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The biological significance of mtNOS modulation

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TABLE OF CONTENTS

1. ABSTRACT

In the last years, nitric oxide synthases (NOS) have been localized in mitochondria. At this site, NO yield directly regulates the activity of cytochrome oxidase, O(2) uptake and the production of reactive oxygen species. Recent studies showed that translocated neuronal nitric oxide synthase (nNOS) is posttranslationally modified including phosphorylation at Ser 1412 (in mice) and myristoylation in an internal residue. Different studies confirm that modified nNOS alpha is the main modulable isoform in mitochondria. Modulation of mtNOS was observed in different situations, like adaptation to reduced O(2) availability and hypoxia, adaptation to low environmental temperature, and processes linked to life and death by effects on kinases and transcription factors. We present here evidence about the role of mtNOS in the analyzed conditions.

2. INTRODUCTION

Since 1996 different investigators proposed an association of nitric oxide synthase (NOS) with mitochondria. Early, Bates *et al* found endothelial NOS (eNOS) in mitochondria by immunoelectron microscopy (1). Later, two groups found NOS activity in the inner mitocondrial membrane (2,3), a fact reproduced in numerous studies (4-7). However, others could not detect NOS existence in the organelles (8,9) and therefore, some criticism emerged based upon the idea of contamination of isolated organelles with the cytosol canonical enzymes. This interpretation does not however rule out the notion about mitochondrial NOS (mtNOS), as shown by several related findings. It is clear that NOS is a low abundance protein in mitochondria; moreover, NOS is highly hydrophobic and results to be integrated in the lipophilic inner membrane; in some tissues, disruption of protein-

protein and lipid-protein interactions may be required to detect mtNOS. Furthermore, we and others described the effects of NO on heme and copper centers of cytochrome oxidase (COX) which produces a reversible inhibition of electron transfer to O_2 (10,11). In the physiological setting, matrix NO ranges between 20 and 100 nM, an steady-state concentration capable of inhibiting COX activity and O_2 uptake by 10-30 %. Association of NO to COX proceeds with a very fast reaction rate in the 10^8 $M.s^{-1}$ order (12), while dissociation is a relatively slower reaction (0.004 s^{-1}). In addition, NO effects include proportional production of superoxide anion (O_2^-) to react with NO producing peroxynitrite ($ONOO^-$) and to clear NO reversing the COX inhibition. It is then surmised that high mitochondrial NOS content conducts to NO excess and to high production of harmful $ONOO^-$ oxidant. Boczkowski *et al* showed that diaphragm muscle inducible NOS (iNOS) induction in endotoxemia leads to endogenous $ONOO^-$ production and mitochondrial protein nitration (13). In addition, deficit of NOS-binding dystrophin in heart of *mdx* mice induces loss of nNOS retention and translocation to mitochondria (5), probably contributing to energy deficit and to cardiac failure in muscle dystrophy. On the contrary, deficit of mtNOS and low production of reactive oxygen species could be a platform to sustain uncontrolled proliferative behavior in mice lung and mammary tumors (14); since NO stimulates mitochondriogenesis, low NO concentration impedes an increase of the number of mitochondria and of hydrogen peroxide (H_2O_2) levels characteristic of quiescent adult stage (15).

These findings proposed us a strict modulation of nNOS. As exposed in this chapter, mtNOS has to be carefully adjusted to evoke different responses with enormous significance in the course of life and death. The control of O_2 uptake is a mechanism to adapt ATP synthesis to demand and the efficiency of muscle contraction but also a signal for proliferation and for kinases activation or inactivation. The adjustment of mtNOS content makes necessary to observe its mitochondrial effects in a *particular physiological situation* and specially, out of stable conditions or with a challenge, like development, differentiation, hypoxia or cold environment.

