\(\alpha\)-lipoic acid promotes the growth of rat hepatic pre-neoplastic lesions in the choline-deficient model

Andrea Perra, Monica Pibiri, Pia Sulas, Gabriella Simbula, Giovanna Maria Ledda-Columbano and Amedeo Columbano

Department of Toxicology, Oncology and Molecular Pathology Unit, University of Cagliari, Via Porcell 4, 09124 Cagliari, Italy

To whom correspondence should be addressed. Tel: +39 070 6758345; Fax: +39 070 666062; Email: columbano@unica.it

\(\alpha\)-lipoic acid (\(\alpha\)-LA) is an antioxidant used in a number of conditions related to liver diseases. Herein, we investigated the effect of \(\alpha\)-LA on the development of rat pre-neoplastic lesions generated by a model of hepatocarcinogenesis, which has similarities in its histopathological sequence to human hepatocellular carcinoma development with cirrhosis. Initiation of hepatocytes was achieved by treatment with a single dose of diethyltriamine and promotion by feeding a choline–methionine-deficient diet (CMD), with or without \(\alpha\)-LA. Pre-neoplastic lesions were identified by their positivity to the placental form of glutathione S-transferase (GSTP) or to gamma glutamyl transpeptidase. \(\alpha\)-LA given to rats fed a CMD for 6 weeks dramatically increased the number of GSTP-positive foci as compared with rats fed a CMD alone (96/cm² versus 7/cm²), the mean foci area (0.033 \text{mm}² versus 0.008 \text{mm}²) and the percentage of GSTP-positive liver tissue (3.01 versus 0.07%). Essentially similar results were obtained after 10 weeks of treatment. Co-treatment with CMD + \(\alpha\)-LA also resulted in the enhancement of fat accumulation, lipid peroxidation and hepatocyte death; increased expression of tumor necrosis factor-\(\alpha\), cytochrome 2E1 and cyclooxygenase-2, enhanced activation of c-jun N-terminal kinase and signal transducer activator of transcription 3, and chronic hepatocyte proliferation was also observed. No such effects were observed when \(\alpha\)-LA was added to a choline-supplemented diet. In conclusion, administration of \(\alpha\)-LA in conditions associated with hepatic damage aggravates liver injury and stimulates the development of pre-neoplastic lesions; the results also suggest caution in its use in the presence of chronic liver injury.

Introduction

Hepatocellular carcinoma (HCC) is the fifth leading cancer in the world with an increasing rate of incidence and mortality and a poor prognosis (1). Although, during the past 25 years striking advances have been made in our understanding of HCC, including the development of new approaches to the heterogeneous nature of HCC (2), current treatments are not effective, and the identification of relevant pathways and novel therapeutic agents are much needed.

The availability of substances, including natural antioxidants, which are non-toxic for normal liver while having a direct or indirect effect on tumor cell growth, may provide useful for the development of antitumoral strategies aimed at inhibiting tumor growth and/or selectively eliminating cancer cells.

\(\alpha\)-Lipoic acid (\(\alpha\)-LA) is a naturally occurring, short-chain fatty acid containing two sulfur molecules. It is an essential cofactor of mitochondrial respiratory enzymes (3), taken up and reduced to dihydrolipoic acid, which can be released into the extracellular compartment. \(\alpha\)-LA and dihydrolipoic acid can serve as powerful antioxidants through several mechanisms, including scavenging of free radicals, chelation of metal ions and regeneration of endogenous and exogenous antioxidants, such as ubiquinon, vitamins C and E and glutathione (4). Pharmacologically, \(\alpha\)-LA has been widely used as a therapeutic agent in the treatment and prevention of several pathological conditions, such as diabetic polyneuropathy, hypertension, insulin resistance and bone loss (5–7). Furthermore, \(\alpha\)-LA has also been used in a number of conditions related to liver diseases, such as alcohol-induced damage, mushroom poisoning, metal intoxication, \(\text{CCl}_{4}\) poisoning and hyperdynamic circulation in biliary cirrhosis (8–10) and has been reported to have a beneficial effect in patients with advanced cancer by increasing the glutathione peroxidase activity and by reducing oxidative stress (11). Interestingly, while \(\alpha\)-LA exerts a protective effect against apoptosis in normal hepatocytes, similarly to what described in several cancer cell lines (12–14), it possesses a strong pro-apoptotic effect on human and murine hepatoma cells (15), suggesting that \(\alpha\)-LA may play a role in the hepatocarcinogenic process.

