D-002 on antioxidant enzymes in liver and brain of rats

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Recibido: 16 de mayo del 2001. Aceptado: 12 de septiembre del 2001.

Palabras clave: D-002, enzymes antioxidant, peroxidation lipid, alcoholes primarios, cera de abejas.
Key words: D-002, antioxidant enzymes, lipid peroxidation, primary alcohols, beeswax.

RESUMEN. El D002 es una mezcla de ácidos alifáticos de alto peso molecular aislada y purificada de la cera de abeja. El triacontanol es el componente mayoritario (26,63 %), seguido por el octacosanol (17,4 %), dotriacontanol (16,95 %), hexacosanol (13,4 %), tetracosanol (13,24 %) y el tetratriacontanol (2,23 %). En el presente trabajo se estudió el efecto de la administración oral del D002 durante dos semanas en ratas *Sprague Dawley* sobre las enzimas antioxidantes usando la dosis de 25 mg/kg del producto. el efecto de la administración oral de esta doisis sobre las enzimas antioxidantes en hígado y cerebro. Se observo en los niveles de superoxido dismutasa (SOD) que se elevaron en ambos tejidos. Sin embargo el efecto más marcado se observó en la SOD mitocondrial, alcanzando diferencias significativas al compararse con el grupo control. Ambas dosis indujeron un aumento significativo en la actividad de la glutation peroxidasa (GSH-PX). No se observó efecto del producto sobre la actividad de la glutation reductasa (GSSG-R) ni de la catalasa hépatica. Los resultados sugieren que el D002 puede prevenir la peroxidación lípida en hígado y cerebro mediante un incremento de la actividad de las enzimas Mn-SOD y GSH-peroxidasa. Sin embargo, el papel que desempeña el D002 en la modificación de las enzimas involucradas en el metabolismo de los radicales libres es aun limitado por lo que se requieren investigationes futuras para esclarecer el mecanismo de la acción antioxidante del D002

ABSTRACT. Previous studies have demonstrated that oral administration of D-002 (5 to 100 mg/kg), a mixture of high molecular weight aliphatic alcohols isolated and purified from beeswax, inhibited in a dose dependent manner liver and brain *in vivo* and *in vitro* lipid peroxidation in rats. Triacontanol is its main component (26.63 %), followed by octacosanol (17.4 %), dotriacontanol (16.95 %), hexacosanol (13.4 %), tetracosanol (13.24 %) and tetratriacontanol (2.23 %). In the present work, the effect of D-002 on the antioxidant enzymes in liver and brain was studied. Superoxide dismutase (SOD) was elevated in the soluble fraction from both tissues after D-002 administration. However, D-002 produced a more remarkable effect on mitochondrial (SOD) activity, reaching significant differences when compared with control group. Also, a significant increase in glutathione peroxidase (GPX) was observed in both tissues. However, D-002 had no effect on glutathione reductase (GR) from liver and brain and on liver catalase (CA) activity. The results suggest that D002 can prevent lipid peroxidation in liver and brain by increasing the activity of Mn-SOD and GSH-peroxidase. However, information of the role of D002 in modifying the enzymes involved in free radical metabolism is still limited and further investigations are required to define the mechanism of action of the antiperoxidative effects of D002.

INTRODUCTION

D002 is a well-defined mixture of higher primary alcohols isolated and purified from bee wax. Triacontanol is its main component (26.63 %), followed by octacosanol (17.4 %), dotriacontanol (16.95 %), hexacosanol (13.4 %), tetracosanol (13.24 %) and tetratriacontanol (2.23 %). Previous studies have demonstrated that D002 orally administered with a dose of 5 and 100 mg/kg body weight inhibited dose-dependent carbon tetrachloride-and toluene *in vivo* of lipid peroxidation in liver and brain ,also inhibited enzymatic and non-enzymatic *in vitro* lipid peroxidation in liver and brain microsomes, both cases the maximal protection occurred at a dose of 25 mg/kg . When D002 was orally administered to rats (5, 25 and 100 mg/kg) for two weeks, a partial inhibition of the *in vitro* enzimatic and non-enzimatic liver and brain lipid peroxidation was observed. D002 decreased basal level of MDA in both tissues and behaved differently depending on both, the presence of NADPH and the integrity of liver microsomes, which suggest that under conditions where microsomal metabolism was favored the protective effect of D002 was increased.[1] Therefore, an effect on the antioxidant enzymes should not be ruled out.

