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Effects of D-amino acids on lipoperoxidation in rat liver and kidney mitochondria

C. Cortés-Rojo, M. Clemente-Guerrero, and **A. Saavedra-Molina**

Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, México

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Summary. The effects of the amino acids D-ser, D-asp, and D-ala on lipoperoxidation under conditions of hypertension, alcoholism, and ammonemia in rat liver and kidney mitochondria were studied. Under normal conditions, D-alanine increased in 54% free radicals production in liver mitochondria ($p < 0.05$). The D-amino acids had no effect on kidney mitochondria. D-ser and D-ala increased lipoperoxidation in spontaneously hypertensive rats (SHR) as compared with their normotensive genetic control Wistar-Kyoto (WKY) rats ($p < 0.05$). During hypertension and in oxidative stress in the presence of calcium, only D-ala produced 46% and 29% free radicals in liver and kidney mitochondria ($p < 0.05$), respectively. During chronic alcoholism, D-ser increased lipoperoxidation in 80% in kidney mitochondria ($p < 0.05$), as compared to control. During ammonemia, D-ser produced 41% free radicals.

Keywords: Lipoperoxidation – D-amino acids – Hypertension – Oxidative stress – Chronic alcoholism – Liver mitochondria – Kidney mitochondria

Introduction

It is well established that mammalian proteins are composed predominantly of amino acids with "L" configuration. However, modern analytical techniques (Li and Zhang, 2000; Voss and Galensa, 2000; Hamase et al., 2005) have detected the presence of "D" amino acids (D-aa), free or bonded to proteins in mammals and other life forms (Oguri et al., 1999). HPLC fluorometric methods are the best in view of their high sensitivity and reliability. Picomole levels have been detected in biological tissues (Fisher et al., 2001). D-serine (D-ser) and D-aspartate (D-asp) have been found consistently in significant amounts in mammalian tissue (Wang et al., 2002), whereas D-alanine (D-ala) is present in peptidoglycans, a component of bacterial cell walls, and contributes to resisting digestion by proteolytic enzymes in the digestive system (Friedman, 1999). In this manner, D-ala is susceptible to intestinal absorption. It has been found that D-amino acids (D-aa) as well as the L-amino acids (L-aa) are easily absorbed into the small intestine using a Na^+-dependent transport system in the brush border intestinal epithelium (Fernández et al., 2005). Amino acids transport across the plasma membrane is mediated both by Na^+-dependent and Na^+-independent transporters (Rajan et al., 2000). Recently, a Na^+-independent amino acids transport, which transports D-isomers of neutral amino acids, has been described (Gandhi et al., 2004; Fernández et al., 2005).

D-aa are metabolized in mammals by the D-amino acid oxidase (DAO) and the D-aspartate oxidase, primarily found in the liver and kidney (D'Aniello et al., 1993, 2005). These enzymes catalyze an oxidative deamination of the D-aa to the correspondent α-keto acids, NH_3, and H_2O_2. The later compound, which is not considered a free radical, can generate the hydroxyl radical ($OH^{\bullet}$) by the Haber-Weiss cycle in the presence of free Fe^{2+} or in the presence of iron content from the Fe-S centers of the proton carrier system in mitochondria and the Fe-S centers of the dehydratases of the tricarboxylic acids cycle (Keyer and Imlay, 1996). Rosenfeld and co-workers detected activity of the D-amino acid oxidase in the mitochondrial matrix of *Neurospora crassa* (Rosenfeld and Leiter, 1977), as González-Hernández (2002) did in liver mitochondria.

Specific functions for D-aa such as D-arginine (D-arg) have been proposed in the urea cycle (Saavedra-Molina and Piña, 1991). In addition, D-ser regulates N-methyl D-aspartate (NMDA) receptors in the brain (Nishikawa, 2005) and inhibits sphingolipids biosynthesis in rats (Hanada et al., 2000). D-aspartate (D-asp), on the other

hand, 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 synthesis by stimulating the cholesterol translocation system through the inner mitochondrial membrane (Furuchi and Homma, 2005). In addition, D-aa have been involved in pathological diseases, such as Alzheimer's (D'Aniello et al., 1998), in which significantly decreased amounts of D-asp and significant increased amounts of D-ala have been found. A high accumulation of [^{14}C]-D-ser has been found in kidneys associated with acute renal necrosis. A high accumulation of D-amino acids has also been found during aging of several tissues, including human teeth, eye cataracts, and brain (Fujii, 2005).

