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# Functional characterization of brain mitochondrial nitric oxide synthase during hypertension and aging

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**Summary.** Nitric oxide ($NO^{\bullet}$) plays an important role in various physiological processes. The aim of the present study was to investigate if brain mitochondrial nitric oxide synthase (mtNOS) is active and functional during hypertension. L-citrulline production, an indicator of nitric oxide synthesis, was concentration-dependent on L-arginine in all strains and all ages tested, and was inhibited by 7-Nitroindazole (7-NI). Brain mitochondria of 1 month-old (prehypertensive) spontaneously hypertensive rats (SHR) exhibited a significantly ($p < 0.05$) low basal L-citrulline content as compared to age-matched Wistar (W) and Wistar-Kyoto (WKY) rats. L-citrulline synthesis in SHR rats showed a significant ($p < 0.01$) low response to L-arginine in 3 and 7 months-old rats. Respiratory rates in states 3 and 4 increased with low L-arginine concentration in all strains and all ages. The results suggest that in rat brain mitochondria, L-citrulline synthesis is constant once age-related hypertension is installed and $NO^{\bullet}$ does not regulate oxidative phosphorylation.

**Keywords:** Mitochondrial L-citrulline – Rat brain – Spontaneously hypertensive rats – Aging – L-Arginine – Nitric oxide synthase

## Introduction

In the last few years a number of reports appeared showing that a putatively new isoform of nitric oxide synthase (NOS) occurs inside rat liver mitochondria (mtNOS) (Ghafourifar and Richter, 1997; Giulivi et al., 1998; Tatoyan and Giulivi, 1998). Its activity has been also detected in mitochondria from brain, heart and kidney (Tatoyan and Giulivi, 1998; French et al., 2001; Kanai et al., 2001; Lacza et al., 2001; Manzo-Ávalos et al., 2002; Aguilera-Aguirre et al., 2002). However, in very recent reports mtNOS has been identified as brain NOS$\alpha$ suggesting the existence of a new pathway (Kanai et al., 2001; Elfering et al., 2002). Mitochondrial nitric oxide ($NO^{\bullet}$) production has been involved in modulation of several organelle functions, such as transmembrane potential and matrix pH, inhibition of respiration by competitive inhibition with oxygen in cytochrome c oxidase, inhibition of ATP synthesis, permeability transition pore (PTP) opening, apoptosis and cell death (Giulivi et al., 1998; Ghafourifar and Richter, 1999; Ghafourifar et al., 1999; Shiva et al., 2001; Aguilera-Aguirre et al., 2002; Saavedra-Molina et al., 2003). During aging and hypertension, activation of endothelial cells produce and release contractile factors that affect the relaxing physiological action of $NO^{\bullet}$ (Nakahara et al., 1998; Taddei et al., 1998), and affect NOS expression (Chou et al., 1998). Since the enzyme has been localized in the inner mitochondrial membrane (Elfering et al., 2002), it has become important to search for its role in different physiological and pathological conditions. In this regard, previous data have shown that basal mtNOS activity has no physiological relevance in porcine heart (French et al., 2001), but it regulates several mitochondrial functions in the rat (Ghafourifar and Richter, 1997; Giulivi et al., 1998; Tatoyan and Giulivi, 1998; Ghafourifar and Richter, 1999; Ghafourifar et al., 1999; Aguilera-Aguirre et al., 2002). These mtNOS-modulating events have been implicated in disease states such as hypertension (Aguilera-Aguirre et al., 2002), hypoxia (Shiva et al., 2001) and a natural process: development (Riobó et al., 2002). The aim of the present report was to characterize $NO^{\bullet}$ production, as L-citrulline formation, and to determine its influence in the

respiratory rate in rat brain mitochondria, both in the normal state and during hypertension. In addition, the influence aging has in this condition was explored.

## Materials and methods

### *Chemicals*

All reagents and substances were from Sigma Chemical Co. (St. Louis, MO, USA).

### *Biological materials*

Male Wistar (W), normotensive genetic control Wistar-Kyoto (WKY) and spontaneously hypertensive rats (SHR) of 1, 3 and 7 months of age were fed *ad libitum* and kept under controlled conditions of light:darkness in our animal facilities. All animal procedures were conducted in accordance with our Federal Regulations for the Use and Care of Animals (NOM-062-ZOO-1999, Ministry of Agriculture, México), and were approved by the Institutional Committee of the Universidad Michoacana de San Nicolás de Hidalgo, for the use of animals. Systolic blood pressure was determined by plethysmography, yielding values of $134 \pm 3$ and $122 \pm 3$ mmHg (WKY) and $183 \pm 5$ and $203 \pm 14$ mmHg (SHR), for 3 and 7 month-old rats, respectively. At 1 month of age rats from all strains were normotensive. In experiments with young rats, the brains from 4 animals were pooled.

