Neurochem Res
DOI 10.1007/s11064-013-1172-3

ORIGINAL PAPER

Mitochondrial Dysfunction in Brain Cortex Mitochondria of STZ-Diabetic Rats: Effect of L-Arginine

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Received: 12 June 2013 / Revised: 27 September 2013 / Accepted: 3 October 2013

Abstract Mitochondrial dysfunction has been implicated in many diseases, including diabetes. It is well known that oxygen free radical species are produced endogenously by mitochondria, and also nitric oxide (NO) by nitric oxide synthases (NOS) associated to mitochondrial membranes, in consequence these organelles constitute main targets for oxidative damage. The aim of this study was to analyze mitochondrial physiology and NO production in brain cortex mitochondria of streptozotocin (STZ) diabetic rats in an early stage of diabetes and the potential effect of L-arginine administration. The diabetic condition was characterized by a clear hyperglycaemic state with loose of body weight after 4 days of STZ injection. This hyperglycaemic state was associated with mitochondrial dysfunction that was evident by an impairment of the respiratory activity, increased production of superoxide anion and a clear mitochondrial depolarization. In addition, the alteration in mitochondrial physiology was associated with a significant decrease in both NO production and nitric oxide synthase type I (NOS I) expression associated to the mitochondrial membranes. An increased level of thiobarbituric acid-reactive substances (TBARS) in brain cortex homogenates from STZ-diabetic rats indicated the presence of lipid peroxidation. L-arginine treatment to diabetic rats did not change blood glucose levels but significantly ameliorated the oxidative stress evidenced by lower TBARS and a lower level of superoxide anion. This effect was paralleled by improvement of mitochondrial respiratory function and a partial mitochondrial repolarization.In addition, the administration of L-arginine to diabetic rats prevented the decrease in NO production and NOSI expression. These results could indicate that exogenously administered L-arginine may have beneficial effects on mitochondrial function, oxidative stress and NO production in brain cortex mitochondria of STZ-diabetic rats.

Keywords Brain cortex mitochondria · Diabetes · Mitochondrial respiration · Nitric oxide · L-Arginine

Introduction

Type I diabetes mellitus is associated with gradually developing end-organ damage in the central nervous system [1, 2]. Data from the literature indicate an association between type I diabetes and several brain conditions, such as cerebral ischaemia, macrovascular disease, microangiopathy, cognitive decline and brain atrophy [3, 4]. Increased oxidative stress has been implicated in the etiology (especially type 1) and pathology (both type 1 and type 2) of diabetic complications [5, 6]. Emerging evidences show that the increased oxidative stress and consequent oxidative damage observed in hyperglycaemic conditions begin in the mitochondria, which is one of the main sites of reactive oxygen species production (ROS) [7, 8]. The overproduction of ROS induced by enhanced glucose oxidation might overwhelm the antioxidant defenses, leading to cell damage [9]. It must be noted that ROS are

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Published online: 05 November 2013

also produced within mitochondria at sites other than the inner mitochondrial membrane by the monoamine oxidase (MAO) system. This process produce significant amounts of hydrogen peroxide (H_2O_2) [10].

In addition, nitric oxide (NO) generated by specific nitric oxide synthases (NOS) contribute to a large number of physiological processes. nNOS (NOS1) and eNOS (NOS3) are nuclear coded and are constitutively expressed in the brain, producing NO in nM concentrations. The inducible iNOS or NOS 2 can produce NO in μM concentrations [11]. All three enzymes can be associated to the outer mitochondrial membrane, and be regulated by a variety of stimuli and by several physiological conditions. It is well known that NO production by these cellular systems has important implications for the mitochondrial respiratory function due to its ability to induce a reversible inhibition of the cytochrome oxidase (complex IV) [12]. In special conditions such as in the absence of BH4 or in the presence of increased endogenously metilated L-arginine (ADMA), NOS enzymes can be uncoupled producing free radicals such as superoxide anion instead of NO. Interesting to note is that this condition has been described in diabetes [13].

NO can react with superoxide (O_2^-) to form a potent oxidizing agent, peroxynitrite ($ONOO^-$) which contributes to cellular damage and oxidative stress [14]. $ONOO^-$ can cause protein tyrosine nitration and lipid peroxidation that eventually leads to extensive membrane damage and cellular dysfunction [15]. L-arginine is a semi essential amino acid and the main substrate for the generation of NO via NOS. The beneficial effect of L-arginine administration in experimental diabetes mellitus induced by alloxan has been observed in the liver and in the brain. This effect was attributed to both its NO-dependent and independent antioxidant capacities. Administration of L-arginine either before or after alloxan significantly abolishes the increase in TBARS concentration. This effect was accompanied by higher reduced glutathione (GSH) concentration and activities of superoxide dismutase (SOD) and catalase [16]. Moreover, Vasilijevic et al. [17] evidenced multiple beneficial effects of the L-arginine-NO-producing pathway in alloxan-induced diabetes mellitus. They showed that GSH content in the pancreas of diabetic rats increased upon L-arginine treatment.

Additionally, L-arginine decreases oxidative stress by reduction of the vascular O_2^- production with improvement of endothelial function in hypercholesterolaemic patients [18].

The aim of this study was to evaluate the respiratory function, oxygen free radical production, MAO activity, NO production and NOS I expression in brain cortex mitochondria of streptozotocin (STZ) diabetic rats in an early phase of diabetes. The potential effect of L-arginine administration in order to improve mitochondrial physiology and NO metabolism in the hyperglycaemic condition was also studied.

