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Neurochemical Research

Neurochem Res (2008) 33:1216–1223
DOI 10.1007/s11064-007-9570-z

ORIGINAL PAPER

Age Related Changes from Youth to Adulthood in Rat Brain Cortex: Nitric Oxide Synthase and Mitochondrial Respiratory Function

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Accepted: 12 December 2007 / Published online: 8 February 2008

Abstract Age related changes in brain cortex NO metabolism were investigated in mitochondria and cytosolic extracts from youth to adulthood. Decreases of 19%, 40% and 71% in NO production were observed in mitochondrial fractions from 3, 7, and 14 months old rats, respectively, as compared with 1-month-old rats. Decreased nNOS protein expression in 14 months old rats was also observed in mitochondria as compared with the nNOS protein expression in 1-month-old rats. Low levels of eNOS protein expression close to the detection limits and no iNOS protein expression were significantly detected in mitochondrial fraction for both groups of age. NO production in the cytosolic extracts also showed a marked decreasing tendency, showing higher levels than those observed in mitochondrial fractions for all groups of age. In the cytosolic extracts, however, the levels were stabilized in adult animals from 7 to 14 months. nNOS protein expression showed a similar age-pattern in cytosolic extracts for both groups of age, while the protein expression pattern for eNOS was higher expressed in adult rats (14 months) than in young animals. As well as in mitochondrial extracts iNOS protein expression was not significantly detected in cytosolic extracts at any age. RT-PCR assays indicated increased levels of nNOS mRNA in 1-month-old rats as compared with 14 months old rats, showing a similar pattern to that one observed for protein nNOS expression. A different aged pattern was observed for eNOS mRNA expression, being lower in 1-month-old rats as compared with 14 months old animals. iNOS mRNA was very low expressed in both groups of age, showing a residual iNOS mRNA that was not significantly detected. State 3 respiration rates were 78% and 85% higher when succinate and malate-glutamate were used as substrates, respectively, in 14 months rats as compared with 1-month-old rats. No changes were observed in state 4 respiration rates. These results could indicate 1 that nNOS and eNOS mRNA and protein expression can be age-dependent, and confirmed the nNOS origin for the mitochondrial NOS. During rat growth, the respiratory function seems to be modulated by NO produced by the different NOS enzymes: nNOS, eNOS and mtNOS present in the cytosol and in the mitochondria.

Keywords Age related changes · Brain cortex cytosol and mitochondria · Nitric oxide · Electron transport chain · NOS-mRNA

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Introduction

The brain cortex region has been related with a variety of functions such as memory, learning and behavior cognition which are significantly altered with aging [1, 2]. Several hypotheses have been proposed in order to explain the causes of the growing and aging processes, some of them consistent with the free radical theory of aging, with the nitric oxide hypothesis of aging [3–5] and with the closely related Ca^{2+} hypothesis of brain aging [6]. Neuronal dysfunction associated with the aging process leads to modification of cell excitability and synaptic function, decreasing learning capacity and memory [6].

Free radicals, superoxide and nitric oxide are important molecules, mediating numerous signaling pathways

associated with different cellular functions such as growth, differentiation, and death. Alteration of their metabolism may lead to damaging processes in cells and tissues. Nitric oxide, a gaseous molecule, generated by the different nitric oxide isoforms from L-arginine [7] constitute an important neuronal mediator acting as a neurotransmitter in the nervous system [8].

Several studies suggest that impairment in NO production could be associated with damage and alteration of different brain functions during aging [9, 10, 5] which has lead to establish the NO hypothesis of aging, despite its positive roles in physiological regulation of different processes.

The presence of a nitric oxide synthase in mitochondria [11, 12] and the ability of NO to inhibit several components of the respiratory chain [13, 14] suggest that the endogenous production of this molecule in mitochondria should be associated with a rapid regulatory mechanism of the electron transfer chain, involved in the continuous supply of energy, which is central for neuronal activity and survival. Previous results from our laboratory showed that in the immune system the respiratory function is closely related with the presence of NO in mitochondria during different cellular events associated with cell death [15, 16]. We have also reported a modulation of mitochondrial function by NO after drug treatment [17, 18].