Animal models have greatly contributed to the understanding of the carcinogenic process in the liver. In particular, short-term in vivo assay systems with suitably short duration but sufficient effect in inducing measurable lesions, have been widely accepted as early indicators of neoplastic development, as they provide results closely comparable with those of long-term carcinogenicity testing (16–18). Among the several models developed in the past years, a very useful one is the high-fat choline–methionine-deficient diet (CMD), which is known to cause fatty change, hepatocyte injury, fibrosis, cirrhosis and generation of oxidative DNA damage via \(\text{S-hydroxydeoxyguanosine}\), in rodents (19,20). Administration of CMD to rats, following a single initiating dose of carcinogen, enhances both induction of enzyme-altered pre-neoplastic foci and subsequent progression to HCC (21). Acceleration of development of hepatic neoplasms by CMD has also been reported in mice (22). This provides us with a useful experimental model for promotion of hepatocarcinogenesis caused by endogenous factors, and which has similarities in its histopathological sequence to human HCC development with cirrhosis (23).

Based on the wide therapeutic use of \(\alpha\)-LA in several diseases (5–7), including conditions related to liver diseases (8–10), and since no data are available on the effect of \(\alpha\)-LA on liver cancer development, the aim of the present study was to investigate the effect of \(\alpha\)-LA on the occurrence of putative pre-neoplastic lesions induced by a short-term in vivo assay consisting of a single dose of the hepatocarcinogen diethyltriamine (DENA) and promotion with the CMD. We report here that addition of \(\alpha\)-LA to the CMD strongly promoted the growth of hepatic pre-neoplastic foci positive for the placental form of glutathione S-transferase (GSTP) and gamma glutamyl transpeptidase (GGT), the two most reliable markers for rat pre-neoplasia (24,25), and potentiates accumulation of triglycerides in the liver and hepatocellular damage, suggesting caution about its use in conditions associated with pre-existing liver damage.

Materials and methods

**Animals and reagents**

Eight-week-old Fischer rats (F-344, 175–200 g, Charles River, Milano, Italy) were used for the experiments. All procedures were performed in accordance with the Universities Federation for Animal Welfare Handbook on the Care and Management of Laboratory Animals and the guidelines of the animal ethics committee of this University. DENA was purchased from Sigma Chemical Co. (St Louis, MO). \(\alpha\)-LA (Sigma) was given in drinking water at the
concentration of 0.01, 0.05, 0.1 and 0.2% for the times indicated in the Experimental protocol I or added to the diet at a final concentration of 0.2% (Experimental protocols II and III).

**Experimental protocol I**

Rats were injected intraperitoneally with a single dose of DENA (150 mg/kg body wt). After a 2 week recovery period, rats were fed a CMD for 6 weeks. At the beginning of the CMD, α-LA was given in drinking water at the following concentrations: 0.01, 0.05, 0.1 and 0.2%. The same doses of α-LA were given to rats fed a choline-supplemented diet (CS).

**Experimental protocol II**

Two weeks after treatment with DENA, rats were divided in four groups: group 1 was placed on a CMD; group 2 on a CMD supplemented with 0.2% α-LA (α-LA + CMD); group 3 on a CS; group 4 on a CS supplemented with 0.2% α-LA (CS + α-LA). All animals were killed 6 or 10 weeks after starting of the diet.

