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Severe Total Hepatic Ischemia and Reperfusion: Relationship between very High α-Tocopherol Uptake and Lipid Peroxidation

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Reperfusion injury of the liver occurs in liver transplantation and in major hepatectomies. It triggers a severe oxidative stress that leads to increased lipid peroxidation. In our study we examined the effect of parenteral supranutritional administration of α-tocopherol, a vitamin that plays a key role in the endogenous antioxidant system, to rats subjected to severe ischemia/reperfusion (I/R) injury of the liver. α-Tocopherol was administered to the animals at doses of 30 and 300 mg/kg bw, whereas total hepatic ischemia was induced for 60 min followed by 120 min reperfusion. Tissue and blood samples were collected for malonyldialdehyde (MDA) and serum α-tocopherol assay, respectively. In the sham operation group, mean MDA level in liver was 1.14 nmole/g wet tissue in the control subgroup, and 1.01 or 0.74 nmole/g wet tissue in the subgroups given 30 or 300 mg/kg α-tocopherol. In the I/R group, mean MDA level was 1.57 nmole/g wet tissue in the control subgroup, and 0.97 and 0.77 nmole/g wet tissue in the subgroups given 30 or 300 mg/kg α-tocopherol. Mean levels of α-tocopherol in serum (μmole/l) were 10.20 and 1.80 in the control subgroups, 25.28 and 11.25 in the subgroups treated with 30 and 300 mg/kg bw of α-tocopherol, and 31.00 and 13.02 in the subgroups treated with 30 and 300 mg/kg bw of α-tocopherol, within the sham-operation and I/R groups, respectively. A significant decrease of MDA accompanied by a significant increase of serum α-tocopherol was documented in the α-tocopherol-treated rats within both groups. Ischemia/reperfusion triggered a significant increase of the MDA level in the liver of the rats not treated with α-tocopherol as compares with the treated animals.

Keywords: Lipid peroxidation, malonyldialdehyde, ischemia/reperfusion, liver, α-tocopherol

INTRODUCTION

An important clinical problem concerning liver transplantation and hepatic surgery, is ischemia/reperfusion (I/R) injury of the liver. Even though in recent years significant research is in progress, it has not been yet totally clarified the

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pathophysiological mechanism of I/R injury and the potential protective interventions. The early activation of Kupffer cells and their ability to enhanced formation of reactive oxygen species and pro-inflammatory mediators such as tumor necrosis factor alpha, interleukin-1, proteases, along with the migration and activation of the polymorphonuclear leukocytes that secrete inflammatory cytokines, adhesion molecules, and reactive oxygen species, are considered to play key role in the pathophysiology of reperfusion injury.[1–8] Enhanced formation of reactive oxygen species is considered to be responsible for triggering a severe oxidative stress and lipid peroxidation.[9–15]

Lipid peroxidation results from a chain reaction involving removal of proton from unsaturated fatty acids to form oxygen free radicals followed by the formation of peroxy radicals and lipid peroxides. A major target site of lipid peroxidation damage is the cellular membrane because of the presence of polyunsaturated fatty acids in membrane phospholipids.[16–18] Toxicity of partially reduced oxygen species arises from peroxidation of polyunsaturated fatty acids moieties of membrane phospholipids, which may cause membrane disintegration.[16,19] The consequent cellular damage of liver by ischemia and reperfusion results in mitochondrial dysfunction and loss of the cellular calcium homeostasis.[1] Additionally the effects on lipid metabolisms and especially arachidonic acid metabolism can induce microcirculatory disturbances.[20] In this direction, the endogenous antioxidant defense systems that protect the organism from reactive oxygen species injury are of great importance. These systems consists mainly of enzymes such as catalase, superoxide dismutase and glutathione peroxidase, and antioxidant vitamins such as α-tocopherol and ascorbic acid.[21]

α-Tocopherol plays a key role as an endogenous antioxidant in the biological systems and it is thought to be protective for the cell membranes from the oxidative stress and lipid peroxidation.[22,23] It is transported by lipoproteins and the main way of action is as a chain breaking antioxidant in lipid bilayers of the cellular membranes.[18,22] Previous studies have provided evidence that administration of α-tocopherol results in increased protection of the hepatic parenchyma by diminishing lipid peroxidation.[24] Recently a new role has been proposed for α-tocopherol that is independent from its antioxidant properties. It concerns documented inhibitory effects of α-tocopherol on protein kinase C, on growth regulation of some cells, and in transcription of CD36 scavenger receptor expressions.[25–28]

