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Biochimica et Biophysica Acta 1767 (2007) 1118 – 1125
Biochimica et Biophysica Acta
BBA
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Dopamine enhances mtNOS activity: Implications in mitochondrial function

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Received 13 February 2007; received in revised form 20 June 2007; accepted 6 July 2007
Available online 24 July 2007

Abstract

Dopamine and nitric oxide systems can interact in different processes in the central nervous system. Dopamine and oxidation products have been related to mitochondrial dysfunction. In the present study, intact mitochondria and submitochondrial membranes were incubated with different DA concentrations for 5 min. Dopamine (1 mM) increased nitric oxide production in submitochondrial membranes and this effect was partially prevented in the presence of both DA and NOS inhibitor N_ω-nitro-L-arginine (L-NNA). A 46% decrease in state 3 oxygen uptake (active respiration state) was found after 15 mM dopamine incubation. When mitochondria were incubated with 15 mM dopamine in the presence of L-NNA, state 3 respiratory rate was decreased by only 17% showing the involvement of NO. As shown for O_2 consumption, the inhibition of cytochrome oxidase by 1 mM DA was mediated by NO. Hydrogen peroxide production significantly increased after 15 mM DA incubation, being mainly due to its metabolism by MAO. Also, DA-induced depolarization was prevented by the addition of L-NNA showing the involvement of nitric oxide in this process too. This work provides evidence that in the studied conditions, dopamine modifies mitochondrial function by a nitric oxide-dependent pathway.

Keywords: Dopamine; Mitochondrial respiration; Brain mitochondria; Nitric oxide; Mitochondrial membrane potential

1. Introduction

It is generally known that dopamine (DA) loss represents the main neurochemical alteration in Parkinson's disease. However, DA itself has been shown to have a role in dopaminergic neurodegeneration. The most commonly described mechanism of DA toxicity is its own enzymatic oxidation by monoamine oxidase, leading to hydrogen peroxide (H_2O_2) generation which causes oxidative stress and increases lipid and protein peroxidation [1]. The disruption of mitochondrial processes by catecholamines has been implicated in the mechanisms underlying neurodegeneration in Parkinson's disease [2]. It has been shown that dopamine (DA) is able to induce mitochondrial dysfunction by a decrease in respiratory complex I activity, induction of mitochondrial swelling and alteration of mitochondrial membrane potential [2–4]. Different mechanisms of action of dopamine on mitochondrial function have been described depending on drug concentration and time of exposure [5,6]. For instance, Gluck et al. [4] have proposed that inhibition of brain mitochondrial respiration by 0.5 mM DA would be mediated by H_2O_2 generation by MAO, while incubation of brain mitochondria with 5–20 mM DA can inhibit mitochondrial oxygen consumption mainly by quinone oxidation [7].

Dopamine, acetylcholine and NO systems interact to induce corticostriatal synaptic plasticity [8]. Also, Melis and colleagues [9] reported that dopamine agonists increase nitric oxide synthase (NOS) activity in the cell bodies of paraventricular nucleus of the hypothalamus. Recently, it has been described that phasis dopaminergic transmission increases NO efflux in the rat dorsal striatum via a neuronal NOS [10].

Nitric oxide (NO) is produced in mitochondria by a mitochondrial nitric oxide synthase called mtNOS [11,12] and it has important implications for the metabolism of cellular energy [13] mainly by acting as a modulator of mitochondrial respiratory chain.

The aim of this study was to evaluate the possible interaction between dopamine and nitric oxide systems, and its impact on mitochondrial function using a broad range of dopamine concentrations. Oxygen consumption, hydrogen peroxide production, cytochrome oxidase activity and mitochondrial membrane

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doi:10.1016/j.bbabio.2007.07.005

potential were determined as parameters of mitochondrial function.

2. Materials and methods

2.1. Animals

Three months female Swiss mice (20–25 g) from the animal facility of the School of Pharmacy and Biochemistry were used. Animals were housed in an environmentally controlled room and allowed free access to food and water. Animal treatment was carried out in accordance with the guidelines of the National Institute of Health (USA) for the care and use of laboratory animals (NIH Publ. 8023, 1996). All efforts were made to minimize suffering and reduce the number of animals used.