Materials and Methods

Chemicals

The reagents used in Western blot analysis were from Bio-Rad (Calif., USA). All other chemicals and reagents used in this study, unless otherwise indicated, were from Sigma-Aldrich Corporation (St. Louis., USA).

Animal Treatment

Male Wistar rats weighing 250–300 g from the animal house of the School of Pharmacy and Biochemistry were maintained with a 12-h light/dark cycle at 21 °C and allowed free access to a standard rat chow and tap water. All efforts were made to minimize animal suffering and reduce the number of animals used. Diabetes was induced by a single intraperitoneal injection of STZ (70 mg/kg body weight) diluted in citrate buffer 0.1 M, pH 4.5. In order to avoid the hypoglycemic coma, which occurs within 24 h following STZ injection and to minimize mortality, animals were injected intraperitoneally with 5 % glucose.

Several investigators reported induction of diabetes in rats by intraperitoneal injection of STZ at a dose between 65 and 70 mg/kg [19–22]. In our experiments, no mortality was observed in the rats injected intraperitoneally with STZ at a dose of 70 mg/kg.

Controls were injected with the citrate buffer alone. Rats were divided in four groups: (1) Control (C), (2) Diabetic (D), (3) Control + L-arginine (C+A) and (4) Diabetic + L-arginine (D+A). L-arginine was administered to C+A and D+A groups in the drinking water at a dose of 622 mg/kg/day [23, 24]. The concentration of L-arginine in the drinking water was adjusted considering the previous day's water drinking of each animal in order to eliminate changes due to different intakes.

The protocol was approved by the Evaluating Committee Board of the University of Buenos Aires. 4 days after the induction of diabetes, animals were anaesthetized with ether and blood samples were immediately obtained by cardiac puncture until exsanguination, in accordance with the guidelines of the Council of Laboratory Science (ICLAS).To confirm diabetes, blood glucose was measured by a glucose oxidase method (Betachek, National Diagnostic Products, Sydney, Australia). STZ-treated animals with glucose levels below 200 mg/dl were not included in the experimental protocol.

TBARS Production

The amount of thiobarbituric acid-reacting substances (TBARS), a commonly used marker for lipid peroxidation and malondialdehyde (MDA) production, was determined by a fluorescence assay [25] in brain cortex homogenates. Briefly, homogenates were treated with 1 ml 0.1 N sodium dodecyl sulfate, 0.1 N HCl, 0.15 ml of 10 % phosphotungstic acid and 0.5 ml of 0.7 % 2-thiobarbituric acid. After 1 h boiling, the samples were cooled and 2.5 ml of butanol were added. Fluorescence of the butanol layer was measured at 515 nm for excitation and 555 nm for emission. The values were expressed as nmoles of MDA per mg of protein, using a MDA standard prepared from 1,1,3,3-tetramethoxypropane [26].

Isolation of Brain Cortex Mitochondria

Brain cortex was rapidly removed and minced on ice, resuspended in MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.4) supplemented with 1 mM EDTA and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600xg for 10 min at 4 °C. The supernatant was decanted and centrifuged again at 8,000xg for 10 min; the mitochondrial pellet was washed twice in MSH [27]. Protein was determined by the Lowry assay [28]. The isolated mitochondria correspond to synaptic and non-synaptic cortex mitochondria mainly from neurones and glial cells. Mitochondrial samples were less than 2–4 % contaminated with cytosolic components according to the amount of lactate dehydrogenase present in the samples. Submitochondrial membranes were obtained by twice freezing and thawing the mitochondrial preparation and were homogenized by passage through a tuberculin syringe with a needle [29]. This preparation consisted in a fraction of outer and inner membranes which do not present restriction to substrate access. The mitochondrial fraction obtained from brain cortex tissue showed a respiratory control ratio (RCR) between 4.0 and 6.0 determined with malate plus glutamate as substrates and was not affected by the concentration of the potentiometric probes used.

Mitochondrial Respiratory Function

Oxygen consumption by isolated brain cortex mitochondria was measured with a high-resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria). Mitochondrial protein (0.5–1 mg/ml) was placed in a reaction medium consisting of 0.23 M mannitol, 0.07 M sucrose, 20 mM Tris–HCl, 5 mM malate plus glutamate, 5 mM PO_4H_2K, 4 mM $MgCl_2$, pH 7.4, and 0.2 % bovine serum albumin at 30° C. State 3 was estimated by the addition of 1 mM ADP and the RCR was calculated from the ratio of the state 3/state 4 respiratory rates with and without ADP, respectively [30–32].

Mitochondrial Superoxide Anion Level

$O_2^{\cdot-}$anion level was measured in intact isolated mitochondria by flow cytometry after loaded in the dark, with 2 μM of MitoSOX (Molecular Probes, Eugene, OR, USA) during 20 min at 37 °C in MSH buffer supplemented with 5 mM malate, 5 mM glutamate and 1 mM phosphate. Mitochondria 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 10 μM antymicin treatment was measured as negative and positive controls respectively.

Hydrogen Peroxide Production

H_2O_2 production was determined in intact isolated mitochondria (0.1–0.3 mg/ml) by the scopoletin- horseradish-peroxidase (HPR) method, following the decrease in fluorescence intensity at 365–450 nm (λexc–λem) at 37 °C.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, 7 mM succinate, 6 mM glutamate and 0.3 μM SOD. This method allows determining the H_2O_2 that diffuses from mitochondria. Calibration was made using a H_2O_2 standard curve in order to express the fluorescence changes as nmol H_2O_2/min mg protein [33].