Brain mitochondria were isolated by differential centrifugation in a Percoll gradient as described (Thakar and Hassan, 1988; Sims, 1990). Briefly, rats were decapitated and the brain was extracted and placed in a cold medium that contained 210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 0.5% bovine serum albumin, 10 mM MOPS (pH 7.4). Brain was homogenized manually in a glass homogenizer and centrifuged at $400 \times g$; the supernatant was centrifuged at $9000 \times g$. Centrifugations were carried out for 10 min at 4°C. The pellet was resuspended in 15% Percoll and placed in a discontinuous gradient of Percoll (23% and 40%). The gradient was centrifuged at $30{,}700 \times g$, during 6 min, band 3 was extracted, diluted 1:4, centrifuged and washed at $16{,}700 \times g$ in the isolation medium added with 0.5% bovine serum albumin, following centrifugation at $6{,}900 \times g$ for 10 min. Mitochondrial protein concentration was measured by a slight modification of the biuret reaction (Gornall et al., 1949).

### *L-Citrulline synthesis*

L-Citrulline production was determined as described (Knipp and Vasak, 2000) in a medium that contained 190 mM mannitol, 5 mM $KH_2PO_4$, 15 mM KCl, 3 mM $MgCl_2$, 1 mM EGTA, 10 mM MOPS (pH 7.4), plus