2.2. Preparation of synaptosome-free mitochondria

Experimental groups of 6 mice were used. Brains from 2 mice were pooled for each experimental point. Tissues were weighed and homogenized (1:5 w/v) in a medium consisting of 0.23 M mannitol, 0.07 M sucrose, 10 mM Tris–HCl and 1 mM EDTA, pH 7.4. Homogenates were centrifuged at 700×*g* for 10 min to discard nuclei and cell debris and the pellet was washed to enrich the supernatant that was centrifuged at 8000×*g* for 10 min. The resulting pellet, containing both synaptic and non-synaptic mitochondria, was able to carry out oxidative phosphorylation [14]. Further mitochondrial purification was performed by Ficoll gradient [15]. All the procedure was carried out at 0–2 °C. Mitochondria were submitted to twice freezing and thawing conditions and passed through hypodermic needle [16], and the preparation obtained consisted of a fraction of submitochondrial membranes which do not present any sideness or restriction to substrate access. Protein content was assayed by using the Folin phenol reagent and bovine serum albumin as standard [17].

For mitochondrial membrane potential determinations, brains were removed and homogenized in MSH buffer (0.21 M mannitol, 0.07 M sucrose, 5 mM HEPES, pH 7.4) supplemented with 1 mM EDTA. Homogenates were centrifuged at 600×*g* for 10 min at 4 °C. The supernatant was decanted and centrifuged at 6000×*g* for 10 min to form a mitochondrial pellet that was resuspended in MSH buffer without EDTA and centrifuged again at 6000×*g* for 10 min [18]. The final pellet was resuspended in MSH buffer at a protein concentration of 20–25 mg/ml.

2.3. Measurement of nitric oxide production

Nitric oxide production was measured in submitochondrial membranes using a spectrophotometric method by following the oxidation of oxyhemoglobin (HbO_2) to methemoglobin (metHb) at 37 °C. The NO assay was performed using a Beckman-Coulter Serie DU 7400 diode array spectrophotometer in which the active wavelength is set at 577 nm and the reference wavelength at the isosbestic point at 591 nm ($\varepsilon = 11.2\ mM^{-1}\ cm^{-1}$) [16]. The method is based on the original assay developed by Murphy and Noack [19] for perfused organs in which the HbO_2 γ band is used to follow NO production. The α band is more suitable for high light scattering conditions of cellular and mitochondrial suspensions due to the close vicinity of the active (577 nm) and the reference (591 nm) wavelengths [16].

The measurements were carried out in a reaction medium containing 50 mM phosphate buffer pH 5.8, 1 mM $CaCl_2$, 50 μM L-arginine, 100 μM NADPH, 10 μM DTT, 4 μM Cu–Zn superoxide dismutase (SOD), 0.1 μM catalase, 0.5–1.0 mg submitochondrial protein/ml and 25 μM oxyhemoglobin (expressed per heme group).

Oxyhemoglobin reacts efficiently with NO if the reaction between NO and superoxide anion (O_2^-) is prevented. Superoxide dismutase is added to abrogate any other reaction with O_2^-, including a direct oxidation of HbO_2 to metHb or reduction of metHb to Hb. In the presence of SOD, H_2O_2 could be produced. H_2O_2 can oxidize both HbO_2 and metHb to higher oxidation states. For this reason, catalase is added to the assay [19].

Controls adding 0.5 mM N_ω-nitro-L-arginine (L-NNA) and 0.5 mM N-nitro-L-arginine-methyl-ester (L-NAME) as NOS inhibitors were performed in all cases to give specificity to the assay; addition of L-NNA and L-NAME inhibited by about 73% and 50% the rate of hemoglobin oxidation respectively.

2.4. Evaluation of respiratory complexes activity

NADH-cytochrome *c* reductase activity (complex I+III) was measured in brain submitochondrial membranes by following spectrophotometrically the reduction of cytochrome *c* at 30 °C at 550 nm ($\varepsilon = 19.6\ mM^{-1}\ cm^{-1}$) in a reaction medium containing 100 mM phosphate buffer (pH 7.4), 0.2 mM NADH, 0.1 mM cytochrome *c* and 0.5 mM KCN. Enzyme activity was expressed in nmoles cytochrome *c* reduced per minute per mg of protein. Succinate cytochrome *c* reductase activity (complex II+III) was similarly determined and expressed, except that NADH was substituted by 20 mM succinate.

Cytochrome oxidase activity (complex IV) was assayed spectrophotometrically at 550 nm by following the rate of oxidation of 50 μM ferrocytochrome *c* [20]. The activity was expressed as nmoles ferrocytochrome *c* oxidized per minute per mg of protein.

2.5. Oxygen consumption

A two-channel respirometer for high-resolution respirometry (Oroboros Oxygraph, Paar KG, Graz, Austria) was used. Mitochondrial respiratory rates were measured in a reaction medium containing 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 4 mM $MgCl_2$, 5 mM phosphate and 0.2% bovine serum albumin at 37 °C. Malate 6 mM and glutamate 6 mM were used as substrates to measure state 4 respiration and 1 mM ADP was added to measure state 3 respiration [21].

The respiratory control ratio (state 3 respiration/state 4 respiration) was determined [22].