Membrane Potential in Brain Cortex Mitochondria

Mitochondria of C, D, C+A and D+A rats were loaded with 30 nM of the potentiometric fluorescent probe DiOC6 in the dark, during 20 min at 37 °C in MSH buffer supplemented with 5 mM malate, 5 mM glutamate and 1 mM phosphate. Immediately after, mitochondria were acquired by a FAC-SCAN flow cytometer equipped with a 488 nm argon laser and a 615 nm red diode laser. Mitochondrial autofluorescence (no probe) and after 0.5 μM carbonyl cyanide *4*-(trifluoromethoxy)phenylhydrazone (FCCP) treatment were measured as negative and positive controls, respectively. Data from the experiments were collected in a Becton–Dickinson FACS Calibur and analyzed using the WinMDI 2.6 version software [34].

Monoamine Oxidase Activity

Monoamine oxidase activity was measured in submitochondrial membranes (0.5 mg/ml) by following spectrophotometrically the oxidation of 100 μM kynuramine in a reaction medium containing 50 mM phosphate buffer pH

7.4 at 30 °C. Kinetics studies were followed at 360 nm ($\varepsilon = 4.28$ mM/cm) [35].

Measurement of Nitric Oxide Production

NO production associated to the mitochondrial membrane was measured in submitochondrial particles 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/cm). 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 dithiothreitol, 4 μM Cu–Zn SOD, 0.1 μM catalase, 0.5–1.0 mg submitochondrial protein/ml and 25 μM HbO_2 (expressed per heme group). HbO_2 reacts efficiently with NO if the reaction between NO and $O_2^{\cdot-}$ is prevented. SOD is added to abrogate any other reaction with $O_2^{\cdot-}$, 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 must be added to the assay. Controls adding 0.5 mM Nω-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 [29, 36].

Western Blotting Analysis

Western blotting was used to analyze the expression of NOS I attached to brain cortex mitochondrial membranes. Aliquots of sample containing 50 μg of proteins were loaded on 7.5 % SDS polyacrylamide gels and then blotted on to PVDF membranes (GE Healthcare, Amersham Hybond-P) at 60 V for 2 h. The membranes were washed in TBST buffer (50 mmol/l Tris- based saline, pH 7.4, containing 0.1 % Tween 20) and blocked with 7,5 % skimmed milk in TBST for 1 h. Blots were then incubated overnight at 4 °C with rabbit polyclonal antibody against NOS Type I diluted 1:1,000 (BD Biosciences, Transduction Laboratories, Franklin Lakes, NJ, USA). Then the membranes were incubated with a biotynilated donkey anti rabbit IgG (1:3,000) (Jackson Immuno Research, Baltimore Pike, PA, USA) in TBST for 1 h. Blots were stained using Vectastain ABC kit and DAB substrate for peroxidase (both from Vector Labs, Calif., USA). Rabbit polyclonal to β-tubulin (Abcam, Kendall Square, Cambridge, USA) was used as a loading control. The relative protein levels were determined by analyzing the bands with GEL PRO Analyzer 3.1 for windows and expression was calculated as ratio of NOS I to β-tubulin, a protein present in the mitochondria [37].

Table 1 Changes in the blood glucose level and body weight of control and diabetic rats

Group	Glycemia (mg/dl)	Body weight (initial) (g)	Body weight (final) (g)
C	100.6 ± 8.9	307.3 ± 11.1	355.1 ± 17.2
D	288.8 ± 32.7***	320.6 ± 21.7	270.1 ± 19.4*
C+A	90.4 ± 8.1	311 ± 12	347.9 ± 22.5
D+A	283.4 ± 20.9***	327.2 ± 20.5	275.6 ± 16.1*

* $p < 0.05$ compared to control rats, *** $p < 0.001$ compared to control rats. Results are expressed as the mean ± SEM (n = 5)

C control rats, *D* streptozotocin (STZ)-diabetic rats, *C+A* control rats treated with L-arginine, *D+A* STZ- diabetic rats treated with L-arginine. Results are expressed as the mean ± SEM (n = 5)

Statistical Analysis

Data are presented as mean and standard error (mean ± SEM) of at least 4 experiments. Sets of results were compared using a one-way analysis of variance (ANOVA) with Bonferroni′s post test correction for multiple comparisons using Graph Pad Prism version 5.0 for windows, Graph Pad Software (San Diego, CA, USA). A probability value of less than 0.05 was taken as significant.

Results

Blood Glucose and Body Weight

Twenty four and seventy two hours after STZ injection, blood glucose levels were significantly elevated in diabetic rats, confirming the onset of diabetes. Twenty-four hours after STZ injection, the glycaemia (mg/dl) levels were already increased (C = 94.5 ± 6.6; D = 290.5 ± 11.2 $p < 0.001$ vs C and C+A; D+A = 298.0 ± 9.7 $p < 0.001$ vs C and C+A; C+A = 98.6 ± 7.3) and remained increased on day 3 (C = 100.0 ± 5.3; D = 293.1 ± 10.9 $p < 0.001$ vs C and C+A; D+A = 297.0 ± 10.2 $p < 0.001$ vs C and C+A; C+A = 97.7 ± 6.6). These results confirmed the onset of diabetes. On day four, blood glucose (mg/dl) was increased to the same extent in both diabetic groups (C = 100.6 ± 8.9; D = 288.8 ± 32.7 $p < 0.001$ vs C and C+A; D+A = 283.4 ± 20.9 $p < 0.001$ vs C and C+A; C+A = 90.4 ± 8.1).