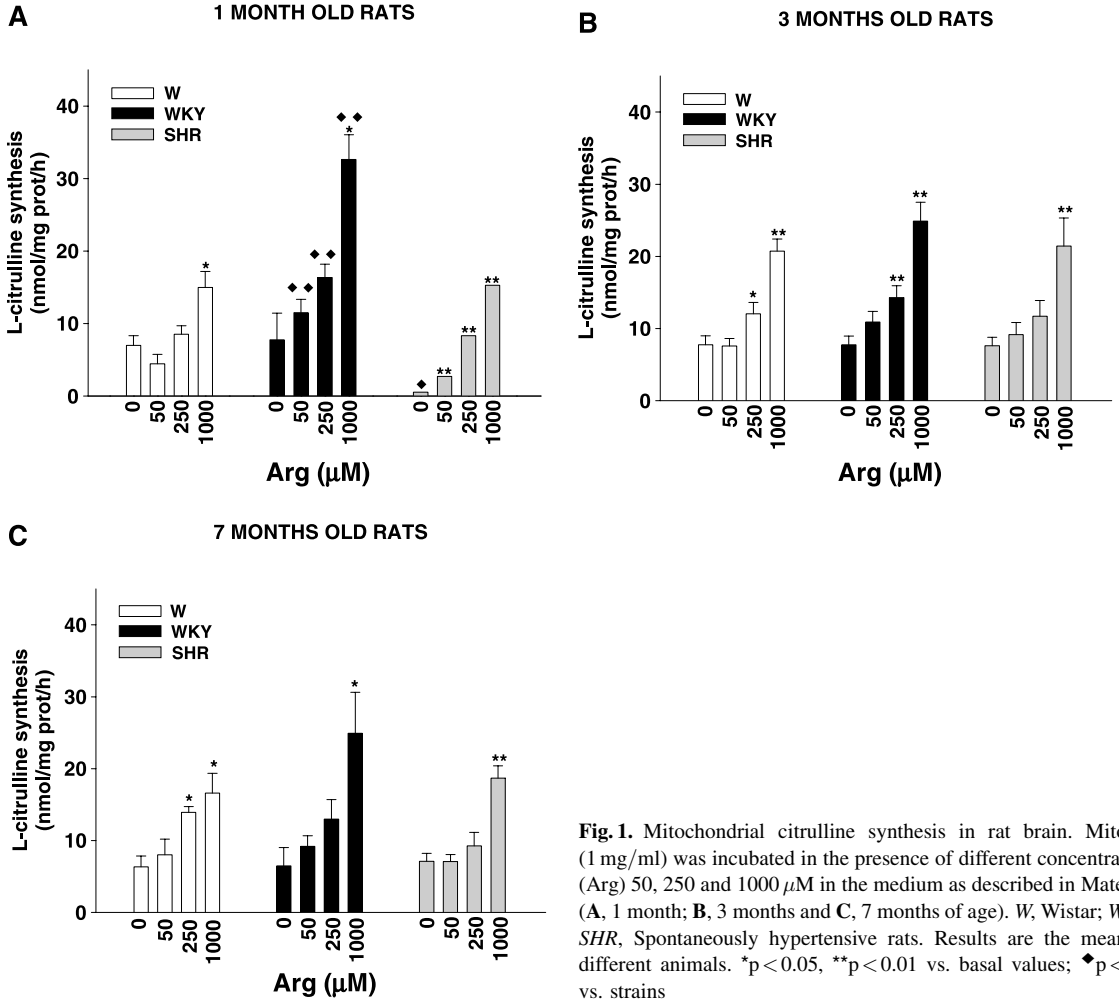


**Fig. 1.** Mitochondrial citrulline synthesis in rat brain. Mitochondrial protein (1 mg/ml) was incubated in the presence of different concentrations of L-arginine (Arg) 50, 250 and 1000 $\mu$M in the medium as described in Materials and methods. (**A**, 1 month; **B**, 3 months and **C**, 7 months of age). *W*, Wistar; *WKY*, Wistar-Kyoto; *SHR*, Spontaneously hypertensive rats. Results are the mean ± SEM of 4–10 different animals. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. basal values; $^{\blacklozenge}p < 0.05$, $^{\blacklozenge\blacklozenge}p < 0.01$ vs. strains

10 mM succinate and 2 $\mu$M rotenone. Brain mitochondria were incubated 1 h at 30°C, in a shaking bath (30 rpm), in the presence of the nitric oxide synthase cofactors 0.1 mM NADPH, 10 $\mu$M tetrahydrobiopterin, 5 $\mu$M $FAD^+$ and 40 $\mu$M $CaCl_2$ and 50, 250 or 1000 $\mu$M of the substrate L-arginine, with or without 100 $\mu$M 7-Nitroindazole (7-NI). Basal values of L-citrulline were obtained with endogenous L-arginine. L-Citrulline was quantified in a Perkin-Elmer Lambda 10 spectrophotometer at 530 nm using a calibration curve.

*Mitochondrial respiration*

Oxygen consumption was measured in a chamber fitted with a Clark-type oxygen electrode. The incubation buffer contained 210 mM mannitol, 70 mM sucrose, 0.5 mM EGTA, 10 mM $K_2HPO_4$ and 10 mM MOPS (pH 7.4), plus respiration substrate (10 mM succinate and 2 $\mu$M rotenone). State 3 was obtained by adding 300 $\mu$M ADP. Different concentrations of L-arginine were used to determine the effect of $NO^\bullet$ in mitochondrial respiration with or without 7-NI.

*Data analysis*

Values represent the mean ± SEM from 3–17 animals. Data were subjected to Student's *t* test to determine the statistical significance ($p < 0.05$).

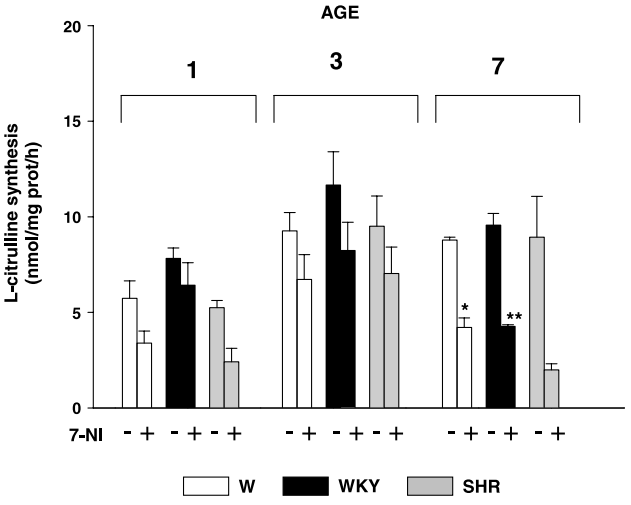


**Fig. 2.** Mitochondrial citrulline synthesis in rat brain at different ages in the presence of 7-Nitroindazole. Mitochondrial protein (1 mg/ml) was incubated (7-NI) 100 $\mu$M in the medium as described in Materials and methods. *W*, Wistar; *WKY*, Wistar-Kyoto; *SHR*, Spontaneously hypertensive rats. Results are the means ± SEM of 4–10 different animals. $^*p < 0.05$, $^{**}p < 0.01$ vs. basal values

## Results

*L-Citrulline determination*

Nitric oxide synthase catalyze the oxidation of L-arginine to L-citrulline and $NO^\bullet$. Therefore, the production of L-citrulline is used as an indirect determination of $NO^\bullet$. In this work, we studied the effect of the stimulation and inhibition of the synthesis of L-citrulline by the addition of the substrate L-arginine, and the NOS noncompetitive inhibitor 7-NI, respectively. Figure 1 shows the results of L-citrulline production in the three rat strains of different ages. Wistar, WKY and SHR rat brain mitochondria from 1 month-old rats exhibited L-arginine concentration-dependent L-citrulline synthesis (Fig. 1A). Wistar basal value was 7 ± 1 nmol L-citrulline/mg prot/h, which increased to 16 ± 2 nmol L-citrulline/mg prot/h ($p < 0.05$), at 1 mM L-arginine. However, in WKY brain mitochondria the basal value of L-citrulline was 8 ± 4 nmol/mg prot/h., which increased ≈4 times (33 ± 3 nmol/mg prot/h, $p < 0.05$), in the presence of 1 mM of L-arginine. In contrast, a very low basal value of L-citrulline (0.53 nmol/mg prot/h), which was significantly different ($p < 0.05$) to the other strains, was obtained in SHR brain mitochondria (Fig. 1A). However, these mitochondria also exhibited a significant ($p < 0.01$) concentration-dependent response to L-arginine (up to 15 nmol/mg prot/h at 1 mM substrate).

