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Mitochondrial nitric oxide synthase, mitochondrial brain dysfunction in aging, and mitochondria-targeted antioxidants ☆

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ABSTRACT

This paper reviews the current ideas on nitric oxide (NO) physiology in brain and other mammalian organs and on the subcellular distribution of nitric oxide synthases (NOS) emphasizing on the evidence of a mitochondrial NOS isoform (mtNOS) that exhibits a mean activity of 0.86±0.09 nmol NO/min×mg protein in 13 mouse and rat organs. Mammalian brain aging is associated with mitochondrial dysfunction, determined as decreased electron transfer and enzymatic activities and as an increased content of phospholipid oxidation products and of protein oxidation/nitration products. Brain mtNOS is the most decreased enzymatic activity upon aging; decreased levels of NO are interpreted as the cause of decreased mitochondrial biogenesis in aged brain. The beneficial effect of high doses of vitamin E on mice survival and neurological function are related to its effect as antioxidant in brain mitochondria and to the preservation of mtNOS activity. Mitochondria-targeted antioxidants, phosphonium cation derivatives and antioxidant tetrapeptides, are reviewed in terms of structures and biological effects.

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1. Nitric oxide and the intracellular sources of nitric oxide

Nitric oxide (NO) is a recent newcomer to the group of small molecules produced in biological oxidations, as superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2), that as normal metabolites with signaling functions are present in all aerobic cells from microorganisms to mammals. Inoue et al. have described the competition of NO and molecular oxygen (O_2) at the active center of cytochrome oxidase, the biological reduction of O_2 to O_2^-, and the interaction of NO and O_2^-, a reaction that annihilates the two free radicals, as a "cross-talk" between the three chemical species for the basic regulation of aerobic life [1]. Nitric oxide is a free radical considering the odd number of electrons in the molecule; however, from the chemical point of view NO is quite un-reactive as free radical; it does not participate in hydrogen abstraction reactions and only yields addition reactions with other molecules or chemical centers that also have unpaired electrons, such as O_2^-, O_2 and heme

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iron that, of note, are essential biochemical features of aerobic life. From a biological point of view, NO has dual effects. On one hand, as a signaling molecule and at low concentrations (10–150 nM), NO has physiological functions as an intracellular and intercellular regulatory messenger. On the other hand, NO behaves as a cytotoxic molecule at high levels (>300 nM) in pathological and pathophysiological phenomena [2]. In this sense, NO is similar to H_2O_2, both molecules are highly diffusible through biological membranes and suited at low concentrations for the role of cellular messengers and at relatively high concentrations are able to start free-radical dependent processes, likely by generation of hydroxyl radical (HO·) [3–5]. It is worth noting that the 1998 Nobel Prize in Physiology and Medicine was awarded to R.L. Furchgott, L.J. Ignarro and F. Murad for their discovery of NO as a signaling molecule in the cardiovascular system [6].

Nitric oxide is involved in numerous physiological phenomena in the circulatory, immunological and nervous systems. Considering the brain and the central nervous system, NO concentration gradients have been considered regulatory in intra- and extracellular neuronal compartments and, hence, in pre-synaptic, synaptic, and post-synaptic regions [7], exerting regulatory functions in brain synaptic plasticity [8], neurodegeneration [9–11] and aging [12–15]. It is well known that NO plays the central role in the regulation of circulation in small blood vessels and in the synaptic plasticity involved in cognitive processes and in memory by a soluble guanylate cyclase and cGMP-dependent mechanism [16–18]. For the NO cytotoxicity in neurodegenerative disorders, such as Alzheimer's and Parkinson's diseases, the favored hypothesis implies the formation of peroxynitrite ($ONOO^-$) with subsequent protein nitration and/or nitrosation [9–11]. Current claims for the NO mechanistic role in aging include situations of either NO hyperproduction or NO hypoproduction. On the one hand, an excessive production of NO by the inducible form of nitric oxide synthase (iNOS) due to pro-inflammatory responses has been considered a feature of neurodegenerative disorders and brain aging [12]. On the other hand, a decreased production of NO by the mitochondrial form of nitric oxide synthase (mtNOS) has been recently proposed as the cause of decreased mitochondrial biogenesis and turnover with direct implication in brain aging [13,14].

Nitric oxide is produced in biological systems by the general enzymatic reaction of nitric oxide synthase (NOS) [16]:

$$NADPH_2 + \text{arginine} + O_2 \Rightarrow \text{citrulline} + H_2O + NO$$

The NOS reaction is catalyzed by a family of enzymes in mammalian aerobic cells, codified in the human genome as neuronal NOS (nNOS, NOS-1), inducible NOS (iNOS, NOS-2) and endothelial NOS (eNOS, NOS-3) [16,19]. The three genomic NOS are localized as follows: nNOS in chromosome 12 [20], iNOS in chromosome 17 [21] and eNOS in chromosome 7 [22], with the understanding that there is no room for other NOS in the human genome, and that the NOS are expressed by transcription mechanisms with post-translational modifications that give a wide array of proteins that catalyze the NOS reaction. There is an ample expression of the three genomic NOS in mammalian tissues and for a while there was a belief that the three types of NOS were expressed in all mammalian aerobic cells. The common way of identification of nNOS, eNOS and iNOS in tissue slices, isolated cells and homogenates is by the use of the immunoreaction with anti-NOS antibodies directed to the three types of genomic NOS. However, there is a growing awareness that the specificity of commercial antibodies is highly variable and rather poor, considering the cross-reactions. Other distinctions between the enzymes members of the NOS family have been made based on their level in the tissues, on the Ca^{2+} requirement or on the subcellular localization [16]. According to their level in the tissues, NOS are divided in constitutive and inducible; the classical constitutive enzymes are the nNOS of the nervous system and the eNOS of the vascular endothelium, whereas the classical inducible enzyme is macrophage iNOS, considering that stimulated macrophages show 5–10 times enhanced levels of iNOS activity [16]. However, the constitutive nNOS and eNOS have been observed to also show important changes in their activities, usually in the range of 50 to 200%, caused by physiologically relevant effectors. Another distinction is made between the NOS that require calmodulin and Ca^{2+} for their activities (nNOS and eNOS) and the calmodulin and Ca^{2+}-independent isoform (iNOS) [16]. The third type of classification, more meaningful in terms of cell physiology, is made according to their subcellular localization, with mitochondrial (mtNOS) and cytosolic isoforms [8,23–27]. The cytosolic types are another family by themselves, considering the sarcoplasmic NOS of the heart and muscle [25–27], the endoplasmic reticulum isoform of liver [23] and thymus [28] and the NOS in the synaptosomes of the brain and central nervous system [8]. The mitochondrial and cytosolic types of NOS share the common property of being associated to membranes, with covalently attached fatty acids that anchor the enzyme to the membrane phospholipid bilayer [29]. It is apparent that all aerobic mammalian cells express two types of functional NOS, one isoform, the mtNOS, located in the mitochondrial compartment and the another isoform in the cytosolic space, as the eNOS of heart, muscle, liver and thymus and the nNOS of the nervous system (Fig. 1). The physiological function of this bipolar distribution of the NO-producing enzymes in the cell and the mechanisms that regulate NOS activities in mitochondria and cytosol are at the moment starting to be studied and a matter of speculation.

