Amino Acids (2003) 24: 163–169
DOI 10.1007/s00726-002-0317-5

Amino
Acids
Printed in Austria

Effect of D-amino acids on some mitochondrial functions in rat liver

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Received January 15, 2002
Accepted April 14, 2002
Published online September 4, 2002;

Summary. We studied the role of the D-amino acids (D-aa) D-serine, D-alanine, D-methionine, D-aspartate, D-tyrosine and D-arginine on rat liver mitochondria. The stability of D-amino acids, mitochondrial swelling, transmembrane potential and oxygen consumption were studied under oxidative stress conditions in rat liver mitochondria. In the presence of glutamate-malate all D-aas salts increased mitochondrial swelling, while in the presence of succinate plus rotenone only D-ala, D-arg and D-ser, induced mitochondrial swelling. The transmembrane potential ($\Delta\Psi$) was decreased in the presence of 1 μM Ca^{2+}. The D-aas inhibited oxygen consumption in state 3. The D-aa studied exerted effects on mitochondria via an increase of free radicals production.

Keywords: Mitochondria – Liver – D-Amino acids – Membrane potential – Mitochondrial Swelling – Free radicals

Introduction

Mammals neither synthesize nor posses D-amino acids (D-aa), while invertebrates, amphibians and microorganisms use D-aa in a variety of metabolical reactions such as the production of antibiotics or D-amino acid-containing peptides (Nagata et al., 1987; Iida et al., 2001). The natural presence of D-aa suggests that they are significant in evolution, since the emergence of biochemical homochirality was a key step in the origin of life (Hazen et al., 2001). D-amino acids present its maximum levorotatory effect, which also is specific for their isoelectric point (Corrigan, 1969). Racemization is present when the amino acids are subjected to high temperatures and alkaline pH (Harris et al., 1999). D-aa may play a role in animal metabolism and cell differentiation in insects (Corrigan, 1969). It is now increasingly being realized that trace amounts of the D-aa may have some, yet unknown biological function. However, for the most part the role D-aa still undefined (Corrigan, 1969). Enzymatic (Nagata et al., 1985), chromatographic (Bhushan et al., 1993) and immunohistochemical methods (Lee et al., 1997) have been developed to investigate the presence of D-amino acids in biological samples. Of these methods, HPLC fluorometric methods are the most superior in view of their high sensitivity and reliability. Picomole levels have been detected in biological tissues (Fisher et al., 2001). D-aa are present in several animal tissues including teeth and brain (Nagata et al., 1998). In fact, they can also be accumulated during renal dysfunctions or aging (Sela and Zisman, 1997). D-aa or peptides containing D-aa can be introduced to mammals via circulation of synthetic analogs, hormones or peptidic antibiotics, or synthetic peptide-based vaccines where certain L-amino acids (L-aa) are replaced by their D-enantiomers resulting with an increase in activity or elongation of its biological life (Sela and Zisman, 1997).

D-amino acid oxidase (DAO; EC 1.4.3.3) is a flavoprotein oxidase that is not inhibited by L-amino acids (Pilone, 2000), that uses flavin adenine dinucleotide as a prosthetic group, and that catalyzes the dehydrogenation of D-amino acids to the corresponding imino acids (subsequently hydrolysed to α-ketoacids and ammonia) with concomitant reduction of the FAD cofactor (Pollegioni et al., 2001). DAO has been detected in kidney, liver, brain, leucocytes and heart (Nagata et al., 1988; Fukui et al., 1988). Its function is to metabolise in vivo D-aa derived from endogenous or exogenous compounds in animals (Nagata et al., 1988; Fukui et al., 1988; D'Aniello et al., 1993; Pilone, 2000).