In Fig. 1B and C L-citrulline values for 3 and 7 month-old rats are shown. These results also showed an L-arginine concentration-dependent increase in L-citrulline formation. Basal L-citrulline values were 7 and 8 nmol/mg prot/h in 3 and 7 months old rats, respectively (Fig. 1B and C). L-Citrulline synthesis increased to 20 ± 2 (W), 26 ± 2 (WKY) and 22 ± 4 nmol/mg prot/h (SHR) in rats of 3 months of age, in the presence of 1 mM L-arginine (Fig. 1B). While in older rats the values found were 17 ± 3 (W), 25 ± 5 (WKY) and 19 ± 2 nmol L-citrulline/mg prot/h (SHR) with 1 mM of L-arginine (Fig. 1C). These results show that there are not differences between rat strains at these ages (Fig. 1B and C). SHR of 3 and 7 months of age increased basal L-citrulline values with respect to 1 month old (Fig. 1A–C).

The activity of mtNOS was assayed in the presence of 7-Nitroindazole, which is a noncompetitive inhibitor of NOS with high selectivity for the brain nNOS enzyme (Wolff et al., 1994). The results showed an inhibition of basal values of L-citrulline in the three ages and the three rat strains of 20–50% ($p < 0.05$) (Fig. 2).

*Mitochondrial respiration*

Nitric oxide can regulate oxygen consumption (Ghafourifar and Richter, 1997). Therefore, it was important to know its effect on this mitochondrial function in rat brain. Oxygen consumption was determined in the presence of different concentrations of L-arginine and 7-NI. In Fig. 3A–C, oxygen consumption values in states 3 and 4 in W, WKY and SHR rats of 1 month of age, are shown. Oxygen consumption in Wistar rats increased in