These studies have demonstrated the antioxidant effect of D002 after oral administration. To provide an improved understanding of the mode of action of D002 on liver and brain lipid peroxidation, it was

examined the effect of oral administration of D002 (25 mg/kg) on liver and brain antiperoxidative enzymes SOD, GSH-PX, GSSG-R and catalase (CA).

MATERIAL AND METHODS

Wistar rats were purchased from the Centro Nacional para la Producción de Animales de Laboratorio (Havana, Cuba).

Animals and treatment

Male Wistar rats (180-200 g) were used throughout the study. D002 was suspended in a 2 % Tween-20-water vehicle and administered orally (25 mg/kg) for two weeks by gastric gavage (1 mL/200 g). A control group received an equivalent volume of the vehicle.

Enzyme assays

SOD activity was assayed according to Beauchamp and Fridovich.[2] Fresh livers and brains from control and treated rats (25 mg/kg) were homogenized in a four volumes of ice-cold 50 mmol/L potassium phosphate buffer (pH 7.8) by a motor-driven Teflon-pestle homogenizer. Homogenates were sonicated in 1 min bursts for a total of 5 min at maximum output of the Vibracell™ 72434 (Bioblock Scientific). For mitochondria SOD determinations, homogenates were centrifuged in phosphate buffer, frozen and thawed once and sonicated again by 4 x 15 s on ice. Supernatant containing cytosol obtained after centrifugation at 105 000g was used for the assay of cytosolic SOD activity. The assay mixture contained 0.5 mol/L carbonate buffer (pH 10.2), 0.1 mmol/L EDTA, 0.25 mmol/L nitroblue tetrazolium (NBT), 0.1 mol/L xantine, XO ($7.6 \cdot 10^{-3}$ units) and sample at a final volume of 3 mL . The activity of SOD is expressed in units of SOD/mg. The activity of SOD, which inhibited the reduction of NBT for 50 % was defined as 1 unit.

GSSG-R was assayed according to Carlberg and Mannervik.[3] Fresh livers and brains from control and treated rats were homogenized in 10 volumes of ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 1 mmol/L EDTA. Homogenates were centrifuged at 105 000 x g and supernatant was used for the assay. The assay mixture contained 0.1 mol/L phosphate buffer (pH 7.0), 0.5 mol/L EDTA, 2 mmol/L GSSG, 0.1 mmol/L NADPH and sample in a final volume of 1 mL . The amount of GR was measured by following the decrease of the absorbance at 340 nm due to oxidation of NADPH. Activity was expressed as mmol NADPH $\cdot$ min^{-1} $\cdot$ mg^{-1} protein.

GSH-peroxidase (EC 1.11.1.0) was assayed as described Plaglia and Valentine.[4] Tissue homogenates (1:10 w/v) from control and D002 treated rats were prepared in ice-cold 0.25 mol/L sucrose and differential centrifugation was performed to obtain cytosolic fraction. The assay mixture contained 50 mmol/L phosphate buffer (pH 7.0), 1 mmol/L EDTA, 1 mmol/L sodium azide, 1 mmol/L GSH, 0.2 mmol/L NADPH, 1 unit of GSSG-R, 1.5 mmol/L cumene hydroperoxide previously prepared in phosphate buffer, in a final volume of 1 mL . The decrease of NADPH was determined at a wavelength of 340 nm for 5 min and the activity was calculated as mmol NADPH oxidized $\cdot$ min^{-1} $\cdot$ mg^{-1}.

CA (EC 1.11.1.6) was assayed according to Sazuka.[5] Liver cytosolic fraction (10 mL) were added to 3 mL H_2O_2 (0.45 mmol/L) previously prepared in 0.1 mol/L phosphate buffer pH 7. Exactly after 30 s and 60 s 500 mL of the mixture were added to 2 mL of bencidine-horseradish peroxidase-sodium azide (20 mg, 8.1 mg and 1.25 mg respectively/100 mL of phosphate buffer (0.1 mol/L, pH 7). Then, after 10 min, 2.5 mL of concentrated H_2SO_4 were added. Afterward, the absorbance (530 nm) was measured. The enzyme activity (k') was calculated by the following equation:

$$k' = \frac{2.303}{t\left(\log\frac{a}{a-x}\right)}$$

where, (a) is the initial concentration of H_2O_2 and (a – x) is the remnant concentration of H_2O_2 after the incubation during 10 min . The activity was expressed in k'/mg of protein. Protein was determined by a modification of the Lowry procedure. [6]