Specific functions for D-aa such as D-arginine (D-arg) have been proposed in the urea cycle (Saavedra-Molina and Piña, 1991). D-aspartate (D-asp) on the other hand, which was detected in newborn chicken and rodents, may play a specific role in vertebrate development (Asakura and Konno, 1997). For example, D-asp is synthesized in purified rat Leydig-cells increasing testosterone production (Nagata et al., 1999). Recently, it was reported that N-methyl-D-aspartic acid stimulates luteinizing hormone-release in the rat hypothalamus, and that D-asp may be a candidate precursor for its synthesis (D'Aniello et al., 2000). Moreover, in patients with Alzheimer's disease, the levels of D-asp and D-ser decrease significantly (Long et al., 1998; Schell et al., 1997; Fisher et al., 1998). In the differentiation of rodent neurons, D-asp changes its intracellular localization as well (Schell et al., 1997; Long et al., 1998; Sakai et al., 1998). By contrast, D-serine (D-ser), D-asp and D-alanine (D-ala) are abundant in brain and it has been suggested that they enhance the activity of NMDA-type receptors (Nagata et al., 1997; Snyder and Kim, 2000; Morikawa et al., 2001). Finally, high levels of D-methionine (D-met) and D-ala were detected in serum and urine as a result of dietary changes of the intestinal bacterial metabolism (Nagata et al., 1997).

To date there are few studies on the effects of D-aa on mitochondrial metabolism. Therefore, the specific aim of this work is to assess the effect of D-aa on mitochondrial swelling, membrane potential and oxygen consumption of state 3 respiration in rat liver. In addition, nanomolar concentrations of the D-aa were used due to the very small levels detected in biological tissues (Fisher et al., 2001). Our results indicate that D-aas may inhibit mitochondrial functions. Moreover, the mechanism of inhibition appears to involve an increase of free radicals production.

Materials and methods

Materials

All chemicals were of the purest analytical grade. The D-aa: aspartate, arginine, alanine, methionine, serine, tyrosine, as well as L-glutamate and the substances L-malate, succinic acid, MOPS, EGTA, serum albumin (BSA), sucrose, D-mannitol, bovine serum albumin (BSA), KCl, KH_2PO_4, $MgCl_2$, $CaCl_2$, 1-butanol, thiobarbituric acid, pyridine, dimethylformamide, ADP, safranine, rotenone and m-chlorophenylhydrazone (CCCP) were purchased from Sigma Chemical (St. Louis, MO. USA).

To assess if D-amino acids presented natural racemization to L-enantiomers under our assay conditions, we evaluated the specific rotation of each D-aa. In this study D-aa racemization was not a problem due to the controlled experimental conditions of temperature and pH and their freshly preparation during the study. Only D-ser and D-tyr racemized after 24 hs. From these controls, D-aa were always prepared daily.

Preparation of mitochondria

Male Wistar rats weighing 200–250 g were fed *ad libitum* and kept under controlled conditions of light : darkness cycles. All animal procedures were conducted in accordance with our *Federal Regulations for Use and Care of Animals* (Ministry of Agriculture, SAGAR, 2001. México). Mitochondria were isolated by standard differential centrifugation as described (Saavedra-Molina and Devlin, 1997). Briefly, the liver was gently homogenized in a medium containing 220 mM mannitol, 70 mM sucrose, 10 mM MOPS (pH 7.4), 1 mM EGTA and 0.2% BSA (Medium 1). After differential centrifugation the mitochondrial pellet was resuspended in a medium containing 220 mM mannitol, 70 mM sucrose, 10 mM MOPS (pH 7.4), 0.2% BSA (Medium 2). All centrifugations were carried out at 4°C. Protein content was assayed according to Gornall et al., with slight modifications (Gornall et al., 1949).

Oxymetry assays

To assure functionality of mitochondria, oxymetry assays were performed by measuring consumption of oxygen at 25°C by a YSI-5300 oxygen monitor (Yellow Springs Instrument, Yellow Springs, OH, USA), equipped with a Clark electrode (Estabrook, 1967). The medium used contained 130 mM KCl, 2 mM KH_2PO_4, 1 mM $MgCl_2$, 10 mM MOPS and 5 mM succinate plus rotenone or 5 mM glutamate-malate, pH 7.4. In all oxymetry assays 1 mg mitochondrial protein/ml was used and 300 μM ADP was added to stimulate oxidative phosphorylation. To determine the effect of D-aa on state 3 respiration, a concentration of 20 nM of the D-aa was always used and the stress condition was elicited by adding 1 or 30 μM Ca^{2+}. The free concentration of Ca^{2+} in the incubation medium was calculated with the computer program WinMax 2.0 (Patton, 1999).