2.6. Measurement of hydrogen peroxide production

Hydrogen peroxide generation was determined in intact brain mitochondria (0.1–0.3 mg protein/ml) by the scopoletin-HRP method, following the decrease in fluorescence intensity at 365–450 nm (λexc–λem) at 37 °C [23]. The reaction medium consisted of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl (pH 7.4), 0.8 μM HRP, 1 μM scopoletin, 6 mM malate, 6 mM glutamate and 0.3 μM SOD. Superoxide dismutase was added in order to minimize $ONOO^-$ production by interaction of NO with O_2^-. A calibration curve was made using H_2O_2 (0.05–0.35 μM) as standard to express the fluorescence changes as nmol H_2O_2/min mg protein.

2.7. Mitochondrial membrane potential

Mitochondria (0.25 μg/ml) were loaded with 30 nM of the potentiometric cationic probe $DiOC_6$ during 20 min at 37 °C and immediately 20000 events were acquired by a FAC-SCAN flow cytometer equipped with a 488-nm argon laser and a 615-nm red diode laser. Mitochondrial fluorescence with no probe and after FCCP treatment was measured as negative and positive controls respectively. Data from the experiments were analyzed using the CellQuest software (Becton and Dickinson).

2.8. Drugs and chemicals

Dopamine, ADP, L-arginine, catalase, dithiothreitol, EDTA, glutamic acid, malic acid, mannitol, NADPH, N_ω-nitro-L-arginine, N-nitro-L-arginine-methyl-ester, haemoglobin, scopoletin, horseradish peroxidase, succinate, sucrose, superoxide dismutase, cytochrome *c,* trizma base and were purchased from Sigma Chemical Co. (St. Louis, Missouri). Other reagents were of analytical grade.

2.9. Statistics

Results are expressed as mean values±SEM. Student's *t* test was used to analyze the significance of differences between paired groups. ANOVA was used to analyze differences between mean values of more than two groups, as described at the bottom of the tables.

3. Results

3.1. Effect of dopamine on mitochondrial enzyme activity

3.1.1. Nitric oxide synthase activity

Nitric oxide production was measured in control brain submitochondrial membranes and after 5 min incubation at 37 °C with different DA concentrations. Fig. 1a shows that low concentrations of DA (2–500 μM) were not able to modify NO production as compared with control submitochondrial membranes. However, incubation of submitochondrial membranes with 1 mM and 15 mM DA significantly increased NO production by approximately 2-fold and 3-fold respectively as compared with control values (0.30±0.04 nmol/min mg protein).

In order to evaluate the direct effect of DA on mtNOS activity, we measured NO production in the presence of two different NOS inhibitors: L-NNA and L-NAME. A typical response of inhibition of NO production was observed in control submitochondrial membranes after incubation with both 0.5 mM L-NNA and 0.5 mM L-NAME (Fig. 1b). The inhibitory effect of L-NNA on mitochondrial NO production was more important than the effect of L-NAME, possibly due to its higher specificity for brain nNOS inhibition as reported [24]. In the presence of L-NNA, incubation of submitochondrial membranes with 1 mM and 15 mM DA increased NO production by 87% and 162% respectively, as compared with control submitochondrial membranes incubated with L-NNA. Also, in the presence of L-NAME and 1 mM dopamine an 80% increase in NO production was observed, as compared with control+L-NAME values.

Similar results were obtained by measuring NO production using a spectrophotometric method based on the detection of the NO–HRP complex (data not shown) [25].

3.1.2. Respiratory complexes activity

Incubation of submitochondrial membranes with 1 mM DA inhibited NADH-cytochrome *c* reductase activity by 20% as compared with control submitochondrial membranes (326±7 nmol/min mg protein). In contrast, no significant changes