There are no reports on the relationship between the production of free radicals during D-aa metabolism and mitochondria; in addition, there is a lack of information to correlate mitochondrial D-amino acid (D-aa) metabolism with certain pathologies or diseases, where an increase in D-aa has been detected. In this study, our objective was to test the effect of D-ser, D-asp, and D-ala on lipoperoxidation in rat liver and kidney mitochondria under oxidative stress, hypertension, chronic alcoholism, and ammonemic conditions. Liver and kidney are target tissues of these pathologies. The results indicate that these pathologies are sensitive to the presence of the studied D-amino acids.

Materials and methods

Materials

All chemicals were of the purest analytical grade and purchased from Sigma Chemical (St. Louis, MO, USA). To assess whether 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 fresh preparation during the study. Only D-ser racemized after 24 hours. From these controls, D-aa were always prepared daily.

Biological materials

All animal procedures were conducted in accordance with our *Federal Regulations for Use and Care of Animals* (NOM-062-ZOO-1999, Ministry of Agriculture, SAGAR, 2001. México), and were approved by the Institutional Committee of the Universidad Michoacana de San Nicolás de Hidalgo, for the use of animals. Male Wistar rats weighing 200–250 g were fed ad libitum and kept under controlled conditions of light:darkness cycles. For the hypertension experiments, we employed male Spontaneously Hypertensive Rats (SHR) and their normotensive genetic control Wistar-Kyoto rats (WKY) obtained from the Departamento de Farmacobiología, CINVESTAV-IPN, Sede Sur. D.F. Systolic blood pressure was determined by pletysmography, yielding values of 134 ± 3 (WKY) and 183 ± 5 (SHR) mmHg.

Preparation of mitochondria

Mitochondria were isolated by standard differential centrifugation as described (Saavedra-Molina and Devlin, 1997). Briefly, the liver and/or kidney, separately, were 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. (1949), with slight modifications.

Induction of alcoholism and ammonemia

Chronic alcoholism in rat was induced by adding 10% ethanol to the drinking water during 4 weeks, as described (Zentella de Piña et al., 1989). To induce ammonemia in rats, an intraperitoneal injection of 350 mg/kg of ammonium acetate was used, 30 min before sacrificing animals, as described (Maracaida et al., 1992). This amount was determined through a specific doses-response curve, by which the animals showed characteristic NH_3 intoxication symptoms (Maracaida et al., 1992).

Determination of free radical production

The amount of generated aldehydic products was measured with the thiobarbituric acid (TBA) assay (Buege and Aust, 1978), assessing lipid peroxidation through free radicals production, as described (González-Hernández et al., 2002).

Statistical analysis

Data were subjected to ANOVA and Student's *t*-tests to determine statistical significance ($p < 0.05$, $p < 0.001$). Points represent the mean $\pm$ SEM with the number of experiments in parenthesis in duplicate and separately.

Results

Effect of D-amino acids on free radical production

To prove the efficiency of our experimental conditions on free radical production, we measured the reactive substances using thiobarbituric acid (TBARS) in liver mitochondria under normal conditions and in the presence of the ADP/Fe^{2+} complex, an inducer of free radicals, as described (Gutteridge et al., 1990). During 30 min of incubation, TBARS production was about 0.3 nmoles/mg, induced by electron transfer in the respiratory chain (Pérez-Vázquez et al., 2002), an amount that was considered as basal production of free radicals in the presence of the normal antioxidant mechanisms in mitochondria. In the presence of ADP/Fe^{2+} free radical production increased to 0.8 nmoles/mg at 20 min incubation, and to 1.5 nmoles/mg after 30 min incubation.

We evaluated lipoperoxidation as free radical production in rat liver and kidney mitochondria in the presence of D-ser, D-asp, and D-ala. D-ala promoted a significant increase ($p < 0.05$) of 54% in liver mitochondria (Fig. 1A), whereas D-asp and D-ser elicited a lesser response.