Measurement of the transmembrane potential

Generation of the transmembrane potential ($\Delta\Psi$) was according to Åkerman and Wikström (1976) using safranine orange as probe. The assay solution contained 200 mM sucrose, 10 mM MOPS, 10 mM KCl, 2 mM K-phosphate, 5 mM glutamate-malate, 10 μM safranine, 1 μM Ca^{2+} and 20 nM D-aa at pH 7.4. Where indicated, the uncoupler CCCP was dissolved in dimethylformamide and was added at a final concentration of 6 μM. Changes in absorbance were recorded with a DW-2000 spectrophotometer and double beam at 511–533 nm.

Determination of mitochondrial swelling

Mitochondrial swelling was assayed according to Beavis et al. (1985) by monitoring diminution of absorbance at 540 nm at 25°C in a u.v./vis. Perkin Elmer (Lambda 18) spectrophotometer. Mitochondria (1 mg/ml) were suspended in a solution contained 200 mM sucrose, 10 mM MOPS (pH 7.4), 5 mM succinate plus 1 μM rotenone or 5 mM glutamate-malate plus 20 nM of the D-aa salt in a final volume of 3 ml. After optical density was stabilized, swelling was initiated with the addition of the D-aa (20 nM) alone or in the presence of Ca^{2+} (30 μM).

Determination of free radical production

The amount of aldehydic products generated was measured with the thiobarbituric acid (TBA) assay (Buege and Aust, 1978) by lipid

peroxidation as a consequence of free radicals production. Briefly, 1 mg/ml of mitochondrial protein was incubated in plastic tubes for 30 min at 30°C in a medium containing 250 mM sucrose, 4 mM MOPS (pH 7.4) in the presence of 20 nM of the D-amino acid and 30 μM $CaCl_2$. The reaction was stopped with 1.5 ml of acetic acid (20% (w/w), pH 2.5) and 1.5 ml of TBA (0.8% (v/v)). Tubes were heated at 100°C in a water bath for 45 min and chilled in ice. To each tube, 1 ml KCl (2%) and 5 ml of a 1-butanol/pyridine mixture (15 : 1 v/v) was added and tubes were agitated manually. Later, tubes were centrifuged at 5000 r.p.m. for 5 min and the supernatant was extracted and the absorbance was determined at 532 nm in a u.v./vis. Perkin-Elmer (Lambda 10) spectrophotometer. The results were calculated based on the molar extinction coefficient of malondialdehyde reported by Buege and Aust (1978) and expressed in terms of nanomoles of TBA reactants (TBARS) per mg protein.

Results

Mitochondrial swelling is an indirect measurement of transport of certain solutes across the inner membrane to the matrix (Haworth and Hunter, 1979). This complex phenomenon may be due to the opening of a nonspecific pore in which the membrane permeability transition change is in reversibly manner by simply removing the Ca^{2+} via chelation with EGTA (Haworth and Hunter, 1979). Chernyak and Bernardi (1996) found that 1 μM Ca^{2+} is sufficient to induce measurable permeabilization. In fact, a maximal and still reversible effect of this stress condition was achieved with 30 μM Ca^{2+}. In this study, an irreversible stress condition was induced by adding 150 μM Ca^{2+} to demonstrate the maximal swelling rate (data not shown). Regarding this approach, we performed swelling experiments with liver mitochondria in order to measure transport of D-aa to the matrix in the presence of 30 μM Ca^{2+} (Fig. 1). When the D-aas were prepared as chloride salts and succinate was used as a respiratory substrate in the presence of rotenone, D-ala showed the maximum degree of swelling (165%, $p < 0.05$) (Fig. 1). D-asp does not affect swelling and the other D-aa induced swelling to a small degree. The order of mitochondrial swelling caused by the D-aa tested was as follows: D-ala (165%) > D-ser (90%) > D-tyr (67%) > D-met (67%) > D-arg (46%) > D-asp (0%). As control, there was no effect of rotenone on mitochondrial swelling as another possibility to observe these phenomena (data not shown).