The regulatory actions of NO are currently explained by the physiological intracellular concentrations of NO, in the range of 10 to 200 nM, and three well known NO-dependent cell signaling and regulatory pathways. The first signaling pathway is the NO activation of the cytosolic and soluble guanylate cyclase that yields cGMP as second messenger and results in signal amplification and activation of cGMP-dependent protein kinases [16,18,30]. The second regulatory pathway is the reversible and O_2 competitive inhibition of mitochondrial cytochrome oxidase [31–37]. The third pathway are the NO-dependent protein modifications, mainly *S*-nitrosylation of cysteine residues [38,39] and nitration of tyrosine residues [40–42]. It has been observed that when the cellular NO concentration exceeds the physiological levels, as in the case of induction of astrocyte iNOS upon pro-inflammatory responses, NO-derived molecules of higher

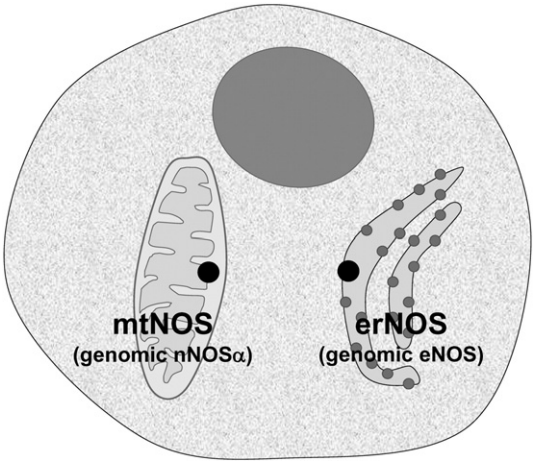

Fig. 1. Scheme showing the bipolar distribution of the NOS in mammalian cells. (a) mtNOS, identified as a mitochondrial inner membrane constituent protein with the sequence of the nNOS variant α phosphorylated and miristylated [29]; (b) erNOS, identified as an endoplasmic reticulum constitutive enzyme in liver [23] and thymus [28], as a sarcoplasmic reticulum and caveolae constitutive enzyme in the heart [26,86] and as a synaptosomal enzyme in brain [7]. The regulation of the activities of the mitochondrial and cytosolic NOS is considered an essential cross talk for cellular homeostasis.

chemical reactivity (reactive nitrogen species, RNS) are generated and impair protein function [43].

The NO-cytochrome oxidase regulation, referred above as the second pathway, establishes the critical principle of the *mitochondrial redox-energy axis* for cell function [44]. Nitric oxide has been recognized as a physiological inhibitor of cytochrome oxidase activity in mitochondria, whole cells and tissues. This NO action is of special interest in brain physiology because of the strong dependence of the brain on respiration and oxygen supply. The current knowledge is that oxidative phosphorylation is regulated in mitochondria by the availability of three small molecules, ADP, O_2 and NO, in the matrix and at the N-side of the mitochondrial inner membrane [45]. The ADP availability to F_1ATPase is the rate limiting step that sets mitochondria from the resting state (state 4) to the active state (state 3) with a markedly faster (4–10 times) respiration rate. In turn, O_2 and NO intramitochondrial concentrations compete for the active center of cytochrome oxidase and regulate oxygen uptake [45]. Slightly higher levels of NO exert an antimycin-like effect by inhibiting electron transfer at complex III, in the mitochondrial bc_1 segment of the respiratory chain, with concomitant production of O_2^- and H_2O_2 [34]. Interestingly, the NO higher levels that promote O_2^- production set a feed back mechanism that, by formation of $ONOO^-$ and removal of NO, releases the reversible inhibition of cytochrome oxidase [34]. These NO effects on mitochondria can be accomplished by cytosol-generated NO diffusing to mitochondria or by the activity of mtNOS.

Nitric oxide signaling involving protein post-translational modifications became relevant for mitochondrial physiology as an *in vivo* phenomenon after that proteomic analysis showed that rat aging is associated with an almost specific nitration of the Tyr^{269} of the β-subunit of F_1-ATPase [44]. This is not surprising considering that mitochondria are major sites of O_2^- production and the diffusion-controlled reaction of O_2^- with NO to yield $ONOO^-$ [3]. Hence, it might be surmised that mitochondrial formation of $ONOO^-$ would be favored over cytosolic formation of $ONOO^-$ by the NO diffusion, with no barrier between mitochondrial and cytosolic compartments, and by the about one order of magnitude higher concentration of O_2^- in mitochondria ($^{10-10}$ M) than in the cytosol ($^{10-11}$ M) [46]. An intramitochondrial steady-state concentration of about 2 nM $ONOO^-$ was calculated for the matrix, taking into account the rate constants of $ONOO^-$ reactions and the concentrations of mitochondrial $ONOO^-$ reductants (NADH, UQH_2 and GSH) [47]. Peroxynitrite is a strong oxidizing and nitrating species [3,4] and added to isolated mitochondria produces a marked inhibition of NADH-ubiquinone reductase (complex I) activity [48,49], a fact considered with implications for Parkinson's disease [50]. 3-Nitrotyrosine, a footprint of $ONOO^-$-mediated protein nitration has been reported elevated in several neurodegenerative disorders (Alzheimer's, Parkinson's, and Huntington's diseases [50,51]. Mitochondrial proteins, as ATP synthase, creatine kinase and aconitase are inhibited by nitration or $ONOO^-$-mediated oxidation of cysteinyl residues [44,52]. Peroxynitrite also induces mitochondrial membrane depolarization [53] and lipid peroxidation [54]. The modifications of mitochondrial proteins by oxidation, with formation of protein carbonyls [13,14,55], or by NO-mediated reactions (*S*-nitrosylation and nitration) lead to a mitochondrial dysfunction associated with decreased electron transfer, decreased inner mitochondrial membrane potential and decreased selective permeability with eventual induction of mitochondrial permeability transition [13,14]. These processes are recognized as direct causes of the mitochondrion-driven apoptosis which is made irreversible by cytochrome *c* release and activation of the downstream signaling apoptotic cascades [53,56,57].