In this work we analyzed the brain cortex NO metabolism and the function of the mitochondrial electron transport chain in order to explore if they could be age-regulated processes. To address these issues, mitochondrial respiratory rate and the respiratory control in the presence of different substrates were studied together with mtNOS activity. Protein expression levels of the three different NOS isoforms present in mitochondrial and cytosolic fractions of cortical tissues and mRNA expression levels of the three different NOS isoforms were analyzed in the 1 and 14 months old animals.

Experimental Procedures

Materials

Ethylenediaminetetraacetic acid, oxyhemoglobin, N_{ω}-nitro-L-arginine methyl ester (L-NNA), catalase, superoxide dismutase and Folin reagent were obtained from Sigma Chemical Co. (Saint Louis, MO, USA); RPMI 1640 and fetal calf serum were purchased from GIBCO Laboratories, (Rockville, MD). Rapid hybridization buffer (NIF939) was from Amersham Pharmacia Biotech Inc. (Uppsala, Sweden) and Trizol reagent from Life Technologies (Grand Island, NY, USA). Other reagents were of analytical grade. All the antibodies, including those against the different NOS isoforms were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

Animals

Groups of 4 female Wistar rats of 1, 3, 7, and 14 months old, housed in a humidity and-temperature-controlled environment with an automatic 12:12-h light–dark cycle and fed standard rat chow and tap water ad libitum were used. Animal handling for the different experiments was in accordance with the American Physiological Society "Guiding Principles in the Care and Use of Animals" and with the 6344/96 regulation of Argentinian National Drug Food and Medical Technology Administration (ANMAT).

Isolation of Mitochondrial and Cytosolic Fractions from Brain Cortex

Brain cortex was isolated on ice after decapitation, a 5% homogenate (w/v) was immediately prepared in a glass homogenizer with Teflon pestle in MSTE buffer (0.23 M mannitol, 0.07 M sucrose, 1 mM EDTA and 10 mM Tris–HCl, pH 7.2) with a cocktail of protease inhibitors (1 μg/ml pepstatin, 1 μg/ml leupeptin, 0.4 mM phenylmethylsulfonyl fluoride and 1 μg/ml aprotinin). After centrifugation at 600*g* and 8,000*g* for 10 min the 8,000*g* supernatant was collected and saved at 4°C until used (cytosolic fraction). The pellet (mitochondrial fraction) was washed with the same buffer, purified by Ficoll gradient [19], suspended at 20–30 mg protein/ml and immediately used for oxygen uptake determination. This brain cortex mitochondrial preparation corresponds to an enriched non-synaptic mitochondrial fraction which corresponds mainly to mitochondria from neurons and glia. The criterion for using the non-synaptic fraction is because this mitochondrial fraction is the most representative of brain mitochondria, and also due to our previous observation of a higher nNOS protein expression in non-synaptic mitochondria than in synaptosomes reported in Czerniczyniec et al. [20]. The respiratory control ratio of these mitochondrial samples (RCR) was between 4.0 and 6.0 ($n = 5$) determined with malate plus glutamate as substrates. Sub-mitochondrial particles (SMP) were obtained after three cycles of freezing and thawing. All isolation procedures were performed at 0–4°C.