When rat liver mitochondrial swelling was assayed in the presence of the respiratory substrates glutamate-malate and the D-aa were prepared as the chloride salts (Fig. 2), again D-ala (115%, $p < 0.05$) induced the maximum swelling rates. D-tyr induced swelling (100%) in the same range as in the presence

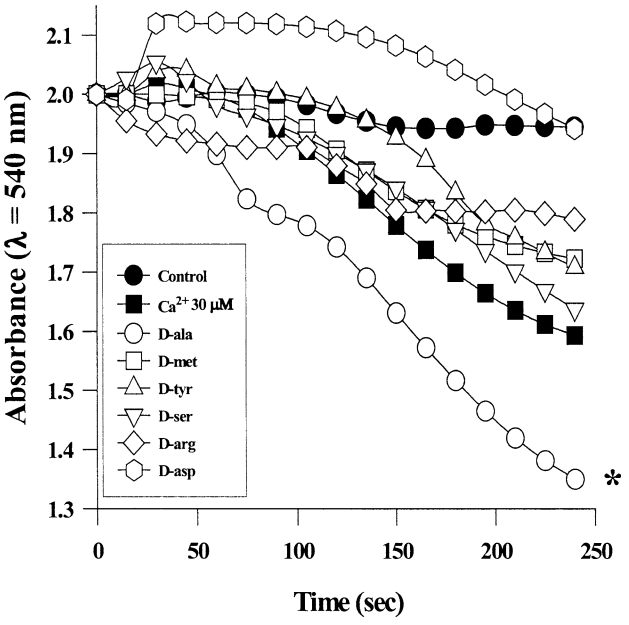

Fig. 1. Effect of D-amino acids on rat liver mitochondrial swelling. Mitochondria (1 mg/ml) were incubated in a medium containing 200 mM sucrose, 5 mM succinate plus 1 μM rotenone, 10 mM MOPS (pH 7.4) and 20 nM of the chloride D-amino acid salt added. After optical density was stabilized, swelling initiated when the correspondent D-aa (20 nM) was added. The results represent the mean ± SEM of n = 5. *$p < 0.05$

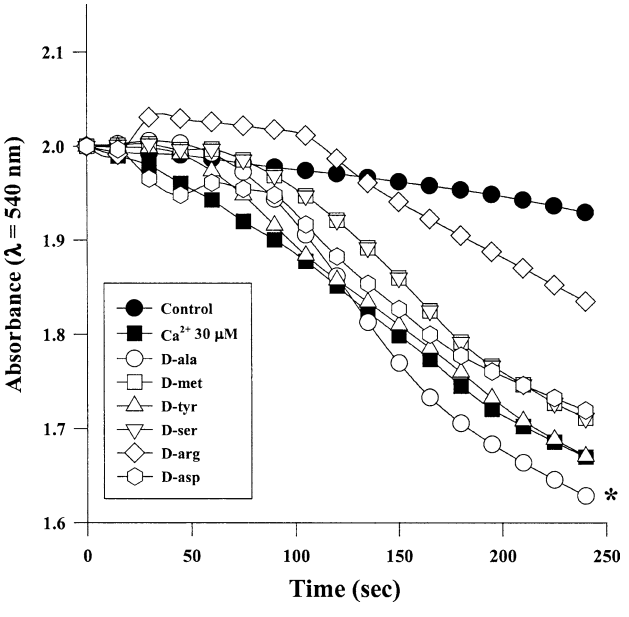

Fig. 2. Effect of D-amino acids on rat liver mitochondrial swelling. Mitochondria (1 mg/ml) were incubated in a medium containing 200 mM sucrose, 5 mM glutamate-malate, 10 mM MOPS (pH 7.4) and 20 nM of the chloride D-amino acid salt added. After optical density was stabilized, swelling initiated when the correspondent D-aa (20 nM) was added. The results represent the mean ± SEM of n = 5. *$p < 0.05$

Table 1. Effect of D-amino acids on transmembrane potential in rat liver mitochondria[1,2]

D-amino acid	Condition	ΔΨ (mV)	ΔΨ (%)
	$-Ca^{2+}$	−180	100
	$+Ca^{2+}$ 1 μM	−164	91
	$+Ca^{2+}$ 150 μM	0	0
D-ala	$+Ca^{2+}$ 1 μM	−143 ± 4.0 (5)	79
D-met	$+Ca^{2+}$ 1 μM	−148 ± 3.0 (5)	82
D-asp	$+Ca^{2+}$ 1 μM	−154 ± 6.0 (6)	85
D-ser	$+Ca^{2+}$ 1 μM	−159 ± 0 (3)	88
D-arg	$+Ca^{2+}$ 1 μM	−159 ± 1.0 (5)	88
D-tyr	$+Ca^{2+}$ 1 μM	−164 ± 0.3 (3)	91

[1] Results represent the mean ± SEM with the number of experiments in parenthesis

[2] The different concentrations of Ca^{2+} used (0 and 1 μM) were added by using EGTA-Ca^{2+} buffers as described (Patton, 1999)

of 30 μM Ca^{2+}. The mitochondrial swelling rate caused by the other D-aa assayed was as follows: D-asp, D-ser and D-met induced the same swelling (80%), and finally D-arg (36%).