**Experimental protocol III**

Rats were fed CMD with or without α-LA (final concentration in the diet 0.2%), in the absence of carcinogen treatment, for 10 weeks.

**Histology and immunohistochemistry**

Sections of the liver were fixed in 10% buffered formalin and processed for staining with hematoxylin-eosin. Other sections were used for immunohistochemical detection of bromodeoxyuridine (BrDU), GSTP and GGT as described previously (26). The remaining liver was snap frozen in liquid nitrogen and kept at −80°C until use.

**Double labeling of BrDU and GSTP-positive hepatocytes in DENA-initiated rat liver**

BrDU (Sigma Chemical Co.) dissolved in drinking water (1 mg/ml) was given throughout the last week of treatment (26). BrDU incorporation into nuclei and the location of GSTP was determined as described previously (27). The sites of the location of GSTP was determined as described previously (27).

**Measurement of GSTP- and GGT-positive foci**

GSTP- and GGT-positive foci were measured with a computer-assisted image processor, according to Abramoff et al. (28). For each animal, three different sections from three distinct lobes were measured. Only foci >75 μm in diameter were scored.

**Determination of labeling index**

Random microscope fields were scored for BrDU-positive hepatocytes. The labeling index was calculated as BrDU-positive hepatocyte nuclei per 100 nuclei. At least 5000 hepatocyte nuclei per liver were scored.

**Determination of apoptotic index**

The incidence of apoptotic bodies was determined by scoring 3000–4000 hepatocytes per liver, as described previously (29). The apoptotic index was expressed as number of apoptotic bodies per 100 hepatocytes.

**Serum alanine aminotransferase**

Blood samples were centrifuged at 1500 r.p.m. for 15 min and the serum was tested for alanine aminotransferase, using a commercially available kit from Ortho-Clinical Diagnostics, Rochester, NY.

**Lipid peroxidation assay**

Approximately 100 mg of liver tissue were homogenized with a Ultra Turrax T8 stirrer in ice-cold 20 mM Tris–HCl buffer, pH 7.4, to produce a 1/10 homogenate. The crude homogenate was centrifuged at 7,000 r.p.m. for 15 min at 4°C. Aliquots of the supernatant were used for protein total determination or to calculate lipid peroxidation (LPO). Malondialdehyde and 4-hydroxyalkenals concentrations provide a convenient index of LPO (30).

**Western blot analysis**

Total cell extracts were prepared from frozen livers as described previously (31). For immunoblot analysis, equal amount (from 100 to 150 μg per lane) of proteins were electrophoresed on sodium dodecyl sulfate 12 or 8% polyacrylamide gels (Sigma Chemical Co.). After gels’ electrotransfer onto nitrocellulose membranes (Osmonics, Westborough, MA) to ensure equivalent protein loading and transfer in all lanes, the membranes and the gels were stained with 0.5% (wt/vol) Ponceau S red (ICN Biomedicals) in 1% acetic acid, and with Coomassie blue (ICN Biomedicals) in 10% acetic acid, respectively. After blocking in Tris-buffered saline + 5% non-fat dried milk, membranes were washed in Tris-buffered saline-Tween 20 and then incubated with the appropriate primary antibodies. Filters were incubated with anti-mouse or anti-rabbit or anti-goat horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoreactive bands were identified with chemiluminescence detection system (Supersignal Substrate; Pierce, Rockford, IL).