3. nNOS TRANSLOCATION TO MITOCHONDRIA: THE mtNOS

It is now clear that mtNOS is nNOS alpha translocated to mitochondria with co –or posttranslational modifications. Elfering *et al* (16) demonstrated that mtNOS is myristoylated at the N-termini domain by an ester bond (not by classic glycine-NH_2 in an amide bond) as detected by mass spectrometry of fatty acids extracted from pure mtNOS with and organic solvent; differently to eNOS, mtNOS should not be palmitoylated. Usually, myristoylation indicates a targeting signal to subcellular structures, and could increase hydrophobicity and the attachment to the internal membrane and finally, its increased translocation to mitochondria. Instead, eNOS could be simultaneously acylated by myristic and palmitic acids through N-linkage. The findings could explain a different subcellular traffic of canonical NOS in cells. It is our feeling, that as shown by Bates *et al* (1) and Gao *et al* (17), wild type or mutated eNOS may be attached to mitochondrial outer membrane but hardly reaches the inner one. Likewise, we reported that iNOS attaches to mitochondria and accumulates in the intermembrane space, which suggest a differential contribution of mitochondrial translocon pathways.

In addition, MALDI-TOF studies performed by Elfering *et al* (16) showed mtNOS phosphorylation at C-termini. Similarly to eNOS, phosphorylation of Ser1412 at an protein kinase B (Akt) motif is a prominent feature of mice mtNOS and could therefore be linked to its mitochondrial import; previous studies reported the absolute requirement of phosphorylation for import of CYP2E1 (18), a phylogenetic ancestry of NOS with homology with their reductase domain (19). Since nNOS has no presequence for mitochondrial import, it is likely that internal hydrophobic motifs determine the traffic to the organelles in the same fashion that the electron transfer components of the inner membrane (20). Interestingly, once NOS is translocated to the inner membrane, it could interact with redox components of the electron transfer chain. We recently reported that mtNOS interacts with complex I proteins, according to our immunoprecipitation studies (21) and Persechini *et al* reported that mtNOS interacts with cytochrome oxidase through PDZ domains (22), this latter finding confirmed by ourselves in rat liver.

In addition, nNOS translocation depends on its interaction with cytosol-retaining enzymes. As mentioned, binding to dystrophin by PDZ domains is an important mechanism of stabilization of nNOS in skeletal muscle and heart (5). Although not defined yet, a role of heat shock protein (HSP) 90 and 70 as chaperones is expected in the control of NOS subcellular traffic; HSP90 is tightly bound to nNOS and increases its activity (23). It is then concluded that physiological or pathological modulation of nNOS translocation could involve a myriad of steps like myrystoyl transferases activities, Akt phosphorylation, PI3K activation, or expression of chaperones (Figure 1).

4. ADAPTATION TO O_2 LEVELS

Considering the regulatory properties of NO on O_2 uptake, a role of mitochondrial NO in energy conservative mechanisms is expected. In isolated mitochondria inhibited by L-NMMA and in absence of NO, O_2 uptake depends exclusively on available [ADP], [O_2] and [substrates]. In this condition, O_2 uptake is sustained up to very low [O_2] which means high mitochondrial oxidation at any available [O_2] (the *all-or -nothing* paradigm, *ref*. 24); likewise, this situation has some metabolic disadvantages: a) uncontrolled O_2 uptake rapidly decreases available O_2 and contributes to anoxia; b) efficiency of contractile tissues, like muscle or heart, represented by developed work to utilized O_2 is very low (25); and c) a maximal rate of O_2 uptake is associated to high uncontrolled production rates of O_2^- and H_2O_2 (2% of O_2 uptake is driven to formation of reactive oxygen species; *ref*. 26) contributing

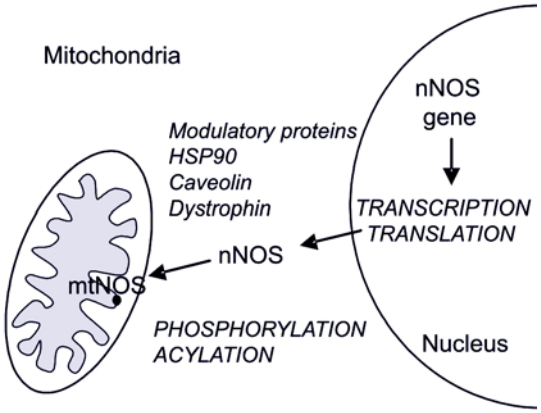

Figure 1. Scheme of the modulation of nNOS translocation to mitochondria.