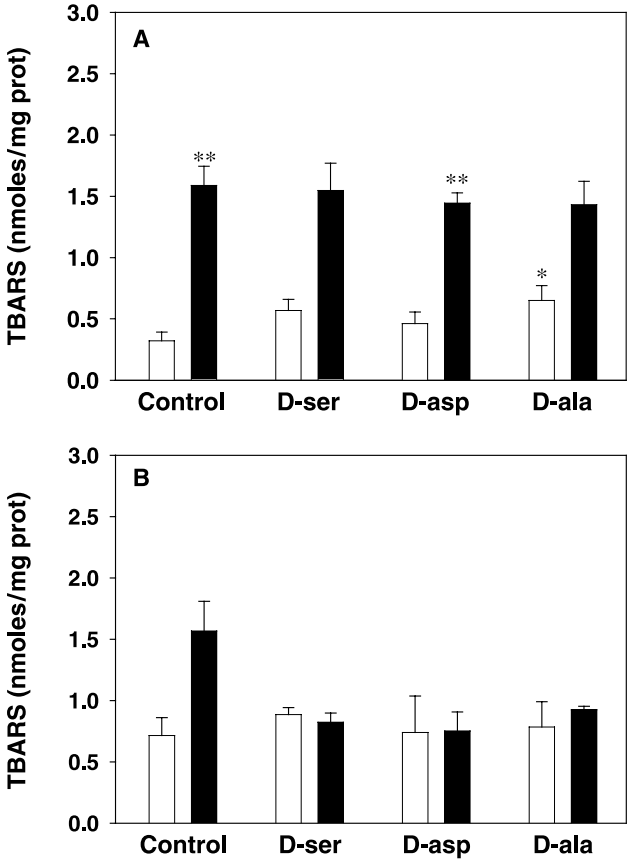

Fig. 1. Effect of D-amino acids on lipoperoxidation in rat liver and kidney mitochondria. Mitochondria (1 mg/ml) were incubated in the presence of 20 nM D-amino acid. **A** Rat liver mitochondria; **B** rat kidney mitochondria. Where present, 40 μM $CaCl_2$ (■). Other conditions as described under Materials and methods. $n = 4 \pm$ SEM. $^{*}p < 0.05$ compared to control. $^{**}p < 0.001$ compared to its control

High concentrations of Ca^{2+} can damage cells and induce apoptosis or necrosis under oxidative stress conditions (Chávez et al., 2003). When lipoperoxidation was assayed in the presence of calcium (40 μM), TBARS production increased significantly ($p < 0.001$) up to 1.55 nmoles/mg, as compared to 0.34 nmoles/mg without calcium (Fig. 1A). Under these oxidative stress conditions, only D-asp induced significant ($p < 0.001$) TBARS production when added together with calcium, as compared with the addition of the D-amino acid alone. The other D-aa increased TBARS production without significant differences (Fig. 1A). Rat kidney mitochondria produced 0.73 nmoles/mg TBARS (Fig. 1B). These values represent a 59% increase as compared to basal values of liver mitochondria (Fig. 1A), due to a lesser activity of antioxidant systems, such as superoxide dismutase, glutathione peroxidase, and a smaller capacity of the kidney to store antioxidants as compared with the liver (Halliwell and Gutteridge, 1999). In kidney mitochondria, addition of D-aa produced no significant changes even when calcium was present in the incubation mixture (Fig. 1B).

Effect of D-amino acids on free radical production in hypertension

Several reports suggested an abnormal increment in reactive oxygen species (ROS) in hypertension in SHR rats and in humans (Pettit et al., 2002). Figure 2A shows, in liver mitochondria, basal values of TBARS production of 0.49 ± 0.06 nmoles/mg and 0.77 ± 0.10 nmoles/mg, both in WKY and SHR rats, respectively, and a production of about 3.0 nmoles/mg TBARS in the presence of D-ser, D-asp, and D-ala in WKY rats; whereas, in SHR rats, the presence of D-ser and D-ala protected significantly ($p < 0.05$) against TBARS production (22 and 21%, respectively). In liver mitochondria from SHR rats, in the presence of calcium to produce oxidative stress condition, D-ser, D-asp and D-ala increased TBARS production (46, 81 and 86%, respectively, $p < 0.05$) (Fig. 2B). In rat kidney mitochondria from both WKY and SHR rats, TBARS production was about 3.5 nmoles/mg (Fig. 2C), with the exception of D-ser, which increased TBARS production almost twice. In the presence of calcium, in kidney mitochondria from SHR rats, only D-ala increased TBARS significantly ($p < 0.05$) about 4.9 nmoles/mg (Fig. 2D). This result is similar to that found in hypertensive liver mitochondria in the presence of calcium.