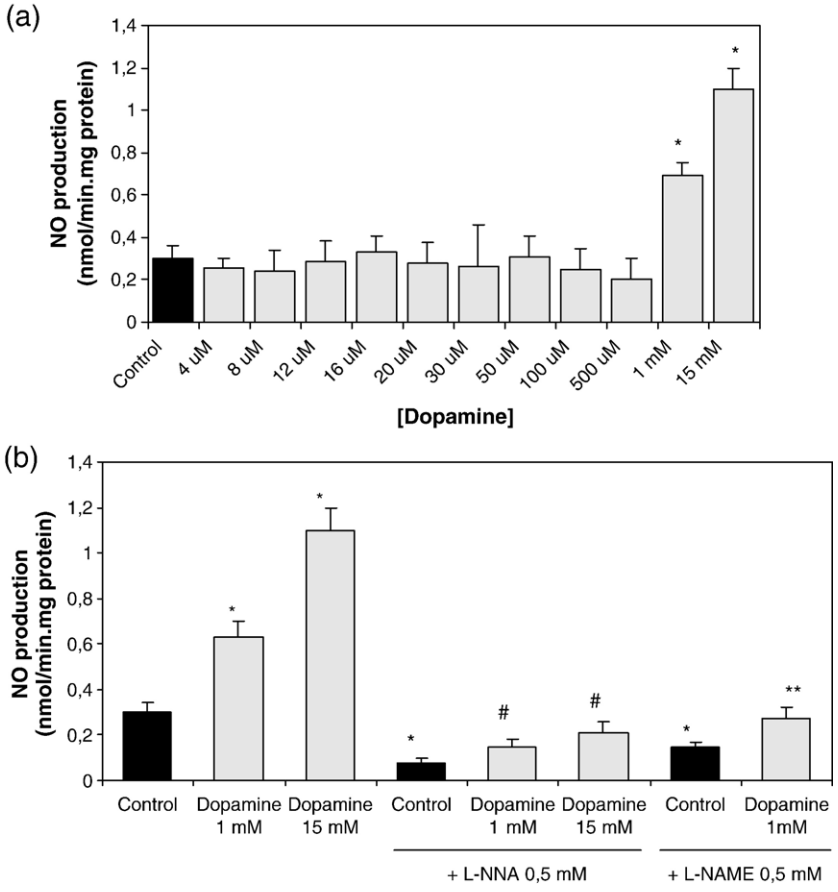

Fig. 1. mtNOS activity in control and dopamine-incubated brain submitochondrial membranes. (a) Effect of dopamine incubation on NO production activity in brain submitochondrial membranes. Submitochondrial membranes were incubated for 5 min with different DA concentrations at 37 °C. Bars represent the mean±SEM of 3 individual submitochondrial membranes samples, each obtained from a pool of two brains. ANOVA followed by Dunnett test was used ($F=6.464$). *$p<0.05$, as compared with control submitochondrial membranes. (b) Effect of incubation of brain submitochondrial membranes with both dopamine and L-NNA or L-NAME on NO production. Submitochondrial membranes were incubated for 5 minutes with 1 mM and 15 mM DA in the presence or absence of 0.5 mM L-NNA or 0.5 mM L-NAME at 37 °C. Bars represent the mean±SEM of 3 individual submitochondrial membranes samples, each obtained from a pool of two brains. ANOVA followed by Tukey test was used ($F=21.557$) *$p<0.01$, significantly different from control submitochondrial membranes. #$p<0.01$, significantly different from control submitochondrial membranes+L-NNA. **$p<0.05$, significantly different from control submitochondrial membranes+L-NAME.

Table 1
Effect of dopamine incubation on activity of mitochondrial respiratory chain complexes in brain submitochondrial membranes

Activity (nmol/min mg protein)	Control	Dopamine (1 mM)
NADH-cytochrome *c* reductase	326±7	290±3*
Succinate-cytochrome *c* reductase	176±5	155±6

Submitochondrial membranes were incubated for 5 minutes with 1 mM DA at 30 °C before measurements.
Values represent the mean±SEM of 3 individual submitochondrial membranes samples, each obtained from a pool of two brains.
Student's test was used (t=2.689).
*p<0.05, significantly different from control submitochondrial membranes.

were observed in succinate-cytochrome *c* reductase activity after DA incubation (Table 1).

Cytochrome oxidase activity was significantly inhibited by 21% after incubation of submitochondrial membranes with 1 mM DA. To evaluate the involvement of NO in this process we measured enzyme activity in the presence of both DA and L-NNA. Fig. 2 shows that the inhibitory effect of DA on cytochrome oxidase observed previously was partially prevented by L-NNA addition.

3.2. Effect off dopamine on mitochondrial function

3.2.1. Mitochondrial respiration

Malate-glutamate-dependent oxygen consumption rate was measured in state 3 and state 4. State 3, termed active respiration, is defined as respiration in the presence of an oxidizable substrate and ADP and thus is a measure of the coupled respiration. State 4, or resting respiration, is the rate of respiration in the presence of substrate but without ADP and thus is a measure of the rate of uncoupled respiration [21].

Low DA concentrations (25–75 μM) were not able to modify mitochondrial respiratory rates, as compared with control mitochondria (Table 2).