As expected, STZ treatment significantly raised blood glucose levels by 187.3 % and decreased body weight by 15.7 % 4 days after injection when compared with control

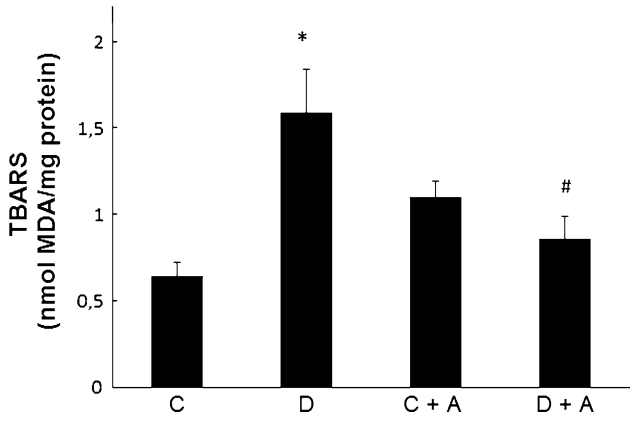


Fig. 1 Thiobarbituric acid-reacting substances (TBARS) production in brain cortex homogenates. *Bar graph* indicating the TBARS production. C: control rats; D: streptozotocin (STZ)-diabetic rats; C+A: control rats treated with L-arginine; D+A: STZ-diabetic rats treated with L-arginine. $^{*}p < 0.05$ compared to control rats; $^{\#}p < 0.05$ compared to diabetic rats. Results are expressed as the mean ± SEM (n = 5)

rats. In addition, other study shows that STZ is rapidly eliminated from the body: about 80 % appeared in the urine within 6 h [38].

Treatment with L-arginine did not produce any significant alteration in blood glucose levels and body weight in diabetic rats. L-arginine administration to control rats (C+A) did not produce any significant alteration in both parameters (Table 1).

Brain Cortex Lipid Peroxidation

A significant increase (148 %) in lipid peroxidation measured as TBARS production was observed in brain cortex homogenates 4 days after the induction of diabetes mellitus. Administration of L-arginine to diabetic rats prevented the increase in TBARS production. L-arginine supplementation to control rats did not show significant changes in lipid peroxidation (Fig. 1).

Respiratory Function From Brain Cortex Mitochondria

State 4 respiratory rate was increased by 52 % and state 3 respiratory rate was decreased by 38.6 %in brain cortex mitochondria of diabetic rats compared to control rats. CR was significantly decreased in the diabetic rats, indicating a clear mitochondrial dysfunction.

L-Arginine administration to the diabetic rats prevented the increase in state 4 but did not produce any significant changes in state 3 respiratory rate. These results evidence that L-arginine administration could induce a partial recovery of the mitochondrial respiratory function in the diabetic rats but did not restore the RCR observed in control rats. L-arginine administration to control rats did not affect the mitochondrial respiratory function. These results can be observed in Table 2.

Table 2 Oxygen consumption in brain cortex mitochondria of control and diabetic rats

Oxygen consumption (ng-atO/min mg protein)			
Group	State 4	State 3	Respiratory control ratio
C	5.2 ± 0.5	35.5 ± 4.2	7.0 ± 0.5
D	8.0 ± 1.0*	21.8 ± 3.4*	2.7 ± 0.2*
C+A	7.0 ± 2.0	43.1 ± 6.0	7.3 ± 2.0
D+A	5.7 ± 2.0$^{\#}$	18.5 ± 7.1*	3.1 ± 0.4*

$^{*}p < 0.05$ compared to control rats; $^{\#}p < 0.05$ compared to diabetic rats

C control rats, *D* streptozotocin (STZ)-diabetic rats, *C+A* control rats treated with L-arginine, *D+A* STZ- diabetic rats treated with L-arginine. Results are expressed as the mean ± SEM (n = 5)

Reactive Oxygen Species Production

Superoxide Anion Production

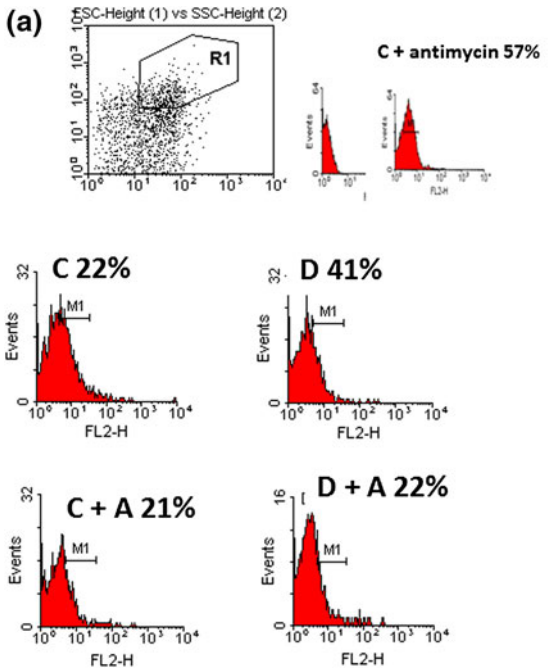
The data presented in Fig. 2 a and b shows that $O_2^{\cdot-}$ level was increased by 86 % in brain mitochondria of diabetic rats as compared with control rats. It was observed that L-arginine administration to the diabetic rats markedly prevented the increase in $O_2^{\cdot-}$ level. L-arginine administration to control rats did not affect $O_2^{\cdot-}$ production (Fig. 2 a and b).