Production of NO by Mitochondrial and Cytosolic Fractions

Sub-mitochondrial particles (SMP) were used at 0.5 mg protein/ml for measuring NO production by the oxidation

of oxyhemoglobin (30 μM in heme) to methemoglobin in a double-beam dual-wavelength spectrophotometer at 577–591 nm ($\Delta\varepsilon_{577-591} = 11\ mM^{-1}\ cm^{-1}$), in the presence of 0.1 mM NADPH, 0.3 mM L-arginine, 1 mM $CaCl_2$, 1 μM superoxide dismutase, and 0.5 μM catalase, in 50 mM phosphate buffer, (pH 5.8) at 37°C [17, 21]. NO production was expressed as nmol/min mg protein. In order to measure the degree of oxyhemoglobin oxidation sensitive to NOS inhibitors, parallel experiments were performed in samples pre-incubated with 1 mM L-NNA. Similar results were obtained replacing oxyhemoglobin (HbO_2) by 10 μM horseradish peroxidase, this assay uses horseradish peroxidase instead of HbO_2 as a NO trap, and registers 15% less NO production than with HbO_2 [22, 23]. Similar procedure was employed for the production of NO by cytosolic fractions.

Western Blotting and Chemiluminescence

Brain cortex NOS immunoreactivity was determined in non-synaptic mitochondria and cytosolic fraction as described: equal total protein amounts of both fractions were separated by SDS-PAGE (7.5%), blotted into a nitrocellulose membrane (Bio-Rad, München, Germany) and probed primarily with rabbit polyclonal antibodies (dilution 1:500) for the three isoforms of nitric oxide synthases: neuronal constitutive form (n-NOS), epitope corresponding to amino acids 2–300, mapping to the amino terminus of NOS I; inducible constitutive form (i-NOS), epitope mapping to the carboxy terminus of NOS II; and endothelial form (e-NOS), epitope mapping to the amino terminus of NOS III. The nitrocellulose membranes were then incubated with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (dilution 1:5,000), followed by development of chemiluminescence with the ECL reagent for 2–4 min [16, 24, 25]. Analysis of protein levels for the different NOS isoforms in the mitochondrial and cytosolic fractions were performed after normalization with cytochrome c and β-actin antibodies, respectively.

Tissue mRNA Preparation and RT-PCR

Total RNA was isolated from the right parietal cerebral cortex of Wistar rats at two different ages: 1 and 14 months old animals. Briefly: 30–50 mg of tissue was homogenized in Trizol® reagent (Invitrogen, BsAs, Argentina). RNA concentration was determined in each sample by measuring its absorbance at 260 nm. RNA samples (2 μg) were pretreated with RNase-free DNase (deoxyribonuclease I, amplification grade, Invitrogen, BsAs, Argentina), to remove any residual genomic DNA contamination, heated at 70°C for 10 min, placed on ice for 1 min, and then incubated with a mixture containing 0.5 mM dNTPs mix, 25 ng/μl (8 μM) random primers, 1× first strand buffer, 25 units of rRNase inhibitor, 200 units of MMLV (Moloney murine leukemia virus) reverse transcriptase (Promega Corp., Madison, WI, USA), and water to a final volume of 25 μl for 1 h at 42°C. The reaction was stopped by heating at 90°C for 5 min. The reaction mixture was brought to 100 μl with diethylpyrocarbonate-treated water and stored at −70°C. In selected tubes the reverse transcriptase was omitted as a control of amplification from contaminating cDNA or genomic DNA.