Next, we aim to test the possible effects of D-aa on the transmembrane potential (ΔΨ), which can be affected by the transport of solutes both under mild and reversible stress conditions. Due to the sensitivity of the method that used safranine as a probe (Åkerman and Wikström, 1976), we performed the addition of the D-amino acid in the presence of 1 μM $CaCl_2$ as oxidative stress condition and 150 μM $CaCl_2$ to abolish the transmembrane potential (Table 1). In the absence of calcium, the maximum increase of the transmembrane potential was obtained equivalent to −180 mV (according to the Nernst equation applied) (Åkerman and Wikström, 1976), which was abolished completely by the addition of the uncoupler CCCP or 150 μM $CaCl_2$. When we added 1 μM $CaCl_2$ the ΔΨ diminished 9% (−164 mV) only, which still is considered in physiological range. In fact, this value indicates that rat liver mitochondria maintain metabolic reactions functioning satisfactorily. The effects of the addition of different D-amino acids, without the addition of calcium, on mitochondrial ΔΨ were: D-asp (9%), D-ala (3%) and D-tyr (6%) (data not shown). The other D-aa tested did not induce changes on the ΔΨ. When low concentration of calcium (1 μM) was added in the presence of each D-aa, ΔΨ changed as follows: D-ala 21%, D-met 18%, D-asp 15%, D-arg 12%, D-ser 12%, D-tyr 9% (Table 1).

Mitochondrial respiration coupled with energy production is another important function that was analysed as a possible target for the addition of D-amino acids. With this in mind, we performed experiments assaying this effect on state 3 respiration i.e. the oxidative phosphorylation system (Table 2). Experiments assaying the presence of calcium in the low (1 μM) or high (30 μM) but reversible stress conditions were maintained as above. In all experiments, liver mitochondria displayed a respiratory control ratio of 5.0 at least. As controls, rat liver mitochondria were assayed for state 3 of respiration in the presence of low concentration of Ca^{2+} (1 μM) with a 15% decrease (85% ± 0.7, n = 8) compared with control (100%) values. When state 3 of respiration was assayed in the presence of the high concentration of Ca^{2+} (30 μM) and reversible stress condition (Haworth and Hunter, 1979), a 45% (55% ± 2.0, n = 9) decrease in the state 3 of respiration was observed. When D-aa were added during the state 3 of mitochondrial respiration with glutamate-malate as substrates, only D-asp (83% ± 4), D-ser (79% ± 1) and D-ala (78% ± 4) exerted significant ($p < 0.001$) inhibition of 17%, 21% and 22%, respectively, compared with control values (100%) without D-aa (Table 2). D-arg (9%), D-met (5%) and D-tyr (3%) induced very low and not significant inhibition on state 3 of mitochondrial respiration. When the addition of the low concentration of calcium (1 μM) was tested and the correspondent chloride salt of the D-amino acid on the state 3 of mitochondrial respiration was measured, only D-ser (Table 2) induced a significant ($p < 0.01$) change (96% ± 2) of protection compared with the control values (85% ± 0.7). Under this mild oxidative stress condition (1 μM Ca^{2+}), the other D-aa did not induce significant

Table 2. Effect of D-amino acids on state 3 of mitochondrial respiration[1,2,3]

D-amino acid	D-amino acid (20 nM)	D-amino acid + Ca^{2+} (1 μM)	D-amino acid + Ca^{2+} (30 μM)
D-ala	78 ± 4.0 (8)*	78 ± 3.0 (10)	74 ± 1.0 (6)
D-arg	91 ± 2.0 (5)	95 ± 2.0 (4)	78 ± 3.0 (4)
D-asp	83 ± 4.0 (4)*	81 ± 4.0 (4)	71 ± 4.0 (4)
D-met	95 ± 2.0 (4)	90 ± 2.0 (3)	67 ± 1.0 (3)
D-ser	79 ± 1.0 (5)*	96 ± 2.0 (6)**	67 ± 2.0 (5)
D-Tyr	97 ± 2.0 (9)	84 ± 0.3 (7)	74 ± 1.0 (8)