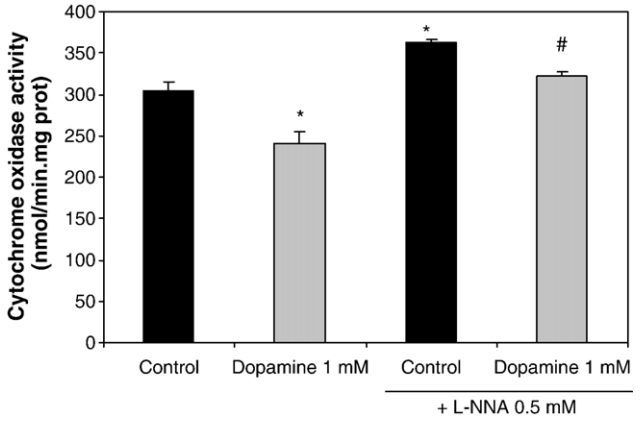

Fig. 2. Dopamine effect on cytochrome oxidase activity. Submitochondrial membranes were incubated for 5 min with 1 mM DA in the presence or absence of 0.5 mM L-NNA at 30 °C. Bars represent the mean±SEM of 3 individual submitochondrial membranes samples, each obtained from a pool of two brains. ANOVA followed by Tukey test was used (F=31.965) *p<0.01, significantly different from control submitochondrial membranes. #p<0.05, significantly different from control submitochondrial membranes+L-NNA.

Table 2
Effect of dopamine on brain mitochondrial respiration

Condition	Oxygen consumption (ng-at O/min mg protein)	Respiratory control
Control (state 4)	18±4	
+ ADP (state 3)	92±4	5.1±0.2
Dopamine 25 μM (state 4)	17±5	
+ ADP (state 3)	80±6	4.7±0.3
Dopamine 50 μM (state 4)	15±7	
+ ADP (state 3)	84±3	5.5±0.5
Dopamine 75 μM (state 4)	13±3	
+ ADP (state 3)	90±7	5.4±0.2
Dopamine 5 mM (state 4)	21±4	
+ ADP (state 3)	78±4	3.7±0.2
Dopamine 10 mM (state 4)	20±1	
+ ADP (state 3)	68±3*	3.40±0.06
Dopamine 15 mM (state 4)	19.5±0.4	
+ ADP (state 3)	52±5*	2.67±0.09#

Brain mitochondria were incubated with different DA concentrations during 5 minutes at 30 °C before measurements.
Values represent the mean±SEM of 3 individual mitochondria samples, each obtained from pools of two brains.
ANOVA followed by Tukey tests was used (F=9.047).
*p<0.01, as compared to basal state 3 control value.
#p<0.05, as compared to control value.

Incubation of intact brain mitochondria with different DA concentrations (5–10–15 mM) for 5 min inhibited state 3 oxygen consumption in intact brain mitochondria by 19%, 28% and 46% respectively, but no significant changes were observed in state 4 after DA incubation (Table 2). Respiratory control is a parameter of integrity and mitochondrial functionality [21]. A significant decrease in mitochondrial respiratory control was observed after incubation with 15 mM DA as compared with control mitochondria, indicating mitochondrial dysfunction.

In order to evaluate the involvement of NO on the inhibition of mitochondrial state 3 respiratory rate by DA, we measured state 3 oxygen consumption in the presence of L-NNA both in control or DA-incubated mitochondria. L-NNA addition alone increased state 3 mitochondrial respiratory rate by 35%, as compared with control state 3 respiratory rate. This agrees with previous results from our laboratory showing that NO exerts a regulatory effect on mitochondrial respiration [14]. When mitochondria were incubated with 15 mM DA in the presence of L-NNA, state 3 respiratory rate was decreased by 17%, as compared with control+L-NNA (Fig. 3).

Note that intact brain mitochondria required higher dopamine concentrations than submitochondrial membranes, probably because inner mitochondrial enzymes are less accessible to the drug. All the experiments were carried out considering this point. So, for further studies, intact mitochondria and submitochondrial membranes were incubated with 15 mM and 1 mM DA respectively.

3.2.2. Hydrogen peroxide production

Incubation of intact brain mitochondria with 15 mM DA was able to increase H_2O_2 production rates by 87%, as compared with control H_2O_2 production rates (0.30±0.04 nmol/min mg

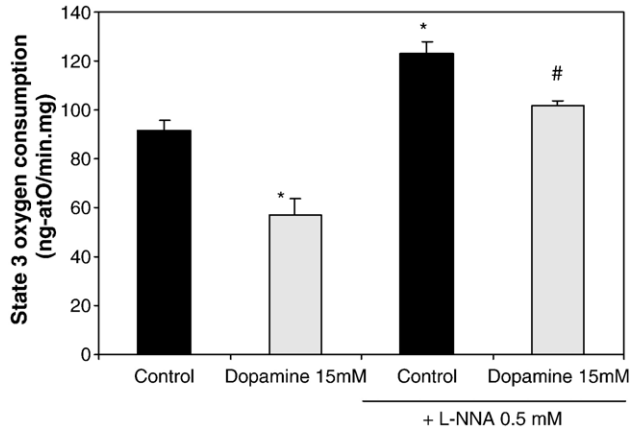

Fig. 3. Evaluation of state 3 respiration after dopamine incubation. Intact brain mitochondria were incubated during 5 min at 30 °C with 15 mM DA in the presence or absence of 0.5 mM L-NNA. Bars represent the mean±SEM of 3 individual submitochondrial membrane samples, each obtained from a pool of two brains. ANOVA followed by Tukey test was used ($F=36.274$). *$p<0.01$, significantly different from control submitochondrial membranes. #$p<0.01$, significantly different from control submitochondrial membranes+L-NNA.