Due to the importance of α-tocopherol as a potent antioxidant, there have been many approaches in defining the best doses, way of administration and the duration of the treatment.[29] Short-term oral or intravenous administration of α-tocopherol can lead to elevation in serum mitochondrial and liver concentration.[30,31] On the other hand, chronic oral administration of α-tocopherol results in significant increase in serum and liver concentration of the vitamin.[32] Recently Lehr *et al.*[32] questioned the long-term results of chronic α-tocopherol administration, suggesting that it may jeopardize findings in laboratory animals in models of disease.

The aim of this study was to investigate whether a correlation exists between serum α-tocopherol levels and the lipid peroxidation induced by ischemia/reperfusion of liver, and to examine further the relationship between the α-tocopherol levels attained in serum after high or very high intramuscular administration of this vitamin to rats.

MATERIALS AND METHODS

Experimental Protocol

Sixty male Wistar rats weighing 300–350 g each, were used in this study. The rats, cared for in accordance with the Guide for the Care and Use of Laboratory Animals,[33] were maintained

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under a 12-h light/dark cycle, and permitted *ad libitum* access to standard laboratory rodent chow and tap water for 2 weeks before beginning the experimental procedure.

The animals were divided into two groups with three subgroups each, to be subjected to hepatic ischemia/reperfusion (I/R) or to sham operation, respectively. Within each group, one of the subgroups served as the control; the rest two subgroups were intramuscularly treated at 48 h, 24 h and 1 h before the operation, with a low (30 mg/kg bw) or a high (300 mg/kg bw) dose of α-tocopherol, respectively.

The surgical procedure was carried out by anesthetizing the rats with Ketamine (Ketalar®, Park-Davis, U.S.A.) and Fentanyl (Fentanyl®, Janssen Pharmaceutica, Beerse, Belgium) at doses of 50 and 0.012 mg/kg bw, respectively. In the I/R group, a subcalvian bilateral laparotomy was performed and heparin (Heparin® 5000, Astra Pharmaceuticals, Sweden) at 200 IU/kg bw was administered intravenously. Hepatic ischemia was induced for 60 min by cross-clamping the hepatic artery, portal vein and bile duct (Pringles' maneuver) with a vascular microclip (Scanlan® International, St. Paul, Minnesota, U.S.A.). Hepatic blood reflow was achieved by removing the microclip. Following 120 min of reperfusion, the liver was surgically removed for MDA assay, and blood samples were obtained by cardiac puncture for α-tocopherol assay.

In the sham operation group, the abdomen was opened, heparin at 200 IU/kg bw was administered intravenously, the liver was surgically removed, and blood samples were obtained by cardiac puncture for α-tocopherol assay. Blood samples from both groups were centrifuged at 1200 g for 10 min, the serum was obtained, and stored at −40 °C pending analysis.

Assay of MDA in Liver Tissue

Determination of malonyldialdehyde, the compound used as an index of lipid peroxidation, was carried out by a selective third-order derivative method[34] recently developed in our laboratories. According to this, a 2-g sample was thoroughly homogenized (Polytron homogenizer, PCU, Switzerland) with 5 ml of 5% aqueous trichloroacetic acid (Merck, Germany), and 2 ml of 0.8% butylated hydroxytoluene (Sigma Chemical Co., St Louis, MO) in hexane (Merck, Germany), and centrifuged. The top layer was discarded, and a 2.5-ml aliquot from the bottom layer was mixed with 1.5 ml of 0.8% aqueous 2-thiobarbituric acid (Sigma Chemical Co., St Louis, MO) to be further incubated at 70 °C for 30 min. Following incubation, the mixture was cooled to room temperature and submitted to conventional spectrophotometry (Shimadzu, Model UV-160A, Tokyo, Japan) in the range 400–650 nm with a scanning speed of 480 nm/min. Third-order derivative spectra were obtained by electronic differentiation (derivative difference setting, 21 nm) of the conventional absorption spectra of samples from both control and drug-treated rats. The concentration of MDA (nmol/g wet tissue) was calculated on the basis of the third-order derivative peak height at 532 nm by referring to slope and intercept data of the computed least-squares fit of standard calibration curve.

