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Increased Unsaturated Fatty Acid Levels in Liver and Muscle of Guinea Pig Induced by Vitamin-E, ALA and Linalool

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Abstract: This study aimed to determine the protective effects of intraperitoneally administered vitamin E, lipoic acid and linalool on the levels of fatty acid in guinea pig liver and muscle under oxidative stress induced by hydrogen peroxide. While the level of total unsaturated fatty acid in the vitamin E, lipoic acid and linalool groups significantly increased ($p < 0.01$) as compared to the H_2O_2 group but its level slightly increased ($p < 0.05$) as compared with the control, H_2O_2 + vitamin E, H_2O_2 + lipoic acid and H_2O_2 + linalool groups in liver. The level of polyunsaturated fatty acid (PUFA) in H_2O_2 + vitamin E, H_2O_2 + lipoic acid and H_2O_2 + linalool groups increased in comparison with H_2O_2, decreased in comparison with vitamin E and lipoic acid groups in both tissues. In muscle, the level of unsaturated fatty acid in the H_2O_2 + vitamin E, H_2O_2 + lipoic acid and H_2O_2 + linalool groups increased in comparison with H_2O_2 group, but its level in the same groups decreased in comparison with vitamin E, lipoic acid and linalool groups. Vitamin E, lipoic acid and linalool could be used in therapeutic approaches, aimed at limiting the damages from oxidation reactions in unsaturated fatty acids.

Keywords: Vitamin E, lipoic acid, linalool, guinea pig, liver

Introduction

Reactive oxygen species (ROS) are known to play multiple roles in physiological and pathological states and are constantly produced in living organisms (Darr and Fridovich, 1994). The highly reactive superoxide radical and hydrogen peroxide may be toxic to cells by direct attack at the molecular level or indirectly by generating secondary reactive species such as the hydroxyl radical (Brenneisen *et al*., 1997). These radicals may cause oxidative damage virtually to any biomolecule (Kowaltowski and Vercesi, 1999). Lipids, especially polyunsaturated fatty acids, are preferential targets for such oxidative damage (Douillet *et al*., 1993). If unsaturated fatty acids react with ROS in living cells, the resulting defects in membrane function may cause cell death (Gurr and Harwood, 1991). Despite the destructive potential of ROS, cells have developed defence mechanisms to prevent or limit oxidative injury. These mechanisms include several enzyme systems and antioxidants such as vitamin E and carotenoid, which prevent lipid peroxidation (Sobajic *et al*., 1998).

Vitamin E is present in the lipid bilayers of biological membranes and prevents oxidation of various compounds including unsaturated fatty acid (McCay, 1985). α-Lipoic acid is a naturally occurring free radical scavenger that has been shown to regenerate endogenous antioxidants, such as vitamin E and increase glutathione formation (Bienwenga, 1997). Recent reports indicate that lipoate exerts its therapeutic effect in pathological conditions involving free radicals (Kozlov *et al*., 1999; Rudich *et al*, 1999).

Linalool is a monoterpene compound reported to be a major component of essential oils in various aromatic species. Several linalool producing species are used in traditional medicines. Among these is *Ueolanthus suaveolens G. Dom (Labiatae)*, which is used as an anticonvulsant in the Brazilian Amazon. Psychopharmacological evaluations of these compounds *in vivo* showed that they have marked dose-dependent sedative effects on the central nervous system, including hypnotic, anticonvulsant and hypothermic properties (Re *et al*., 2000). However, protective effect of linalool against the decrease of unsaturated fatty acid are not reported in the literature.

The objective of our work was to study the protective effects of intraperitoneally administrated vitamin E, lipoic acid and linalool on the levels of fatty acids in liver and muscle of guinea pig with oxidative stress induced by hydrogen peroxide.

Materials and Methods

Animals: Ninty seven guinea pigs were used during the experiment. At the start of the experiment, the guinea pigs weighed 460-640 g and were aged 11 months. The animals were divided into eight groups and kept at 20°C. They were fed *ad libitum* diet including the ingredients shown in Table 1 during the experiment.