Hydrogen Peroxide Production

H_2O_2 production was measured in brain cortex mitochondria from C, D, C+A and D+A rats. Mitochondrial H_2O_2 production rate was significantly decreased by 31 % in diabetic rats (0.22 ± 0.03 nmol/min mg protein) as compared with control H_2O_2 production rate (0.32 ± 0.03 nmol/min mg protein) and L-arginine supplementation to diabetic rats prevented this decrease. No significant changes were observed in C+A group. Data are shown in Fig. 3.

Mitochondrial Membrane Potential

In this study clear differences in mitochondrial membrane potential were observed as DIOC6 fluorescence changes between the different groups (Fig. 4 a). Quantification of DiOC6 fluorescence under the different conditions was presented in Fig. 4b, showing 20 % decrease in diabetic rats as compared with the control group. This result indicates a clear mitochondrial depolarization, possibly as a consequence of the increased oxidative stress in the diabetic condition. Interesting to note was that administration of



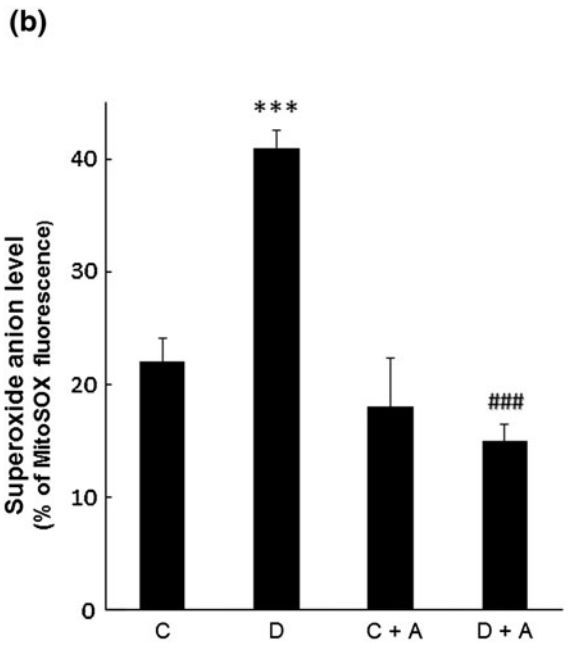


Fig. 2 Mitochondrial superoxide anion level. **a** dotblot of forward scatter (FSC) versus side scatter (SSC) indicating the gated mitochondrial population, autofluorescence and antimycin as negative and positive controls (shown only for control samples). Histograms of MitoSox relative fluorescence intensity (FL-2) from C: control rats; D: streptozotocin (STZ)-diabetic rats; C+A: control rats treated with L-arginine; D+A: STZ-diabetic rats treated with L-arginine. Each histogram represents a typical experiment, which was performed in triplicate. **b** *Bar graph* quantification of MitoSox fluorescence. $^{***}p < 0.001$ compared to control rats; $^{\#\#\#}p < 0.001$ compared to diabetic rats. Results are expressed as the mean ± SEM (n = 5)

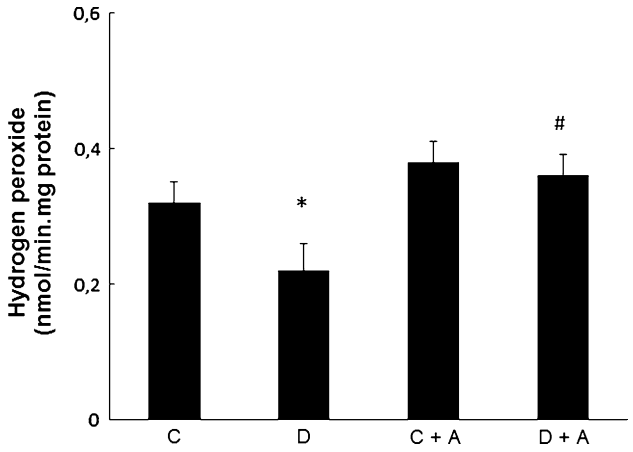


Fig. 3 Hydrogen peroxide production in brain cortex mitochondria. C: control rats; D: streptozotocin (STZ)-diabetic rats; C+A: control rats treated with L-arginine; D+A: STZ-diabetic rats treated with L-arginine.$^{*}p < 0.05$ compared to control rats; $^{\#}p < 0.05$ compared to diabetic rats. Results are expressed as the mean ± SEM (n = 5)

L-arginine to the diabetic rats prevented the decrease in membrane potential. On the other hand, L-arginine administration to the control rats did not affect this parameter. As expected, significant mitochondrial depolarization was detected in all samples after 0.5 μM FCCP addition(only control data are shown in Fig. 4).

MAO Activity

Analysis of MAO activity in brain cortex mitochondria showed a 54 % decrease in diabetic rats (4.4 ± 0.1 nmol/min mg protein) as compared with the control group (9.6 ± 0.9 nmol/min mg protein). L-arginine administration to diabetic rats prevented the decrease in MAO activity. L-arginine administration to control rats enhanced MAO activity as compared with control rats (Fig 5).