Assay of α-Tocopherol in Serum

Determination of α-tocopherol in serum of the wistar rats was carried out by the method proposed by Arnaud *et al.*[35] According to this method, a 200-μl volume of serum was mixed in an Ependorf tube with 200 μl of ethanol and 500 μl of hexane. The mixture was homogenized (Polytron homogenizer, PCU, Switzerland) for 2 min, and centrifuged at 35000g for 7 min at 4 °C. The hexane layer were transferred to another tube, evaporated to dryness under a mild stream of nitrogen, and the remaining residue was reconstituted in the mobile phase to be injected into the liquid chromatograph.

Analysis of α-tocopherol in the injected extract was carried out by reverse phase liquid

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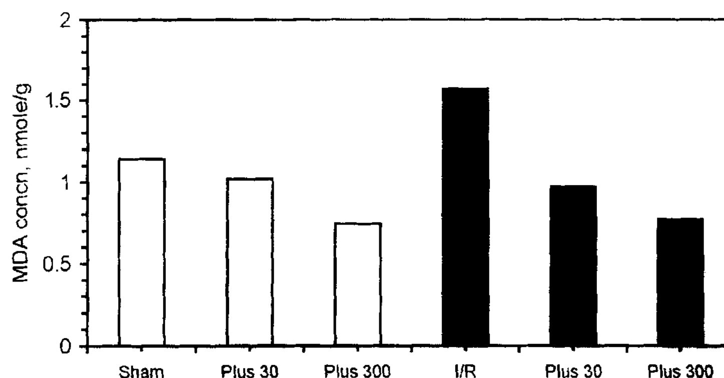

FIGURE 1 Mean concentrations of MDA in liver of rats not treated or intramuscularly treated with 30 or 300 mg/kg bw of α-tocopherol and, then, subjected to ischemia/reperfusion (I/R, grey bars) of the liver or to sham (white bars) operation.

chromatography (Shimadzu, Model LC-6A, Tokyo, Japan) using a 5-µm, 25 × 0.46 cm I.D. column (Nucleosil ODS, Jones Chromatography, London, UK), and a mobile phase consisting of dichloromethane, acetonitrile and methanol at proportions of 20:70:10 (v:v:v), respectively. Mobile-phase flow was maintained at 1 ml/min, while monitoring of the column effluents was performed at 292 nm.

Statistical Analysis

Statistical evaluation of the data drawn was performed with one way analysis of variance (ANOVA) followed by Student's *t*-test, where $p < .05$.

RESULTS

MDA Assay

In the sham-operation group, mean MDA values were found to be 1.14 ± 0.05 nmole/g wet liver tissue in the control subgroup, and 1.018 ± 0.08 and 0.74 ± 0.08 nmole/g wet liver tissue in the subgroups treated with 30 and 300 mg/kg bw of α-tocopherol, respectively. In the ischemia/reperfusion group, mean MDA values reached 1.57 ± 0.16 nmole/g wet liver tissue in the control subgroup, and 0.97 ± 0.04 and 0.77 ± 0.08 nmole/g wet liver tissue in the subgroups treated with 30 and 300 mg/kg bw of α-tocopherol, respectively (Figure 1).

Statistical analysis of the data showed that ischemia and reperfusion of the liver resulted in significant ($p < .001$) increase of MDA in animals non-treated with α-tocopherol. Administration of α-tocopherol resulted in statistically significant ($p < .001$) decrease of MDA compared with the non-treated rats in both the sham operation and I/R groups. The MDA levels observed within the subgroups treated with the same dose of α-tocopherol did not differ significantly ($p > .05$) between the sham operation and the I/R groups.

α-Tocopherol Assay

In the sham-operation group, mean α-tocopherol values were found to be 10.20 ± 0.20 µmole/l of serum in the control subgroup, and 25.28 ± 0.35 and 31.00 ± 0.42 µmole/l of serum in the subgroups treated with 30 and 300 mg/kg bw of α-tocopherol, respectively. In the ischemia/reperfusion group, mean α-tocopherol values were 1.80 ± 0.21 µmole/l of serum in the control subgroup, and 11.25 ± 0.45 and 13.02 ± 0.31 µmole/l of serum in the subgroups treated with 30 and 300 mg/kg bw of α-tocopherol, respectively (Figure 2).