Effect of D-amino acids on free radical production in chronic alcoholism

Reactive oxygen species have been involved as a possible mechanism of the toxicity promoted by chronic ethanol consumption (Adachi and Ishii, 2002). In the present study, administration of 10% ethanol to drinking water during 4 weeks was a reasonable model to study the effects of moderate chronic ethanol consumption in rats as previously described (Zentella de Piña et al., 1989). The results in Fig. 3A show that, in rat liver mitochondria, TBARS production increased to 0.89 nmoles/mg protein, as compared to 0.34 nmoles/mg protein TBARS found in untreated rats (Fig. 1A). There are no significant changes when the different D-aa were added separately, nor when the oxidative stress conditions (Fig. 3A) were achieved by adding calcium to the incubation medium. In this latter condition, the damage to liver mitochondria exerted by chronic ethanol consumption is already present. In isolated rat kidney mitochondria, under moderate chronic ethanol

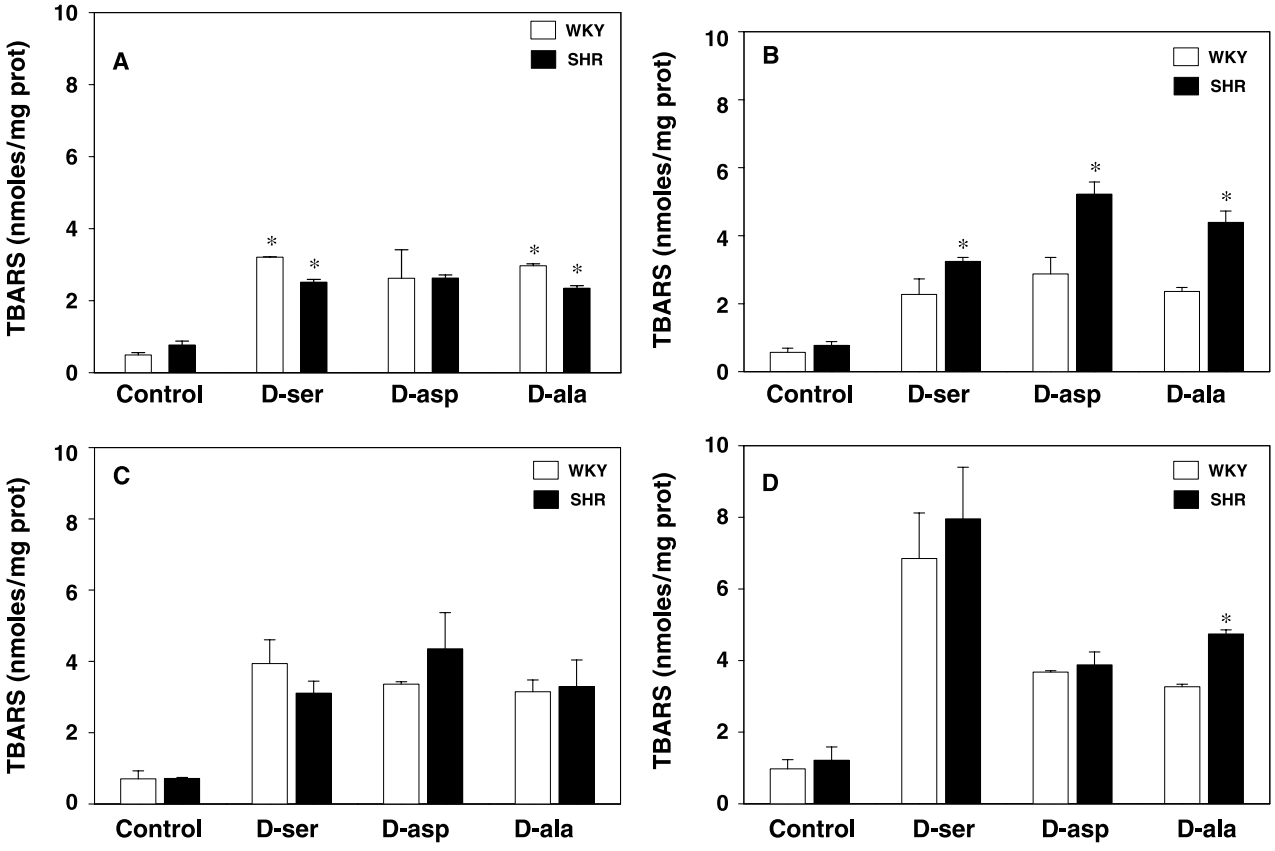

Fig. 2. Effect of D-amino acids on lipoperoxidation in hypertension. Mitochondria (1 mg/ml) were incubated in the presence of 20 nM D-amino acid. **A** WKY vs. SHR rat liver mitochondria. **B** WKY vs. SHR rat liver mitochondria, plus 40 μM $CaCl_2$. **C** WKY vs. SHR rat kidney mitochondria. **D** WKY vs. SHR rat kidney mitochondria, plus 40 μM $CaCl_2$. *SHR* Spontaneous hypertensive rats; *WKY* Wistar-Kyoto normotensive genetic rats. Other conditions as described under Materials and methods. $n = 4 \pm$ SEM. $^{*}p < 0.05$ compared to control

consumption (Fig. 3B), a significant ($p < 0.05$) increase to 1.42 nmoles/mg protein TBARS was obtained when D-ser was added, as compared to 0.79 nmoles/mg protein TBARS of control values. D-asp and D-ala only exerted a slight and non-significant increasing effect. Again, as in liver mitochondria (Fig. 3B), addition of calcium to promote an oxidative stress condition induced a slight increase in TBARS values (Fig. 3B).