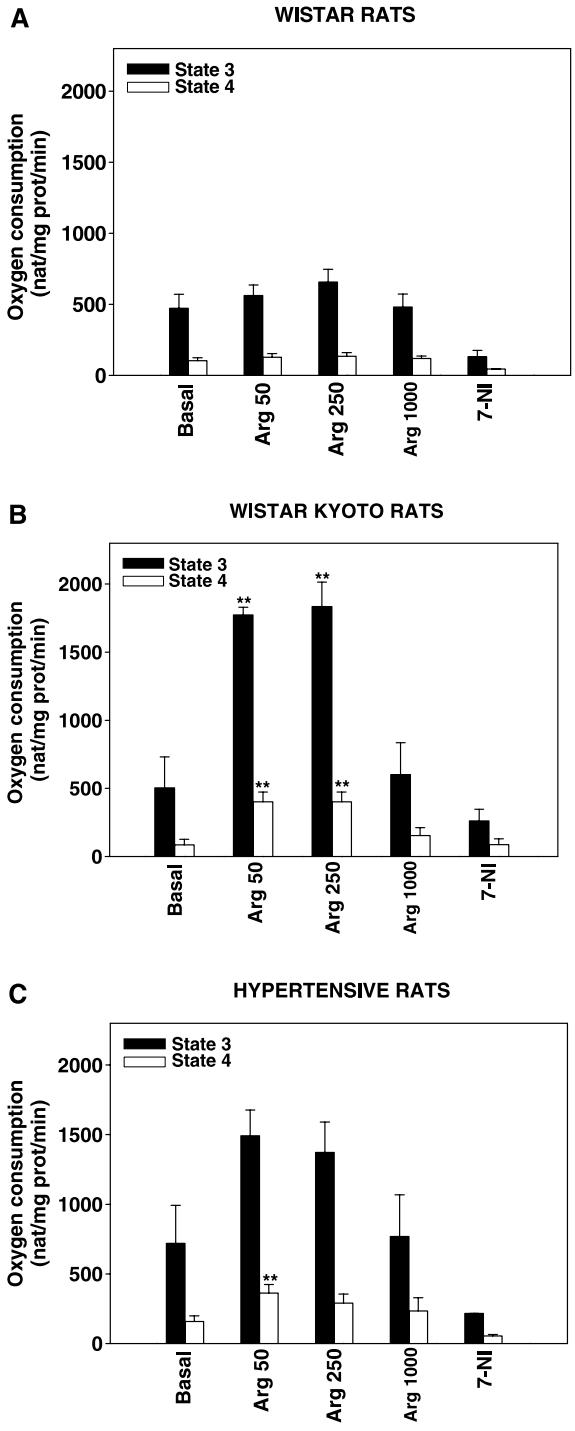


**Fig. 3.** Effect of L-Arginine and 7-NI on mitochondrial respiration in 1 month-old rats. Oxygen uptake of purified mitochondrial in rat brain of different strains. Mitochondrial protein (0.5 mg/ml) was incubated in the presence of different concentrations of L-arginine (*Arg*), 50, 250 and 1000 $\mu$M, and 100 $\mu$M 7-NI in the medium as described in Materials and methods. **A**, Wistar; **B**, WKY; **C**, SHR rats. Results are the means ± SEM of 4–10 different animals. $^{**}p < 0.01$ vs. basal

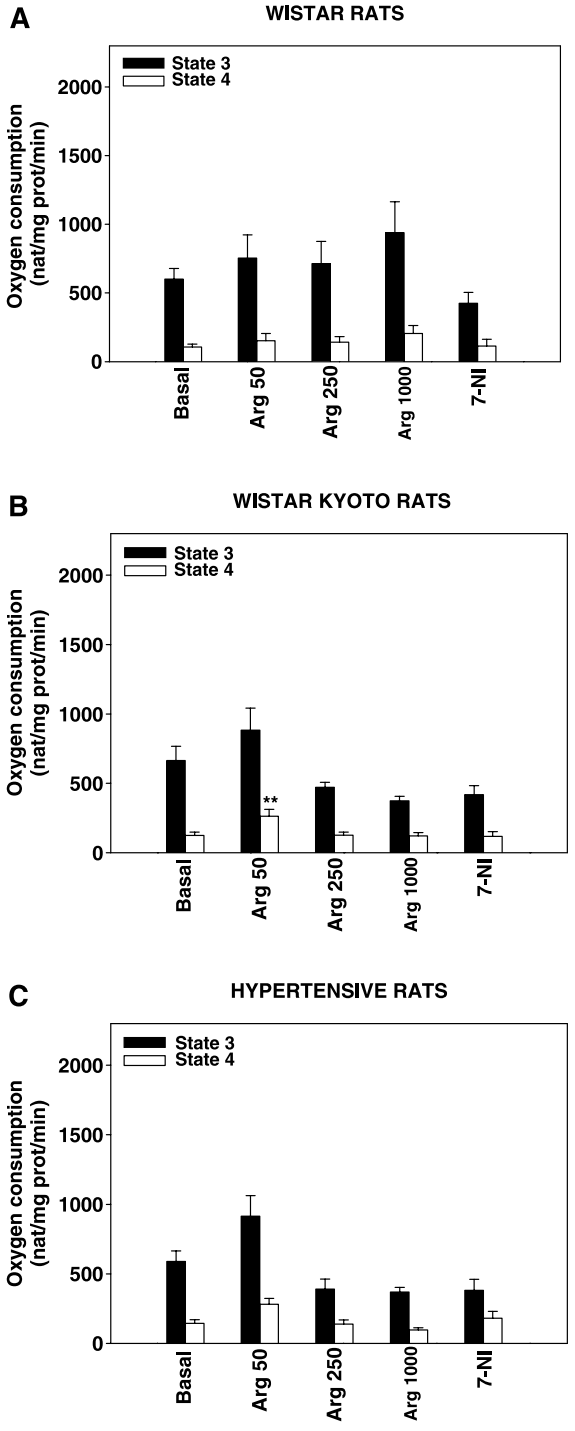


**Fig. 4.** Effect of L-Arginine and 7-NI on mitochondrial respiration in 3 month-old rats. Oxygen uptake of purified mitochondrial in rat brain of different strains. Mitochondrial protein (0.5 mg/ml) was incubated in the presence of different concentrations of L-arginine (*Arg*), 50, 250 and 1000 $\mu$M, and 100 $\mu$M 7-NI in the medium as described in Materials and methods. **A**, Wistar; **B**, WKY; **C**, SHR rats. Results are the means ± SEM of 4–10 different animals. $^{**}p < 0.01$ vs. basal

the presence of 50 and 250 $\mu$M of L-arginine (Fig. 3A). Similar results were observed in WKY rats, where oxygen consumption basal value was $504 \pm 200$ nat/mg prot/min and increased to $1773 \pm 58$ and $1834 \pm 179$ nat/mg prot/min in the presence of 50 and 250 $\mu$M of L-arginine, respectively, in state 3 (Fig. 3B). Basal oxygen consumption was $86 \pm 39$ nat/mg prot/min, while 50 and 250 $\mu$M L-arginine increased to $401 \pm 72$ nat/mg prot/min, in state 4 (Fig. 3B). The increase in oxygen consumption also was observed in states 3 and 4 for SHR rats (Fig. 3C).