protein) (Table 3). L-NNA addition decreased H_2O_2 production by 30% in control brain mitochondria as previously shown by our laboratory [14]. In the presence of L-NNA, DA increased H_2O_2 production rates by 68% (Table 3).

3.2.3. Mitochondrial membrane potential

After incubation with 15 mM DA an important decrease in FL-1-DiOC$_6$ fluorescence was observed (Fig. 4d) as compared with control mitochondria (Fig. 4c), indicating mitochondrial depolarization. Incubation with 50 μM DA did not disturb mitochondrial polarization (Fig. 4e). The involvement of NO on these effects was evaluated by incubation of mitochondria with both DA and L-NNA. No changes in mitochondrial polarization were observed after L-NNA incubation in control mitochondria (Fig. 4f). A clear inhibition of the 15-mM DA-induced depolarization was observed in the presence of L-NNA (Fig. 4g) showing the possible role of NO in DA-induced loss of mitochondrial membrane potential. No changes were observed after both 50 μM DA and L-NNA incubation (Fig. 4h). Addition of the ionophore FCCP (0.5 μM) to brain mitochondria showed stronger decrease in transmembrane potential (Fig. 4i). Unloaded control mitochondria present a low auto fluorescence as observed in Fig. 4b.

Statistical values of the relative fluorescence intensity showed that control mitochondria presented 84±3% (Fig. 4c) and after 15 mM and 50 μM DA incubation, FL-1-DiOC$_6$ fluorescence was 20±5% and 83±4% respectively (Fig. 4d, e).

The data presented in Fig. 4j and k show that NO participate in the 15-mM DA-induced depolarization due to the fact that L-NNA was able to maintain mitochondrial polarization.

4. Discussion

Dopamine plays an important role in the physiopathology of several psychiatric and neurological disorders such as schizophrenia and Parkinson's disease and in those cases disruption of mitochondrial processes was observed [2].

Several studies on mitochondrial function following exposure to DA have produced variable results. An inhibition of respiration and a decrease in complex I activity after incubation (5–15 min) with DA have been reported as a result of the deleterious actions of H_2O_2 and oxyradicals [2,4,5,7]. Also, Khan and colleagues [6] reported that extended periods of incubation with DA produce mitochondrial dysfunction by DA oxidation products. Due to the fact that catecholamines may modify NO levels in central nervous system, in this study we investigated the effects of incubation of mitochondria with DA on NO production and the consequences on mitochondrial function.

Due to the discrepancies concerning DA concentrations observed in the literature, in our study, submitochondrial membranes were incubated with a broad range of DA concentrations (from μM to mM range) to evaluate its effects on NO production. According to our results, low DA concentrations (range μM) did not alter mitochondrial NO production. However, a significant increase was observed in NO production after 1 mM and 15 mM DA incubation. The striatal concentration of DA is estimated to be about 70 μM, whereas the dopaminergic concentration in the neuronal endings and during the phasic release is of the order of mM [26]. Cells with high DA concentrations such as dopaminergic neurons are highly vulnerable to degeneration. It has been proposed that if DA is not efficiently stored in vesicles or if vesicles are disrupted, it can achieve high intracellular concentrations and may overcome the antioxidant capacity of the cell [27]. Results reported in our study are related to DA concentrations probably present in pathological situations where an imbalance between cytosolic and vesicular DA occurs.

Brain mtNOS has been previously identified by Western Blot as nNOS isoform [28] and its activity can be modified by drug treatments [16]. DA-induced increase in NO production was largely prevented by pre-treatment with NOS inhibitors, thus showing a specific action of DA on mtNOS activity. Regarding the mechanism of action of DA on mtNOS activity, we can speculate that DA might interact with the enzyme in a site different from the catalytic site, activating the enzyme, and thus acting as an allosteric modulator. Further investigations on enzymatic kinetics would help to elucidate the probable mechanism of this activation.