PCR reactions were carried out in a Tpersonal Thermocycler (Biometra biomedizinische Analytik GmbH, Göttingen, Germany) and were performed using 2 μl cDNA for each amplification reaction. The cDNA was added to 18 μl of the following reaction mixture: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 μl of [^{32}P]dCTP, 500 nM of each specific oligonucleotide primer, and 0.625 U *Taq* polymerase (Life Technologies, Bs As, Argentina). The sequence for the oligonucleotide primers were based on published sequences for murine nNOS and eNOS isoforms (GenBank Accession No. D14552 and U53142, respectively) and rat iNOS isoform (GenBank Accession No. M87039) [26]. Primer pairs for nNOS were 5-TTTCTGTCCGTCTCTTCAAACGCAAAG TGG-3 (nucleotides 145–174) for the forward sense primer and 5-GCGGGAGACTGTTCGTTCTCTGAATACGGG-3 (nucleotides 943–914) for the reverse anti-sense primer. Primer pairs for iNOS were 5-CACGGAGAACAGCA GAGTTGG-3 (nucleotides 212–232) for the forward sense primer and 5-GGAACACAGTAATGGCCGACC-3 (nucleotides 979–959) for the reverse anti-sense primer. For eNOS the primer pairs used were 5-CTGTGTCC AACATGCTGCTAGAAATTG-3 (nucleotides 1,008–1,034) for the forward sense primer and 5-TAAAGGTCTT CTTCCTGGTGATGCC-3 (nucleotides 1,493–1,469) for the reverse anti-sense primer. The appropriate number of cycles was determined by performing a linear amplification curve and choosing a cycle number within the linear amplification range. The relative amount of NOS mRNAs was calculated by densitometry using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal control. The GAPDH forward primer was: 5′-TCCCTCAAGAT TGTCAGCAA-3′ and as a reverse primer: 5′-AGAT CCACAACGGATACATT-3′ (309 bp fragment). For amplifying NOS and GAPDH gene sequences, PCRs were carried out with a first step at 94°C for 10 min and then *n* cycles (21 cycles for GAPDH and 27 cycles for NOS) of denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and elongation at 72°C for 1 min, plus a final incubation at 72°C for 10 min. PCR products were separated on a 6%

acrylamide gel, dried, and autoradiographed. The optical density of the bands was assessed by densitometry.

Mitochondrial Respiration

Mitochondrial respiratory function was measured in isolated brain cortex mitochondria (50 μg protein/ml) by using an oxygen electrode fitted to a 1.5 ml water-jacketed closed chamber (Oroboros Oxygraph, Paar KG, Graz, Austria) maintained at 30°C, in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 5 mM PO_4H_2K, 4 mM $MgCl_2$ (pH 7.4), and 0.2% bovine serum albumin [17]. Combination of 5 mM Succinate and 7 mM malate plus 7 mM glutamate were used as substrates to measure state 4 respiratory rate and state 3 was initiated by 1 mM ADP addition. The respiratory control ratio (state 3 respiration (ADP dependent state)/state 4 respiration (only substrate dependent)) was determined [27].

Statistics

Values and data from tables and figures are expressed as mean values ± SEM. Student's *t* test was used to analyze the significance of differences between paired groups.

The Western blotting experiments were typical results of three different experiments.

Results

NOS Activity

NO production by mitochondria and cytosol from brain cortex was characterized as described in Table 1. The specificity of the metHB formation in the presence of substrates and cofactors shows the requirements for NOS activity in brain cortex mitochondria and cytosol. In the absence of the specific substrate, L-arginine, a little amount of NO is still produced, probably due to the high L-arginine content present in the tissue [17]. The addition of L-NNA, nNOS inhibitor, decreases mitochondrial and cytosolic NO production by 62% and 68%, respectively. NO production was not detected in the absence of NADPH.

Table 1 Characterization of NO production

Reaction mixture	MetHB formation (nmol/min mg protein)	
	Mitochondria	Cytosol
Complete	0.80 ± 0.03	1.22 ± 0.05
Minus L-arg	0.17 ± 0.02	0.24 ± 0.03
Minus NADPH	N.D.	N.D.
Minus Ca^{2+}	0.10 ± 0.09	0.20 ± 0.05
Plus 1 mM L-NNA	0.31 ± 0.02	0.32 ± 0.07

Characterization of the NO production by brain cortex mitochondrial and cytosolic fraction of 1 months old rats. Values are means ± SEM

