Original Research

High dosing of α-tocopherol inhibits rat liver regeneration by modifying signal transducer and activator of transcription protein expression and its correlation with cell redox state and retinoid metabolism

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Abstract

Lipid peroxidation (LP) promoted by partial hepatectomy (PH) is qualitatively distinct among subcellular fractions and temporally transient, probably being a necessary physiological event for rat liver regeneration. In fact, α-tocopherol (vitamin E [VE]) exerts adverse effects, partially inhibiting PH-induced rat liver regeneration and inducing decreased cyclin D1 expression. The phosphorylation of signal transducer and activator of transcription (STAT) factors 1 and 3 are involved in DNA synthesis and cyclin D1 expression after PH, which is stimulated by production of retinoic acid (RA). Hence, this study was aimed at addressing these events, and its association with cell redox state and oxidative stress, probably underlying VE effects on rat liver regeneration. PH-enhanced activation of STAT proteins, mainly as activated STAT-3, significantly change the cytoplasmic pool for STATs. The latter was associated to a more reduced cytoplasmic redox state and increased alcohol dehydrogenase (ADH)-mediated retinol oxidation to RA. Whereas α-tocopherol promoted minor changes in the parameters tested when administered to sham (control)-animals, pretreatment with VE blocked the PH-induced increase of reactive oxygen species (ROS), altering the pattern of STAT protein activation, blunting RA formation by decreased ADH activity, inducing higher liver caspase-3 activity and increasing tumor necrosis factor-α concentrations, while levels of interleukin-6 were decreased; altogether coinciding with disturbed PH-promoted changes on the liver redox state. In conclusion, altered activation and translocation of STAT-1 and -3 proteins and inhibited retinoid metabolism seem to be involved in the VE-induced inhibition of rat liver regeneration. Data suggest that a PH-induced increase of ROS could participate in the activation of STAT factors, retinoid metabolism and changes in the cell redox state during proliferation of liver cells.

Keywords: vitamin E, cell proliferation, apoptosis, tumor necrosis factor, NAD/NADH ratio, carcinogenesis

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Introduction

The liver is capable of recovering from damage or greater losses of its mass by means of proliferative activity, restoring it to a normal size.[1] The oxidant and antioxidant status are now recognized as putative controlling mechanisms in the progression of partial hepatectomy (PH)-induced rat liver regeneration.[2–4] We have demonstrated that increased lipid peroxidation (LP), induced by PH in rats, could function as a physiological event, possibly triggering a synchronized proliferative response in the regenerating rat liver.[2]

Vitamin E (VE) consists of four tocopherols (α, β, γ- and δ) and the corresponding tocotrienols. The γ-tocopherol is the predominant form of tocopherol found in the Western diet, while α-tocopherol is the predominant form of VE found in human plasma, and the most effective antioxidant tocopherol ($\alpha > \beta > \gamma > \delta$).[5] We have shown that treatment with the α-tocopherol form of VE exerts deleterious effects on cell proliferation, apparently by promoting an early termination of priming cell events, culminating in a partial inhibition of PH-induced rat liver regeneration,[4] as well as in the compensatory hyperplasia that follows an ethanol-induced gastritis in rats.[6] In the proliferating rat liver, α-tocopherol reduces expression of the proliferating cell nuclear antigen (PCNA), and significantly decreases cyclin D1 translocation into the nucleus.[4] Moreover, VE

promotes apoptosis in the gastric mucosa even in the presence of enhanced levels of endogenous antioxidants.[6] Hence, the present study is an attempt to assess the involvement of molecules participating in cell redox signaling as putative underlying targets for the VE actions. Liver regeneration is a complex process that involves signaling pathways, including cytokines,[7] reactive oxygen species (ROS)[2–4] and, among others, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), nuclear factor-κB (NF-κB) and activation of signal transducer and activator of transcription-3 (STAT-3) protein.[8,9] ROS can function as small messenger molecules during physiological processes, since they can activate the transcription of NF-κB and activator protein 1 (AP-1).[10]

One of the best-understood systems of signal transduction is the activation of STAT proteins. Different stimuli induce activation of different combinations of STAT and STAT-associated proteins in a process mediated by the Janus activated kinase family of protein tyrosine kinases, inducing gene transcription.[9,11] After PH, a pronounced activation of STAT-3 is accompanied by elevated serum levels for IL-6.[12] In constrast to normal cells, constitutively aberrant activation of STAT-3 is often associated with cell survival, proliferation and transformation.[13]

Retinoic acid (RA) is formed from retinal, and retinol and exerts different effects such as inhibition of proliferation, induction of genes related with differentiation and immunomodulation.[14] RA and interferons are potent regulatory molecules capable of activating transcription factor STAT-3, and to a lesser degree, STAT-1, through the mediation of IL-6.[15] After PH, liver retinol decreases, suggesting that its changes correspond to the proliferative activity of stellate cells, rather than reflect that of hepatocytes.[16] In addition, liver alcohol dehydrogenase (ADH) activity is decreased toward exogenous substrates after PH, probably due to putative endogenous substrates, involving retinoid metabolism, which is apparently required during rat liver regeneration,[17] and coincides with PH-induced modifications in the cytoplasmic and mitochondrial redox states.[18] Hence, we propose that PH induces early changes in cell redox state, affecting enzyme activities such as cytoplasmic ADH and, consequently, retinoid metabolism, which, in turn, could regulate expression of transcription factors like the STAT protein family, this being mediated by fluctuations in the production of IL-6 and of TNF-α.

Materials and methods

Materials

All-trans-retinol, all-trans-retinal, retinoic acid, NAD, NADH, ADH from liver horse, thiobarbituric acid and α-tocopherol were obtained from Sigma-Aldrich Chemical Co. (Sigma-Aldrich de México, Mexico City, Mexico). All other chemicals were high-pressure liquid chromatography (HPLC) grade or the highest purity available.

Animals and treatments

Male Wistar rats ($n = 120$), weighing 240–270 g, were housed under a 12 h dark/light cycle with free access to food and water. Animals were randomly divided into two groups: rats receiving a daily intragastric administration of 6 IU/kg of α-tocopherol (approximately 4 mg/kg of VE) diluted in peanut oil (1 mL/rat), and those receiving only the α-tocopherol vehicle. Treatments were applied for a period of three days, as previously described by Trejo-Solís *et al.*[4] After completing the treatment with α-tocopherol, animals were again divided according to their surgical status. A 70% PH was performed according to Higgins and Anderson,[19] and sham-operated animals provided a surgical control. Afterwards (6–96 h postsurgery), rats were euthanized by decapitation under sodium pentobarbital anesthesia and liver and serum samples were obtained. All manipulations were conducted according to our *Institutional Guide for Animal Experimentation and Care* (National University of Mexico).