to excessive protein and lipid oxidation. The presence of nNOS *within* mitochondria assures a continuous NO flux to the matrix. Moreover, although NO is as diffusible as O_2, cytosol eNOS or nNOS act intermittently in response to Ca^{2+} pulses elicited by autacoids or synaptic mediators; hence, inhibition of O_2 uptake is transient though probably adapted to adjust this variable to specific purposes, like modifying the neural resting potential and synaptic transmission. At same demands but in the presence of NO and in order to the respective association constants, physiologic COX activity and O_2 uptake are achieved at 5000 nM [O_2] and 20 nM [NO]; a ratio = 250 determines 10-20% inhibition of O_2 uptake and, inverse variations of [O_2] and [NO] further increase or decrease O_2 uptake, in accord to cell requirements (26).

The mtNOS-dependent modulation of O_2 uptake could be important in the distribution of blood flow. Many years ago, we suggested that NO yielding allows prolonging O_2 gradient stepwise to distal circulation (27). In accord, some works recently reported that $^{\cdot}$NO modifies the signaling consequences of hypoxia (28). During hypoxia, NO participates in the inhibition of mitochondrial respiration and promotes O_2 redistribution to the neighboring cells with stabilization of hypoxia-inducible factor 1-alpha (HIF) through a prolyl hydroxylase-dependent degradation. Recently, $^{\cdot}$NO has also shown long-term effects, leading to biogenesis of functionally active mitochondria in addition to its oxygen sensing function (29). Moreover, in hypoxic conditions $^{\cdot}$NO-dependent decrease of oxygen consumption is enhanced by mtNOS activity through the existence of higher Ca^{2+} levels in the matrix (30).

During hypobaric hypoxia, rat heart mtNOS participates in about 56% of total cellular $^{\cdot}$NO production; Valdez *et al* reported that mtNOS expression is selectively regulated by O_2 availability (31). At high altitude, myocardial mtNOS activity is selectively increased, while neither heart cytosolic eNOS, nor liver mtNOS were affected. Heart mtNOS activity exhibited a linear relationship with hematocrit at high altitude and it was suggested that it triggered a physiological and tissue specific adaptive response that upregulated heart mtNOS activity (32).

The actual evidences suggest us that $^{\cdot}$NO produces a sort of "Robin Hood effect" that saves oxygen in "rich cells" close to the circulatory bed while brings O_2 to the "poor cells" placed remotely from blood supply. This mechanism seems to be particularly useful in disturbances of O_2 delivery, like ischemia or shock with subsequent tissue reperfusion. In the hypoxia-reoxygenation conditions, accumulated NO in intact rat heart mitochondria inhibits O_2 consumption and ATP synthesis and, at physiological free Ca^{2+} concentrations, it protects mitochondria from secondary damage (33).