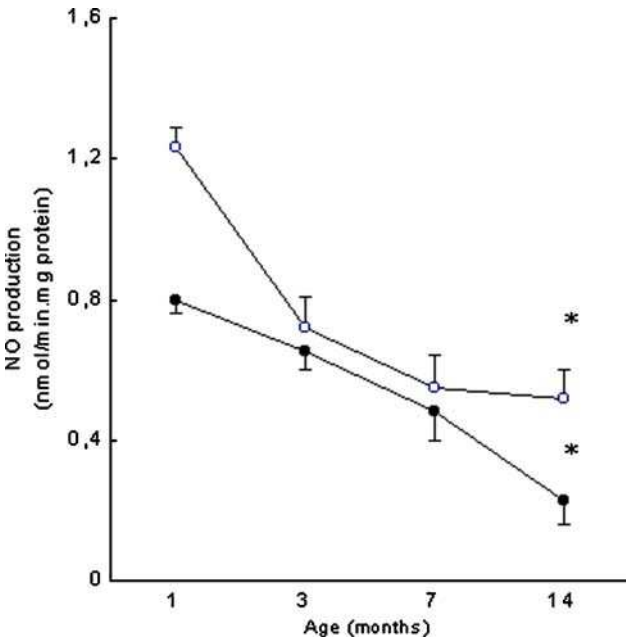

Fig. 1 The influence of age on nitric oxide production. Mitochondrial (•) and cytosolic (o) fractions were incubated at 37°C for spectrophotometric determination of NO, as described previously in Results section, values are mean ± SEM from three different experiments. *$P < 0.05$, compared with control 1-month-old rats

Clear decreases in mitochondrial NO production of 19%, 40%, and 71% were observed in 3, 7, and 14 months old animals, respectively, as compared with 1-month-old rats (Fig. 1). Cytosolic NO production was also reduced with aging by 27%, 45%, and 46% in 3, 7, and 14 months old rats, respectively, as compared with 1-month-old rats (Fig. 1). However, different kinetics were evident in the cytosolic fraction, with a fast decrease in young animals, stabilization at adult stages, and with no significant changes from 7 to 14 months aged rats (Fig. 1).

NOS Protein Expression

In order to better understand the decreasing age-associated NO production from young to adult rats, protein and mRNA NOS expression analysis of cytosolic and mitochondrial fractions, was performed in 1 and 14 months aged animals. The densitometric analysis of NOS expression in mitochondrial fractions showed higher ratios of nNOS/cyt c when compared with the other NOS isoforms. Its expression was 36% higher in young rats (1 months

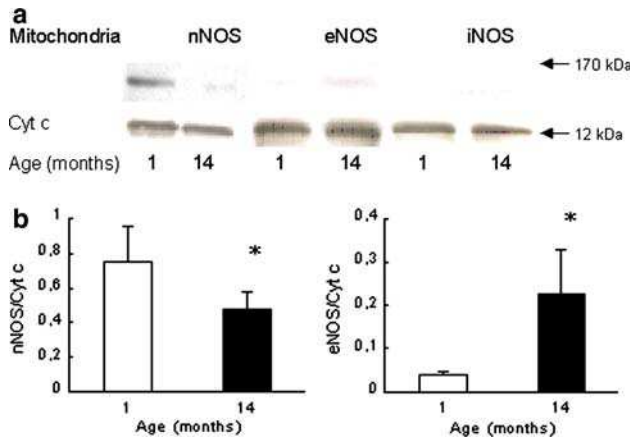

Fig. 2 Young and adult mitochondria NOS protein expression. Western-blot bands of the three different NOS isoforms were analyzed in brain cortex mitochondrial extracts after electrophoresis and transbloting onto membranes, which were then probed with anti-nNOS, anti-eNOS and anti-iNOS antibodies as described in Experimental Procedures section. (**a**) Representative blot after ECL development. (**b**) Mean values ± SEM from three different experiments (*$P < 0.05$). Results were normalized with an antibody against cytochrome c