Biochemical analysis

The cytosolic fraction was obtained by differential centrifugation, as described by Aguilar-Delfín *et al.*[2] Cytosolic malondialdehyde (MDA), measured as thiobarbituric acid reactive substances (TBARS), was determined, as described in detail elsewhere.[20] Cytosolic ROS levels were estimated through the method described by Viarengo *et al.*,[21] using the fluorescence signal generated by ROS reacting with 2′,7′-dichlorodihydrofluorescein di-acetate (H_2DCF-DA; Molecular Probes, Eugene, OR, USA). The activity of thymidine kinase (TK), a marker of DNA synthesis, was determined according to Sauer and Willmans.[22] Perchloric acid extracts were neutralized with 5 mol/L of K_2CO_3 and used for metabolite determinations by enzymatic methods;[18] lactate (LAC) and pyruvate (PYR), as well as β-hydroxybutyrate (B-OH-B) and acetoacetate (AcAc), were used to calculate the cytoplasmic and mitochondrial redox states, respectively.[18] The VE content in liver homogenates from control and α-tocopherol-treated animals was determined through HPLC, according to the method described by Huang and Shaw.[23]

Caspase activities, and levels of TNF-α and of IL-6

Caspase activities in the cytosolic fraction (caspase-3, -8 and -9) were assayed with colorimetric kits (Chemicon, Temecula, CA, USA), based on the principles described by Thornberry.[24] Liver and serum concentrations for TNF-α and for IL-6 were determined using enzyme-linked immunosorbent assay kits from Chemicon.

Western-blot analysis for total and phosphorylated STAT-1 and -3 proteins in nuclei and cytosol

Isolated nuclei from liver were purified by using sucrose gradients and suspended in a HEPES–$MgCl_2$–sucrose solution.[25] Fifteen micrograms per well of nuclear or cytosolic protein were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, with the voltage increasing every 10 min, starting at 65 V and ending at 110 V. After separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad Co., Hercules, CA, USA) in Tobwin buffer at 250 mA for two hours at 4°C. To block

unspecific sites, we incubated the membrane in a 0.3% phosphate-buffered saline (PBS)-Tween buffer (pH = 7.4) containing 1% casein and 0.3% gelatin for 60 min, followed by an overnight incubation with primary antibody (diluted to 20 ng/mL). Non-phosphorylated STAT-1 and -3, as well as polyclonal anti phospho-STAT-1 and phospho-STAT-3 antibodies, were also determined (Cell Signaling Technology, Danvers, MA, USA). Secondary antibody coupled to horseradish peroxidase was used at a concentration of 1 ng/mL, and incubated for 60 min at room temperature. Equal loading of protein was demonstrated by probing the membranes with a rabbit anti-lamina B1 or anti-β-actin polyclonal antibodies for nuclear or cytosolic assays, respectively. After incubations, membranes were washed extensively with 0.3% PBS-Tween buffer to reduce background. Membranes were incubated with a chemiluminescence kit following the manufacturer's instructions and exposed to photographic film. Images were captured with a GelDoc XR photo documenter and analyzed using Quantity One Software version 4.6 (Bio-Rad). Data were exported to GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, CA, USA) to calculate statistical significance.

ADH assay toward retinoids

The oxidation of retinol and reduction of retinal through ADH activity (as validated by their inhibition with 1mmol/L 4-methylpyrazole) was tested according to Parés and Juliá.[26]

Quantification of retinoids by HPLC

Liver levels of retinoids were measured using HPLC. Total liver homogenate (200 μL) was extracted with 2 mL of methanol/acetone 1:1 v/v). After centrifugation at 10,000 *g* for 10 min (4°C), the residue was suspended in 200 μL of methanol/dimetihyl sulfoxide (1:1 v/v). Samples were run and analyzed by HPLC, as described previously in detail.[17]

In vitro generation of cytosolic LP by-products and their effect on ADH activity

Liver cytosolic fractions from our experimental groups (5 mg of protein) were incubated in a final volume of 2 mL of 10 mmols/L Tris-HCl buffer (pH 7.4) for 90 min at 37°C in sealed tubes. Afterwards, incubation was stopped with the addition of perchloric acid (8% w/v, final concentration), and the samples were neutralized and placed in a buffer of 0.1 mol/L glycine (pH 9.7). Cytosolic ADH activity was determined in the presence or absence of added neutralized perchloric acid from the cytosolic fractions.

Calculations and statistics

ADH kinetic parameters were calculated through the non-linear Enzfitter program (1989), and the cytosolic and mitochondrial redox states were calculated as described in detail.[18] Statistical significance of the differences was assessed by two-way analysis of variance and by the Newman–Keuls test.

Results

Effects of VE treatment on production of ROS and on parameters indicative of rat liver regeneration

As previously reported for LP by-products, such as MDA and conjugated dienes,[2–4] we found a transient increase of DCF-detectable cytosolic ROS after PH, with two clear maximal peaks at six and 24 h postsurgery, respectively (Table 1). Whereas α-tocopherol had no effect on cytosolic ROS levels in the controls, it readily diminished ROS generation practically at all times tested in PH animals, except at the early peak (6 h) induced by surgery (Table 1). It has been previously reported that VE partially inhibits PH-induced liver regeneration.[4] Here, α-tocopherol administered to rats subjected to PH elicited a diminished TK activity (24 h), as well as mitotic index (48 h; Table 1), when compared with PH animals treated with the vehicle. However, an unexpected increase in liver TK activity was noted in PH animals treated with VE (6–12 h after surgery; Table 1). As previously reported,[4] we found an increase of basal liver content of α-tocopherol in animals given VE repetitively, when compared with sham-operated and PH rats without VE treatment (Table 1). After stopping pretreatment with VE in sham-operated rats, α-tocopherol levels gradually returned to basal levels; right before PH, the liver α-tocopherol content was 41.4 ± 4.2 nmol/mg of membrane protein, and drastically decayed with progression of restitution of liver mass (Table 1), which was normalized thereafter (Table 1). Therefore, it was clear that α-tocopherol metabolism is accelerated during PH-induced rat liver regeneration, which, in turn, is associated with decreased cytosolic ROS generation and TK activity, further supporting that VE inhibits rat proliferating liver after PH.