Table 1: Diet composition

Ingredients	%
Wheat	10.0
Corn	22.0
Barley	15.0
Wheat bran	8.0
Soybean	26.0
Fish flour	8.0
Meat-bone flour	4.0
Pelted	5.0
Salt	0.8
Vitamin mineral mix[a]	0.2

[a]Vit. A, B_3, E, K_3, B_1, B_2, B_6, B_{12}, nicotinamid, folic acid, biotin, Mn, Fe, Zn, Cu, I, Co, Se, antioxidant and Ca.

The first group was the control (9 animals), the second was intraperitoneally administered hydrogen peroxide (16 animals, 24 mg kg^{-1}), the third group received vitamin E (12 animals, dl-α-tocopheryl acetate, 24 mg kg^{-1}), the fourth group hydrogen peroxide plus vitamin E (12 animals, 12 mg kg^{-1} H_2O_2 + 24 mg kg^{-1} vitamin E), the fifth group dl-α-lipoic acid (12 animals, 3 mg kg^{-1}), the sixth group H_2O_2 plus dl-α-lipoic acid (12 animals, 12 mg kg^{-1} H_2O_2 + 3 mg kg^{-1} lipoic acid), the seventh group linalool (12 animals, 120 mg kg^{-1}) and the eighth group received H_2O_2 plus linalool (12 animals, 12 mg kg^{-1} H_2O_2 + 120 mg kg^{-1} linalool) daily. This intraperitoneal administration was performed every day for 6 weeks.

Samples were prepared from animals at the end of the treatment period, after overnight fasting. Each experimental guinea pig was anaesthetised with ether and tissue samples were collected. These samples were kept at -25°C until lipid extraction and further analyses were performed.

Lipid extraction: Total lipids were extracted with chloroform-methanol (2:1, v/v) using the method of Folch *et al*. (1957). The tissue samples were homogenized and 3 g of them was mixed with chloroform-methanol (2:1, v/v). Non-lipid contaminants in the lipid extracts were removed with a 0.88 % KCl solution. The extracts were evaporated in a rotary evaporator and stored at -25°C.

Determination of total lipid: Total lipid was determined by the method of Frings *et al*. (1972). Twenty μl of each solution of

extracted lipids was treated with 200 μl of concentrated H_2SO_4 and boiled for 10 min. After cooling, 10 ml phosphovanilin reagent was added. The mixture was incubated at 37°C for 15 min and the absorbance of the samples was then read at 540 nm (CECIL 5000 series spectrophotometer). A high quality grade of olive oil (Sigma, St. Louis, MO, USA) was used as a standard. The total lipid was calculated by reference to the standard curve.

Fatty acid analysis: Fatty acids in the lipid extracts were converted into methyl esters by means of 2% H_2SO_4 (v/v) in methanol (Christie, 1990). The fatty acid methyl esters were extracted three times with n-hexane, then separated and quantified using a gas chromatograph equipped with a flame-ionization detector (Unicam 610 gas chromatograph) attached to a Unicam 4815 computing recorder. Chromatography was performed with a capillary column (25 m in length and 0.22 mm in diameter, B 10x70), using hydrogen as the carrier gas (flow rate 0.5 ml min^{-1}). The temperature of the column, detector and injection port was 185, 280 and 240 °C, respectively. Identification of the individual methyl esters was performed by frequent comparison with authentic standard mixtures analysed under the same conditions.

Statistical analysis: Data were reported as means ± SE. Statistical analysis was performed using SPSS 6.0 Software. Analysis of variance (ANOVA) and LSD test were used for comparison between groups.

Results

At the end of the 6th week, the mean body mass of each experimental group did not significantly differ. Mortality in control, vitamin E and lipoic acid groups after 6 weeks was nil but reached 25% in H_2O_2 and H_2O_2+linalool groups and 17% in linalool, H_2O_2+vitamin E and H_2O_2+lipoic acid groups.

The effect of vitamin E, lipoic acid and linalool on the levels of fatty acids in liver due to oxidative stress-induced by H_2O_2 are reported so (Table 2). While the level of stearic (18:0) acids in the H_2O_2 and H_2O_2+lipoic acid groups increased significantly ($p < 0.01$) compared with the control, vitamin E, lipoic acid and linalool groups, they increased slightly ($p < 0.05$) in comparison with the H_2O_2+vitamin E and H_2O_2+linalool groups. The levels of total saturated fatty acids did not differ between the control, H_2O_2+vitamin E, lipoic acid, H_2O_2+lipoic acid and H_2O_2+linalool groups, but were highest in the H_2O_2 group.