[1] Control: 100%; Control + Ca^{2+} (1 μM): 85 ± 0.7 (8); Control + Ca^{2+} (30 μM): 55 ± 2.0 (9). The different concentrations of Ca^{2+} used (1 μM and 30 μM) were added by using EGTA-Ca^{2+} buffers as described (Patton, 1999)

[2] Results represent the mean ± SEM with the number of experiments in parenthesis

[3] * $p < 0.001$. ** $p < 0.01$

changes on state 3 respiration compared to control in the presence of calcium (Table 2). At 30 μM $CaCl_2$, the D-aa assayed did not exert significant changes on the state 3 mitochondrial respiration, compared with the correspondent control (Table 2). The low values obtained reflect only the calcium effect on this mitochondrial function but without affecting the oxidative phosphorylating reaction in irreversible manner.

In this study, we assess that a possible explanation of the different effects obtained by the addition of D-aa on certain mitochondrial functions could be due to the generation of free radicals. At least one product of the DAO activity, which has been found in mitochondria (Rosenfeld and Leiter, 1977), is H_2O_2. H_2O_2 can react with the mitochondrial superoxide anion and produce another pro-oxidant, the hydroxyl radical. All these reactive oxygen species (ROS) can be determined by the thiobarbituric acid assay (Buege and Aust, 1978). Therefore, experiments were designed with only three D-aa, namely D-asp, D-ser and D-ala. These D-aas in turn produced the most pronounced inhibitory effects on the above mitochondrial reactions studied (Fig. 3). The other D-aa did not affect the free radical production (data not shown). The basal lipid peroxidation data obtained in our experimental conditions were 0.30 $\pm$ 0.08 nmoles TBARS/mg prot. (100%). This value changed to 0.69 $\pm$ 0.1 nmoles TBARS/mg prot. (230%, $p < 0.05$) in the presence of D-ser; with D-asp was 0.50 $\pm$ 0.05 nmoles TBARS/mg prot. (166%) and 0.67 $\pm$ 0.1 nmoles TBARS/mg prot. (223%, $p < 0.05$) for D-ala (Fig. 3A). When the same reaction was tested under reversible oxidative stress conditions of 30 μM Ca^{2+} (Fig. 3B) (Chernyak and Bernardi, 1996), the basal values of lipid peroxidation obtained were 1.08 $\pm$ 0.09 nmoles TBARS/mg prot. (100%, $p < 0.05$; compared to the control without calcium, Fig. 3A). However, the addition of D-ser resulted in 2.03 $\pm$ 0.09 nmoles TBARS/mg. prot. (188%, $p < 0.05$ compared with control plus Ca^{2+}). The levels observed with D-asp were 1.5 $\pm$ 0.07 nmoles TBARS/mg. prot. (134%, $p < 0.05$); with D-ala 1.56 $\pm$ 0.1 nmoles TBARS/mg. prot. (144%, $p < 0.05$) (Fig. 3B).