Table 1 Effect of PH and treatment with α-tocopherol on parameters indicative of liver regeneration

Treatment	TK activity	Mitotic index	Recovery of liver mass
Sham	0.25 ± 0.03	0.4 ± 0.1	–
PH (6 h)	0.24 ± 0.04	0.5 ± 0.1	–
PH (12 h)	0.29 ± 0.03	0.7 ± 0.1	2.0 ± 0.3
PH (24 h)	$5.90 \pm 0.33^{*}$	$11.9 \pm 1.3^{*}$	$12.3 \pm 1.4^{*}$
PH (48 h)	$3.35 \pm 0.36^{*}$	$17.6 \pm 3.7^{*}$	$29.1 \pm 5.2^{*}$
PH (72 h)	0.55 ± 0.15	5.0 ± 1.4	$46.5 \pm 7.0^{*}$
Plus treatment with α-tocopherol			
Sham	0.23 ± 0.02	0.3 ± 0.1	–
PH (6 h)	$1.20 \pm 0.13^{*,**}$	0.4 ± 0.1	–
PH (12 h)	$3.30 \pm 0.23^{*,**}$	0.5 ± 0.1	1.5 ± 0.3
PH (24 h)	$1.50 \pm 0.18^{*,**}$	$7.6 \pm 0.9^{*,**}$	$5.7 \pm 1.8^{*,**}$
PH (48 h)	$1.18 \pm 0.35^{*,**}$	$10.5 \pm 1.9^{*,**}$	$17.7 \pm 3.0^{*,**}$
PH (72 h)	0.53 ± 0.10	3.2 ± 0.6	$28.5 \pm 5.8^{*,**}$

PH, partial hepatectomy

Results are expressed as mean $\pm$ SE of six individual determinations per experimental group for thymidine kinase activity (*n* mols of formed $AMP \cdot min^{-1} \cdot mg^{-1}$ of cytosolic protein), and mitotic index as number of mitotic figures per microscopic field counted in 50 fields per slide. The gain of liver mass (recovery) is indicated as percentage (%). Statistical significance: $^{*}P < 0.01$ as compared with controls rats and $^{**}P < 0.01$ versus animals without treatment with α-tocopherol

Effects of α-tocopherol treatment on the expression of STAT-1 and -3 after 70% PH

We measured the time course and magnitude of the pattern for translocation of phosphorylated STAT-1 and -3 into nuclei (Figure 1), as well as their cytosolic pool (Figure 2). PH alone increased the nuclear amount of phosphorylated STAT-1 after 30 min postsurgery, and coincided with an elevated total amount of this STAT protein, remaining up to 60 min after PH and becoming normalized thereafter (Figure 1a). Levels of STAT-3 were increased, showing two clear peaks at 30 and 180 min after PH, respectively (Figure 1b), without significantly changing total STAT-3 content. Interestingly, α-tocopherol treatment to sham controls promoted higher values for phosphorylated STAT-1, which were even more increased in PH animals (Figure 1a). On the contrary, VE decreased both peaks for phosphorylated STAT-3 (30 and 180 min); in fact, α-tocopherol induced an increased activation of STAT-3 in control rats, which was quite similar in magnitude to that found in PH animals (Figure 1b). As to the cytosolic pool for STAT-1, the PH elicited much less changes in this protein than those found in its phosphorylated form, while that of STAT-3 did not change significantly after surgery (Figure 2). Treatment with VE promoted an increased cytosolic pool for STAT-1 protein at 60 min after PH, but the vitamin readily decreased total liver amount of STAT-3, except at the 60-min post-PH time (Figure 2). Densitometry analyses were adjusted with lamina B1 for the nuclear fraction and β-actin for the cytoslic fraction, as controls for protein load, respectively (Figures 1 and 2). Therefore, α-tocopherol did not significantly modify the expression of STAT-1, but it did increase its activation and translocation into the nucleus from the regenerating liver, while VE readily diminished both liver expression and activation of STAT-3, when administered to animals subjected to PH.

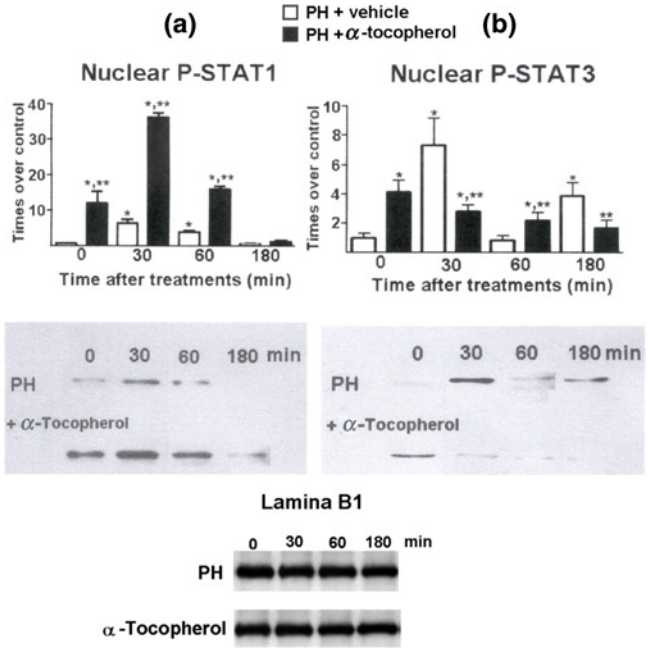

Figure 1 Representative results of immunoblotting and protein detection using antibodies to tyrosine-phosphorylated STAT-1 and -3 in isolated nuclear extracts. Protein loading was normalized using Western blot analysis for lamina B1, depicted at the bottom of the figure. Determinations were done during the first three hours after surgery. Densitometry analysis was performed for STAT-1 (a) and for STAT-3 (b). Symbols for the experimental groups as indicated at the top of the figure. $^{*}P < 0.01$ against the control (sham-operated) group and $^{**}P < 0.01$ versus the only-PH group. STAT, signal transducer and activator of transcription; PH, partial hepatectomy

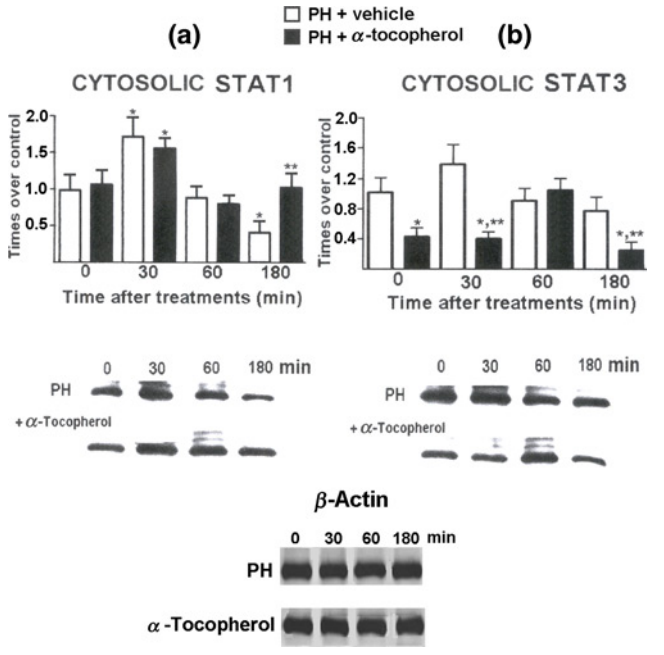

Figure 2 Representative results of immunoblotting and protein detection using antibodies against STAT-1 and -3 proteins. Protein loading was normalized using β-actin as standard, shown at the bottom of the figure. Determinations were done during the first three hours after surgery. Densitometry analysis was performed for STAT-1 (a) and for STAT-3 (b). Symbols and statistics as indicated in Figure 1. STAT, signal transducer and activator of transcription; PH, partial hepatectomy