The total saturated fatty acid levels were reduced ($p < 0.05$) in the vitamin E and linalool groups compared to the H_2O_2+vitamin E and H_2O_2+linalool groups. The proportion of linoleic acid (18:2) in the control, vitamin E, H_2O_2+vitamin E, lipoic acid, H_2O_2+lipoic acid and linalool groups increased slightly ($p < 0.05$) in comparison with H_2O_2+linalool group, which increased significantly ($p < 0.01$) in the same groups in comparison with the H_2O_2 group. In addition, the level of arachidonic acid (20:4) in the vitamin E, lipoic acid, H_2O_2+vitamin E and linalool groups increased slightly ($p < 0.05$) in comparison with the lipoic acid, H_2O_2+lipoic acid and H_2O_2+linalool groups, but increased significantly ($p < 0.01$) in comparison with the control and H_2O_2 groups.

Docosahexaenoic acid (22:6) in the control and H_2O_2 groups decreased in comparison with other groups. Total PUFA and unsaturated fatty acid levels in the H_2O_2 group were minimal. On the other hand, total unsaturated fatty acid levels did not differ significantly between the control, H_2O_2+vitamin E, H_2O_2+lipoic acid and H_2O_2+linalool groups, but levels in the vitamin E, lipoic acid and linalool groups increased slightly ($p < 0.05$) in comparison with these same groups. Total PUFA levels did not differ between the control, H_2O_2+vitamin E, lipoic acid, H_2O_2+lipoic acid, linalool and H_2O_2+linalool groups. Levels in the H_2O_2+vitamin E and H_2O_2+linalool groups were reduced in comparison with the vitamin E and linalool groups. The effects of vitamin E, lipoic acid and linalool on the level of fatty acid in muscle tissue with oxidative stress-induced H_2O_2 are reported (Table 3). Myristic acid (14:0) was highest in the H_2O_2 group. Its level did not differ between the control, vitamin E, lipoic acid and H_2O_2+lipoic acid groups. While 16:0 levels in the control, H_2O_2+lipoic acid and H_2O_2+linalool groups increased in comparison with the vitamin E, H_2O_2+vitamin E and lipoic acid groups, they decreased slightly ($p < 0.05$) in comparison with the H_2O_2 group. Levels of 18:0 did not differ between the control, H_2O_2, H_2O_2+vitamin E and linalool groups, but increased in the lipoic acid, H_2O_2+ lipoic acid and H_2O_2+linalool groups.

The proportion of 18:2 in the lipoic acid and control groups increased in comparison with the H_2O_2, H_2O_2+lipoic acid and H_2O_2+linalool groups, but decreased in comparison with the vitamin E, H_2O_2+vitamin E and linalool groups. The level of 20:4 was lowest in the H_2O_2 group. However, its level in the H_2O_2+vitamin E and H_2O_2+linalool groups were reduced as compared in the vitamin E and linalool groups. The level of 22:6 was also lowest in the H_2O_2 group. While the level of 22:6 in the vitamin E and linalool groups increased significantly ($p < 0.01$) in comparison with the H_2O_2+vitamin E, control and H_2O_2+linalool groups. Total saturated fatty acid levels were highest in the H_2O_2 groups, but minimum in the vitamin E and linalool groups.

The level of unsaturated fatty acid was minimum in the H_2O_2 group. Its level in the vitamin E and linalool groups increased slightly ($p < 0.05$) in comparison with the H_2O_2+vitamin E and H_2O_2+linalool groups, but increased significantly ($p < 0.01$) in comparison with the control and H_2O_2+lipoic acid groups. The level of PUFA did not differ between the control, H_2O_2+vitamin E, lipoic acid, linalool and H_2O_2+linalool groups. However, its level in the H_2O_2+lipoic acid and H_2O_2+vitamin E groups was reduced in comparison with the vitamin E and lipoic acid groups.