**Antibodies**

Primary antibodies used in this study were mouse monoclonal antibodies against cyclin D1 (72-13G; Santa Cruz Biotechnology), actin (clone AC40; Sigma Chemical Co.) proliferating cell nuclear antigen (PC-10; Santa Cruz Biotechnology), Bax (B-9; Santa Cruz Biotechnology) and caspase-9 (C-9; Cell Signalling Technology, Beverly, MA); rabbit polyclonal antibodies against signal transducer activator of transcription 3 (STAT3), phospho-STAT3 (Tyr705), Stress activated protein kinase (SAPK/JNK and Poly(ADP-ribose)polymerase (Cell Signalling Technology), pro-caspase-8 (H-134; Santa Cruz Biotechnology); goat polyclonal antibodies directed against cyclooxygenase (COX)-1 (M-20) and COX-2 (M-19) (Santa Cruz Biotechnology). For immunohistochemistry, the antibodies used were rabbit polyclonal antibody anti-GSTP (MBL, Woburn, MA) and anti-GGT (a kind gift from A.Pompea and A.Paolicchi, University of Pisa).

**Statistical analysis**

Instant software (GraphPad Prism 5, San Diego, CA) was used to analyze the data. One-way analysis of variance with post hoc analysis using Tukey’s multiple comparison test was used for parametric data. The results of multiple observations were presented as the means ± SE of at least two separate experiments. A P value of <0.05 was regarded as a significant difference between groups.

**Table I. Effect of various concentrations of α-LA on the induction of GSTP-positive foci in rat liver initiated by DENA**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSTP-positive foci (No./cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENA + CS</td>
<td>3.75 ± 0.61</td>
</tr>
<tr>
<td>DENA + CS + α-LA</td>
<td>4.12 ± 0.92</td>
</tr>
<tr>
<td>(0.01%)</td>
<td>3.99 ± 1.12</td>
</tr>
<tr>
<td>(0.05%)</td>
<td>4.25 ± 0.83</td>
</tr>
<tr>
<td>(0.1%)</td>
<td>4.50 ± 0.32</td>
</tr>
<tr>
<td>(0.2%)</td>
<td>13.52 ± 2.61*</td>
</tr>
<tr>
<td>DENA + CMD</td>
<td>11.9 ± 1.13</td>
</tr>
<tr>
<td>(0.01%)</td>
<td>19.9 ± 3.12</td>
</tr>
<tr>
<td>(0.1%)</td>
<td>31.3 ± 2.60*</td>
</tr>
<tr>
<td>(0.2%)</td>
<td>38.8 ± 4.82*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE of five to six animals per group.

*Significantly different from CS; P < 0.005.

*Significantly different from CMD; P < 0.001.

*Significantly different from CMD; P < 0.050.
Values are expressed as means ± SE of five to six animals per group. BW, body weight; LW, liver weight.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BW (g) ± SD</th>
<th>LW/BW (%)</th>
<th>GSTP-positive foci/cm²</th>
<th>Mean GSTP+ area (mm²)</th>
<th>GSTP+ area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DENA + CS (6 weeks)</td>
<td>291 ± 4</td>
<td>3.63 ± 0.08</td>
<td>1.8 ± 0.6</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DENA + CS + α-LA (6 weeks)</td>
<td>267 ± 4²</td>
<td>3.48 ± 0.11</td>
<td>3.6 ± 0.5</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>DENA + CMD (6 weeks)</td>
<td>281 ± 5</td>
<td>4.50 ± 0.11³</td>
<td>6.7 ± 1.9⁰</td>
<td>0.0026 ± 0.0009³</td>
<td>3.01</td>
</tr>
<tr>
<td>DENA + CMD + α-LA (6 weeks)</td>
<td>229 ± 7³</td>
<td>6.15 ± 0.33³</td>
<td>95.7 ± 12.1³²</td>
<td>0.0067 ± 0.0008³</td>
<td>ND</td>
</tr>
<tr>
<td>DENA + CS (10 weeks)</td>
<td>311 ± 9</td>
<td>3.08 ± 0.04</td>
<td>5.3 ± 1.1</td>
<td>0.0067 ± 0.0008³</td>
<td>0.04</td>
</tr>
<tr>
<td>DENA + CS + α-LA (10 weeks)</td>
<td>280 ± 7₇</td>
<td>3.46 ± 0.09⁹</td>
<td>16.2 ± 5.8</td>
<td>0.0072 ± 0.0002</td>
<td>0.13</td>
</tr>
<tr>
<td>DENA + CMD (10 weeks)</td>
<td>302 ± 8</td>
<td>5.00 ± 0.03⁷</td>
<td>66.5 ± 14.1⁷⁹</td>
<td>0.0278 ± 0.0040³</td>
<td>1.94</td>
</tr>
<tr>
<td>DENA + CMD + α-LA (10 weeks)</td>
<td>255 ± 3³</td>
<td>5.54 ± 0.13³</td>
<td>155.0 ± 12.7³⁴</td>
<td>0.0742 ± 0.0147³</td>
<td>11.27</td>
</tr>
</tbody>
</table>