Retinoid concentrations in the regenerating liver after α-tocopherol treatment

In an attempt to correlate changes on activation of STAT proteins with modifications of retinoid levels present in the regenerating liver, the concentrations of retinol, retinal and RA were quantified by HPLC. Administration of VE significantly decreased retinol while augmenting retinal concentrations in control animals (Figure 3). After 70% PH, retinoid concentrations were overall increased; retinol concentrations were initially diminished but they increased, reaching a peak at 24–48 h (Figure 3a). Retinal was increased earlier (12–24 h; Figure 3b), while RA levels temporally coincided with the retinol levels (Figure 3c). In PH animals treated with α-tocopherol, increases of retinoids were practically abolished. The PH-induced increase of liver retinol was reduced by α-tocopherol (Figure 3a), and retinal showed only a delayed peak after PH (Figure 3b). As for RA, α-tocopherol treatment flattened the increased levels induced by PH (Figure 3c). Thus, after the first 12 h after PH, retinol was increased and was thought to be

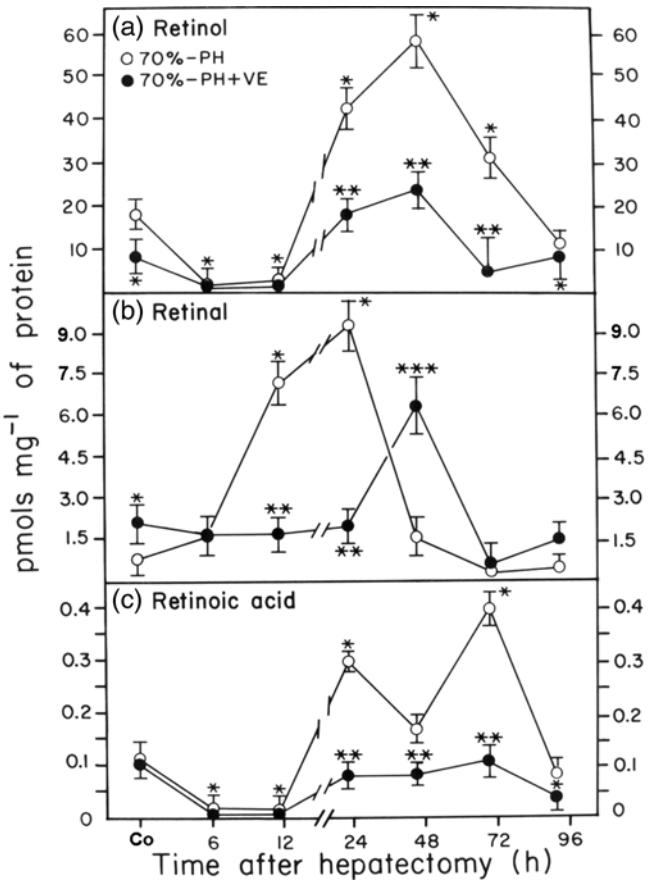

Figure 3 Results are the mean ± SE of six individual determinations for each experimental point. Retinoids (retinol, retinal and retinoic acid) were determined by HPLC in samples obtained from PH animals and those also treated with α-tocopherol. Symbols for the experimental groups as indicated at the top of the figure. Statistical significance: $^{*}P < 0.01$ against the control (sham-operated) group and $^{**}P < 0.01$ versus the only-PH group. PH, partial hepatectomy; VE, vitamin E; HPLC, high-pressure liquid chromatography

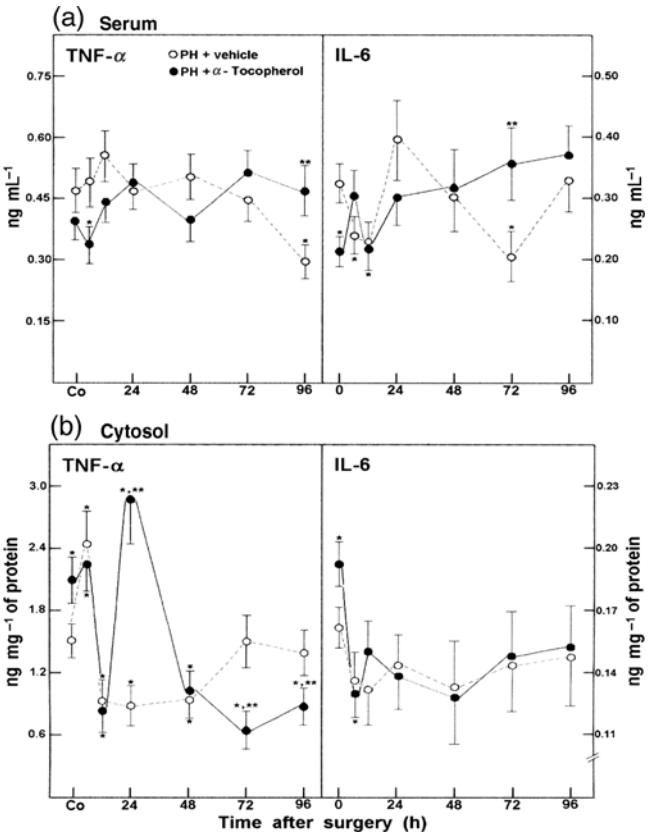

Figure 4 Results are the mean ± SE of six individual determinations for each experimental time point. (a) Serum concentrations of TNF-α and IL-6 in animals subjected to PH and in PH rats treated with α-tocopherol; (b) liver TNF-α and IL-6 concentrations for the same experimental groups. Symbols for the experimental groups and statistical significance as indicated in Figure 3. TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; PH, partial hepatectomy

oxidized to retinal, which is further converted to RA; administration of VE clearly delayed and diminished this sequential retinoid metabolism.

Serum and liver levels of TNF-α and IL-6 in the regenerating liver after VE treatment

VE did not modify the serum concentations of TNF-α, and, after surgery, α-tocopherol induced an early decrease in serum TNF-α that was normalized at later times (Figure 4a). In contrast, administration of VE in control rats elicited an augmented liver TNF-α level; PH promoted an early peak for liver TNF-α content (6 h) that rapidly normalized thereafter (Figure 4b). Pretreatment with α-tocopherol elicited a mirror image for liver TNF-α fluctuations throughout the surgical times, when compared with animals subjected to PH only (Figure 4b). As for IL-6, serum levels of this protein moved oppositely to those of TNF-α, during the first 12 h after surgery, and normalized up to 24 h post-PH; however, a second decrement was noted at 48 h after surgery (Figure 4a). On the contrary, liver levels for IL-6 were not significantly modified by PH (Figure 4b). Pre-treatment with VE indeed changed the PH-induced levels of IL-6 in animals subjected to PH; α-tocopherol readily diminished serum availability of IL-6 in controls, while, after surgery, it elicited a mirror image pattern when compared with animals subjected to PH only (Figure 4a). In contrast, PH-rats treated with VE had increased levels of liver IL-6, but the subsequent profile after surgery was not significantly different than that seen in PH animals receiving the VE vehicle (Figure 4b). α-Tocopherol induced a delayed increase in serum levels of both TNF-α and IL-6, but whereas VE had no effect on the liver content for IL-6, it did alter the liver TNF-α level during progress of the regenerating process.