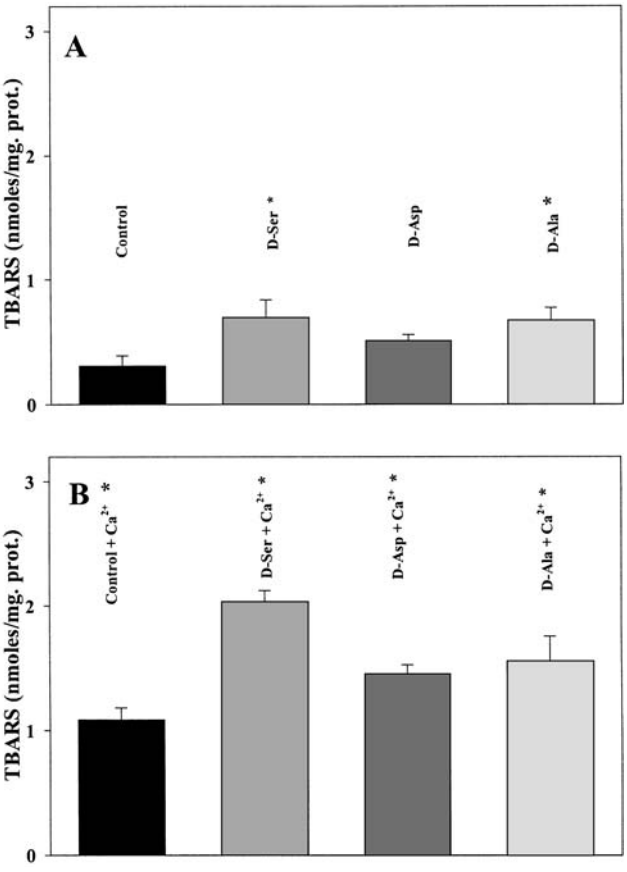

Fig. 3. Effect of D-amino acids on mitochondrial free radicals production. Mitochondrial protein (1 mg/ml) was incubated in the presence of the correspondent D-aa (20 nM). In **A** is the D-aa alone; in **B** is the D-aa plus 30 μM Ca^{2+}. The results represent the mean $\pm$ SEM of n = 4. *$p < 0.05$

Discussion

D-amino acids and D-amino acid oxidase have been found distributed in several mammalian organs (Asakura and Konno, 1997; Pilone, 2000; Morikawa et al., 2001), and within an intracellular localization (Rosenfeld and Leiter, 1977; Villalobos-Molina et al., 1987). One report on this issue is from Villalobos-Molina et al. (1987), who reported that D- and DL-arginine were transported to rat liver mitochondria due to an electric potential generated by calcium. Same results are in our data on the effect of D-aa on mitochondrial respiration (Table 2), where the presence of calcium combined with the addition of D-aa resulted in a protective effect of the state 3 respiration when it was present an oxidative stress condition, situation observed with all of the D-aa tested. A possible explanation of these results is that an increase on the matrix free calcium concentrations is critical to exert damage. In our case, the transport of the D-amino acid through the matrix could be accompanied with a release of calcium and so calcium is maintained in the proper concentration to continue the mitochondria functioning as described (Saavedra-Molina et al., 1990; Saavedra-Molina and Devlin, 1997).

Bernardi et al. (1993) proposed that an augmentation on the matrix free calcium concentration and alkalinization of the mitochondrial matrix (Bernardi, 1991) are necessary factors to induce membrane permeability transition, which in turn exert a diminution on the membrane potential. Our data obtained with the effects of D-aa on membrane potential (Table 1) indicates that all D-aa assayed diminished the membrane potential; in that order D-ser and D-tyr resulted with the lowest but not collapsed values of membrane potential with a great possibility of its high reactivity that can produce the membrane damage. In stress conditions, the D-aa transport were more pronounced through the inner membrane which means that calcium ions can induce other enzymatic activations like the phospholipase A_2. In turn, its products, the lysophospholipids and the peroxide fatty acids (peroxidation intermediaries), participate in the regulation of the permeability transition pore (Bernardi et al., 1992; Pfeifer et al., 1979). Stegman et al. (1998) raised the question that increased oxidation of D-amino acids could produce sufficient reactive oxygen species, a cytotoxic oxidative stress conditions to kill tumour cells.

Amino acid transport across the plasma membrane is mediated both by Na^+-dependent and Na^+-independent transporters (Christensen, 1990). Recently, a Na^+-independent amino acid transport, which transports D-isomers of the neutral amino acids, has been described (Fukasawa et al., 2000). The results that D-asp and D-arg (in the acetate salt preparation) (data not shown) affected the membrane potential suggest that an electrogenic transport is involved (Villalobos-Molina et al., 1987). In addition, it is suggested the same mechanism for the other D-aa assayed because it has been described a similar transport mechanism in certain bacteria (Ingraham and Scaecher, 1987) or the same co-transport reported in mitochondria for glutamate-H^+/aspartate (Munk, 1997). More research is needed in this field to clarify the possible physiological effects of D-amino acids on cellular metabolic reactions.

Acknowledgements

The authors appreciated the partial economical support from Mexican grants of CONACYT (to A.S.-M. during its sabbatical) and CIC-UMSNH (2.5) and critical readings from Rafael Álvarez-González.

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