Values expressed as means ± SE of five to six animals per group. BW, body weight; LW, liver weight.

Significantly different from CMD (6 weeks); P < 0.005.
Significantly different from CMD (10 weeks); P < 0.001.
Significantly different from CS (6 weeks) for at least; P < 0.050.
Significantly different from CS (10 weeks) for at least; P < 0.001.

**Results**

**Experimental protocol I**

A concentration of 0.2% of α-LA does not cause any toxic effect on the liver while improving indices of metabolic activity as well as lowering oxidative stress and damage caused by aging in rat liver (32). To evaluate the effect of α-LA on the development of pre-neoplastic hepatic lesions, rats fed CMD received α-LA in drinking water at concentrations of 0.2% or lower (0.1, 0.05 and 0.01) for 6 weeks. No rats died in any of the groups during the experimental period. As shown in Table I, the three highest concentrations of α-LA exerted a promoting effect on the development of GSTP-positive foci compared with the group given CMD alone. Indeed, quantification of the number of GSTP-positive foci showed that CMD-fed rats given 0.2, 0.1 and 0.05% of α-LA exhibited a significant increase in the number of GSTP-positive foci (38, 31 and 20/cm², respectively versus 0.008 mm² of rats given CMD alone). Very few GSTP-positive foci were observed in the liver of rats treated with DENA and fed CS, and this number was not modified by any of the doses of α-LA.

**Experimental protocol II**

As the concentration of 0.2% of α-LA appeared to be the most effective in promoting the occurrence of GSTP-positive foci, next experiments were performed using a CMD supplemented with this concentration of α-LA for 6 or 10 weeks. As in experimental protocol I, no mortality was observed. A decreased body weight of rats given α-LA fed on either the CS or CMD for 6 weeks was found, compared with CMD or CS alone (Table II). The ratio of liver weight to body weight increased in CMD-fed rats compared with CS and was further enhanced in CMD + α-LA-fed rats.

Under histological examination, the liver of rats fed CMD showed distinct nodular foci of hepatocytes, which, on this basis, could be readily distinguished from the surrounding fatty parenchyma. The hepatocytes in the foci were arranged irregularly without a distinct sinusoidal pattern and in plates more than one cell thick, had basophilic cytoplasm, contained droplets of fat, albeit at a lower level than in the control. As shown in Table I, the three highest concentrations of α-LA exerted a promoting effect on the development of GSTP-positive foci compared with the group given CMD alone. Indeed, quantification of the number of GSTP-positive foci showed that CMD-fed rats given 0.2, 0.1 and 0.05% of α-LA exhibited a significant increase in the number of GSTP-positive foci (38, 31 and 20/cm², respectively versus 0.008 mm² of rats given CMD alone). Very few GSTP-positive foci were observed in the liver of rats treated with DENA and fed CS, and this number was not modified by any of the doses of α-LA.
area GSTP positive (1.94%) and in the average area size (0.028 mm²) (Table II and Figure 1E). However, supplementation with α-LA for the same period of time did result in a further increase in the number (155 GSTP-positive foci/cm²), percentage liver area (11.3%) and average foci area (0.074 mm²), clearly demonstrating the powerful enhancing effect of α-LA on tumor promotion by CMD (Table II and Figure 1F). Although GSTP is considered to be the most reliable marker for pre-neoplastic lesions induced by genotoxic carcinogens (24), it does not identify all putative pre-neoplastic lesions. Therefore, pre-neoplastic lesions were also identified by their positivity for GGT, the second best marker for pre-neoplasia (25). The results confirmed that feeding a CMD + α-LA diet for 10 weeks caused a striking increase in the number of pre-neoplastic lesions compared with the animals fed the CMD alone (72.1 ± 13 foci/cm² versus 25.4 ± 7 foci/cm²), although, in agreement with the literature (25), the number of lesions detected by GGT staining was lower than that determined by GSTP staining.