ADH activity toward retinol oxidation and retinal reduction in the regenerating liver after α-tocopherol treatment

Since we have previously shown that activity of ADH, a cytoplasmic NAD-dependent enzyme, is largely affected by PH,[17] we compared ADH activity towards oxidation and/or reduction of retinol and retinal in remnant livers after PH. Figure 5 shows cytosolic ADH activity towards retinol oxidation (with NAD+), as well as to retinal

reduction (in the presence of NADH). In the controls, VE did not significantly change liver ADH activity towards retinoid, but after 70% PH, oxidation of 70 μmol/L retinol was progressively increased up to 24 h, normalizing thereafter, at later times postsurgery (72–96 h; Figure 5a). Reduction of 25 μmol/L retinal was also enhanced but only at 12 h post-PH (Figure 5b). α-Tocopherol administration blocked the first peak for retinol oxidation, shifting at 48 h the maximal ADH activity towards oxidizing retinol (Figure 5a); in contrast, VE induced a more sustained increase in retinal reduction in PH animals (Figure 5b). Correlating with liver content for retinoid after PH (Figure 3), α-tocopherol partially inhibited the ADH-mediated oxidation of retinol to retinal, while the backwards reaction (retinal reduction) was much less affected by VE in the regenerating liver.

Changes in the cell redox (NAD/NADH) state in the regenerating liver after α-tocopherol treatment

Administration of VE did not significantly modify cell redox state (cytoplasmic and mitochondrial) in sham-operated control rats (not shown). PH and treatment with α-tocopherol elicited changes in liver concentrations of LAC and PYR, thus, modifying the cytoplasmic redox NAD/NADH potential (Figure 6). In animals subjected to PH, we found a reduced cytoplasmic redox state (6 h post-PH, represented by a higher LAC/PYR ratio and a consequent decreased NAD/NADH ratio), which returned to the control value and became again reduced at 24 h after PH, represented by a lowered NAD/NADH ratio up to 72 h (Figure 6a). However, in PH-rats also receiving VE, the drastic reduction of the cytopasmic redox state was progressively normalized (Figure 6a). As to the liver mitochondrial redox state (B-OH-B/AcAc redox-pair; Figure 6b), PH induced an opposite pattern when compared with the cytosolic redox state, since an increased mitochondrial NAD/NADH ratio was found (first 72 h post-PH; Figure 6b). Again, α-tocopherol administration to PH animals elicited a mirror image, restoring the mitochondrial redox state earlier (Figure 6b). Data might indicate that VE drastically altered the PH-induced modifications on cell redox state, like a more reduced cytoplasmic linked to a more oxidized mitochondrial redox state, which seems to play an essential role on progression of liver regeneration.

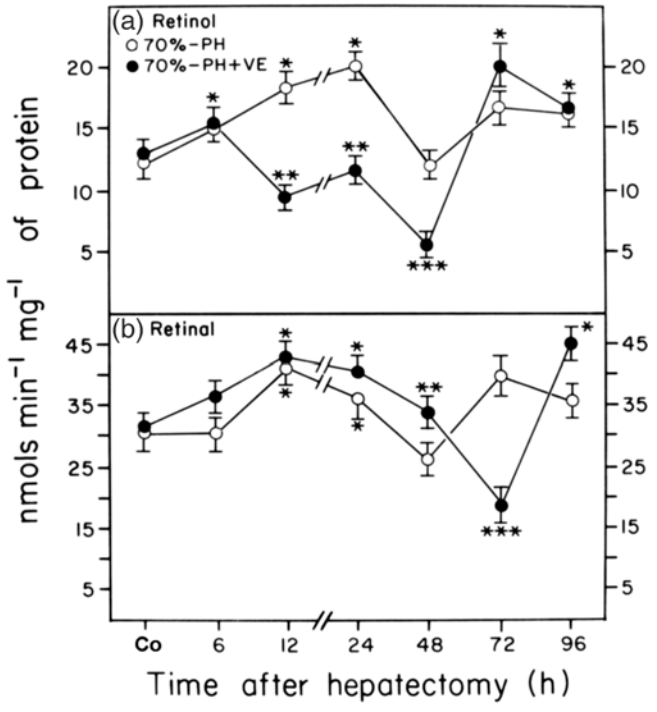

Figure 5 Results are the mean ± SE of six individual determinations for each experimental time point for PH animals and those also treated with α-tocopherol. Statistical significance: $^{*}P < 0.01$ against control group, $^{**}P < 0.01$ versus the PH group without vitamin E administration and $^{***}P < 0.01$ against control group and the group with PH only. PH, partial hepatectomy; VE, vitamin E

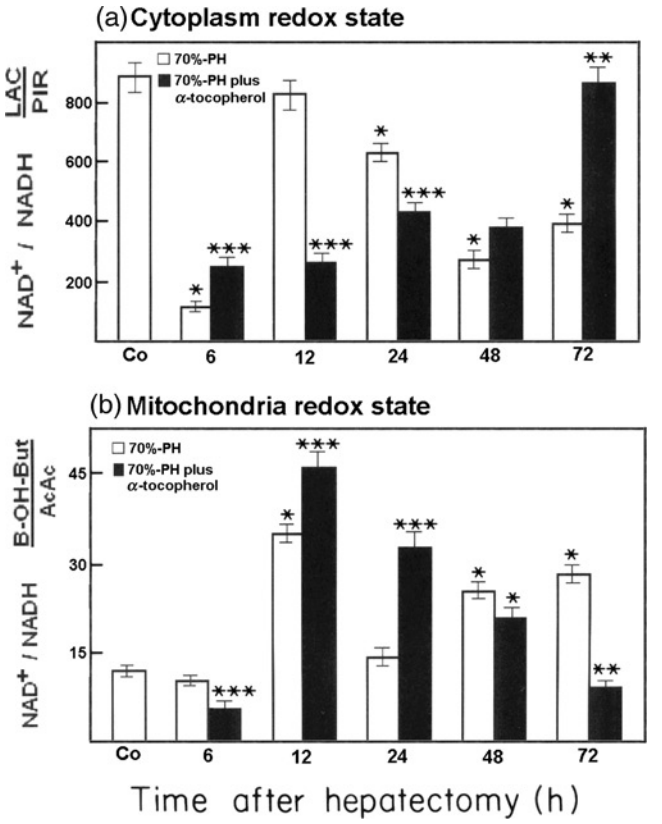

Figure 6 (a) Cytoplasmic and (b) mitochondrial NAD+/NADH redox ratio, calculated from the lactate (LAC)/pyruvate (PYR) and β-hydroxybutyrate (B-OH-But)/acetoacetate (AcAc) ratios, respectively. Each bar is the mean ± SE of six independent experiments for each time point. Symbols at the top of the figure. Symbols for the experimental groups and statistical significance as indicated in Figure 5. PH, partial hepatectomy