2. Mitochondrial nitric oxide synthase (mtNOS)

The mitochondrial NOS activity was simultaneously reported in rat liver mitochondria by Giulivi, Poderoso and Boveris [58] and by Ghafourifar and Richter [59] in 1997–1998. For a few years there was a skeptical attitude towards this new member of the NOS family, since the current understanding at that time was dominated by the concept of the trilogy of the NOS, with nNOS, iNOS and eNOS, and by the ideas of "constitutive and Ca^{2+}-independent" versus "inducible and Ca^{2+}-independent" activities for the NOS. Indeed, there were reports that concluded the inexistence of mtNOS [60–64], confounding the absence of evidence in specific laboratories with the evidence of absence. The new enzyme was identified by its subcellular loclization in mitochondria and reacted mainly with antibodies anti-iNOS, but also with antibodies anti-nNOS, anti-eNOS, as reviewed in [60]. In a few years mtNOS activities were reported, besides the original rat liver mitochondria [23,58,59], in mitochondria isolated from rat and mouse brain [65–67], heart [26,68,69], kidney [70], thymus [24] and skeletal muscle [25,71,72] with activities similar to those of liver mitochondria and then the idea of contamination faded away, because it seemed unreasonable that very different tissues would show a similar level of contamination of the mitochondrial fraction with the cytosolic NOS of very different tissues. Some of the reported mtNOS activities, indeed the experimental evidence produced by a group of few laboratories, are listed in Table 1. An homogeneous mean value for 13 types of mitochondria isolated from rat and mouse organs is 0.86±0.09 nmol NO/min×mg protein. In this consideration, the activities measured by the radioactive assay of L-[^{3}H]arginine and the cases in which mitochondria were isolated from frozen tissue are not considered. It can be added that in April 2008, the mtNOS entry in the PubMed citation data base retrieved 97 research paper titles.

In 2002, Giulivi and co-workers in a seminal contribution [29] sequenced the mtNOS from rat liver mitochondria and identified the enzyme as an inner membrane protein and as the transcript of nNOS, splice variant α, myristylated and phosphorylated. The confuse antibody reactivity [60] is now interpreted as the result of the poor specifity of the commercial antiNOS antibodies and of cross reactions. In addition, processes of cytosolic recognition of the transcripts of the mRNA of nNOS by specific chaperones and of mitochondrial internalization of the pro-mtNOS protein are starting to be understood. Additional support for the mtNOS identification as derived from

Table 1
The activity of mtNOS in mitochondria isolated from mammalian tissues

Mitochondria isolated from:	mtNOS activity (nmol NO/min×mg protein)	Reference
Rat whole brain	0.67±0.04	[73]
Rat whole brain*	0.01±0.001	[65]
Rat brain cortex	0.66±0.04	[67]
Rat hippocampus	0.64±0.04	[67]
Rat liver	0.49; 0.75	[69,73]
Rat liver*	0.004; 0.074	[23,71]
Rat heart	0.69±0.04	[69,74]
Rat kidney	0.14±0.02	[70]
Rat ovary	2.5±0.2	[75]
Rat skeletal muscle	1.12±0.09	[71,74]
Mouse brain	0.63±0.05	[66]
Mouse liver	0.73±0.06	[66]
Mouse heart	0.76±0.05	[74]
Mouse kidney cortex	1.30±0.09	[74]
Mouse skeletal muscle (leg)	0.84±0.06	[74]
Human brain cortex (**)	4.0±0.4	–
Human colorectal carcinoma (initial stage)(**)	5.4±0.7	[76]
Human colorectal carcinoma (advanced stage)(**)	7.9±1.0	[76]
Human HaCaT keratinocytes (**)	13.8±0.7	[77]

The mtNOS enzymatic activity was mainly assayed by the spectrophotometric assay of haemoglobin oxidation to methemoglobin, as described by Boveris et al. [78] with slight modifications. In the cases in which the method used to measure mtNOS activity was the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline, this in indicated by an asterisk (*). Human brain cortex, data from Navarro and Ferrer. In the cases indicated by (**) frozen tissues were utilized to isolate mitochondria.

genomic nNOS was provided by Kanai et al. with the determination of the Ca^{2+}-induced NO release from a single mouse heart mitochondrion by using an electrochemical NO-detector, a process that was abolished in $nNOS^{-/-}$ knock out mice [79].

The biochemical properties of mtNOS are known; the enzyme catalyzes a classic NOS reaction in terms of substrates, products and K_M for the substrates and requires Ca^{2+} for full activity, similarly to the parental nNOS [80]. Brain mtNOS has been carefully identified as a 144 kDa nNOS variant localized in the inner mitochondrial membrane [65,81].

Crossed immunoprecipitation and kinetic evidence suggest, according to the supercomplexes model of the mitochondrial respiratory chain, that mtNOS is structurally attached to both complex I (NADH-ubiquinone reductase) and to complex IV (cytochrome oxidase) [82–85] (Fig. 2). The structural vicinity of mtNOS and cytochrome oxidase add to the consideration of a physiological regulatory role of NO in cell respiration.

Interestingly, the activity of mtNOS has been found regulated by important physiological effectors, such as environmental oxygen [26,27], autonomic regulation [86], angiotensin [68], thyroxin [23], and insulin [87]. In addition mtNOS is considered a key mediator of oxidative damage in ischemia/reperfusion [88] and a marker of brain aging, with younger animals showing higher activities [89,73], and with activities that correlate well with the decreasing neurological performances and with survival in aging mice [13,14,90].

The mitochondrial metabolic state regulates the rate of NO release in coupled mitochondria and mtNOS activity. The regulatory mechanism seems to be the membrane potential with mtNOS as a voltage-dependent enzyme and with a marked regulation at the physiological range of membrane potentials [91,92].

It has been claimed that the regulation of brain development in the pre- and post-natal period, understood as a process that evolves to establish the normal brain homeostasis in the adult brain, is determined by intramitochondrial O_2^- and NO steady state levels, as the result of the coordinated expression and activities of mtNOS and Mn-superoxide dismutase [65]. Further support for the significance of the mitochondrial redox-energy axis is the observed mitochondrial dysfunction with shortage of energy supply as a likely cause of brain dysfunction in aged hippocampus [13,67].