Discussion

We have previously shown that the levels of saturated fatty acids slightly decrease, but unsaturated fatty acids slightly increase in rat and lamb tissues after dietary and intraperitoneally-administered vitamin E, without any other treatment (Yilmaz *et al.*, 1997a; Yilmaz *et al.*, 1997b; Çelik *et al.*, 1999; Dilsiz *et al.*, 1997). This effect of vitamin E on fatty acids becomes clearer during hydrogen peroxide-induced oxidative stress in this study. α- tocopherol is an antioxidant that prevents biological membranes from undergoing oxidative damages, due to its ability to quench lipid peroxides, thereby protecting cellular structures from attack by free radicals (Traber and Sies, 1996). Both isolated polyunsaturated fatty acids and those incorporated into lipid are readily attacked by free radicals, becoming oxidized into lipid peroxides. In contrast, both monounsaturated and saturated fatty acids are more resistant to free radical attack; indeed, it has been suggested that increased consumption of the later instead of PUFA will render circulating lipoproteins less sensitive to peroxidation (Reaven, 1991).

Hydrogen peroxide may be toxic to cells by direct attack at the molecular level, or indirectly by generating secondary reactive species such as hydroxyl radicals (Brenneisen *et al.*, 1997). The significant reduction in PUFA levels in the hydrogen peroxide treated groups observed in this study agrees with previous findings. Chow *et al.* (1999) reported that vitamin E can directly regulate hydrogen peroxide production in mitochondria and suggested that the over-production of mitochondrial ROS is the initial event leading to the tissue damage observed in Vitamin E deficiency syndromes. Hydrogen peroxid may accumulate, leading to a condition of mitochondrial oxidative stress (Kowaltowski *et al.*, 1999). In our study, vitamin E supplementation restored fatty acid distribution close to that of the control group.

These results clearly demonstrate that unsaturated fatty acid peroxidation occurs *in vivo* in H_2O_2-induced oxidative stress in the absence of lipoic acid supplementation. At the same time, intraperitoneal lipoic acid supplementation restores fatty acid distribution in H_2O_2+lipoic acid groups close to that of the control group. Magen *et al.* (1997) reported that feeding rats with the lipoic acid reduced malondialdehyde levels in tissues, which is an indicator of lipid peroxidation. This is a free radical-induced process

Table 2: The effects of vitamin E, lipoic acid and linalool on the level of fatty acids in the liver tissues with oxidative stress induced by H_2O_2 (%)

Fatty acids	Control	H_2O_2	Vitamin E	H_2O_2+VitE	LipoicAcid	H_2O_2+Lip.	Linalool	Lin + H_2O_2
14: 0	2.71 ± 0.64^{d}	1.35 ± 0.13^{b}	1.70 ± 0.30^{c}	1.41 ± 0.21^{b}	1.65 ± 0.58^{c}	0.68 ± 0.11^{a}	1.77 ± 0.16^{c}	1.61 ± 0.13^{c}
16: 0	32.89 ± 3.11^{b}	33.20 ± 2.39^{b}	27.21 ± 2.46^{a}	27.00 ± 1.27^{a}	32.52 ± 3.62^{b}	27.87 ± 2.33^{a}	28.95 ± 1.52^{a}	27.74 ± 2.22^{a}
18: 0	11.94 ± 1.90^{a}	21.57 ± 1.30^{d}	12.26 ± 0.93^{a}	18.10 ± 1.26^{c}	12.87 ± 1.91^{a}	20.19 ± 1.18^{d}	10.91 ± 1.81^{a}	17.71 ± 1.79^{c}
18:1	11.36 ± 0.48^{a}	11.40 ± 0.44^{a}	11.00 ± 0.63^{a}	11.85 ± 1.64^{a}	11.50 ± 2.78^{a}	11.50 ± 3.83^{a}	11.87 ± 3.27^{a}	12.57 ± 2.38^{a}
18: 2	29.94 ± 2.62^{c}	22.97 ± 2.50^{a}	33.26 ± 2.48^{c}	29.11 ± 1.94^{c}	29.42 ± 2.42^{c}	29.00 ± 2.09^{c}	32.69 ± 2.46^{c}	27.34 ± 2.06^{b}
18: 3	3.15 ± 0.64^{d}	1.04 ± 0.63^{a}	3.12 ± 0.22^{d}	1.09 ± 0.36^{a}	1.41 ± 0.23^{b}	0.86 ± 0.16^{a}	2.79 ± 0.32^{c}	2.35 ± 0.17^{c}
20: 4	3.29 ± 0.18^{a}	3.28 ± 0.67^{a}	5.20 ± 0.30^{c}	5.63 ± 0.82^{c}	5.27 ± 0.43^{c}	4.29 ± 0.14^{b}	5.41 ± 0.32^{c}	4.41 ± 0.52^{b}
22: 6	3.68 ± 0.18^{a}	3.77 ± 0.24^{a}	6.14 ± 0.29^{c}	5.80 ± 0.33^{c}	4.92 ± 0.92^{b}	5.61 ± 0.11^{b}	5.95 ± 0.70^{c}	6.24 ± 0.66^{c}
∑Saturated	47.54 ± 3.55^{b}	56.12 ± 2.64^{c}	41.17 ± 3.01^{a}	46.51 ± 1.97^{b}	47.03 ± 3.91^{b}	48.74 ± 2.27^{b}	41.61 ± 2.27^{a}	48.23 ± 2.89^{b}
∑Unsat.	51.42 ± 3.55^{b}	42.46 ± 2.64^{a}	58.72 ± 3.24^{c}	53.49 ± 1.62^{b}	62.97 ± 3.97^{c}	51.26 ± 3.72^{b}	58.71 ± 4.16^{c}	52.91 ± 3.95^{b}
∑PUFA	40.06 ± 2.02^{c}	31.06 ± 2.89^{a}	47.72 ± 3.01^{d}	41.64 ± 1.41^{c}	41.02 ± 3.65^{c}	39.76 ± 4.14^{c}	46.84 ± 4.16^{d}	40.35 ± 3.09^{c}