Caspase activities in cytosol from the regenerating liver after VE treatment

Unexpectedly, the administration of α-tocopherol to control (sham) animals induced an increase of caspase-9 activity, without affecting the other caspases tested (Figure 7). On the other hand, shortly after PH (6 h), we recorded a sharp peak of increased activity for the three caspases, of

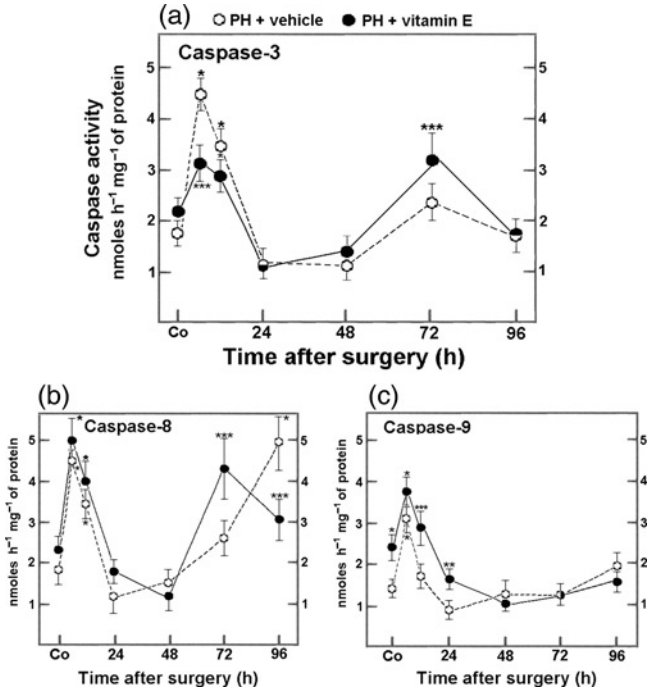

Figure 7 Results are the mean ± SE of six individual determinations for each experimental time point. Symbols for the experimental groups at the top of the figure, for caspase-3 (a), caspase-8 (b) and for caspase-9 (c). Statistics as indicated in Figure 5. PH, partial hepatectomy; VE, vitamin E

which caspase-3 was the most enhanced; this activity remained high 12 h after PH and then decreased to the control value (Figure 7a). A similar pattern of caspase-9 activity was noted after PH (Figure 7c), whereas that of liver caspase-8 (extrinsic apoptotic pathway) showed a biphasic profile, reaching a second and significant peak of activity 96 h post-PH (Figure 7b). Pretreatment with α-tocopherol for PH animals largely diminished the early peak of caspase-3 activity (6 h) induced by PH, while increasing that activity at 72 h after surgery (Figure 7a). During the first 48 h post-PH, VE did not modify the surgery-induced pattern for caspase-8 activity, but promoted a mirror image thereafter (Figure 7b). Moreover, VE increased the control caspase-9 activity, which was also found to be higher within 12–24 h after PH, when compared with the group subjected only to surgery, an effect that declined to the control range thereafter (Figure 7c). Despite the high rate of cell proliferation that characterizes PH-induced liver proliferation, early activation of caspases was observed; here, the antiapoptotic effect ascribed to α-tocopherol was noted, at least in the early activation of caspase-3.

Effects of LP by-products on ADH activity in the regenerating liver after VE treatment

We assessed the putative effects of cytosolic LP by-products (MDA) on ADH activity. First, we incubated cytosolic fractions from our experimental groups, and used the extracted soluble non-protein components generated during this incubation. Cytosolic extracts from PH rats inhibited the control cytosolic ADH activity by up to 80%, whereas only a 40% inhibition was achieved with cytosolic extracts from PH animals treated with α-tocopherol (Figure 8). Determination of TBARS in these extracts showed an inverse correlation between cytosolic ADH activity and TBARS present in this subcellular fraction, which was more evident with the *in vivo* α-tocopherol treatment (Figure 8). We further confirmed the influence of MDA on ADH activity (Figure 9). Through Dixon plots, we found that ADH-mediated ethanol oxidation was competitively inhibited by MDA in controls, with an apparent K_i of 245 μmol/L (Figure 9), whereas in PH-rats, the ADH activity was non-competitively inhibited ($K_i = 150$ μmol/L; Figure 9). Interestingly, in PH rats treated with VE, the inhibition of ADH activity by MDA was similar to that found in control preparations (apparent K_i of 220 μmol/L; Figure 9b), except that the inhibition type was still non-competitive. Therefore, it seems that a cytosolic product generated during progression of PH-induced liver regeneration is able to inhibit ADH activity towards oxidation of alcohols (including retinol). Since *in vivo* administration of α-tocopherol avoided the PH-induced inhibition of ADH activity, avoiding *in vitro* production of MDA, the latter could be an important factor involved in retinoid metabolism mediated through hepatic ADH activity.

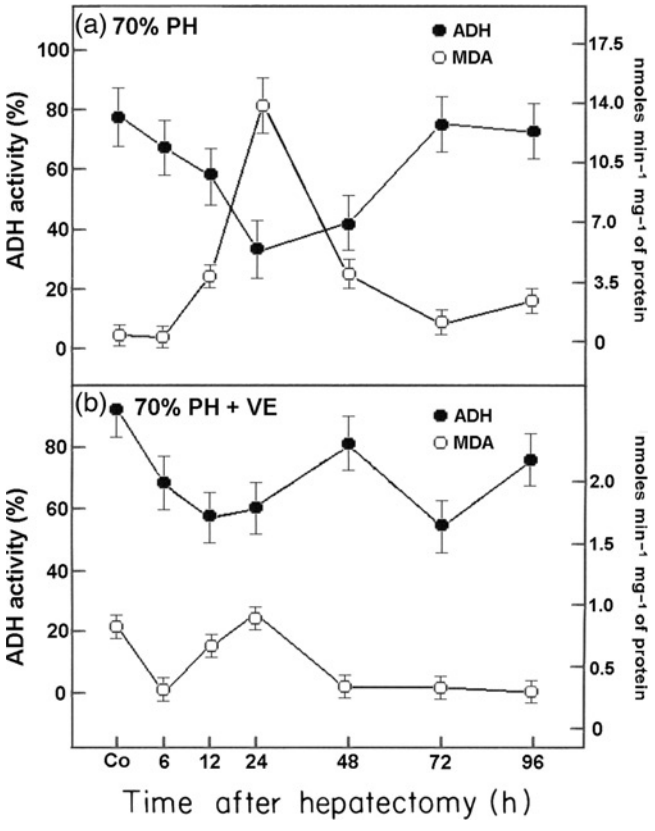

Figure 8 Results are the mean ± SE of six individual determinations for each experimental time point of crude ADH-containing cytosolic fractions from control animals, and incubated with non-protein extracts from cytosol obtained from PH animals (a) and those also treated with α-tocopherol (b). The activity of ADH and the amount of LP derived by-products are indicated by symbols at the top of each panel. PH, partial hepatectomy; VE, vitamin E; ADH, alcohol dehydrogenase; LP, lipid peroxidation; MDA, malondialdehyde