Effect of D-amino acids on free radical production in ammonemic conditions

It has been reported that under acute ammonemic intoxication conditions, the activity of antioxidant enzymes diminishes by increasing superoxide formation in the brain (Kosenko et al., 2003). Based on this reference, we raised the question of how D-amino acids could affect lipoperoxidation in liver and kidney mitochondria under ammonemic conditions. Figure 4A shows, in liver mitochondria, a significant increase ($p < 0.05$) of 0.94 nmoles/mg protein TBARS, as compared to 0.34 nmoles/mg protein TBARS in controls (Fig. 1A) under normal conditions. When the D-aa was added separately, TBARS productions were: 0.93, 1.06, and 1.10 nmoles/mg protein by D-ser, D-asp, and D-ala, respectively. The presence of calcium, to induce the oxidative stress state, again diminished the toxic effects of the ammonemic conditions (Fig. 4A). Under these conditions, produced TBARS were: 0.89, 0.74, and 0.72 nmoles/mg protein in the presence of D-ser, D-asp, and D-ala, respectively (Fig. 4A). In rat kidney mitochondria (Fig. 4B), a slight increase in TBARS production to 0.95 nmoles/mg protein occurred, as compared to 0.73 nmoles/mg protein under normal conditions (Fig. 1B). D-ser and D-asp increased TBARS values to 1.03 ($p < 0.05$) and 1.02 nmoles/mg protein, respectively. Lipoperoxidation, under ammonemic conditions and by adding the D-aa separately, exhibited a protective effect when calcium ions were present in the assay mixture (Fig. 4B), i.e., D-asp and D-ala did not affect TBARS production significantly and only D-ser had a significant ($p < 0.05$)