3. Aging and brain mitochondrial function

Mitochondria were brought to attention in aging biology due to the central role of mitochondria in producing chemical energy (ATP) to meet cellular requirements and to the declines of basal metabolic rate and of physiological performances which are characteristic of aged mammals. Aging is accompanied by a general decline of physiological functions with those functions that depend on the central nervous system being more affected [93]. In a series of mice strains, the higher quality of the integrated responses of the central nervous system were associated with increased life spans and with decreased neurodegeneration [93,89].

Mitochondrial oxidative phosphorylation is a process that encompasses electron transfer between the complexes of the respiratory chain, vectorial H^+ release into the intermembrane space, and H^+ re-entry to the matrix through F_0 with ATP synthesis by F_1-ATP synthase. An age-dependent impairment of mitochondrial function may be due to either decreased electron transfer, or increased H^+ permeability of the inner membrane, or decreased H^+-driven ATP synthesis.

There were speculations about increased H^+ permeability of the inner membrane in aging animals [94] with reports of unchanged [66] and decreased respiratory control [44] in brain mitochondria from aged rats. Aged rats show decreased membrane potential in cortical and striatal mitochondria [95] and in whole brain mitochondria [96], facts that are now interpreted as mainly due to decreased electron transfer.

The H^+-driven ATP synthesis in aging rat brain was reported slightly decreased, as indirectly estimated from the ADP/O ratios determined in coupled brain mitochondria [66]. At variance, a significantly decreased activity of F_1-ATPase was observed in brain mitochondria isolated from aged rats [44].

A common observation in mammalian aging studies is the decreased electron transfer in mitochondria isolated from old animals [13,94]. In the usual aging studies, three experimental groups of mice or rats are used: young animals at full adulthood (20–24 week old), old animals (48–56 week old), and senescent animals (76–90 week old). The relationship that one rodent week equals to one human year is useful for the understanding of the significance of the data. Mitochondria isolated from brain, liver, heart and kidney of old and

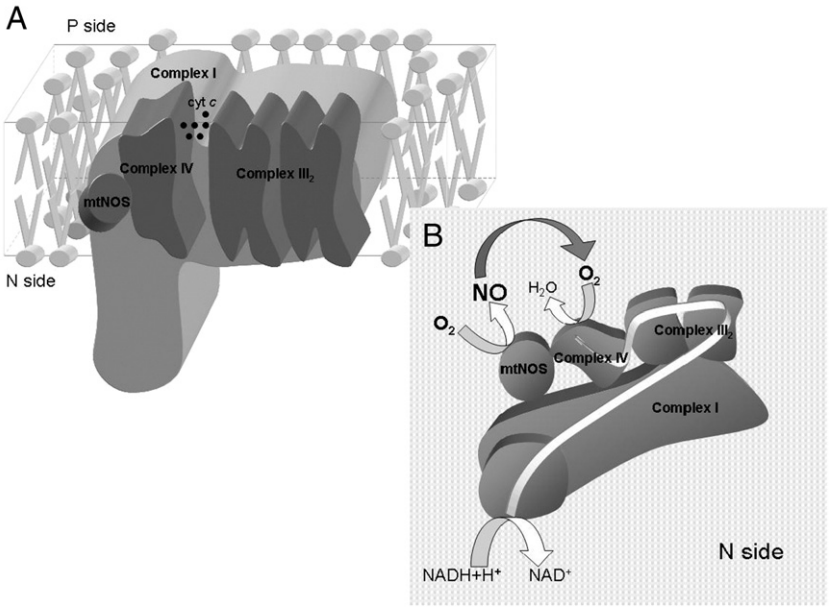

Fig. 2. Solid state model of mtNOS and the supercomplexes of the mitochondrial respiratory chain. Nitric oxide, product of mtNOS activity, regulates mitochondrial respiration by the reversible and O_2-competitive inhibition of cytochrome oxidase. The physiological activity of mtNOS is regulated by inner membrane potential and by the electron transfer through NADH-dehydrogenase. The supercomplex complex I-complex IV-mtNOS constitutes a hydrophobic domain that is sensitive to the mitochondrial oxidative damage characteristic of aging.

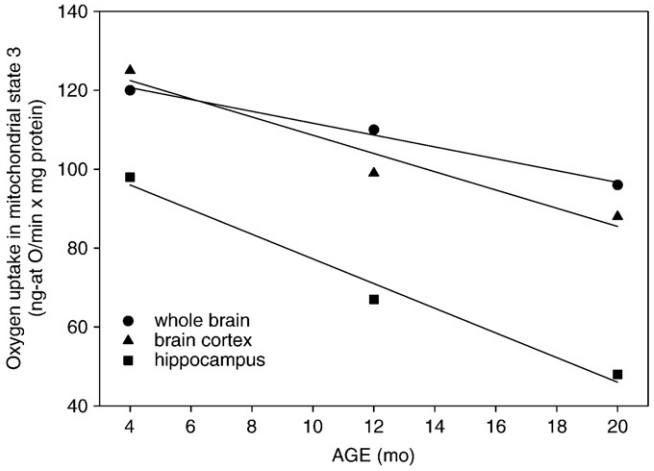

Fig. 3. Correlation between age and state 3 respiration in mitochondria from rat whole brain, brain cortex and hippocampus. Mitochondrial O_2 uptake was measured with a Clark electrode in a 1.5 ml chamber at 30 °C in an air-saturated reaction medium consisting of 230 mM mannitol, 70 mM sucrose, 20 mM Tris–HCl, pH 7.40, 1.0 mM EDTA, 5.0 mM phosphate, 4.0 mM $MgCl_2$, and 0.5–0.7 mg mitochondrial protein/ml, at pH 7.40 [129]. Respiratory rates were determined with 5.0 mM malate-5.0 mM glutamate as substrates. State 3 respiration was established by addition of 0.50 mM ADP. Age and oxygen uptake in state 3: whole brain: $r^2=0.99$ ($p<0.01$); brain cortex: $r^2=0.95$ ($p<0.01$); hippocampus: $r^2=0.98$ ($p<0.01$).

senescent rats and mice show decreased electron transfer activity in complexes I and IV, whereas complexes II and III are largely unaffected [73,97]. The decreased activity of brain complex I is observed as a decreased NADH-cytochrome c reductase activity with a simultaneously unchanged succinate-cytochrome c reductase activity in mitochondrial membranes and as a decreased mitochondrial respiratory rate in state 3 with malate- glutamate and other NAD-dependent substrates (Fig. 3) with a less diminished respiratory rate with succinate [66,98]. Moreover, the inhibition of complex I activity upon aging should occur with increased reduction of complex I flavoprotein and with an increased rate of O_2^- generation by flavoprotein semiquinone auto-oxidation [46]. The decreased activity of complex IV in aged mammalian brain was observed with the assay of the enzymatic activity in mitochondrial fragments [66,13,99] and with the histochemical assay of cytochrome oxidase in human *substantia nigra* [100] and rat hippocampus [101]. Mitochondrial enzymes such as complex I, complex IV, mtNOS [66] (Fig. 4) and carnitine acyltransferase [102] show about 40–65% decreased activities in senescent rodent brain and operate as useful markers of brain aging to study the effect of antioxidants in brain senescence.