NO Metabolism

NO Production

NO production in brain cortex submitochondrial membranes was significantly decreased by 46 % in the diabetic group as compared with control rats (1.3 ± 0.1 nmol/min mg protein vs 2.4 ± 0.2 nmol/min mg protein). The administration of L-arginine to diabetic rats prevented the decrease in NO production, showing similar values to control rats. NO production did not show any significant change in control rats treated with L-arginine as compared with control untreated rats (Figure 6 a).

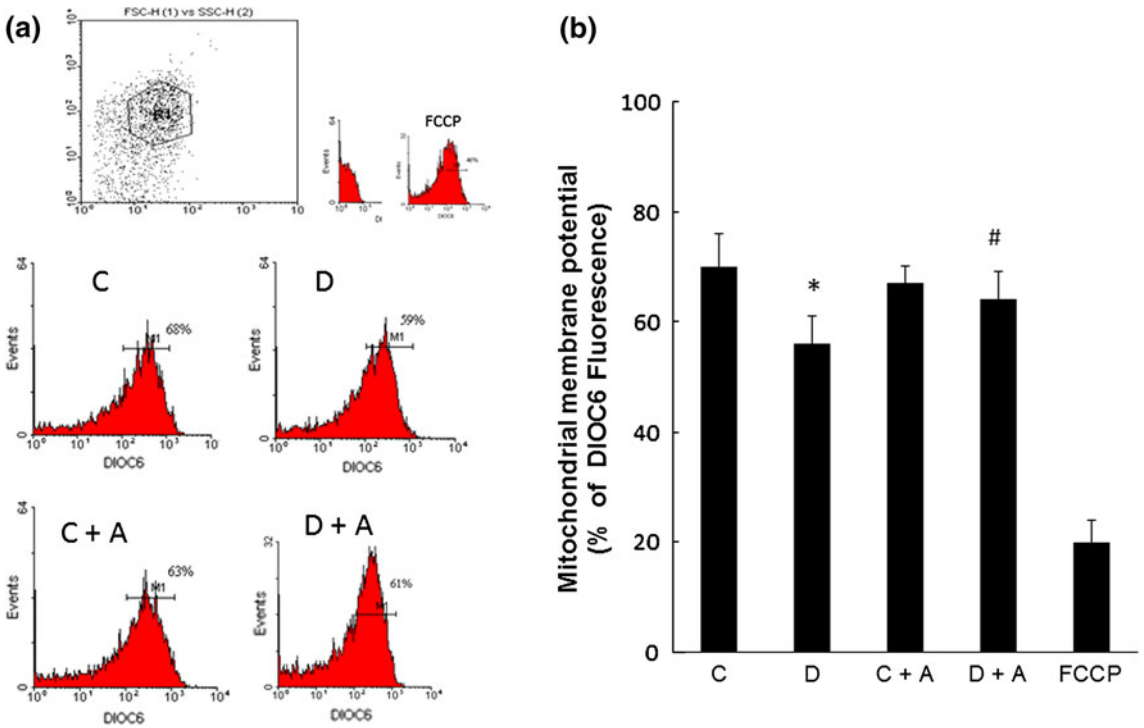


Fig. 4 Mitochondrial membrane potential ($\Delta\Psi_m$). **a** dotblot of forward scatter (FSH) versus side scatter (SSC) indicating the gated mitochondrial population, autofluorescence and carbonyl cyanide 4-(trifluoromethoxy)phenyhydrazone (FCCP) treatment as negative and positive control (shown only for control samples). Histograms of DiOC6 relative fluorescence intensity (FL-2) from C: control rats; D: streptozotocin (STZ)-diabetic rats; C+A: control rats treated with L-arginine; D+A: STZ-diabetic rats treated with L-arginine. Each histogram represents a typical experiment, which was performed in triplicate. **b** *Bar graph* quantification of DiOC6 fluorescence. $^{*}p < 0.05$ compared to control rats; $^{\#}p < 0.05$compared to diabetic rats. Results are expressed as the mean ± SEM (n = 5)

NOS I Expression

The expression of NOS I associated to the mitochondrial membranes (indicated as the ratio NOS I/β-tubulin) in diabetic rats showed a similar profile as observed in NO production. NOS I expression was significantly decreased by 30 % in the diabetic group when compared with untreated control rats. This effect was prevented when L-arginine was administered. No changes were observed in control rats treated with L-arginine compared to control untreated rats. Western blot results are shown in Fig. 6 b.

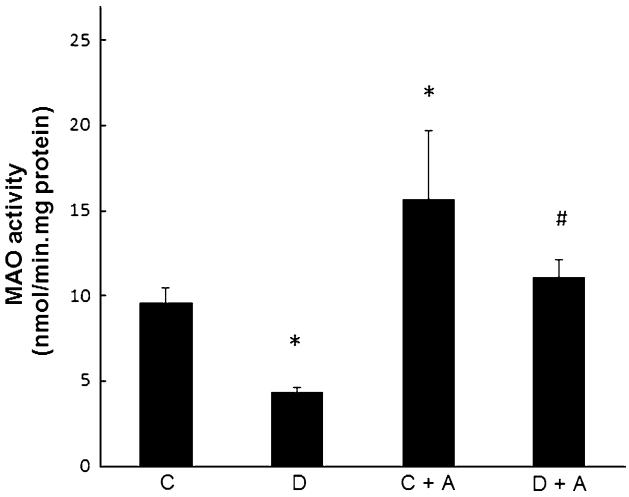


Fig. 5 Mitochondrial MAO activity. MAO activity was performed in mitochondrial fractions from C: control rats; D: streptozotocin (STZ)-diabetic rats; C+A: control rats treated with L-arginine; D+A: STZ-diabetic rats treated with L-arginine. $^{*}p < 0.05$ compared to control rats; $^{\#}p < 0.05$ compared to diabetic rats. Results are expressed as the mean ± SEM (n = 5)