On the other hand, 7-NI inhibited oxygen consumption in states 3 and 4 in the three rat strains at 1 month of age. This inhibition was 60–70% in state 3 and 58–72% in state 4 (Fig. 3A–C).

Similar results were observed in 3 month-old Wistar rats, with an increase in oxygen consumption in both, states 3 and 4 and also the respiratory rate was inhibited by 7-NI (Fig. 4A).

Results obtained in 3 month-old WKY and SHR rats showed an increase in the respiratory rate in the presence of 50 $\mu$M L-arginine (Fig. 4B and C). Basal values were $663 \pm 104$ and $124 \pm 25$ nat/mg prot/min in states 3 and 4, respectively, for WKY rats while they increased to $883 \pm 159$ and $262 \pm 51$ nat/mg prot/min in state 3 and 4 with 50 $\mu$M L-arginine. In SHR rats an increase in oxygen consumption was observed in states 3 and 4, with that L-arginine concentration however, there was a decrease with higher substrate concentrations (Fig. 4C).

Under basal conditions, 7-NI inhibited oxygen consumption in states 3 and 4 in all rat strains of 3 months of age. This inhibition was smaller in 1 month old rats (Fig. 4A–C).

In 7 months old rats an increase in state 3 was observed only at 1 mM L-arginine, with no changes at lower concentrations of the substrate. In these rats 7-NI increased oxygen consumption in all strains (Fig. 5A–C).

Table 1 shows the respiratory control ratio (RCR), where basal values of RCR were 4–8, which corroborate the integrity and functionality of rat brain mitochondria. These results show a decrease of RCR in the presence of L-arginine and 7-NI. This effect was more pronounced in 7 month-old rats of all strains. In Wistar rats RCR diminished to 68, 55 and 46% with 50, 250 and 1000 $\mu$M L-arginine, respectively. When 7-NI was used the RCR value was diminished to 23%, with respect to control.

WKY rats (7 month-old) decreased their RCR with L-arginine (85, 71 and 86% with 50, 250 and 1000 $\mu$M, respectively) and 29% in the presence of 7-NI. In SHR of