Rat and mice brain aging are associated with clear signs of mitochondrial oxidative damage, as determined by the mitochondrial content of protein carbonyls and TBARS (thiobarbituric-acid reactive substances) that are directly proportional to animal age. Interestingly, in brain and other organs the mitochondrial content of oxidation products negatively correlates with the enzymatic activities of complex I, complex IV and mtNOS [13,14].

Northern blot analysis of mice brain mitochondria revealed an increased expression of mitochondrial genes for complexes I, III, IV, and V in 12- and 18-month mice as compared with 2 month-mice, suggesting a compensatory gene up-regulation with an over-production of electron transfer proteins that was exhausted in 24-month mice [57].

Interestingly, mice neurological function, determined by the tightrope test to evaluate neuromuscular coordination and by the T-maze test to evaluate memory and exploratory capacity, was markedly decreased upon aging and linearly related to brain complex I and IV enzymatic activities [13,66,73,97] and, as mentioned, negatively correlated with the mitochondrial content of brain lipid and protein oxidation products [103,89,14]. Applying the concept of rate limiting step in a complex system, it follows that the content of oxidation/nitration products operates negatively as a rate limiting step for mitochondrial enzymatic activity and energy supply in neurons which, in turn, are the rate limiting step for neurological function in physiological aging. The chain of biological phenomena: increased mitochondrial content of oxidation and nitration products, decreased mitochondrial function, neuronal dysfunction and apoptosis, and impaired neurological function clearly illustrates the convenience of the use of antioxidants that are effective in brain mitochondria.

4. Nitric oxide signaling for mitochondrial biogenesis

The NO signaling for mitochondrial biogenesis is emerging as a concept that has straightforward applications for cell turnover and proliferation in liver, heart and brain [104]. Regenerating liver and heart remodeling, as adaptation to chronic hypoxia or as recovery after infarction, are experimental and clinical situations that involve an active cell proliferation. Neuronal division and regeneration are theoretically possible from a genomic point of view and although recognized in very limited situations retain an extraordinary interest for an eventual use in neurodegenerative diseases. Mitochondrial biogenesis and turnover are processes that are included in and the precede cell turnover and proliferation.

Mitochondrial homeostasis, or in other words, mitochondrial turnover, is composed of two processes, mitochondrial biogenesis and mitochondrial death and disposal, a process that normally destroys dysfunctional mitochondria by inclusion in phagolysosomes. The phagolysosomal lysis of mitochondria does not affect the cellular cycle and is absolutely different from mitochondria-driven apoptosis. The normal mitochondrial turnover times are in the range of 7–40 days (liver) to 30–40 days (brain) and are faster than cell turnover.

The current knowledge of the molecular mechanisms that regulate mitochondrial biogenesis is that cytosolic NO, originated by either an extracellular NO donor, or by a cytosolic NOS [104] or by mtNOS [75], activates guanylate cyclase and cGMP production. The increased intracellular levels of cGMP, in turn, activate a series of transcription factors, such as PPAR-GC-1α, nuclear respiratory factors (NRF-1 and NRF-2) and mitochondrial transcription factor A that start the process of mitochondrial biogenesis. This mechanism is supported by observations in brain, kidney, liver, heart, and gastrocnemius muscle [104] and in ovary [75].

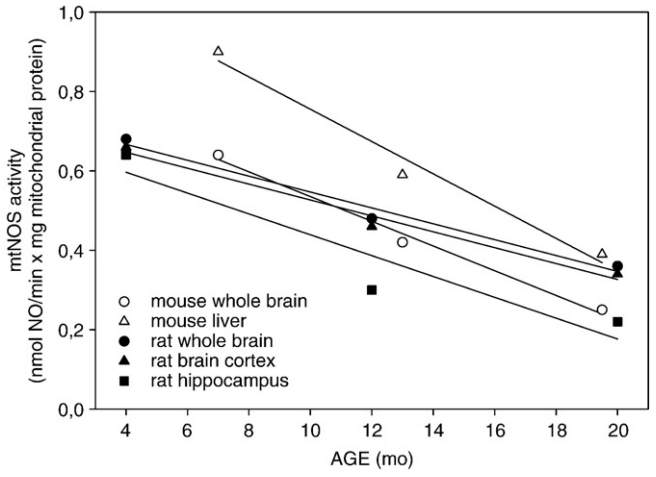

Fig. 4. Correlation between age and the mtNOS activities of mitochondria isolated from mouse whole brain and liver and from rat whole brain, brain cortex and hippocampus. Mitochondrial NO production was determined by the oxyhemoglobin (HbO_2) oxidation assay at 30 °C [78]. Age and mtNOS activity: mouse whole brain: $r^2=0.99$ ($p<0.01$); mouse liver: $r^2=0.98$ ($p<0.01$); rat whole brain: $r^2=0.98$ ($p<0.01$); rat brain cortex: $r^2=0.98$ ($p<0.01$); rat hippocampus: $r^2=0.89$ ($p<0.01$).

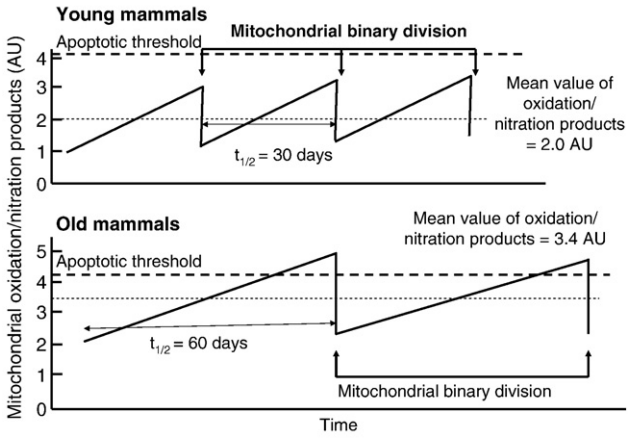

Fig. 5. Hypothesis of the time course of the levels of oxidation and nitration products, associated with mitochondrial turnover, in brain mitochondria as a function of time and age. AU=arbitrary units. For explanation, see text.