Discussion

Streptozotocin is a drug widely employed to induce experimental diabetes. In this study, after 4 days exposure to STZ, a typical type I diabetes was induced and was characterized by an increase in 187 % in blood glucose levels and associated with a 25 % decrease in body weight when compared with control untreated group.

The involvement of mitochondrial function during the diabetic condition has long been stated by different studies [7]. Hyperglycaemic condition has been also associated with oxidative stress due in part to an altered mitochondrial metabolism. Mitochondrial dysfunction can result from an impairment of the mitochondrial

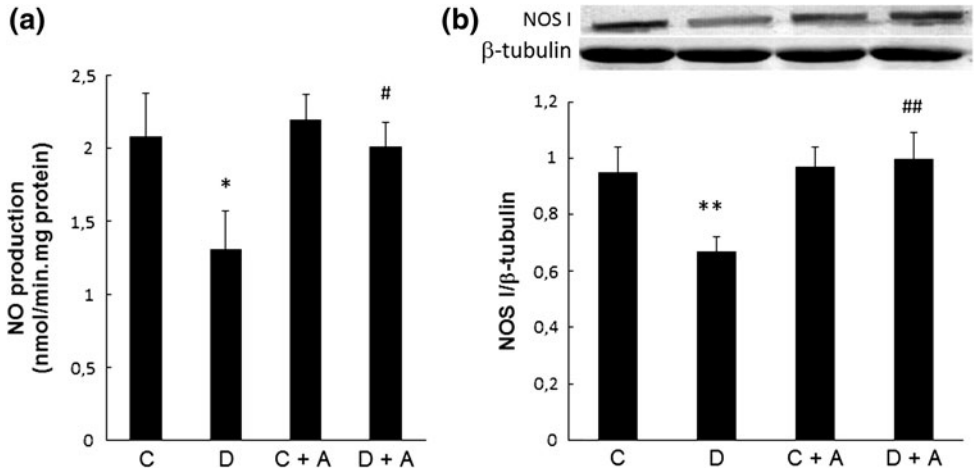


Fig. 6 NO metabolism. **a** *Bar graph* indicating NO production in brain cortex mitochondria from C: control rats; D: streptozotocin (STZ)-diabetic rats; C+A: control rats treated with L-arginine; D+A: STZ- diabetic rats treated with L-arginine. Results are expressed as the mean ± SEM (n = 5). $^{*}p < 0.05$ compared to control rats; $^{\#}p < 0.05$ compared to diabetic rats. **b** Representative Western blot of NOS I associated to mitochondria membranes (155 kDa band) and β-tubulin (50 kDa band) in brain cortex mitochondria and ratio of NOSI/β-tubulin from C: control rats; D: streptozotocin (STZ)-diabetic rats; C+A: control rats treated with L-arginine; D+A: STZ-diabetic rats treated with L-arginine. $^{**}p < 0.01$ compared to control rats; $^{\#\#}p < 0.01$compared to diabetic rats. Results are expressed as the mean ± SEM (n = 5)

respiratory chain, leading to altered proton gradient, inducing changes in ROS production and an uncoupled condition with a high cost in energy production. In this study we observed that STZ-diabetic rats presented mitochondrial dysfunction in brain cortex, mainly due to an abnormal respiratory function. Our results showed increased state 4 and decreased state 3 respiratory rates in brain cortex mitochondria of diabetic rats, reflecting a significant decrease in RCR in these animals, which as described before could be indicative of mitochondrial respiratory dysfunction and a compromised energy metabolism. These results are in agreement with those observed in other diabetic models [39–41].

The results of our study showed that although O_2^- levels were markedly increased, H_2O_2 production was decreased in mitochondria of STZ-diabetic rats as compared with control rats. It is well known that the levels of mitochondrial H_2O_2 result mainly from two different systems, the activity of the electron transport chain at the inner membrane and the MAO system located at the outer mitochondrial membrane. The present results demonstrate that MAO activity in brain cortex mitochondria of diabetic rats was early decreased as compared with mitochondria from control rats. Thus, the observed decrease in H_2O_2 production in diabetic rats could be due, at least in part, to the inhibition of the MAO system. These results are in agreement with the reported decrease in MAO activity at early times after diabetes onset in different regions of the brain [42]. However, we cannot discard the possibility that mitochondrial SOD and/or catalase activity modifications can occur after STZ injection altering also the levels of H_2O_2.

The evaluation of changes in the mitochondrial membrane potential in STZ-diabetic rats showed an important mitochondrial depolarization compared with control rats; these results were closely related with the data obtained from the respiratory function, characterizing a mitochondrial dysfunction that can lead to a low energy production.