5. ADAPTATION TO ENVIRONMENT

In homeotherms, cold exposure to low ambient temperature is a stressful event requiring autonomic, circulatory and metabolic responses to overcome a life-threatening situation. In humans, the physiological responses to cold include changes of energy expenditure, heat production and dissipation, physical activity, and appetite (34). In rodents, the critical defense mechanisms include: shivering, activation of the sympathetic system (35) followed by development of brown adipose tissue (36), increased activity of mitochondrial uncouplers (UCPs) (37) and suppression of white fat leptin production (38). Abruptly exposed to cold, animals increase O_2 uptake and basal metabolic rate (BMR) for some weeks (34) but in some species, a marked lowering of O_2 uptake is observed during hibernation or torpor (39); it is here reminded that other conservative mechanisms like body insulation through accumulation of adipose mass contribute to cold tolerance. Considering that $^{\cdot}$NO modulates mitochondrial O_2 uptake and energy levels, we analyzed in a previous study the effects of cold exposition on expression and activity of liver and skeletal muscle mtNOS of rats living at 4°C during 30 days and the impact on BMR (40). Two periods were delimited during cold exposure: the first period extended from days one to ten, and was associated to high systemic O_2 uptake and weight loss and the second one, from days 10 to 30, with lowering of O_2 uptake and increasing fat deposition. In accordance, activity and expression of mtNOS diminished throughout the first week, but thereafter increased significantly by 60-100% in liver and skeletal muscle. Following NO, mitochondrial O_2 uptake remained initially high in the presence of l-arginine (mtNOS substrate) while further it resulted inhibited by 30-50%. We therefore proposed that, during rat cold acclimation, sequential modulation of mtNOS participated in the adaptive responses, initially favoring calorigenic, and thereafter the conservative energy-saving mechanisms.

Cold modulated translocation of NOS to mitochondria and, in consequence, the $^{\cdot}$NO matrix concentration (40) in liver and skeletal muscle that account for 45% of body mass and represent about 35-40% of total basal metabolic rate (41); it is noteworthy that matrix $^{\cdot}$NO participates in the modulation and distribution of total BMR (4) and that, $^{\cdot}$NO-dependent positive or negative variations of O_2 uptake are a consequence of the amount of

mtNOS operating at the mitochondrial level (4, 42). In the cold, the mitochondrial response to arginine paralleled the modulation of mtNOS, and liver and muscle mtNOS expression and activity shifted directly opposite to systemic O_2 uptake. The results suggest that mtNOS activity is critical for total basal metabolic rate and energy expenditure throughout cold acclimation. Calorigenic effects of different uncouplers like UCP-2, and UCP-3 could be operating early and could counteract the NO inhibiting effects on mitochondrial respiration. Accordingly, Giulivi *et al*, reported a marked decrease of liver mtNOS activity during mitochondrial uncoupling (3). It is then imaginable that NO and UCPs would interplay in mitochondria and that UCPs activity demand the concurrent participation of mtNOS. Though hypothetically we can conjecture that mtNOS expression could be influenced by UCP genes, it was perplexing that UCP-1 ablated mice are sensitive to cold but not obese (43).

The calculated contribution of liver plus muscle to the basal metabolic rate in the acclimation period dropped from 40% to 25%; if we assume that approximately 1.5 moles ATP per animal could be saved and considering that 7 moles of ATP per mol of synthesized triglyceride are required (41) they could be shifted onto fat deposition and weight gain in cold adaptation. Therefore, mtNOS-dependent inhibition of mitochondrial oxidative metabolism allowed a fraction of non-oxidized reduction equivalents to be mostly directed to fat synthesis, critical for thermal insulation and energy reserve. In cold acclimation, body adaptation was mainly powered by mtNOS-synthesized NO, avoiding an expensive energy fuel utilization to maintain isolation. On the other hand, regulation of O_2 uptake by NO could be important in those entities associated to an imbalance between energy intake and expenditure, like obesity (44), diabetes or hypertension (45, 46).