Oxidized and nitrated mitochondrial proteins are starting to be considered as inhibitors of mitochondrial biogenesis and as promoters of mitochondrial death and elimination through phagolysosomal recognition and internalization. The recognition of dysfunctional mitochondria is a process in which decreased membrane potential and oxidized proteins are likely recognized at the outer membrane surface. Mitochondrial protein carbonyls (products of protein oxidation) and TBARS (products of lipid peroxidation) are increased in mitochondria from brain and other organs of aged animals [13,14]. Recently, nitration of the Tyr^{269} of the β-subunit of F_1-ATPase has been observed in a proteomic study in aging rats [44]. This nitration was almost specific, considering the whole mitochondrial proteome, and was associated with a decreased ATPase activity [44]. The concept of mitochondrial aging can be summarized saying that mitochondria simultaneously respire, accumulate oxidation and nitration products and loose enzymatic activity. Physiological mitochondrial respiration entails simultaneous O_2^- and NO production that lead to free-radical chain processes with lipid peroxidation and protein oxidation and nitration [5,46]. The causative link between oxidation and nitration products and the inactivation of respiratory enzymes has been described [14,15]. The whole process of accumulation of oxidized and nitrated products, directly linked to mitochondrial respiration, is illustrated in Fig. 5 by the upward slopes of the dependence of mitochondrial oxidation and nitration products as a function of time. The vertical fall of oxidation and nitration products corresponds to the mitochondrial binary division, since mitochondrial biogenesis is made with proteins and phospholipids *de novo* from non-oxidated and non-nitrated precursor molecules. The level of mitochondrial oxidation products would be usually below the apoptotic threshold in young individuals but those levels, that are consistently increased in old and senescent animals [14,15], would be enough to trigger the neuronal apoptosis that continuously leads brain and nervous system to atrophy. The scheme of Fig. 5 also illustrates the hypothesis of a role for the oxidation products as inhibitors of mitochondrial biogenesis and of an extended time for mitochondrial biogenesis and turnover in aged individuals.

5. Mitochondria-targeted antioxidant therapy

5.1. Vitamin E

Chronic supplementation with high doses of vitamin E extended life span and improved neurological function in aging mice [66] and brain mitochondrial function in aging mice and rats (Table 2). The dietary supplementation with vitamin E increased α-tocopherol levels in the liver (from 27 to 168 nmol/g liver) and in the brain (from 11.5 to 26.2 nmol/g brain) [66]. It is then clear that vitamin E (431 Da) crosses the blood-brain barrier, a process reserved to relatively small molecules (<450 Da) with lipophylic character and less than 10 hydrogen bonds [105,106]. The effect of vitamin E on the prevention of the aging-dependent decline in mitochondrial function was dose-dependent: 2.0 and 5.0 g of α-tocopherol acetate/kg food produced a prevention of 22% and 34% of the decline of respiration in rat hippocampal mitochondria. These two α-tocopherol levels in the rat diet would correspond, on the basis of the ratio: mg α-tocopherol/kJ of basal metabolic rate, to 0.90 and 2.1 g/day in humans.

Other antioxidants, such as acetylcarnitine and lipoic acid [102,107] and flavonoid-rich vegetable extracts [96,108], chronically administered to mice and rats also prevented the age-associated decline in neurological functions and oxidative damage in brain mitochondria. Rats treated chronically with acetylcarnitine showed a lower age-dependent decline in the mitochondrial oxidation rate of NAD-dependent substrates [98] and in the mitochondrial gene expression of complexes I, IV and V and of adenine nucleotide translocase [109].

The effect of high doses of vitamin E on mice survival [66] is to be taken into account in the controversy on the use of vitamin E supplementation in humans. The claim that vitamin E supplementation increases human mortality, based on meta-analysis [110] is challenged by the clinical evidence that vitamin E supplements are safe at high intakes [111] and by the reported effects of vitamins E and C in the reduction of prevalence and incidence of Alzheimer disease in an elderly population [112].

5.2. The Skulachev cations attached to antioxidant molecules

Starting in recent years a new family of mitochondrial antioxidants is being developed. The new mitochondria-targeted antioxidants are covalent derivatives of well known antioxidants, such as vitamin E, ubiquinone and PBN (α-phenyl-N-*tert*-butyl nitrone) that are covalently coupled through an aliphatic carbon chain to a triphenylphosphonium cationic group [113]. The phosphonium derivatives have been used for years for the determination of inner membrane potential and, following to the development and use by Russian

Table 2
Effects of vitamin E on survival, neurological function and mitochondrial function in mice and rats

Survival, neurological function or enzyme activity	Control	Vitamin E treated	Beneficial effect (%)
Mice survival			
Median life span (wk)	61±4	85±4	39
Maximal lifespan (wk)	116±4	136±4	17
Mice neurological function			
Tightrope assay (% of success)	21±3	37±4	76
T-maze assay (% of success)	42±4	58±6	38
Mouse brain mitochondria			
State 3 oxygen uptake (ng-at O/min×mg)	66±5	74±6	12
Complex I activity (nmol cyt *c*/min×mg)	210±10	283±11	35
Complex IV activity (nmol cyt *c*/min×mg)	75±8	102±8	36
mtNOS activity (nmol NO/min×mg)	0.21±0.03	0.41±0.05	95
Rat hippocampal mitochondria			
State 3 oxygen uptake (ng-at O/min×mg)	100±10	134±11	34
Complex I activity (nmol cyt *c*/min×mg)	155±10	210±12	36
Complex IV activity (nmol cyt *c*/min×mg)	95±8	128±10	35
mtNOS activity (nmol NO/min×mg)	0.31±0.03	0.43±0.03	39

Swiss CD1-UCadiz mice and Wistar rats received 5.0 g α-tocopherol acetate/kg food (A04 diet, Panlab LS, Barcelona, Spain). Mice neurological function and mouse mitochondrial activities corresponds to 78 wk mice, mouse brain mitochondrial state 3 respiration corresponds to 52 wk mice, and rat hippocampal mitochondria to 52 wk rats. The enzymatic activities of mouse brain and rat hippocampal mitochondria are expressed in nmol (or ng-at) of substrate or product/min×mg protein.

bionergeticists, are known as the "Skulachev cations". Lipophilic triphenyl-phosphonium cations are actively taken up by mitochondria due to the inner membrane potential, 160–185 mV with the inside negative, that would produce, according to the chemical potential and Nernst equations, an intramitochondrial accumulation of about 700 times [114,115]. The development and use of the phosphonium antioxidants takes advantage of the unique biophysical and biochemical characteristics that mitochondria provide: a negatively charged compartment and the reducing environment that allows regeneration of the free radical scavengers.