Statistical analysis

Data are reported as means ± SD. Statistical analysis was performed by the Mann Whitney U test. $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Tables 1 and 2 show the effect of D002 (25 mg/kg) on the antioxidant enzymes SOD, GSH-PX, GSSG-R and CA on liver and brain. SOD was elevated in both soluble and particulated fraction from both tissues. However, D002 produced a more remarkable effect on mitochondrial SOD activity, reaching significant differences when compared with control group. The 25 mg/kg dose produces higher increase in enzyme activity. A significant increase in GSH-peroxidase activity was observed (25 mg/kg) dose of D002. As occurred for SOD activity, the 25 mg/kg dose produced a higher increase of enzyme activity. D002 had no effect on the GSSG-R from liver and brain and on liver CA activity.

The present results suggest that the inhibition of lipid peroxidation

Table 1. Effect of oral administration of D-002 (25 mg/kg) on antioxidant enzymes in brain.

Enzymes	Control	Treatment
Superoxide dismutase (mitochondria)	0.58 ± 0.03	0.72 ± 0.18*
Superoxide dismutase (cytosolic)	2.56 ± 0.11	2.77 ± 0.36
Glutathione peroxidase	2.87 ± 0.67	4.11 ± 0.85*
Glutathione reductase	0.30 ± 0.08	0.34 ± 0.06

*$p < 0.05$ compared to control (test - U Mann Whitney) (n = 30).

Table 2. Effect of oral administration of D-002 (25 mg/kg) on antioxidant enzymes in liver.

Enzymes	Control	Treatment
Superoxide dismutase (mitochondria)	0.77 ± 0.06	0.95 ± 0.10*
Superoxide dismutase (cytosolic)	10.76 ± 1.44	13.07 ± 1.73
Glutathione peroxidase	34.83 ± 6.27	61.19 ± 6.89*
Glutathione reductase	38.67 ± 9.37	34.80 ± 9.57
Catalase	1.64 ± 0.38	1.66 ± 0.35

*$p < 0.05$ compared to control (test - U Mann Whitney) (n = 30).

by oral administration of D002 would be a consequence at least in part, of its action on antioxidant enzymes. The most important source of O_2^{-}* *in vitro* is the electron transport chain of the mitochondria. Significant elevation of mitochondrial SOD activity by D002 could indicate that D002 can increase the dismutation of superoxide radical once generated from O_2 and hence the reduction of much more deleterious oxygen species and the oxidative damage likely to result from its action.[7] Even when enhanced dismutation of superoxide radical results in the generation of H_2O_2, it is known that GSH-peroxidase can detoxify H_2O_2.[8] Therefore, elevated levels of GSH-peroxidase by D002 can, thus, detoxify enhanced levels of H_2O_2. It should also be considered that GSH-peroxidase can inhibited lipid peroxidation by directly catalyzing the conversion of peroxidated lipid into alcohols. It is known that Se-dependent GSH-peroxidase catalyses the breakdown of inorganic peroxides[8] whereas Se-independent peroxidases catalyzes the breakdown of organic peroxides.[9] Since, GSH-peroxidase activity was measured using cumene hydroperoxide, which determines total peroxidase activity, the significant stimulation of GSH-peroxidase activity by the oral administration of D002 could contribute to the detoxification not only of H_2O_2, but also lipid peroxides produced as a result of oxidative stress. Further, it is also known that GSH-peroxidase can scavenge free radicals which can contribute to the reduction of lipid peroxidation.

CONCLUSION

The results suggest that D002 can prevent lipid peroxidation in liver and brain by increasing the activity of Mn-SOD and GSH-peroxidase. However, information of the role of D002 in modifying the enzymes involved in free radical metabolism is still limited and further investigations are required to define the mechanism of action of the antiperoxidative effects of D002

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ACKNOWLEDGEMENTS

The author thanks Professor J. Núñez Romay for his revision of this paper.

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