Table 3: The effects of vitamin E, lipoic acid and linalool on the level of fatty acids in the muscle tissues with oxidative tress induced by H_2O_2 (%)

Fatty acids	Control	H_2O_2	Vitamin E	H_2O_2+VitE	LipoicAcid	H_2O_2+Lip.	Linalool	Lin + H_2O_2
14: 0	2.51 ± 0.38^{b}	4.01 ± 0.25^{d}	2.12 ± 0.12^{b}	3.79 ± 0.17^{d}	1.73 ± 0.09^{b}	2.04 ± 0.11^{b}	1.22 ± 0.13^{a}	0.85 ± 0.06^{a}
16: 0	34.46 ± 2.90^{c}	41.62 ± 2.63^{d}	28.46 ± 1.80^{a}	30.94 ± 2.09^{a}	32.39 ± 1.87^{b}	34.04 ± 1.98^{c}	30.28 ± 2.16^{a}	32.98 ± 2.18^{b}
18: 0	8.44 ± 0.73^{b}	8.17 ± 0.92^{b}	7.00 ± 0.36^{a}	8.48 ± 0.28^{b}	10.92 ± 0.19^{c}	10.40 ± 0.35^{c}	8.75 ± 1.08^{b}	10.78 ± 1.01^{c}
18:1	14.67 ± 1.44^{a}	18.90 ± 1.60^{c}	15.06 ± 0.68^{a}	14.75 ± 0.98^{a}	17.17 ± 0.70^{b}	18.75 ± 1.13^{c}	15.88 ± 1.17^{a}	17.59 ± 0.78^{b}
18: 2	26.73 ± 2.00^{c}	17.64 ± 1.82^{a}	28.21 ± 2.27^{d}	27.52 ± 2.11^{d}	24.69 ± 1.28^{c}	21.88 ± 2.05^{b}	26.02 ± 1.49^{c}	22.45 ± 1.15^{b}
18: 3	3.00 ± 0.25^{d}	3.17 ± 0.16^{d}	3.12 ± 0.09^{d}	3.07 ± 0.21^{d}	0.96 ± 0.10^{b}	2.20 ± 0.16^{c}	1.58 ± 0.05^{b}	2.52 ± 0.07^{c}
20: 4	3.92 ± 0.26^{b}	2.00 ± 0.12^{a}	6.01 ± 0.31^{d}	4.66 ± 0.11^{c}	5.41 ± 0.21^{c}	5.51 ± 0.06^{c}	6.30 ± 0.21^{d}	5.74 ± 0.18^{c}
22: 6	6.27 ± 0.53^{b}	4.50 ± 0.18^{a}	10.12 ± 0.28^{d}	6.79 ± 0.48^{b}	7.19 ± 0.18^{b}	5.08 ± 0.21^{a}	9.97 ± 0.10^{d}	6.95 ± 0.45^{b}
∑Saturated	45.41 ± 3.05^{c}	53.80 ± 2.83^{d}	37.48 ± 2.76^{a}	43.21 ± 3.12^{b}	45.04 ± 2.38^{c}	46.48 ± 3.05^{c}	40.25 ± 2.27^{a}	42.31 ± 2.76^{b}
∑Unsat.	54.59 ± 2.63^{b}	46.20 ± 2.21^{a}	62.52 ± 3.14^{d}	56.79 ± 2.01^{c}	55.00 ± 2.68^{c}	53.52 ± 3.27^{b}	59.75 ± 3.06^{d}	57.65 ± 3.70^{c}
∑PUFA	39.92 ± 1.59^{c}	27.31 ± 1.98^{a}	47.46 ± 4.12^{d}	42.04 ± 2.68^{c}	37.79 ± 3.10^{c}	34.67 ± 2.68^{b}	43.87 ± 3.09^{c}	40.06 ± 2.19^{c}