6. ENDOCRINE REGULATION

Different hormones participate in the regulation and balance of energy expenditure and conservation. Classically, it is accepted that thyroid hormones are important in the modulation of O_2 uptake. Many years ago, Sestoft indicated that increased BMR in hyperthyroidism may be accounted by the use of chemical energy for metabolic processes and work, and that major contributors are the heart work and futile cycling of free fatty acids into triglyceride in adipose tissue, whereas the maintenance of Na^+ and K^+ concentration gradients across the plasma membranes (main ATP utilization route) are unlikely to play any significant role (47). In this way, different investigators suggested that thyroid-dependent excessive O_2 uptake and heat production was a consequence of mitochondrial uncoupling of oxidative phosphorylation and of excessive H^+ leak. In addition, others reported a transcriptional increase of electron transfer components, as cytochrome oxidase and ATPase that have the major weight on the control of electron transfer rates; direct effects of thyroid hormones and iodothyronines should participate in fast non-genomic variations of mitochondrial O_2 utilization (48). In the same way, low O_2 uptake in hypothyroidism should be dependent on a decrease of mitochondrial cytochromes content and of cardiolipin required for a proper assembly of the respiratory enzymes (49). However, although direct or transcriptional effects have considerable impact on oxidative metabolism, it is not defined how thyroid set the body metabolic rate. Considering NO effects on O_2 utilization, we analyzed the effects of thyroid status on mtNOS content (21). Interestingly, at low 3,3´,5 triiodothyronine (T_3) level nNOS mRNA increased by three-fold and nNOS resulted translocated to mitochondria with concomitant increase of activity. Two effects emerged from NOS confinement. First, decreased O_2 uptake was more sensitive to L-arginine and to NOS inhibitor N^G monomethyl L-arginine (L-NMMA) indicating the modulation of O_2 uptake by mtNOS. Second, high matrix NO conducted to high O_2^- and H_2O_2 yields and to formation of peroxynitrite; in turn, complex I proteins resulted nitrated and a markedly reduced rate of electron transfer to ubiquinol acceptor was appreciated at that level. Complex I derangement was completely reverted by previous administration of N^{ω}-nitro -L-arginine methyl esther (L-NAME) to the hypothyroid rats, as well as the activation of antiproliferating kinases. On these bases, it is concluded that most of O_2 uptake inhibition in hypothyroidism is the consequence of Complex I inhibition by translocated nNOS. It is interesting that lack of T_3 stimulates nNOS gene which suggest the existence of a tonic gene inhibition relying on effects of T_3 receptor dimers on the transcriptional machinery.

7. mtNOS MODULATION IN DEVELOPMENT

Normal cell proliferation requires growth factors, which lead to orderly activation of regulatory proteins that control the transition through G1 phase of the cell cycle. After cells progress through the late G1 restriction point, they can proceed through the cell cycle, even in the absence of mitogens (50). In the adult animal, hepatocytes are highly differentiated and perform numerous essential metabolic functions while fetal hepatocytes proliferate vigorously but lack most mature liver functions. In normal liver, hepatocytes rarely undergo cell division, yet they retain a stem cell-like ability to proliferate in response to injuries that reduce functional hepatic mass, a feature that distinguishes them from other types of differentiated parenchymal cells. The proliferation and differentiation of hepatocytes are regulated by various external signals, such as hormones, cytokines, extracellular matrix, and cell-cell contacts (51). Hydrogen peroxide and the consequent oxidative stress level play an important role in the activation of signaling molecules which control the complex machinery involved in cell proliferation, differentiation, apoptosis and senescence (52). 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 extracellular signal-regulated kinase (ERK) pathway and antagonized by stress-activated p38 mitogen-activated protein kinase (MAPK) cascade (53). During liver development, cyclin D1 content is inversely related to p38 MAPK activity, which in turn may be regulated by reactive oxygen species (54) and NO (55).