Statistical analysis of the data showed a significant decrease ($p < .001$) of serum α-tocoph-

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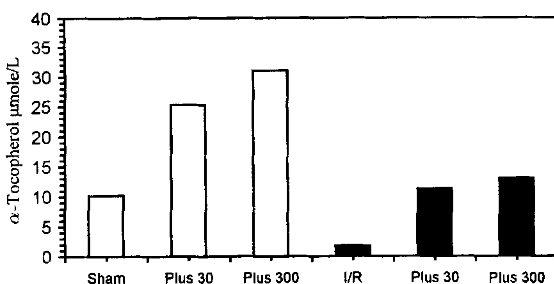

FIGURE 2 Mean concentrations of α-tocopherol in serum of rats not treated or intramuscularly treated with 30 or 300 mg/kg bw of α-tocopherol and, then, subjected to ischemia/reperfusion (I/R, grey bars) of the liver or to sham (white bars) operation.

erol in the I/R group. In this group, a particularly low concentration of α-tocopherol in serum was observed in the subgroup not given α-tocopherol. However, the administration of even the lower dose of α-tocopherol resulted in significant increase of serum α-tocopherol ($p < .001$) in both of the experimental groups. Administration of the higher dose also resulted in higher serum α-tocopherol level ($p < .001$) in both groups.

DISCUSSION

The growing interest in free radical-mediated peroxidation of biomembrane lipids stems from the increasing recognition of the potentially pervasive role of oxidation in tissue damage. Free radicals are generated as byproducts of oxidative metabolism in mitochondria but a critical balance exists between free radical generation and antioxidant defense mechanisms; oxidative damage inflicted by reactive oxygen species simply reflects a shift in the prooxidant–antioxidant balance in favor of the former.[36,37] Our results appear to support the above since malonyldialdehyde was significantly increased in the ischemia/reperfusion group (Figure 1).

MDA is a sensible index in assessing lipid peroxidation even though it is considered nonspecific one. The validity of the MDA assay as an index of lipid peroxidation in biological materials has been clouded by controversy regarding its formation as an artifact of analysis during the test itself, its occurrence in various bound forms, and the specificity of the techniques used for its determination.[38] However, there are theoretical objections to all of the alternative assays that are currently available for investigating free-radical production in the clinical practice[39] and the malonyldialdehyde assay has the merit of simplicity. Moreover, the third-order derivative technique[34] applied in this study for the determination of MDA offers distinct advantages over previous procedures. Its efficient sample preparation procedure suppresses any lipid peroxidation that might arise during the acid-heating stage of the assay, whereas interferences from other reactive compounds are eliminated due to the high discriminative power of the derivative processing.

The results of the MDA assay showed that administration of α-tocopherol prior to sham operation resulted in lower MDA concentrations in the liver tissue. The decrease in the MDA values was significant at both levels of α-tocopherol administration. These results might raise some questions as far as the production of MDA under physiological conditions is concerned. Can lipid peroxidation occur under physiological conditions, and, if yes, could α-tocopherol offer some protection against it? Further research is needed to provide answers to these questions.

The MDA profiles also revealed that ischemia and reperfusion triggered a significant ($p < .001$) increase of the MDA values in the liver of the rats not treated with α-tocopherol, but failed to increase the MDA values ($p > .05$) in the α-tocopherol-treated rats. This implies that the administration of α-tocopherol offered effective protection against the severe oxidative stress initiated by liver ischemia and reperfusion. If we look at the serum α-tocopherol levels we can see a 2- to 3-fold increase of the levels of this vitamin in serum of the rats treated with α-tocopherol.

On the other hand, ischemia and reperfusion caused very high consumption of the level of

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α-tocopherol in serum. This could be readily attributed to increased need for α-tocopherol because of the severe oxidative stress. In the α-tocopherol-treated subgroups some consumption of α-tocopherol could be also observed but there was still significant ($p < .001$) serum α-tocopherol left at the end of liver reperfusion.

In conclusion, our results provide additional support that short-time administration of high or very high doses of α-tocopherol is likely to offer some protection against lipid peroxidation due to ischemia and reperfusion of the liver.

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