This study showed significantly diminished NO production in mitochondria of STZ- diabetic rats compared with those observed in mitochondria from control rats. This result was accompanied with a decrease in the expression of NOS I associated to the mitochondrial membranes in the diabetic rats. This is in agreement with previous results showing that NOS activity and gene expression were decreased in the brain of STZ-diabetic rats [43].The lower NO production by NOS associated to the mitochondrial membranes observed in diabetic rats could be due to NOS uncoupling, which could contribute to increase ROS production, leading to mitochondrial dysfunction. Additionally, the fact that L-arginine treatment ameliorates the mitochondrial respiratory function, restoring NO synthesis and decreasing ROS production, could be due to the recovery of NOS function.

In fact, there is growing evidence that NO deficiency can occur in mitochondrial diseases and can play a major role in the pathogenesis of several complications observed in stroke-like episode, myopathy, diabetes and lactic acidosis [44].

The administration of L-arginine to diabetic rats ameliorated mitochondrial respiration in state 4 (state of low oxygen consumption), and slightly increased RCR. These results suggest that L-arginine supplementation can

partially improve the mitochondrial function by restoring the normal electron transport chain in diabetic rats.

As described before, the endogenous inhibitor of NOS enzymes, ADMA, could be involved in the observed mitochondrial dysfunction. It has been documented in a recent study that elevated ADMA levels in serum of diabetic rats were associated with hepatic mitochondrial dysfunction and that this effect of ADMA was attenuated by treatment with a NO donor [45]. Therefore, it is possible that in this study L-arginine administration to diabetic rats could improve mitochondrial electron transport chain by attenuation of endogenous inhibitors of NOS, thereby stimulating NO synthesis. Recently it was described that the ratio L-arginine/ADMA modulates NOS activity and elevation of ADMA levels may cause relative L-arginine deficiency [46]. So, it is possible that supplying substrate, the increase in NO synthesis outweighs the endogenous inhibition.

Oxidative stress and tissue damage are common phenomena linked to exposure to toxic agents and occurring in several diseases, including diabetes. We observed a marked oxidative impact in STZ-diabetic rats, evidenced by a significant rise in the level of lipid peroxidation in brain cortex homogenates. L-arginine treatment prevented the increase in lipid peroxidation in diabetic rats, indicating a reduction of oxidative stress. Previous studies showed similar results in brain and liver in alloxan diabetic rats, where L-arginine treatment diminished TBARS concentration and restored levels of antioxidants that were markedly diminished in hyperglycaemic condition [16].

The presented data show that L-arginine administration to STZ- diabetic rats resulted in a significant decrease in mitochondrial O_2^- anion levels and augmented H_2O_2 production. This increased H_2O_2 production was associated with an increased MAO activity. Besides, we observed that L-arginine treatment induced a mitochondrial polarization in STZ-diabetic rats similar to control rats, restoring mitochondrial membrane potential.

On the other hand, we demonstrated that L-arginine supplementation prevented the decrease of both, NO mitochondrial production and NOS I expression in diabetic rats, in agreement with the fact that L-arginine ameliorated mitochondrial respiration in state 4 in diabetic rats. Therefore, these results can suggest that in hyperglycaemic conditions, L-arginine may regulate the activity of the respiratory chain by increasing NO levels and decreasing ROS production as a consequence of restoring the normal NOS function.

It is well known, that after just 3 days after STZ treatment a clear hyperglycemic condition is achieved [47]. In addition, the central nervous system (CNS) is considered to be relatively resistant to debilitating effects of diabetes. The basis for this resistance is not known, but it could be due to elevated basal protective processes at early stages of diabetes that decrease after long term of the diabetic condition [48]. We could suggest that the observed mitochondrial dysfunction at early stages in cerebral cortex of STZ-diabetic rats probably did not interfere with the normal function of the central nervous system but it could induce brain metabolic changes at later stages of the diabetic condition.

Taking all the results together we can conclude that after 4 days of STZ administration, the hyperglycaemic condition was associated with a clear mitochondrial dysfunction, characterized by an impairment of the mitochondrial respiratory chain, decreased NO production, increased production of free radicals such as superoxide anion and as a consequence an increased level of peroxidation. The appearance of this mitochondrial dysfunction at early times of induced diabetes before the onset of functional or structural neuropathy indicates that a bioenergetic alteration in neurones could contribute to the development of the complex diabetic neuropathy observed later on the hyperglycaemic condition.

The present results suggest that L-arginine supplementation has beneficial effects on STZ-diabetic rats improving the respiratory function, decreasing oxidative stress and enhancing NO production in brain cortex mitochondria of STZ-diabetic rats, in accordance with the beneficial effects of L-arginine treatment observed in another model of diabetes [17]. However, we cannot discard that L-arginine acting peripherally, improved the animal's condition and secondarily, improved the measured brain parameters.

We can conclude that a clear mitochondrial dysfunction characterized by an increased superoxide production, low MAO activity, decreased NOS I production and diminished NOS I expression associated to the mitochondrial membranes was present in the hyperglycaemic condition induced by STZ. Administration of L-arginine to these rats prevented the oxidative stress, and restored the NO production at early stages of STZ-diabetic rats.

A better understanding of the diabetic condition may help to design new therapeutic approaches to treat pathophysiological changes that occur in the central nervous system.

Acknowledgments This work was funded by Grants UBACYT B087, B025, 20020110200353 and 20020100100511 from the University of Buenos Aires and Grant 112-200801-00653 from CONICET.

Conflict of interest The authors declare no conflicts of interest.

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