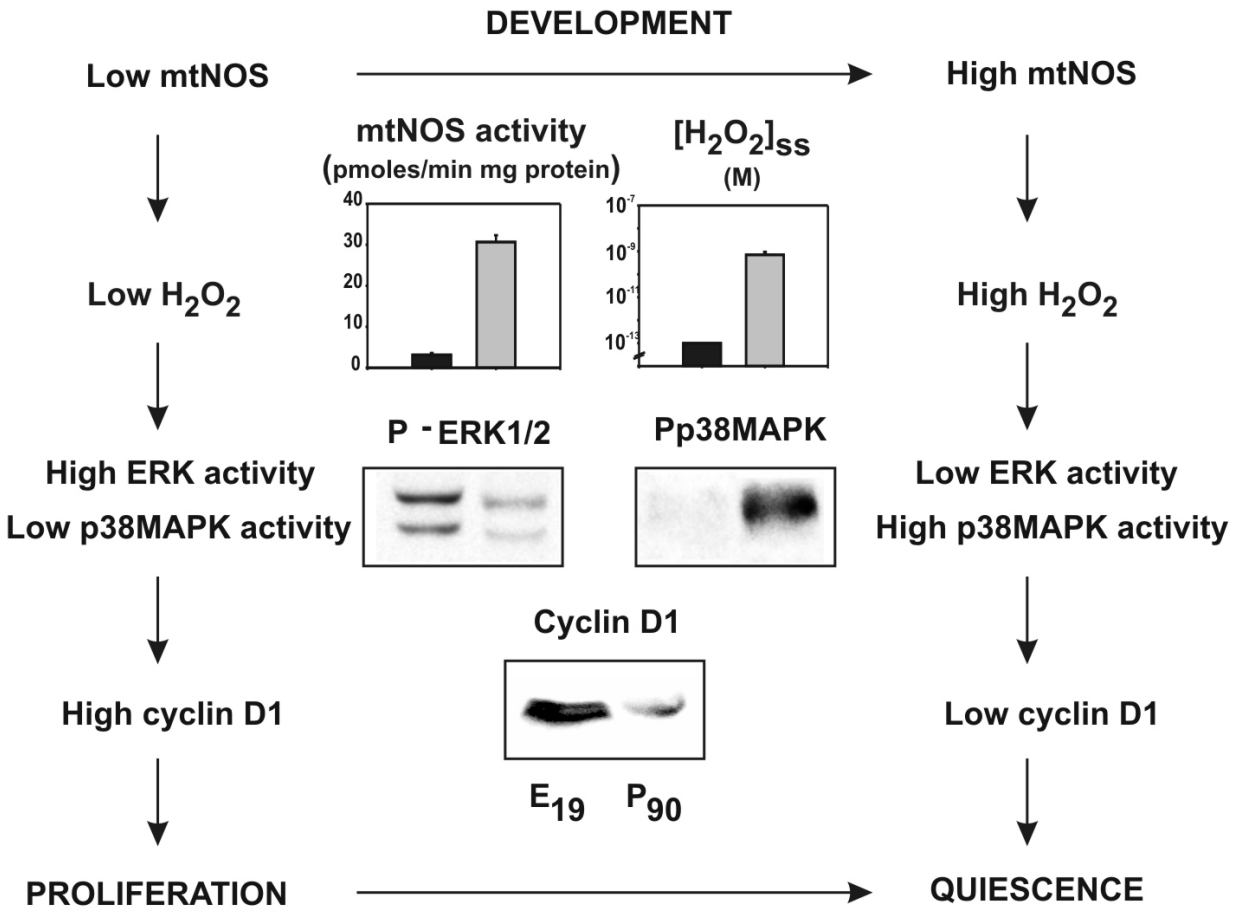

Figure 2. Scheme of the mechanism of the transition of proliferating to quiescent stages during liver development.

In this context, we have observed that during rat liver development, modulation of mtNOS and subsequent redox changes regulate mitogen activated protein kinase cascades and cell cycle regulatory proteins in the sequence of proliferating to quiescent cell stages (Figure 2) (15). Proliferating phenotypes seen in late embryonic stages (E17-E19) and perinatal days (P2-4) are characterized by very low levels of mtNOS activity and expression, with a resulting NO-dependent H_2O_2 steady-state concentration of 10^{-11} to 10^{-12} M, a high expression of cyclin D1 accompanied by high ERK1/2 and low p38 MAPK activities. On the contrary, quiescent phenotypes (postnatal days 15, 30-90) present an opposite pattern with NO-dependent H_2O_2 steady-state concentration of about 10^{-9} M. Interestingly, at the day of birth (E21), where there is a proliferation arrest, NO and H_2O_2 production are similar to the quiescent phenotypes, with the concomitant modulation of the MAPK cascades and cyclin D1. These differences are enhanced by the lower mitochondrial number per gram of liver tissue in proliferating tissues and are paralleled to lower respiratory complex and antioxidant enzyme activities.