The molecule resulting from the coupling of triphenylphosphonium cation (TPP^+) with α-tocopherol, MitoVitE (Fig. 6), was developed to prevent mitochondrial oxidative damage. Mitochondria incubated with 1–20 μM MitoVitE take up the phosphonium cations in about 15–30 min with accumulation ratios of up to 1000 times [116]. Higher levels of MitoVitE in the incubation medium, as 50 μM, have been found cytotoxic for Jurkat cells [116]. It is considered that MitoVitE is inserted in the lipid bilayer of the mitochondrial inner membrane and that the chroman group becomes redox active. The semiquinone, formed after detoxifying a free radical by hydrogen donation, would be reduced either by a collisional reaction with intramitochondrial ascorbic acid or by electron donation or tunnelling from a reduced component of the mitochondrial respiratory chain. Concerning oxidative damage, MitoVitE was reported to reduce H_2O_2-induced caspase activity [117] and to prevent cell death in fibroblasts from patients with Friedrich ataxia patients, an inherited nervous system disease associated with decreased frataxin and increased iron-catalyzed oxidative damage [118], to inhibit cytochrome *c* release and caspase-3 activation, to inhibit complex I inactivation and to restore mitochondrial membrane potential in bovine aortic epithelial cells after oxidative stress [119].

$MitoQ_{10}$ is a similar TPP^+ derivative in this case with ubiquinone-10 (also named UQ_{10} or Q_{10}) (Fig. 6) that is similarly accumulated within mitochondria. Internalized $MitoQ_{10}$ seems to be inmobilized by anchoring the isoprenoid chain into the lipid bilayer and to become redox active: the semiquinone or the oxidized forms are enzymatically reduced by mitochondrial complexes I and II [119]. The ubiquinol molecule inserted in the mitochondrial inner membrane is ready to act as a free radical trap and antioxidant, preventing mitochondrial oxidative damage [119]. $MitoQ_{10}$ has been reported to be effectively reduced by complex II but poorly reduced by complex I; the different reactivity has been explained considering that the bulky TPP^+ moiety sterically hindered the access of the ubiquinone group to complex I reducing site [119].

The superficial position of the TPP^+ moiety and the solubility of $MitoQ_{10}$ in non-polar solvents suggest that $MitoQ_{10}$ concentrates in the membrane core. The ubiquinone moiety of $MitoQ^{10}$ was found to quench fluorophors deep within the membrane, in agreement with its good antioxidant efficacy. In isolated cells, $MitoQ_{10}$ protects from H_2O_2-induced apoptosis but not from the apoptosis induced by staurosporine or TNF-α [113].

MitoPBN is a similar TPP^+ derivative, in this case with phenoxy-butyl-nitrone, that was designed to prevent mitochondrial lipid peroxidation and oxidative damage based on the well known and relatively selective PBN reaction with carbon-centered radicals (R·) and peroxyl radicals (ROO·) [120]. Similarly to the other triphenylphosphonium cations, MitoPBN is rapidly taken up by mitochondria reaching intramitochondrial levels of 2.2–4.0 mM. It has been reported that MitoPBN blocks the oxygen-induced activation of uncoupling proteins [120].

Recently, Poeggeler et al. developed an amphiphilic molecule derived from PBN that is a nitronium cation (LPBNAH) (Fig. 6) and that antagonizes oxidative damage of mitochondrial origin and shows a neuroprotective activity one order of magnitude higher than other nitrone compounds and of the parent compound PBN [121].

Currently, other mitochondria-targeted antioxidants of the phosphonium type are developed and assayed to develop effective mitochondria-targeted antioxidants [122,123]. For instance, the phosphonium derivative with the selenium-containing ebselen moiety that shows hydroperoxide peroxidase activity [124].

5.3. Tetrapeptide antioxidants

The series of the "SS tetrapeptides" is constituted by aromatic-cationic peptides that have the structural motif of alternating aromatic and basic amino acids with 2, 6-dimethyltyrosine residues [116]. These tetrapeptides, that were originally prepared to act as opioid analgesics, are taken up by isolated cells and mitochondria due to their positive charge at physiological pH values and show the antioxidant properties of dimethyltyrosine (similar to 3, 5-dimethylphenol, a phenolic antioxidant) [125,126]. The following SS tetrapeptides have been developed: SS-02, SS-19, SS-20 and SS-31 [116] (Fig. 7). Three structure-property relationships should me mentioned: SS-20 that lacks the dimethyltyrosine of SS-02 also lacks antioxidant properties, SS-31 contains the same amino acids that SS-02, but in a sequence that exhibits better antioxidant properties and SS-19 is more lipophylic than the other analogues and fluorescent due to the anthranyl group. Peptides SS-02 and SS-19 are actively taken up by mouse liver