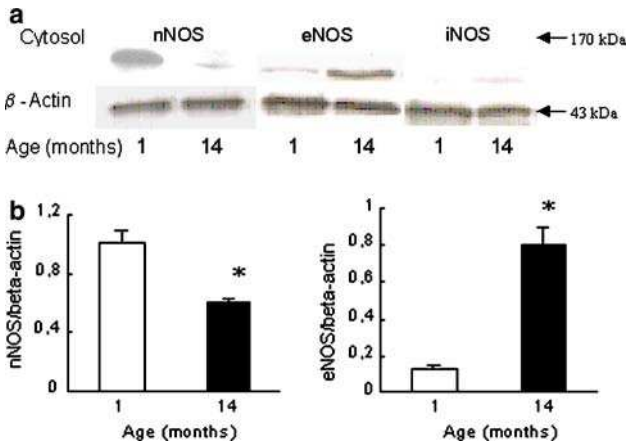

Fig. 3 Young and adult cytosolic NOS protein expression. Similar Western-blot assay as described in Fig. 2, except that results were normalized through β-actin, with an antibody anti β-actin. (**a**) Representative blot after ECL development. (**b**) Mean values ± SEM from three different experiments (*$P < 0.05$)

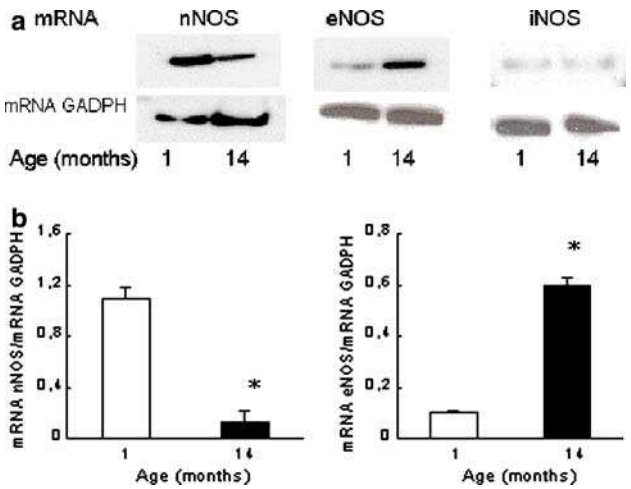

Fig. 4 Young and adult brain cortex NOS mRNA expression. Analysis of the different NOS isoforms mRNA from 1 and 14 months old rats by RT-PCR was performed as described in section ''Tissue mRNA preparation and RT-PCR.'' (**a**) Representative autoradiography of nNOS, eNOS and iNOS and GADPH for 1 and 14 months old animals. (**b**) Mean values ± SEM from three independent experiments (*$P < 0.05$)

aged) than in aged animals (14 months) being 0.75 ± 0.2 and 0.48 ± 0.1, respectively (Fig. 2a, b). The densitometric analysis of eNOS protein associated with this fraction showed much lower values as compared with the nNOS isoform (Fig. 2a, b), confirming the presence of nNOS as the main isoform in this organelle. iNOS was not significantly detected.

NOS protein expression levels in the cytosolic fraction was similarly analyzed [16]. Results also showed a higher nNOS expression level in young animals as compared with adult rats (1.0 ± 0.2 and 0.60 ± 0.04, respectively). On the contrary, eNOS protein expression level was 85% lower in younger animals as compared with adult animals, showing values of 0.12 ± 0.03 and 0.8 ± 0.1, respectively, as observed in Fig. 3a, b. iNOS densitometric values were not significantly detected in young and adult animals.

NOS mRNA Expression

The mRNA analysis of the different NOS isoforms showed also a 88% higher expression in brain cortical tissue in 1 month aged animals, than in adult rats, and nNOS/GADPH ratios of 1.09 ± 0.09 and 0.13 ± 0.089 for 1 and 14 months old rats, respectively. In contrast, eNOS mRNA expression was 83% lower in 1-month-old animals as compared with adult rats, with eNOS/GADPH ratio of 0.1 ± 0.01 and 0.60 ± 0.03 for 1- and 14-month-old, respectively. iNOS mRNA band-densitometry showed a non-significantly detected iNOS/GADPH ratio (Fig. 4a, b).