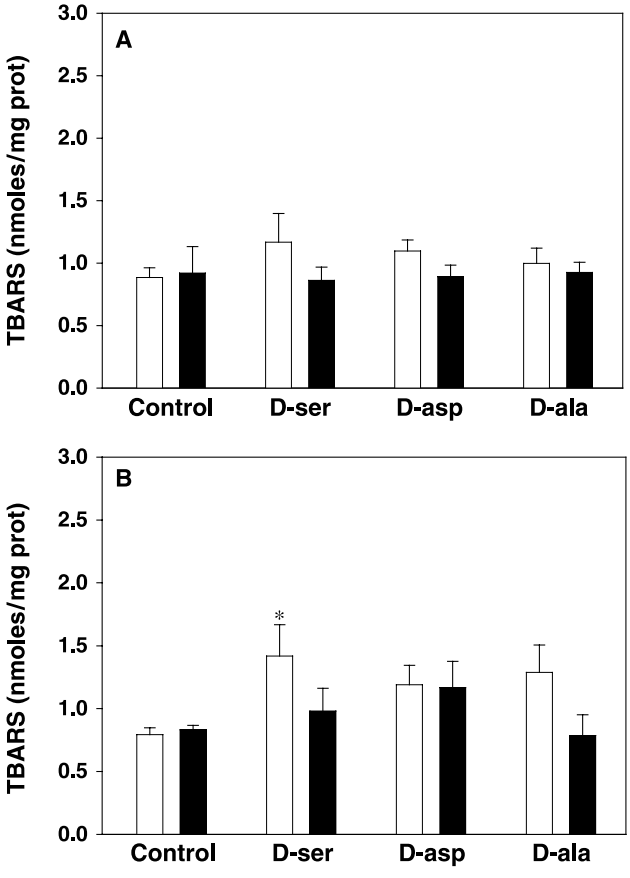

Fig. 3. Effect of D-amino acids on free radical production in chronic alcoholism. Mitochondria (1 mg/ml) were incubated in the presence of 20 nM D-amino acid. **A** Rat liver mitochondria; **B** rat kidney mitochondria. Where present, 40 μM $CaCl_2$ (■). Other conditions as described under Materials and methods. $n = 4 \pm$ SEM. $^*p < 0.05$ compared to control

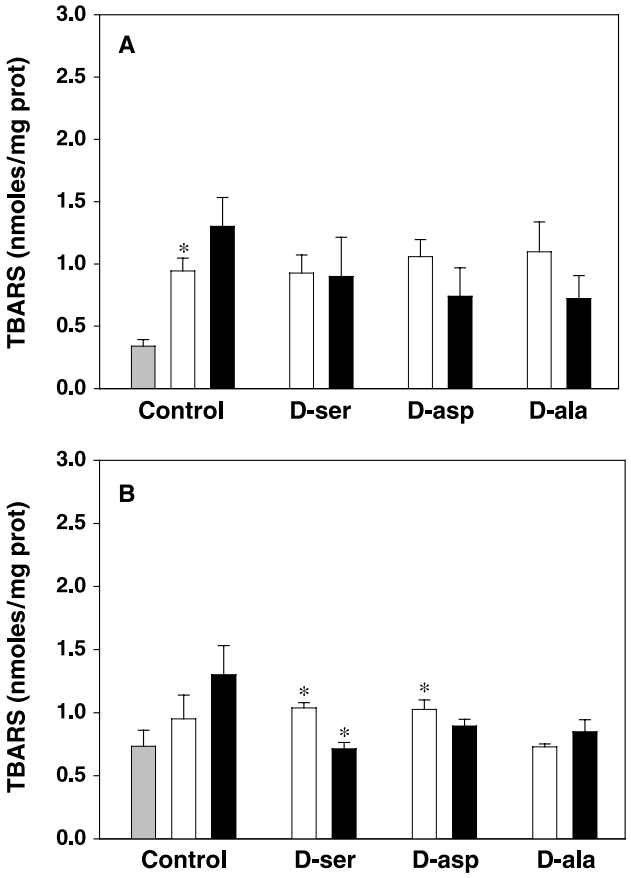

Fig. 4. Effect of D-amino acids on free radical production in ammonemia. Mitochondria (1 mg/ml) were incubated in the presence of 20 nM D-amino acid. **A** Rat liver mitochondria control (▩); in ammonemic condition (□). **B** Rat kidney mitochondria control (▩); in ammonemic condition (□). Where present, 40 μM $CaCl_2$ (■). Other conditions as described under Materials and methods. $n = 4 \pm$ SEM. $^*p < 0.05$ compared to control. **A** $p < 0.05$, compared to control without ammonemic conditions

protective effect when Ca^{2+} was present. These effects could have an important role in the mechanism of ammonia toxicity, when D-amino acids are present, even at these low concentrations.