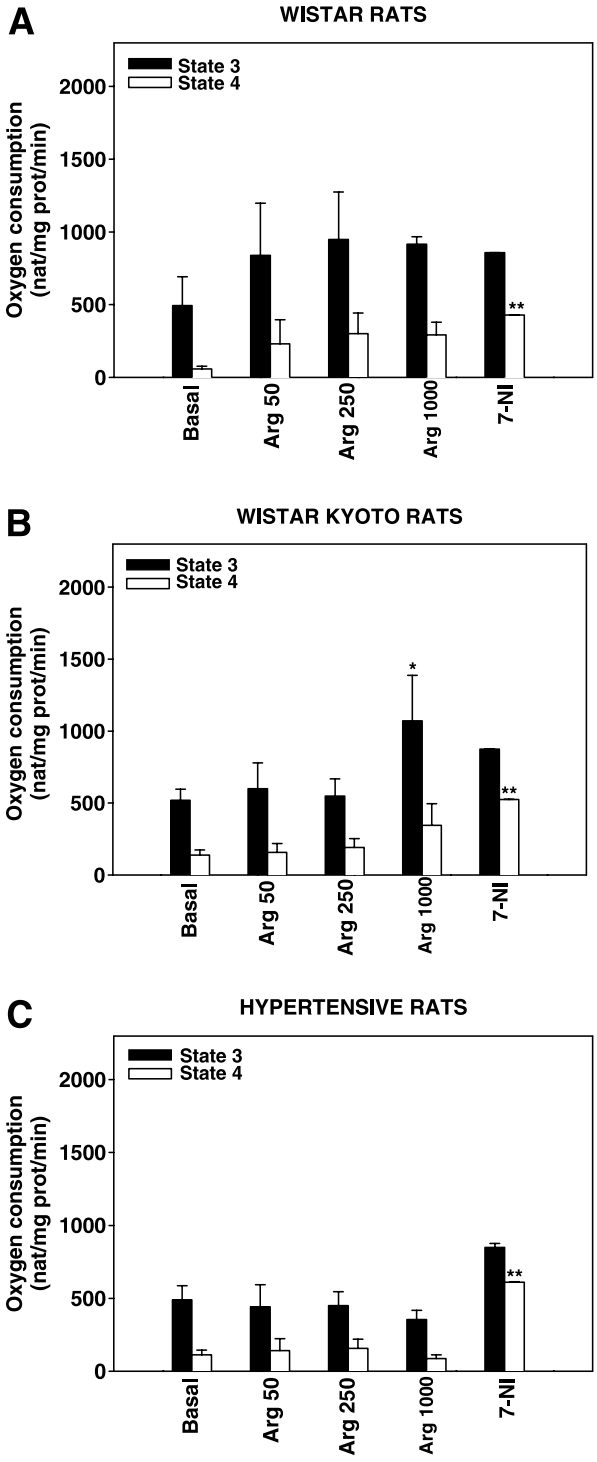


**Fig. 5.** Effect of L-Arginine and 7-NI on mitochondrial respiration in 7 month-old rats. Oxygen uptake of purified mitochondrial in rat brain of different strains. Mitochondrial protein (0.5 mg/ml) was incubated in the presence of different concentrations of L-arginine (*Arg*), 50, 250 and 1000 $\mu$M, and 100 $\mu$M 7-NI in the medium as described in Materials and methods. **A**, Wistar; **B**, WKY; **C**, SHR rats. Results are the means $\pm$ SEM of 4–10 different animals. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. basal

**Table 1.** Effect of L-Arginine and 7-NI on the respiratory control ratio[1,2,3,4]

| Concentration ($\mu$M) | Respiratory control ratio (RCR) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Wistar rats (months) | | | Wistar-Kyoto rats (months) | | | Hypertensive rats (months) | | |
| | 1 | 3 | 7 | 1 | 3 | 7 | 1 | 3 | 7 |
| Control | $4.7 \pm 0.5$ | $6.8 \pm 0.6$ | $8.5 \pm 0.4$ | $6.1 \pm 0.5$ | $6.4 \pm 0.6$ | $5.2 \pm 0.7$ | $4.5 \pm 1.3$ | $5.0 \pm 0.5$ | $4.9 \pm 0.4$ |
| L-arg 50 | $5.0 \pm 0.5$ | $6.9 \pm 1.3$ | $5.8 \pm 1.5$ | $4.8 \pm 0.7$ | $3.6 \pm 0.3^{**}$ | $4.4 \pm 0.9$ | $4.1 \pm 0.1$ | $3.2 \pm 0.5$ | $5.8 \pm 1.7$ |
| L-arg 250 | $5.5 \pm 0.9$ | $5.7 \pm 0.6$ | $4.7 \pm 1.1$ | $4.7 \pm 0.3$ | $4.7 \pm 1.4$ | $3.7 \pm 0.6$ | $5.1 \pm 0.6$ | $3.0 \pm 0.4^{*}$ | $3.8 \pm 0.4$ |
| L-arg 1000 | $3.7 \pm 0.4$ | $6.3 \pm 1.5$ | $3.9 \pm 1.4$ | $4.2 \pm 0.5^{*}$ | $3.3 \pm 0.4^{*}$ | $4.5 \pm 1.4$ | $3.8 \pm 0.8$ | $4.4 \pm 0.8$ | $4.8 \pm 0.8$ |
| 7NI 100 | $3.0 \pm 1.0$ | $5.2 \pm 1.2$ | $2.0 \pm 0.05^{**}$ | $3.3 \pm 0.6^{*}$ | $4.2 \pm 0.5$ | $1.5 \pm 0.08$ | $4.1 \pm 0.8$ | $3.5 \pm 0.6$ | $1.4 \pm 0.03^{**}$ |

[1] The RCR was measured in purified mitochondrial in rat brain of different strains and ages
[2] Mitochondrial protein (0.5 mg/ml) was incubated in the presence of different concentrations of L-arginine (Arg), 50, 250 and 1000 $\mu$M, and 7-NI 100 $\mu$M in the medium as described in Materials and methods
[3] Respiration was stimulated with 300 $\mu$M ADP
[4] Results are the mean $\pm$ SEM of 4–10 different animals. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control

same age, the diminution in RCR were 78, 98 and 29% with 250 and 1000 $\mu$M L-arginine and 7-NI, respectively.

## Discussion

Nitric oxide is an important molecule generated in many organisms. Because of its effects on neurotransmission, vasodilatation, immune responses, modulation of respiration and mitochondrial pH, among others (Giulivi et al., 1998; Tatoyan and Giulivi, 1998; López-Figueroa et al., 2000; Orsi et al., 2000; Ghafourifar et al., 2001; Saavedra-Molina et al., 2003), $NO^{\bullet}$ has become a target for research. $NO^{\bullet}$ production and function is altered in pathological conditions, such as diabetes and hypertension. Hypertension is recognized as an important risk factor for cerebral stroke (Bordet et al., 2000; Marín et al., 2000), which is a key reason for intense research, part of which is focused in this work. Our results show that the production of intramitochondrial $NO^{\bullet}$ could be affected in hypertension and could influence mitochondrial metabolism.