Table 3
Effect of dopamine on brain mitochondrial H_2O_2 production rate

H_2O_2 production (nmol/min mg protein)	Control	Dopamine (15 mM)
Malate–Glutamate	0.30±0.04	0.56±0.02*
+0.5 mM L-NNA	0.21±0.01	0.36±0.04*

Mouse brain mitochondria were incubated with 15 mM DA during 5 min at 37 °C before measurements.
Values represent the mean±SEM of 3 individual mitochondria samples, each obtained from pools of two brains.
ANOVA followed by Tukey tests was used ($F=23.811$).
*$p<0.05$, significantly different from its corresponding control value.

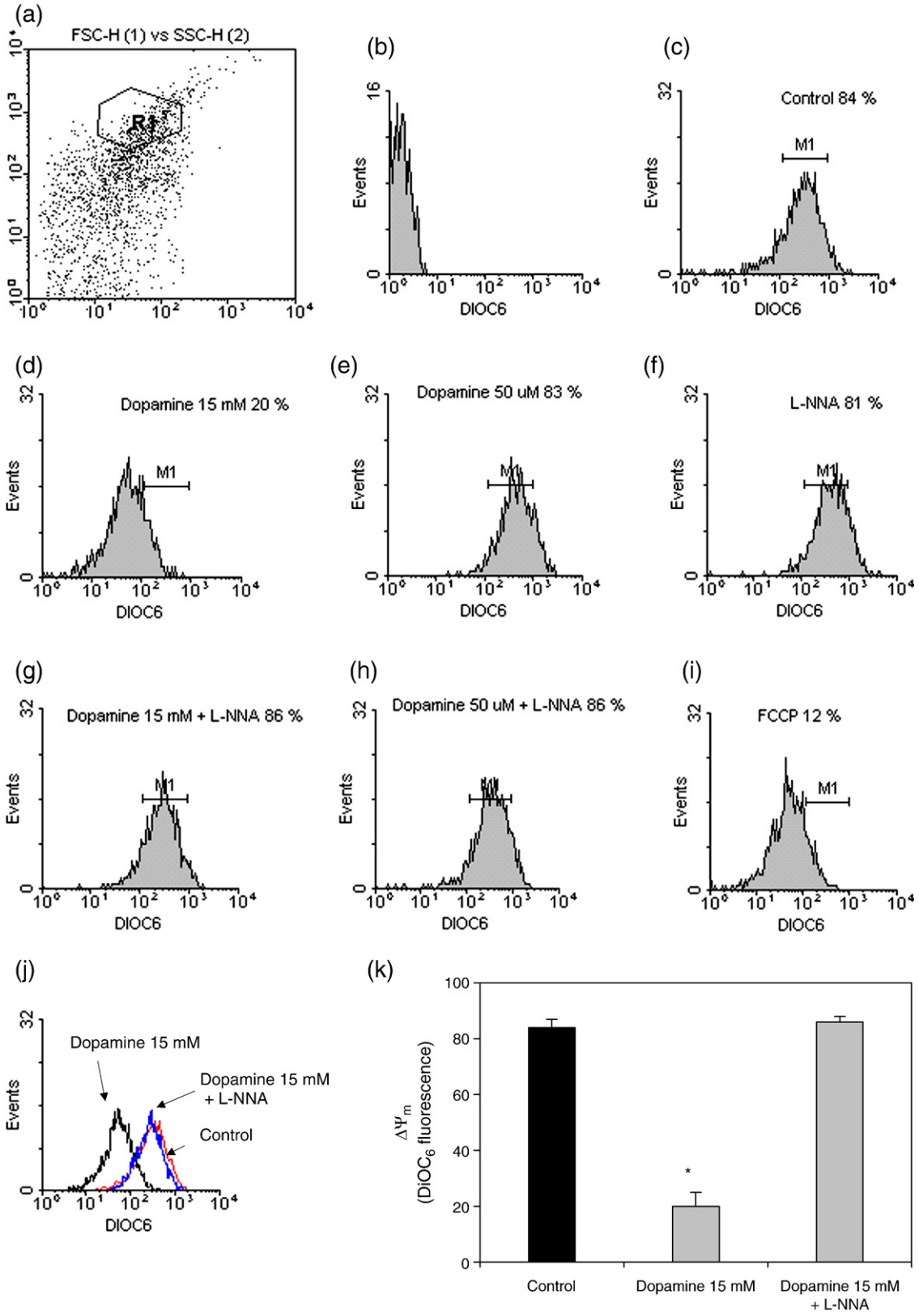

Fig. 4. Dopamine effect on mitochondrial membrane potential. Histograms of gated events (R1) versus relative fluorescence intensity (FL-1) of mitochondrial membrane potential of brain mitochondria described by $DiOC_6$. (a) dotblot of FSC-H vs. SSC-H indicating a gated mitochondrial population, (b) unloaded, (c) control, (d) 15 mM DA, (e) 50 μM DA, (f) 0.5 mM L-NNA, (g) 15 mM DA plus 0.5 mM L-NNA, (h) 50 μM DA plus 0.5 mM L-NNA, (i) 0.5 μM FCCP, (j) overlay histograms of control, 15 mM DA and 15 mM DA plus L-NNA and (k) bars scheme of $\Delta\psi$ mitochondrial ($\Delta\psi_m$) versus different DA treatments (mean values ± SEM); (*$p < 0.01$). Each histogram represents a typical experiment, which was performed in triplicate.