Discussion

In previous studies from our laboratory, it has been demonstrated that D-amino acids have effects on mitochondrial metabolism (Villalobos-Molina et al., 1987; Saavedra-Molina and Piña, 1991; González-Hernández et al., 2002). Here, we considered one of the oxidation products of the D-amino acid oxidase activity, H_2O_2, which is involved in the generation of free radicals by the Haber-Weiss cycle in the presence of Fe^{2+} (Keyer and Imlay, 1996). The data shown in Fig. 1 suggest that even in the presence of nanomole concentrations of the D-aa tested, mitochondria need to maintain calcium concentrations at normal levels because of several factors, such as oxidative stress (Pérez-Vázquez et al., 2002). Oxidative stress conditions increased free radical production, as shown by others (Adachi and Ishii, 2002; González-Hernández et al., 2002), as well as in this study for the D-amino acids tested (Fig. 1). The presence of calcium increased TBARS production especially with D-asp, which yielded the highest and significant ($p < 0.001$) increase. Regarding this finding, a close relationship has been described between Ca^{2+} ions and D-asp: secretion of D-asp from isolated bovine retina is a Ca^{2+}-dependent process even under hypoxic conditions (Ohia et al., 2001). In kidney mitochondria (Fig. 1B), TBARS production was 59% less than in liver mitochondria; in addition, there are smaller amounts of antioxidant enzymatic activities in kidney than in liver, such as superoxide dismutase and glutathione peroxidase, plus a

diminished capacity to store antioxidants (Halliwell and Gutteridge, 1999). The presence of D-aa exerted a protective effect when oxidative conditions were present (Fig. 1B), which suggests a possible interaction between Ca^{2+} and D-aa metabolism in kidney mitochondria that might merit further in-depth studies.

The relation between the presence of D-aa and hypertension when analogues of substance P were synthesized replacing one or two of the amino acid residues with D-amino acids and obtaining a hypotensive response (Dutta et al., 1986) has been poorly reported in the literature. Another report relates a peptide synthesized from D-amino acids that dramatically reduces hypertension (Navab et al., 2002). An additional study suggests that an increase in ROS production is involved in the pathogenesis of hypertension (Nakazono et al., 1991; Pettit et al., 2002). Finally, during hypertension, in SHR rats, a close relationship has been described between intramitochondrial nitric oxide and matrix Ca^{2+} (Aguilera-Aguirre et al., 2002). The data obtained for TBARS production when D-aa were assayed during hypertension reveal insignificant injury (Fig. 2A) as compared with the damage produced during the disease under oxidative stress, i.e., in the presence of calcium. In our results, during hypertension, the three D-amino acids tested increased free radicals production when calcium was present, therefore it is possible that an interaction exists between hypertension conditions and the presence of D-aa.

As pointed out by Adachi and Ishii (2002), ethanol administration affects generation of mitochondrial free radicals in liver due to oxidative stress conditions. In this study, the tendency of D-ser to promote TBARS production in rats under chronic ethanol consumption was higher in isolated kidney mitochondria than in isolated liver mitochondria (Fig. 3). In fact, free radical production in isolated kidney mitochondria from rats subjected to moderate chronic alcoholic conditions increased significantly ($p<0.05$) (Fig. 3B) by producing a necrotic tissue. In this regard, Imai et al. (1998) found accumulation of [^{14}C]-D-ser associated with an acute kidney necrosis in rats induced by the addition of millimolar concentrations of H_2O_2. These oxidative stress conditions reflect the high concentration of the produced ROS.

It has been proposed that, in brain mitochondria, ammonia intoxication induces a depletion of glutathione and an increase in lipid peroxidation (Kosenko et al., 2003). In addition, ammonia can dissipate the proton gradient through the inner mitochondrial membrane, uncouple oxidative phosphorylation, and increase TBARS production (Kosenko et al., 2003). In the present study, in isolated kidney mitochondria from rats in ammonemic conditions (Fig. 4B), the presence of D-ser increased lipoperoxidation as compared with controls; but, surprisingly, when calcium was added, the oxidative stress condition decreased ($p<0.05$) in the presence of D-ser. This protective effect of the D-aa when oxidative stress conditions were present was first described on the mitochondrial transmembrane potential (González-Hernández et al., 2002). Taken together, the data of these studies show direct implications when D-amino acids are present under specific pathological conditions. The results obtained suggest a possible physiological interaction among the D-amino acids tested in the mitochondrial milieu, but many other possibilities have to be resolved and require further experimentation.

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Authors' address: Alfredo Saavedra-Molina, Instituto de Investigaciones Químico-Biológicas, Universidad Michoacana de San Nicolás de Hidalgo, Edificio B-3. C.U., Morelia, Mich. 58030, México,
Fax: 52-443-326-5788, E-mail: saavedra@zeus.umich.mx