Interestingly, at the time points examined, a more severe degree of fatty change was present in the liver of CMD + α-LA diet-fed rats as compared with CMD alone (See Figures 1 and 2). Fatty liver is usually followed by hepatocyte injury (steatohepatitis) which in turns induces compensatory regeneration; repeated cycles of oxidative stress, hepatocyte injury and regeneration (27,33) have been postulated to be involved in the appearance of pre-neoplastic lesions in the CMD model (34). Therefore, we sought to examine whether the promoting effect of α-LA on the development of pre-neoplastic lesions could be the consequence of increased (i) oxidative stress; (ii) liver damage and (iii) chronic liver regeneration.

Cyp2E1, one of the several possible sources of reactive oxygen species (ROS) is increased in the liver of humans with alcohol-induced liver disease (35) and non-alcoholic steatohepatitis (36,37). Furthermore, induction of Cyp2E1 and accumulation of hepatic liperoxides (38) have been shown to occur in rodents with steatohepatitis induced by feeding a CMD. These finding prompted us to investigate whether α-LA could enhance chronic oxidative stress through Cyp2E1 induction and LPO (see Experimental protocol III). The results in Figure 3A show that, in agreement with previous findings (38), Cyp2E1 expression was increased in CMD-fed rats; however, α-LA addition to CMD further enhanced the hepatic levels of Cyp2E1 messenger RNA (mRNA) compared with CMD alone. Very low expression of Cyp2E1 was observed in both CS and CS + α-LA groups. Moreover, α-LA addition to CMD caused a significant increase in LPO, compared with CMD alone (Figure 3B).

The products of LPO can mediate inflammatory recruitment by activating nuclear factor-κB with downstream consequences that include expression of COX. COX catalyzes the conversion of arachidonic acid to prostanoids and thromboxanes, some of which are intensely pro-inflammatory. Since COX-2 acts as pro-inflammatory

**Fig. 2.** Photomicrographs of liver sections from rats treated with DENA (150 mg/kg) and fed CS or CMD with (B and D) and without α-LA (A and C) for 10 weeks. Sections were stained for GSTP and counterstained with hematoxylin (×40). Note the confluence of lipid vacuoles in the liver of rats fed CMD + α-LA and the reduced fat accumulation in the GSTP-positive pre-neoplastic hepatocytes. (E and F) Photomicrographs of liver sections double stained for GSTP and BrdU. Liver sections from rats treated with DENA + CMD (E) and DENA + CMD supplemented with 0.2% α-LA (F). Several labeled nuclei are present in a GSTP-positive nodule generated by a single DENA administration followed by feeding a CMD with α-LA (×40). Note the much higher different labeling in the nodule as compared with the surrounding liver.
mediator in the CMD-induced steatohepatitis model (39), we investigated the effect of α-LA on COX-2 expression. The results shown in Figure 3C demonstrate that CMD increased the expression of COX-2, compared with CS, and that α-LA added to the CMD further increased the hepatic levels of this pro-inflammatory protein. No effect of α-LA was observed when added to the CS. No change in the protein levels of COX-1 was induced by CMD dietary feeding, with or without α-LA. Cyp2E1 over-expression causes an activation of JNK (40). As phosphorylation of JNK regulates hepatocellular injury and insulin resistance, thus mediating the development of steatohepatitis, the expression of JNK was examined in the liver of rats fed CMD with or without α-LA. The results showed that CMD causes an increase of JNK phosphorylation and that α-LA addition further increases activation of this kinase (Figure 3C); northern analysis of Cyp2E1 and TNF-α expression. The results showed that CMD increases TNF-α expression compared with CS, and that α-LA added to the CMD group resulted in increased hepatic levels of TNF-α, a candidate molecule in the transition of steatosis to steatohepatitis and a known JNK activator (Figure 3A); similar results were confirmed by semi-quantitative polymerase chain reaction (data not shown). Very little activation of JNK was observed in the non-injured liver from CS-fed animals, with or without α-LA. STAT3 is known to play a crucial role in inflammation and cell proliferation (41–43). As shown in Figure 3C, hepatic protein levels of phosphorylated STAT3 were increased after 10 weeks of CMD dietary feeding compared with CS. Consistent with the proposed pro-inflammatory role of STAT3, the hepatic levels of phosphorylated STAT3 were further enhanced in rats given α-LA together with CMD whose liver was more severely damaged compared with the CMD group. No STAT3 activation was observed in the liver of rats given α-LA + CS.