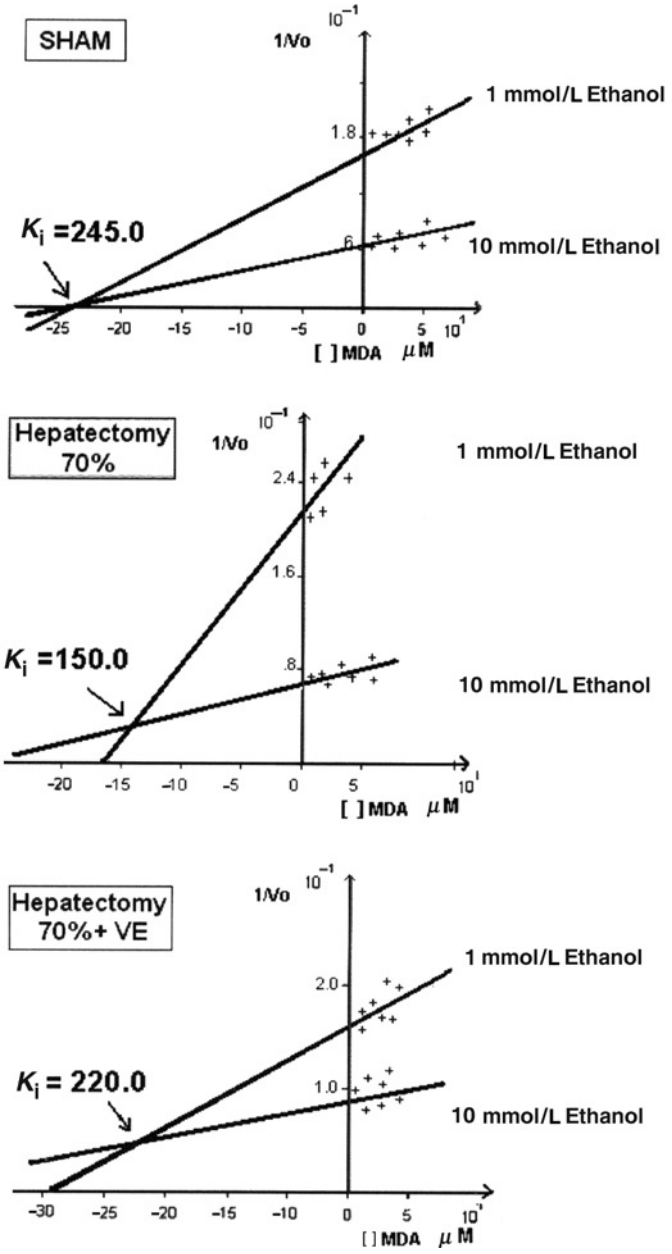

Figure 9 Representative plots resulting from the mean ± SE of six individual determinations for each experimental group: sham (upper panel), PH + vehicle (middle panel) and for PH + α-tocopherol (lower panel) at 24 h after treatments. The Vo (rate) is expressed as nmols min^{-1} mg^{-1} of protein and symbols at the top of the figure and the apparent inhibition constant (K_i) is indicated by an arrow in each case. Note the competitive-like inhibition in preparations from control animals and non-competitive inhibition in preparations from 70% PH animals with or without treatment with α-tocopherol. PH, partial hepatectomy; VE, vitamin E; MDA, malondialdehyde

Discussion

Liver regeneration must undergo changes in major metabolic pathways in order to achieve DNA replication, cell division and restitution of the liver mass.[27] Since the liver is continuously exposed to metabolic products of potential reactivity with unsaturated fatty acids, like ROS, its involvement becomes relevant in the metabolic adjustment of the proliferating liver.[28] We have shown that LP increases selectively in subcellular fractions from the rat regenerating liver, playing a role during the progression of proliferation,[2] which might initiate a general cell-responsive mechanism, activating transcriptional factors to serve as signal transducers.[29]

High doses of ROS are clearly toxic, but the effects of low doses are less clear. Indeed, low levels of ROS have been involved in normal biological processes as putative controlling factors in cell homeostasis,[30] and exposure to it can actually increase the growth response of mammalian cells.[31] The balance between concentrations of ROS is critical for the cell, since pro-oxidant conditions might result in reduction of cellular growth.[32] ROS can regulate activation of NF-κB, which in turn regulates expression of some genes involved in tumorigenesis, such as antiapoptotic genes, bcl-2 and cyclin D1.[33] ROS scavengers can suppress cell proliferation in different systems.[34] Decreasing the magnitude and/or time course of PH-promoted enhanced LP by *in vivo* treatment with α-tocopherol could promote an early termination of prereplication cell events, thus, inhibiting liver proliferation,[4] and it also delays the recovery of the gastric epithelium and restoration of gastric mucosa in an experimental model of ethanol-induced gastritis in rats.[6] Here, it was clear that VE administered to rats undergoing PH led to a decreased ROS production in cytosol, which was associated to inhibited restitution of liver mass (Table 1).

However, α-tocopherol increased earlier TK activity in PH animals (Table 1), which correlates with an anticipated increase in the expression of cyclin D1 and of PCNA after PH.[4] In fact, many of the effects of α-tocopherol occurring within the first 24 h after surgery on the parameters tested can be attributed to its antioxidant action, which was also supported by the active consumption of VE by the regenerating liver. However, the significant effects of α-tocopherol on PH-induced rat liver regeneration beyond 24 h could highlight the relevance of early changes in the oxidative status of the PH-induced rat proliferating liver, since VE administration might induce a 'premature' but ineffective proliferative response. In fact, the α-tocopherol metabolite, carboxy ethyl hydroxy chromanol, also exerts effects on liver tissue, including regulation of multidrug resistance genes, cytochrome P450s and ATP-binding-cassette transporters.[35]

Administration of α-tocopherol prior to 70% PH in rats induced disturbed patterns of translocation of phosphorylated STAT-1 and -3 proteins into the nucleus, without significant changes in the total liver pool for these proteins, and also coincided with an almost abolishment of RA production (Figures 1–3). In fact, quickly after an insult, such as PH, liver early genes are activated immediately by transcription factors such as NF-κB, AP1 and STAT-3, events that approximately last four hours.[36] Reduced liver mass causes increased serum TNF-α levels followed by increased IL-6 and downstream STAT-3 activation, which enable hepatocytes to progress into cell proliferation.[12] In addition, STAT-1 might still play a role in IL-6-type signaling in cell types deficient of STAT-3, inducing formation of the STAT-1/STAT-1 homodimer and significantly mediating gene response, in an opposite fashion to activated STAT-3.[37] Therefore, these targets could be underlying the

inhibitory effect of α-tocopherol in the PH-induced rat proliferating liver. The effects of α-tocopherol on STAT expression, activation and their translocation to the nucleus, could be attributed to a disruption of an unknown ROS-controlled mechanism; however, other possibilities must be considered. RA stimulates interferon-γ, which activates STAT-1 protein in cultured cells that might activate mitogen-activated protein kinases.[38] Conditional deletion of STAT-3 in both hepatocytes and myeloid cells results in elevated activation of STAT-1 and apoptosis of hepatocytes, and a dramatic reduction in survival after PH.[39] Hence, a quite similar liver pattern for STAT activation was found here, through administering α-tocopherol to animals undergoing 70% PH.