$^{a}p > 0.05$, $^{b}p < 0.05$, $^{c}p < 0.01$, $^{d}p < 0.001$, Lin. = linalool, Lip. = lipoic acid, Unsat. = Unsaturated, PUFA = polyunsaturated fatty acid

leading to oxidative deterioration of polyunsaturated lipids. Under normal physiological conditions, low concentrations of lipid peroxides are found in tissues. Free radicals react with lipids and cause peroxidative changes that result in enhanced lipid peroxidation (Girotti, 1985; Pryor, 1973).

Thiols are thought to play a pivotal role in protecting cells against lipid peroxidation (Haenen, 1989). Lipoic acid effectively reduces the amount of hydroxyl radicals generated by the Fenton-type reaction and also scavenges peroxide and superoxide radicals (Sumathi *et al.*, 1993). Our present observations show that lipoic acid administration eventually results in a decrease in 14:0, 16:0 and total saturated fatty acid and an increase in 18:2, 18:3, 20:4, 22:6, total unsaturated fatty acid and PUFA levels, thus substantiating the antioxidant properties of lipoic acid.

The results clearly demonstrate that unsaturated fatty acid peroxidation occurs in the tissues in H_2O_2-induced oxidative stress in the absence of linalool supplementation. The later inhibits the decrease in unsaturated fatty acids and restores fatty acid distribution close to that of the control group.

Although the dose of vitamin E used was approximately 8 times greater than that of lipoic acid, the present study illustrates that lipoate administration may be more effective than vitamin E in preventing lipid peroxidation in tissues. This may be attributed to the bioactivity of lipoic acid directly reacting with various reactive oxygen species, as well as its ability to interfere with oxidation processes in lipid and the aqueous cellular compartment (Packer *et al.*, 1995; Kagan *et al.*, 1992). Lipoic acid is a smaller molecule than vitamin E and is soluble in both water and fat. This is significant because water-soluble antioxidant nutrients (e.g. vitamin C) are found in the cell and fat-soluble antioxidants (e.g. vitamin E) are found on the cell membrane.

Because α-lipoic acid works both inside the cell and at the membrane level, it gives dual protection. Our results indicate that linalool gives considerable protection against hydrogen peroxide induced oxidative stress in tissues. Linalool is a monoterpene compound reported to be a major component of essential oils in various aromatic species. Since linalool, which has a pleasant smell, is an effective antioxidant, it can be used as an additive in foodstuffs to protect against oxidative damage.

In conclusion, these observations suggest that lipoic acid and vitamin E could be used in therapeutic approaches for limiting the damages from oxidation reactions in unsaturated fatty acids and for complementing existing therapies in the treatment of oxidative damage. Linalool was seen to have antioxidant properties similar to vitamin E and lipoic acid. A dose-dependent studies will be necessary to determine whether linalool could have beneficial effects in humans similar to those observed in animals.

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