Mitochondrial Respiration

The mitochondrial respiratory function was studied in different metabolic mitochondrial states: state 4 respiration, a resting substrate dependent state and in state 3 respiration, an ADP dependent state. Succinate and malate plus glutamate were used as substrates in order to analyze

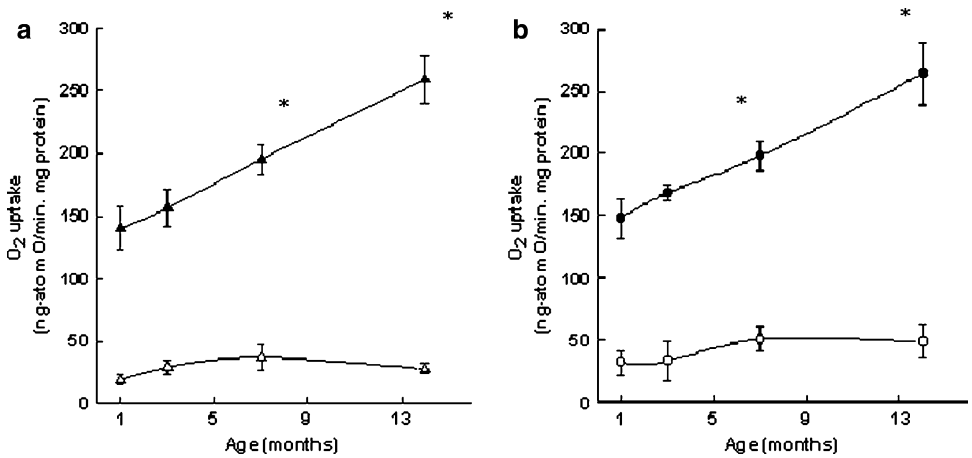

Fig. 5 The influence of age on the respiratory mitochondrial function. Oxygen consumption was determined by a polarographic measurement in brain cortex mitochondria from 1-, 3-, 7-, and 14-month-old rats. (**a**) In metabolic state 3 (▲) and state 4 (Δ) using malate plus glutamate and (**b**) in metabolic state 3 (•) and state 4 (o) using succinate, as substrates (*$P < 0.05$)

the oxygen uptake in 1-, 3-, 7-, and 14-month-old rats and evaluate age related variation in the respiratory function as described in Fig. 5a and b. An increasing tendency in state 3 oxygen uptake was observed from 1 to 14 months old animals for both malate plus glutamate (Fig. 5a) and succinate (Fig. 5b), showing a significant age related change in oxygen uptake in adult animals as compared with young rats. However, a non-significant increase was observed in state 4 respiratory rate with both substrates in the same groups of rats (Fig. 5a, b). Mitochondrial preparation from the four groups of animals (1, 3, 7, and 14 months old) conserved their respiratory function with no significant age-variation in their respiratory control rate (RC), being between 5.3–6.6 for malate plus glutamate and 4.8–5.2 in the case of succinate.

Discussion

Previous studies from different laboratories, including ours, give support to the fact that NO is produced by the mitochondria, probably by a nNOS α isoform [24, 28, 29]. In addition postembbeding immuno-gold electron microscopy experiments have confirmed the presence of a mtNOS in mitochondrial cristae using similar mitochondrial isolation procedure as used in this work [25]. The mtNOS isoform is coded by the same nNOS gene as nNOS knockout mice do not have mtNOS [30]. NO inside mitochondria modulate oxygen consumption by the transient and reversible inhibition of cytochrome c oxidase activity [13, 14] and it has recently been shown that NO production seems to be regulated by other mitochondrial processes such as the modification of calcium uptake [31], and mitochondrial membrane potential ($\Delta\psi_m$) [32]. In this study we investigated the age-related variations from youth to adulthood, of mitochondrial and cytosolic NO production, NOS protein expression and its involvement in the mitochondrial respiratory function in brain cortex of 1, 3, 7, and 14 months old rats. The mRNA NOS levels expression for the three different isoforms was also studied in 1 and 14 months old rats in order to obtain a better understanding of the contribution of NO to the respiratory changes observed from young to adult animals.