The severe fat accumulation and oxidative stress caused by α-LA in rats fed the CMD were associated with increased liver cell death. Indeed, while an almost 3-fold increase of serum alanine aminotransferase levels was found in rats fed CMD for 10 weeks, as compared with CS-fed rats, a 6-fold increase over the control values was observed in α-LA + CMD-fed rats (Figure 3D). The increase in serum transaminase levels was associated with the occurrence of apoptosis. Indeed, a significant increase in the number of apoptotic bodies was observed in CMD + α-LA-fed rats compared with rats fed the CMD alone (apoptotic index was 5.15 ± 0.20% in CMD + α-LA-fed rats versus 1.37 ± 0.010% of rats fed CMD alone) (Figure 4A). Only a negligible number of apoptotic bodies (<0.1%) was observed in CS and CS + α-LA-fed rats. The increased occurrence of apoptosis was associated with enhanced expression of the pro-apoptotic proteins caspase-9, Bax and Poly(ADP-ribose)polymerase (Figure 4B). Hepatocyte injury is followed by liver regeneration. Since cell proliferation is considered to play an important role in the several steps of the carcinogenic process (44–46), we sought to determine whether enhanced hepatocyte replication due to the increased hepatotoxicity caused by feeding CMD + α-LA diet could accompany and sustain the accelerated growth of pre-neoplastic foci observed in the latter group. Figure 5A shows that treatment with α-LA + CMD resulted in a strong increase in the labeling index when compared with rats fed CMD alone (LI was 19.63 versus 8.45% of CMD group). A much lower labeling index was found in rats fed a CS or CS + α-LA (1.5 and 3.0%, respectively). In agreement with the immunohistochemical data, western blot analysis showed an increase in the hepatic levels of two markers of cell proliferation, namely cyclin D1 and proliferating...
cell nuclear antigen (Figure 5B), in the α-LA + CMD group, compared with those of rats fed a CMD alone.

Finally, double staining for BrdU and GSTP showed that the addition of α-LA to the CMD caused a striking increase in DNA synthesis of GSTP-positive pre-neoplastic hepatocytes compared with that of surrounding liver cells and to that of the tiny GSTP-positive hepatocytes generated by the CMD alone (Figure 2E and F).

**Discussion**

The important finding of this study is that, in a nutritional model of steatohepatitis, α-LA, a natural agent widely used in the treatment and prevention of several pathological conditions, such as diabetic polyneuropathy, hypertension, insulin resistance, bone loss, ischemia-reperfusion injury and conditions related to liver diseases, enhances CMD-induced fatty liver, steatohepatitis and the development of pre-neoplastic hepatic lesions. The enhancing effect of α-LA on the development of pre-neoplastic lesions appears to occur only in conditions associated with liver damage, such as fatty liver and steatohepatitis caused by a CMD, but not in normal healthy liver.