Moreover, isolated hepatocyte proliferation rate may be modulated by changes in NO and H_2O_2 steady-state concentrations with NOS inhibitors (L-NAME) or H_2O_2 scavengers (glutathione, N-acetyl-cysteine), or by MAPK inhibitors or stimulators like U0126 (MEK inhibitor), SB202190 (p38 inhibitor) or anisomycin (p38 activator); suggesting that hepatocyte proliferation is related to a fine tuning of H_2O_2 level in the different developmental stages.

In this way, the synchronized increase of mitochondrial activities, mtNOS, and $[H_2O_2]_{ss}$ operates on the balance of signaling pathways to drive the transition from proliferation to quiescence in rat liver development.

8. BRAIN SYNAPTIC PLASTICITY

Neural development and maturation proceed in different pre and postnatal phases. During embriogenesis, brain development is characterized by intense proliferation that is almost arrested before delivery. Neurons and glial cells migrate to cortex and brain, cerebellar and stem nucleus. At the end of pregnancy and during the first 10-15 days after delivery, neonates exhibit an intense activity of neuritogenesis and synaptogenesis in the structural synaptic plasticity period where the neural network is configured. This process involves both neurite growing and the development of specific synaptic connections and apoptosis

of neurons that failed to connect adequately to other neurons and thus to integrate the network. Some authors considered ROS and H_2O_2 as a specific diffusible signaling molecules that modulate Ca^{2+} stores and the activity of protein phosphatases (56). These molecules also participates in dynamic plasticity, like learning and memory and are implicated in aging irrespectively of the existence of Alzheimer disease. Moreover, nNOS is also involved in synaptic plasticity and regulation of synapses (57), particularly the excitatory ones (58). In accordance, continuous administration of NOS inhibitors induced severe alterations of synaptic plasticity and memory in rats (59).

Considering that mtNOS modulates the mitochondrial release of oxygen and nitrogen reactive species, we previously studied the time-course of this enzyme during rat brain development (6). Immunological and functional observations are indicative of a developmental modulation of the brain mtNOS variant, a 144 kDa nNOS protein localized in the inner membrane. In this sense, mtNOS is highly expressed and active in the late stages of fetal development and during the first postnatal days followed by a decreased expression in the adult brain. Concomitantly, classic cytosolic nNOS was poorly detected in embryos or immediately after birth, and its expression increased sharply after postanatal day P6. This study established a link between NO metabolism and the generation of reactive oxygen species by mitochondria during development and a similar temporal pattern of brain mtNOS and Mn superoxide dismutase. The sequential activation of mitochondrial and cytosolic isoforms of nNOS and superoxide dismutase in the brain might play a role in synaptogenesis and synaptic remodeling that follows proliferation arrest. In support of this notion, mtNOS activity persisted until day 15 in the cerebellum in accordance with a longer period of proliferation and plasticity respect to brain. The fine modulation of brain 144 kDa mtNOS and mitochondrial reactive oxygen species during the perinatal period suggests an essential role of NO in the chronological phases of brain maturation and synaptic plasticity.

9. CONCLUDING REMARKS

In the modulation of the mechanisms by which nNOS is translocated to mitochondria relies the marked effects of mtNOS on energy conservation and life processes. Although some investigations proposed that mtNOS is almost undetectable, catalytic production of NO in a small compartment like mitochondria demands a finely adjusted enzyme content to avoid the edge between physiology and tissue damage. In this condition, mitochondrial translocation and activation of nNOS involves a multiplicity of functions like adapting O_2 delivery to O_2 uptake, the control of the production of reactive oxygen species, the modulation of signaling pathways for proliferation, differentiation or apoptosis, and the maturation of brain anatomy and functions. The connection of NO and mitochondria in mammals is an evolutionary pathway since bacteria, plants and invertebrates utilize it for different purposes like denitrification (*Paracoccus denitrificans)* or light emission (firefly).

10. AKNOWLEDGMENTS

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