Our results showed an age-related decreasing tendency of mitochondrial NO production mediated by the presence of an active mtNOS in 1 to 14 months old animals. Previous studies have also shown that brain mtNOS activity was decreased in 27 months old rats as compared with 40 days old rats indicating that mtNOS could be an age modulated enzyme [2]. In agreement, in this study a decrease in nNOS protein and mRNA levels were observed in adult (14 months), as compared with young animals (1 month). An age related decreasing tendency in NO production was also observed in cytosolic fractions, possibly mediated by the decreased nNOS contribution. This is in agreement with the expression pattern for nNOS mRNA obtained in young and adult animals. Low protein and mRNA expression levels for eNOS and iNOS was observed in brain mitochondria and cytosol in young and adult animals, this last result is consistent with the fact that these rats were free of infections and immunogenic problems. The decreased NO production in mitochondria and cytosol that results from a decreased nNOS expression in adult animals could be a consequence of an adaptative mechanism as there is a lesser NO demand in adult rats. This could be in agreement with previous results showing that age-related impairment of cognition could be associated with a decreased neuronal plasticity involving NO-dependent mechanisms [33]. Although this decreased NO mediated plasticity, could be characteristic of this adult stage, the decrease in NOS activity and expression level could be also due to the presence of a mechanism protecting adult brain cortex against an excessive NO synthesis. The observed increase in eNOS protein and mRNA levels in the cytosol from adult rats could be an

adaptative response, to a higher demand of age-related vascular functions during growth.

The close relationship between NO and the mitochondrial physiology where NO exerts a regulatory role in the respiratory function has been already studied in our and other laboratories [13, 14, 34, 35].

In this work we investigated if the metabolic variation of NO production observed in mitochondria and cytosol from young and adults rats was associated with changes in the mitochondrial respiratory function. The results showed that mitochondria from 1, 3, 7, and 14 months old rats were functional with a normal respiratory control (RC), although, an age-related increase in state 3 respiration using both substrates (succinate and malate plus glutamate) was observed in the same specimens. This up-regulation in oxygen uptake capacity in state 3 may be related with the observed decrease in NO production and nNOS expression both in the cytosol and the mitochondria occurring from young to adult animals possibly due to a decreased NO regulation of cytochrome oxidase. Also the increased age-related respiratory tendency in state 3 may be due to a NO decreased diffusion from the cytosol to the mitochondria. The eNOS increase in protein and mRNA expression observed in adult animals as compared with young animals is possibly not enough to counteract the marked observed decrease in NO production mediated by the nNOS protein and mRNA down-regulation observed from 1 to 14 months of age. In addition the increased age-related respiratory tendency in state 3 may be due to an up-regulation of mitochondrial genes coding for electron transport chain polypeptides. Recently it has been described increased mRNA expression of different polypeptides from the electron transport chain in young-to-mid stages and decreases in mid-to-later stages of life, indicating that mitochondrial genes expression is critical for age-related ATP production [36]. However, opposing results have been previously reported by Deshmukh and Patel [37] which showed a reduced state 3 respiratory rate in brain mitochondria from 12- to 24-month-old rats compared with 3-month-old animals, possibly due to differences in the samples used and in the experimental methodology. Changes in free radical production during the mitochondrial metabolic states have been closely associated with the levels of the endogenous mitochondrial NO production [35, 38]. Moreover, results obtained by Navarro et al. showed that a mitochondrial dysfunction due to an impairment in the electron transport system is also an age-related process [39]. We conclude that changes in NOS isoforms activity and expression, and in NO metabolism are closely associated with the mitochondrial activity and strongly dependent on age.

Our data provides support to the concept that NO metabolism and mitochondrial respiratory function are closely related processed that can be modulated in different pathophysiological conditions.

Acknowledgements This work was supported by Grants from University of Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, and Agencia Nacional de Promoción Científica y Tecnológica.

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