The CMD causes not only hepatic steatosis but also an associated inflammatory infiltrate. It therefore seems to be a simple and reproducible animal model of fatty liver disease with inflammation and injury that morphologically resembles the group of human disorders known as non-alcoholic steatohepatitis. Similarly to alcoholic steatohepatitis, the early stages of liver disease in the CMD-fed rats are associated with steatosis mostly in acinar zone 3, with latter extension into zone 2. Cyp2E1 is normally localized within a rim of hepatocytes around the terminal hepatic venule in acinar zone 3 (47,48). The increased Cyp2E1 mRNA levels observed in CMD-fed rats (38) suggest that this cytochrome plays an important role in the production of cellular injury in that it could generate ROS and other radicals in this nutritional model of steatohepatitis. The present finding that α-LA addition to the CMD induces Cyp2E1 mRNA levels, increases LPO and aggravates fat accumulation and necroinflammatory response suggests that concentrations of α-LA which are presumably harmless for normal liver may increase the production of ROS and the progression from steatosis to steatohepatitis, in the presence of pre-existing liver damage. Furthermore, Cyp2E1 overexpression is known to cause JNK activation (40) which in turns regulates hepatocellular injury, thus further contributing to the development of steatohepatitis. Moreover, the increased hepatic levels of TNF-α, a candidate molecule in the transition of steatosis to steatohepatitis and a known JNK activator, may also contribute to liver injury caused by co-treatment with CMD + α-LA. Another interesting finding stemming from this study is the increased expression of COX-2 in CMD + α-LA-fed rats. Previous studies (39) have shown that the expression and the activity of COX-2 are up-regulated in the CMD model of steatohepatitis; the same studies also showed that specific COX-2 inhibitors ameliorate the CMD-induced steatohepatitis. Our finding that the severity of liver damage induced by α-LA is accompanied by increased expression of COX-2 is well in agreement with the possible role of COX-2 in the progression from steatosis to steatohepatitis. The fatty liver is predisposed to forms of injury that involve oxidative stress. Liver steatosis induced by a dietary regimen could therefore provide the setting for steatohepatitis. Our results suggest that the addition of α-LA to the CMD may be the ‘second hit’, which aggravates fatty liver and the necroinflammatory response induced by the dietary regimen. The increased expression of Cyp2E1 mRNA, the production of ROS, the generation of oxidized lipids and
their peroxidation products after treatment with α-LA might be instrumental in worsening and perpetuating the liver damage.

Irrespective of the mechanisms by which α-LA enhances the severity of fat accumulation and liver damage caused by the CMD, one of the consequences is an increased chronic hepatocyte turnover, indicated by the increased serum alanine aminotransferase levels, enhanced apoptotic cell death, BrdU incorporation and expression of cell cycle-related proteins. Within this scenario, it is possible to hypothesize the presence of a selective pressure for the initiated hepatocytes which, on one hand, are known to be more resistant to fat accumulation and death and, on the other hand, can take advantage from the release of growth factors or cytokines, such as TNF-α, produced as a consequence of the liver damage, and considered to be essential for hepatocyte proliferation (49,50). The finding of a very high labeling index in the GSTP-positive hepatocytes, compared with surrounding non-initiated hepatocytes, strongly supports this hypothesis. Interestingly, in spite of its supposed beneficial effect, at no concentrations α-LA proved to exert an inhibitory effect on the development of pre-neoplastic lesions.

In conclusion, the present results demonstrate that α-LA treatment in conditions associated with fatty liver caused by a dietary regimen aggravates liver damage and strongly accelerates the growth of pre-neoplastic lesions. These data also suggest that extreme caution should be taken when considering the therapeutic use of α-LA in conditions characterized by pre-existing liver damage.

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