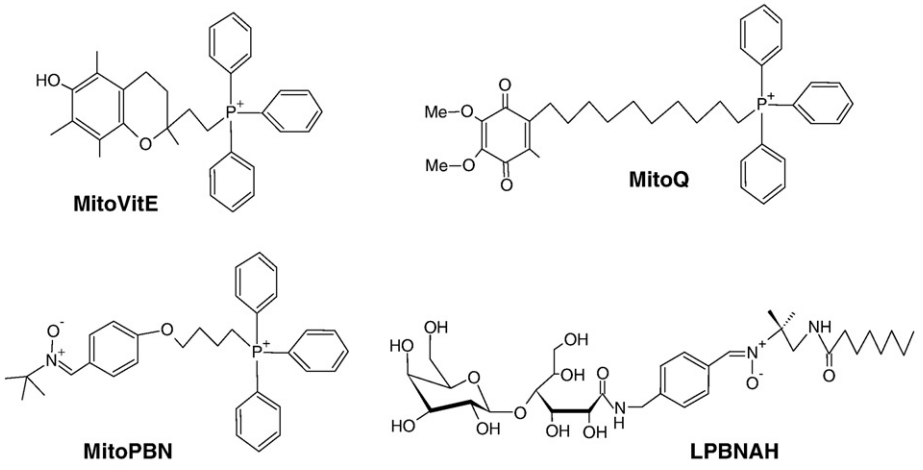

Fig. 6. Chemical structures of mitochondria-targeted antioxidants. Mito Vit E: [2-3,4 dihydro-6-hydroxyl-2,5,7,8-tetra-methyl-2H-1-benzopyran -2-yl]-triphenylphosphonium; $MitoQ_{10}$: 10-(6'-Ubiquinolyl)-decyltriphenylphosphonium; MitoPBN: [4-[4-(1,1-dimethylethyl) oxidoimino]-methyl] phenoxy] butyl]-triphenylphosphonium; and LPBNAH: *N*-[4-(octa-*O*-acetyllactobionamidomethylene) benzylidene]-*N*-[1,1-dimethyl-2-(*N*-octanoyl) amido]-ethylamine *N*-oxide.

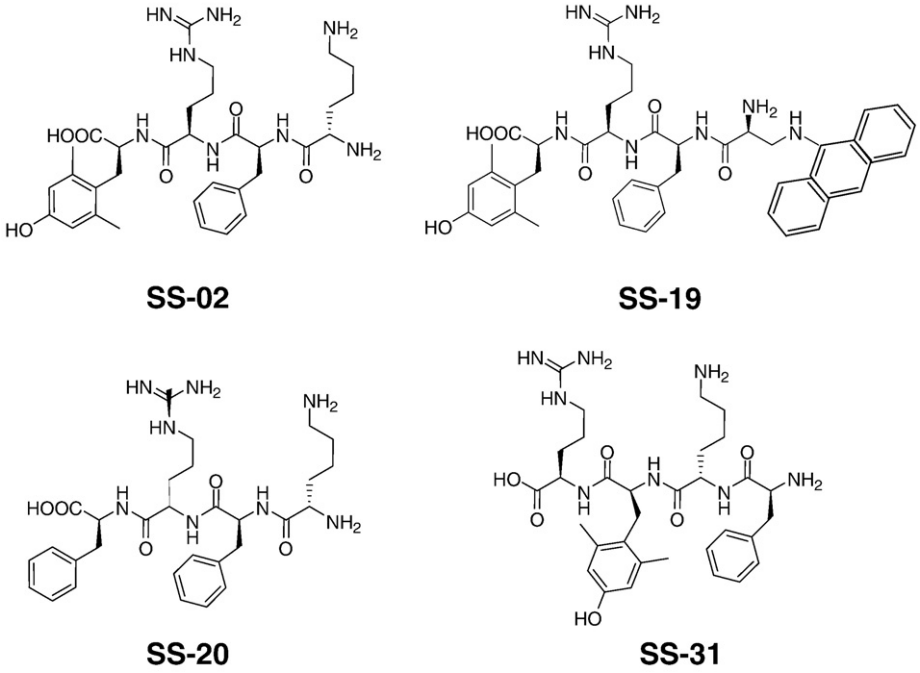

Fig. 7. Chemical structures of the tetrapeptide antioxidants. SS-02: Dmt-D-Arg-Phe-Lys-NH_2; SS-19: Dmt-D-Arg-Phe-atn-L-DAP-NH_2; SS-20: Phe-D-Arg-Phe-Lys-NH2; and SS-31: D-Arg-Dmt-Lys-Phe-NH2. Dmt: 2'6'-dimethyltyrosine; atnDap: β-anthraniloyl-L-α,β-diaminopropionic acid.

mitochondria and human Caco-2 cells, with an intracellular localization in mitochondria and with a 100-fold accumulation in mitochondria. The mitochondrial uptake of SS-19 was decreased by the uncoupler FCCP, indicating a potential dependent accumulation, consistent with the observation that mitoplasts (obtained by digitonin treatment and consisting of inner membrane plus matrix) retain 85% of the SS-02 taken up by mitochondria [127].

Concerning antioxidant properties, SS-02 showed antioxidant properties in a cell free system and inhibited the H_2O_2 promoted oxidation of linoleic acid and of low-density lipoproteins [125] and SS-31 protected against oxidant induced mitochondrial dysfunction and apoptosis in isolated neurons of the N2a and SH-SYSY cell lines [127]. Treatment of the neurons with *t*-butyl-hydroperoxide resulted in increased lipid peroxidation, phosphatidylserine translocation, mitochondrial depolarization, increased caspase activity, nuclear condensation, and cell death by apoptosis The SS-31 peptide was able to prevent the oxidative damage produced by *t*-butyl-hydroperoxide by decreasing intracellular lipid hydroperoxides, keeping mitochondrial membrane potential and preventing apoptosis. The remarkable potency of SS-31 (effects are reported at 1 nM) are explained by extensive cellular uptake and selective partitioning and accumulation in mitochondria (about 5000-fold accumulation in the mitochondrial pellet [128]). Zhao et al. claimed that SS-19 is able to decrease mitochondrial O_2^- production and cellular lipid peroxidation and to improve the contractile force and myocardial stunning in perfused hearts subjected to ischemia-reperfusion [128].

It is clear that peptide antioxidants are a novel platform for the development of mitochondria-targeted antioxidants with a broad therapeutic potential considering the widespread occurrence of diseases and pathological situations associated with old age and neurodegenerative diseases.

6. Conclusions

The last years have witnessed a growing awareness about concepts that make to the knowledge of basic mechanisms of brain aging and that set the basis for a possible useful strategy to retard the manifestations of human aging. Concerning the role of brain dysfunction in human aging, it is clear that the decline in the functions of the central nervous system is by far the most important human incapacity at old age. The work with laboratory animals (mice and rats) identified brain mitochondria as a sensitive subcellular compartment and as a likely pacemaker of mammalian aging. Dysfunctional mitochondria with increased oxidation and nitration products and decreased enzymatic activity, although normally involved and subjected to mitochondrial turnover, accumulate with age. Two recognized facts, the role of NO in mitochondrial biogenesis and the accumulation of oxidation/nitration products in the mitochondria of aged mammals, are the basis for the hypothesis that a decreased process of mitochondrial biogenesis is the basis of neuronal dysfunction and apoptosis and of brain atrophy and involution in human aging. Antioxidants that are active in the mammalian brain compartment, being able to go across the blood-brain barrier and to prevent oxidative mitochondrial damage, are clearly a good strategy for the treatment and prevention of diseases and pathological situations associated with old age.

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