TNF-α can be the main mediator of NF-κB activation that acts in a synergistic manner with STAT-3 expression in hepatocytes and non-parenchymal liver cells, priming hepatocytes to respond to hepatocyte growth factor.[40] Moreover, liver oxidative stress triggers DNA-binding activity of NF-κB, with the consequent increase in the expression of NF-κB-dependent genes for TNF-α and for IL-1α.[41] Interestingly, treatment with α-tocopherol to rats undergoing PH elicited an increased basal liver TNF-α amount, further altering its pattern elicited by PH (Figure 4). Indeed, disruption of STAT-3-mediated intracellular signal transduction pathways might lead to disturbed liver regeneration and repair and increased apoptosis.[39] Marked reduction in cyclin D1 and PCNA levels is accompanied by down-regulation of bcl-2 and augmented titers of activated caspase-3.[42] In agreement with this, α-tocopherol decreases STAT-3 nuclear levels (Figure 1), cyclin D1 and PCNA,[4] prolonging caspase activities, mainly that found in caspase-3 (Figure 7), indicating that α-tocopherol inhibits cell proliferation and favors apoptotic events in the rat proliferating liver. These data also strongly support that α-tocopherol can exert a dual effect of inhibiting proliferation or stimulating cell death *in vivo*,[43] and agree with the fact that some chemopreventive agents with pro-oxidant properties (such as α-tocopherol, among others) cause DNA damage via generation of ROS in the presence of metal ions and endogenous reductants.[44]

Important changes in the liver cell redox state are involved in stimulated cell proliferation linked to metabolic stress, as proven in tumor-bearing tissue.[45] Moreover, a PH-induced shift to a more reduced cytosolic redox state (Figure 5), probably resulting from energy-dependent processes, depends on the transient increases in both portal and arterial blood flows.[46] Since the ADH is a NAD+-dependent dehydrogenase whose activity is highly regulated by the cytoplasmic NAD+/NADH redox state, the PH-induced changes in this parameter (Figure 6) could favor reduction of retinal to retinol and α-tocopherol reverted both phenomena (Figures 5 and 6). Hence, we propose that changes in liver metabolism of retinoids, influenced by the NAD+-dependent ADH activity and, therefore, by the cell redox state, appear to be important for the progression of PH-induced rat liver regeneration, and blocking RA production by α-tocopherol might, consequently, lead to an altered expression of STAT proteins. In this context, RA potently inhibits more proliferation of hypoxic melanoma cells than normoxic cells, diminishing oxidative states, providing a molecular basis for an inverse correlation between RA receptor activity and ROS levels.[47]

Questions arise as to how cytosolic levels of ROS and LP by-products are regulated in order to achieve a transient and strictly controlled enhancement, and how this controlled oxidant stress influences STAT protein activation and retinoid metabolism. Previous work indicates that ADH is a cytosolic enzyme capable of being influenced by LP products, and we proposed that this enzyme plays a role in controlling PH-induced rat liver regeneration.[17] Here, we now show a close inverse relationship between LP by-products generated *in vivo* and *in vitro* and the activity of ADH towards exogenous (ethanol) or putative endogenous (retinoids) substrates (Figures 8 and 9). In addition, we could reproduce the inhibition of ADH-mediated ethanol oxidation by adding *in vitro* increasing concentrations of MDA (Figure 9). ADH activity has already been associated to metabolic characteristics of tumoral cells,[48] and here it was clear that α-tocopherol blocked the PH-induced effects on ADH kinetics. Coincidently, the PH-induced changes in retinoid's metabolism were almost blunted by administering α-tocopherol to PH-rats, suggesting that ADH could use retinoids as significant endogenous substrates (Figures 8 and 9). It was interesting to find that kinetics of MDA inhibition on ADH activity were different when PH animals were treated *in vivo* with VE (Figure 9). These data provide further support to our suggestion that there is a correlation between ADH activity and progression of PH-induced liver compensatory growth.[17] In fact, we think that after 70% PH, proliferating liver cells display lower ADH activity towards ethanol and acetaldehyde, which could correspond to a non-competitive inhibition, probably due to the participation of increased substrates, such as retinoids. Therefore, it seems that the ADH contained in our cytosolic preparations could have different confirmation depending on 'endogenous substrates' probably linked to its catabolic domain. Also, depending on the nature and amount of such putative substrates, the kinetics of competition (inhibition) would be modified, as clearly shown in Figure 9.

The inhibitory effects of α-tocopherol on PH-induced rat liver regeneration seemed to be due to a kind of modulation of cell signaling pathways, through regulating the rate of ROS generation; however, besides being a powerful antioxidant, VE could have other effects that are still uncharacterized. Indeed, there are several issues that clearly have shown that VE can exert a controlling role in the expression or activity of proteins acting in cell signaling cascades. Some of the remarkable actions of VE on cell signaling, such as protecting the production of nitric oxide, prostaglandins and mainly products from the protein kinase activity, could influence the rate of cell proliferation. Therefore, present data could suggest that VE actions on cell metabolism and proliferation go beyond its antioxidant function.[49]

In conclusion, we found that pretreatment with α-tocopherol was capable of increasing the activation and translocation of STAT-1 and -3 proteins, also increasing liver retinal levels and diminishing those of retinol in control rats (sham-operated); moreover, the activity of

caspase-9 was also enhanced. However, in the case of PH-induced proliferating rat liver, VE elicited an altered pattern of activation and nuclear translocation of the STAT proteins tested and inhibited retinoid metabolism, events probably involved in the α-tocopherol-induced inhibition of rat liver regeneration, and related to a blockade on cell redox state elicited by PH. These events were also accompanied by altered profiles for TNF-α, IL-6 and caspase-3 activity. These results might indicate that a controlled increase of ROS can be involved in changes of redox state, ADH activity, modifications of retinoid metabolism and activation of STAT proteins in proliferating cells, with minor effects on those cells maintained in a quiescent state.

Author contributions: All authors participated in the design, interpretation of the studies, analysis of the data and review of the manuscript. LS-S and EM-C conducted most of the experiments and RH-M and LS-S wrote the manuscript.

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