Mitochondrial NO modulates oxygen consumption by a reversible and O_2-competitive inhibition of cytochrome oxidase that slows down electron flow, substrate oxidation and chemical energy store [29]. Oxygen consumption was not affected by low physiological DA concentrations, while high (mM) DA levels decreased O_2 consumption in state 3 and decreased respiratory control in brain intact mitochondria. Gluck et al. [4] have reported that DA at relatively low concentrations (0.5–3 mM) inhibited mitochondrial respiration predominantly by a MAO-dependent mechanism involving H_2O_2 and downstream hydroxyl radical formation. Inhibition of respiration by DA at higher concentration (>3 mM), however, is progressively less dependent on MAO, implying additional coexisting mechanisms that participate in the inhibition of respiration and electron transport chain function. We are giving evidence of another MAO-independent mechanism of DA-induced inhibition of mitochondrial respiration.

In this study, mitochondria pre-treatment with L-NNA partially prevented the decrease in oxygen uptake observed after DA incubation. According to these results, DA was able to inhibit state 3 O_2 consumption by a NO-dependent pathway. The modulatory role of NO on mitochondrial respiratory chain has been extensively described; L-NNA has been shown to increase state 3 respiratory rate in mitochondria isolated from different tissues. Also, previous results from our laboratory gave evidence that drugs that inhibit mtNOS activity induce an increase in state 3 respiratory rate [30].

Due to the fact that in this study DA decreased mitochondrial respiratory rates, we decided to evaluate which mitochondrial respiratory complexes were affected by the drug. As previously reported by several authors [5,7], DA was able to inhibit NADH-cytochrome *c* reductase activity but no changes were observed in succinate-cytochrome *c* reductase activity. Studies of patients with Parkinson's disease demonstrate a 35% decrease in mitochondrial complex I activity [31] showing that DA is a potential candidate that can contribute to both oxidative stress and mitochondrial dysfunction within the dopaminergic neuron leading to cell damage and degeneration. The inhibition of mitochondrial respiration by NO at cytochrome oxidase level has been established as a physiological regulatory mechanism of mitochondrial function [32,33]. Dopamine inhibited cytochrome oxidase activity in submitochondrial membranes. When we measured the activity in the presence of both DA and L-NNA the inhibitory effect of DA on cytochrome oxidase activity was partially prevented, suggesting that inhibition of complex IV activity by DA results from an increase in NO production.

Hydrogen peroxide is normally formed during basal respiration [34] and also is a product of DA metabolism by MAO [35]. We found that 15 mM DA increased H_2O_2 production significantly by 87%, being mainly due to NO-independent mechanisms. Therefore, the main mechanism involved in the increased H_2O_2 generation by DA seems to be caused by its own autooxidation by MAO, while a small fraction of H_2O_2 increased production would be mediated through the inhibition of cytochrome oxidase by DA-induced NO production.

Evaluation of the mitochondrial membrane potential shows that high DA concentrations induce depolarization, while low physiological DA concentrations do not affect mitochondrial membrane potential. This result is in accordance with the lack of effect of low DA concentrations on mitochondrial NO production and on respiratory rates, thus showing that mitochondrial function seems to be preserved at physiological DA concentrations. Our data are consistent with an early work by Berman and Hastings [2] who found that DA induce mitochondrial permeability transition and produce membrane depolarization. In our study, in the presence of both DA and L-NNA an inhibition of 15 mM DA-induced depolarization was observed. It has been reported that NO induce depolarization in isolated neuronal mitochondria [36] and in cortical neurons in culture [37]. The results presented here support the idea that DA induces depolarization by an increase in NO production.

To sum up, the data presented provide evidence that at physiological DA concentrations, mitochondrial function and NO production seem to be preserved. High DA concentrations induce mitochondrial dysfunction as shown by decreased mitochondrial respiratory control and loss of membrane potential mainly by a NO-dependent pathway.

Acknowledgements

This research was supported by grants from Universidad de Buenos Aires (B131), Consejo Nacional de Investigaciones Científicas y Técnicas (PIP 6320) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 01-8710), Argentina.

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