₂O₂ levels activate p38 MAPK, which regulates cyclin D₁ expression, negatively resulting in cell cycle arrest. Representative Western blot of cyclin D₁ and MAPKs in tumoral cells (P07) and in lysates of postnatal day 2 (P2) and day 90 (P90) rat liver are included. p, Phosphorylated.

apoptosis of nonconnected neurons is essential for this purpose.

NO, H₂O₂, AND CELL APOPTOSIS

In the past decades, one of the more fascinating events was the discovery of programmed cell death or apoptosis. The discovery of diverse apoptosis pathways involving signals primarily via the death receptors (extrinsic pathway) or the mitochondria (intrinsic pathway) using caspases as effector molecules has dominated the field. Caspases are cysteine proteases that cleave specific aspartate residues in other regulatory proteins like Bcl-2, Bax, and MEKK (for review, see Ref. 83). In the extrinsic pathway, binding of membrane ligands like TNF- α and Fas to membrane receptors triggers the activation of caspases and the induction of apoptosis (6, 106). In the intrinsic program, mitochondrial damage results in the release of cytochrome *c*, triggering the assembly of the apoptosome complex with apoptotic protease-activating factor-1 as central scaffold protein that directly recruits caspase 9, which in turn elicits caspase 3 effects (31).

It is noteworthy that mitochondria are clearly involved in the process of programmed cell death. Different agents that promote apoptosis are associated with mitochondrial changes. For instance, proapoptotic p53 protein induces gene transcription of redox-related genes encoding proteins that lead to oxidative stress (66). In addition, the increase of intracellular Ca²⁺ levels consecutive to cell injury or dysfunction may open the mitochondrial pore transition and drastically inhibit oxidative phosphorylation (107). The effects of oxidants may rely on the potential changes in thiol groups of caspases.

Oxygen active species and NO are now recognized as important regulators of apoptotic pathways (55, 66, 121). Proapoptotic activities of NO have been demonstrated in different cells and tissues (1, 46). NO can directly induce cytochrome *c* release through mitochondrial membrane potential loss (46) or by tyrosine nitration of cytochrome *c* (67). NO donors or endogenous NO induces apoptotic cell death with the activation of JNK/SAPK and p38 MAPK and caspase 3 or

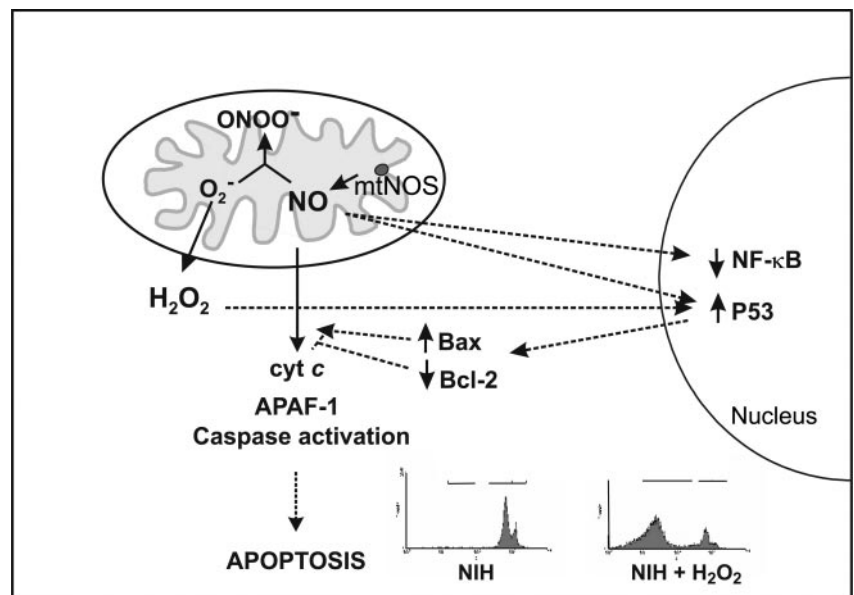
inactivation of NF- κ B. In this way, increased inducible NOS expression and NO production act as a negative regulatory feedback modulator of NF- κ B activity (102).

The stimulation or inhibition of NF- κ B may be related to proliferative or apoptotic effects. NF- κ B is activated by several agents, including cytokines, oxidant free radicals, inhaled particles, ultraviolet irradiation, and bacterial or viral products (42). Inappropriate activation of NF- κ B has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis, and acquired immunodeficiency syndrome. In contrast, complete and persistent inhibition of NF- κ B has been linked directly to apoptosis, inappropriate immune cell development, and delayed cell growth. Disruption of the NF- κ B gene is associated with embryo lethality; many antiapoptotic pathways like Bcl-2 are induced by NF- κ B. Accordingly, new therapeutic strategies have been developed in cancer treatment, including inhibition of the NF- κ B pathway (144).