Riobó et al. (2002) found that basal production of L-citrulline in 1 month old Wistar rats was $\cong$1.25 nmol/mg prot/h, while we found 7–8 nmol/mg prot/h in W and WKY, and 0.53 nmol/mg prot/h in SHR. These results suggest that mtNOS activity is diminished in the first stages of hypertension progression, but once hypertension is established (3 and 7 month-old) mtNOS activity is recovered, and $NO^{\bullet}$ synthesis increased perhaps to offset the damage produced by the pathology.

In intact rat brain mitochondria L-citrulline synthesis was minor than other reports, despite age and strain. In those studies basal mtNOS activity values were 31.2, 61.2, and 62.4 nmol/mg prot/h for rat liver, kidney and heart mitochondria, respectively (Ghafourifar and Richter, 1997; Aguilera-Aguirre et al., 2002). Our results are comparable with those obtained in 3 month-old mice brain and pig heart at the same age (2.0 y 13.6 nmol citrulline/mg prot/h, respectively).

7-Nitroindazole (7-NI) a nNOS specific noncompetitive inhibitor (Wolff et al., 1994), decreased L-citrulline synthesis in brain mitochondria of all rat strains at all ages tested. Results demonstrated that L-citrulline synthesis was mtNOS catalyzed; furthermore, it corroborates that mtNOS is a nNOS (Kanai et al., 2001; Elfering et al., 2002).

$NO^{\bullet}$ is considered to have a regulator effect on respiration, by inhibiting oxygen consumption, both in liver and kidney (Brown and Borutaité, 2002; Ghafourifar and Richter, 1997; Giulivi et al., 1998), but not in porcine heart mitochondria where it has no effect on respiration (French et al., 2001). However, in brain mitochondria we

found a four-fold increase in oxygen consumption in both respiratory states, in all conditions tested. This effect was more evident in young WKY and SHR with 50 and 250 $\mu$M L-arginine; while other authors found a $NO^{\bullet}$ inhibitory action at 1 mM L-arginine in intact liver rat mitochondria (Ghafourifar and Richter, 1997). Our data reveal that oxygen uptake was not inhibited by $NO^{\bullet}$ in rat brain mitochondria, but rather it increased, suggesting an uncoupling of mitochondrial respiration. In this regard, some authors reported that peroxynitrite (ONOO−), an oxidant agent of the reaction of $NO^{\bullet}$ and superoxide anion ($O_2^{\cdot -}$), forms rapidly and can stimulate proton leak through the mitochondrial inner membrane, which could be responsible for this effect (Ghafourifar et al., 2001; Brown and Borutaite, 2002; Radi et al., 2002).

Since nNOS is a multifunctional enzyme that catalyzes $O_2^{\cdot -}$ synthesis in significative amounts, under $BH_4$ and L-arginine non-saturating conditions (Mayer and Hemmens, 1997) and mtNOS is a post-translational modification of nNOS (Kanai et al., 2001; Elfering et al., 2002; Giulivi, 2003), then mtNOS could produce $O_2^{\cdot -}$ with low L-arginine concentrations (50 and 250 $\mu$M). Our results agree with the contention that peroxynitrite could uncouple mitochondrial respiration, and increase oxygen uptake.

On the other hand, because 7-NI inhibited the oxygen uptake in states 3 and 4 in all conditions tested, we suggest that 7-NI acts as an inhibitor of the respiratory chain, probably by binding to hemo group of complex III or lV of the respiratory chain, since 7-NI is a noncompetitive inhibitor at the hemo group of NOS (Wolff et al., 1994).

Other important indicator of mitochondrial function is RCR, where typical values in rat liver coupled mitochondria are 3–15 (Sims, 1990; Giulivi et al., 1998; Thakar and Hassan, 1988). Our RCR values were in that range (4–8), indicating an adequate mitochondrial function; however, others have reported that $NO^{\bullet}$ inhibits respiration and consequently RCR in rat liver mitochondria (Giulivi et al., 1998; Saavedra-Molina et al., 2003). Even though we observed an inhibition in RCR with L-arginine, it is not attributed to $NO^{\bullet}$, since it decreased the oxygen consumption rate; rather, inhibition in RCR could be due to uncoupling of the respiratory chain, because RCR decreased and oxygen consumption increased. A higher effect was observed as the animal ages (7 month-old). Aging is a process that embrace physiological changes occurring during life-time, resulting in a diminished functional capacity of the organism that can be produced by different environmental factors, where free radicals are the most important. Oxidative damage in mitochondria plays an important role in aging, because the organelle is a source of reactive oxygen species, and an increased production favours aging. $O_2^{\cdot -}$ itself or in combinations with other free radicals can cause oxidative stress to mitochondria (Nakahara et al., 1998; Sarkela et al., 2001).

## Conclusions

Our results suggest that basal production of $NO^{\bullet}$ in rat brain mitochondria is not sufficient for regulate oxidative phosphorylation, and show the importance $NO^{\bullet}$ has in different conditions, like hypertension and aging, having specific tissue effects.

## Acknowledgements

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