Many proapoptotic effects of NO may be mediated by peroxynitrite as a by-product of NO and mitochondrial-derived O₂⁻ (15, 108) (Fig. 3). Cytotoxic effects of NO and peroxynitrite on tumor cells have been shown to be the result of DNA damage, which induces the accumulation of p53. NO-mediated p53 accumulation induces cell cycle arrest by p21 upregulation or apoptosis by increase in Bax/Bcl-xL, cytochrome *c* release, and caspase activation (100). Moreover, induction of apoptosis may require the fine biochemical interplay between oxygen and nitrogen species (99); the mechanism could be important in the step control and elimination of transformed cells (11).

On the other hand, NO has antiapoptotic effects that can be associated with cGMP production, which suppresses mitochondrial cytochrome *c* release, ceramide generation, and caspase activation (46, 77). Other antiapoptotic NO pathways include S-nitrosylation and inactivation of caspases thiols and upregulation of antiapoptotic genes like that of heme oxygenase (127) and Hsp70, which protects hepatocytes from apoptosis induced by oxidative and nitrative stress (46).

Fig. 3. NO and H₂O₂ release and activation of intrinsic programmed cell death via apoptotic protease-activating factor-1 and activation of p53. Increased NO and ONOO⁻ result in DNA damage, p53 accumulation, increased Bax/Bcl-2, cytochrome *c* (cyt *c*) release, and caspase activation. Bottom: representative histograms of apoptosis with propidium iodide of NIH/3T3 cells with and without exposure to 250 μ M H₂O₂ for 24 h.



THE LACK OF CELL AND MITOCHONDRIAL NO: A NEW GATEWAY TO PATHOLOGY

Deficient *S*-nitrosylation has also been linked to disease pathogenesis (128). Specifically, increased activity of the denitrosylase *S*-nitrosoglutathione reductase in asthmatic lungs depletes levels of the endogenous bronchodilator *S*-nitrosoglutathione, leading to airway hyperresponsiveness (115). Likewise, in sickle cell anemia, the sickle cell hemoglobin is deficient in the intramolecular and intermolecular transfer of NO moieties. Consequently, red blood cell membrane *S*-nitrosothiol levels are decreased and hypoxic vasodilation is impaired, leading to vasoocclusion in hypoxic tissue (101).

Recently, it was reported that deficient protein *S*-nitrosylation contributes to the development of amyotrophic lateral sclerosis (ALS); ALS is one of the most common adult-onset neurodegenerative diseases and is characterized by degeneration of motoneurons in the spinal cord, brain stem, and cortex (26), and 10–20% of cases are due to mutations in the *SOD1* gene (122, 123). Considering that *SOD1* mutations lead to an increase of the denitrosylase activity of the enzyme, the authors found that deficient *S*-nitrosylation can disrupt the functions of proteins that are regulated by this mechanism (like GAPDH) and that increased denitrosylase activity is a toxic gain of function of *SOD1* mutants.

In the same way but close to the mitochondrial field, we previously reported the existence of defective NOS and mt-NOS in mitochondria from tumor cells (58). Low NO levels were associated with low H₂O₂ yields, high proliferation rate, and activation of ERK1/2, a pattern resembling that shown in embryonic development (32, 44). Because proapoptotic p38 or JNK1/2 did not become phosphorylated, we surmise the results are part of the “reciprocal dance between cancer and fetal development.” In addition, different cell lines normally underwent proliferation arrest when supplemented with 10 μM H₂O₂ (52).

CONCLUDING REMARKS

Many years ago, we proposed that, depending on matrix concentration, NO is utilized in mitochondria with formation of H₂O₂ or peroxyxynitrite (108). Altogether, these species induce profound changes in normal or pathological cell processes. A moderately low level of these mitochondrial activities is compatible with proliferation and mitochondrial biogenesis (95, 98); accordingly, stem proliferating cells have a low number of mitochondria, with resultant low [H₂O₂]_{ss}. Also, embryonic and tumor cells share a distinctive pattern characterized by low mitochondrial and cell NO and H₂O₂ yields. A controlled decrease of mitochondrial respiration accompanies proliferation and tumor behavior (132). Likewise and depending on state 3-to-state 4 O₂ uptake ratios and ATP and nucleotide demands, control of cell proliferation by mitochondrial respiration acts by setting [H₂O₂]_{ss} with differential activation of MAPKs, Akt, and cyclin D. In this sense, very low NO inhibits COX and determines low H₂O₂ yield. Otherwise, selective and persistent high NO yields block complexes I, II, and III and further increase active species production in those sites and associates with cell arrest and/or with activation of the proapoptotic pathways. Interestingly, selective nitration of complex I components (57) could determine the final phenotype in some conditions, like hypothyroidism. On the other

hand, lack of mitochondrial NO drives mitochondria and cells to deregulate modulation of metabolism by *S*-nitrosylation or to restrain H₂O₂ increase, leading to the onset of diseases with high mortality rate, like ALS or cancer.

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