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Original Contribution

α-Tocopherol administration blocks adaptive changes in cell NADH/NAD$^+$ redox state and mitochondrial function leading to inhibition of gastric mucosa cell proliferation in rats

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ABSTRACT

In experimentally induced chronic gastritis, a compensatory mucosal cell proliferation occurs with enhanced glucose oxidative metabolism linked to lipoperoxidative events. Therefore, this study was aimed at assessing the participation of cell NAD/NADH redox state and mitochondrial functions during gastric mucosa proliferation and the effects of in vivo α-tocopherol (vitamin E) administration. Glucose oxidation and oxygen consumption were tested in gastric mucosa samples obtained from rats with gastritis and from those also treated with α-tocopherol. Gastric mucosal mitochondria were isolated and structural and functional parameters were determined. Succinate oxidation, ADP phosphorylation, mitochondrial enzyme activities, and membrane lipid composition were measured. In addition, parameters indicative of cellular NAD/NADH redox state, proliferation, apoptosis, and nitric oxide (NO) metabolism were also determined. After ethanol withdrawal, the damaged gastric mucosa increased glucose and oxygen consumption, events associated with a more reduced cytoplasmic NAD/NADH ratio. Enhanced mitochondrial oxidative phosphorylation and increased mitochondrial enzyme activities occurred early, accompanied by recovery of lost mitochondrial protein and lipid composition in the gastric mucosa, events associated with increased NO production. When mitochondrial function and structural events were normalized, apoptosis was initiated as assessed by the mitochondrial Bax/Bcl2 ratio. Treatment with α-tocopherol inhibited cell proliferation and blocked enhanced glucose utilization, mitochondrial substrate oxidation, and changes in redox state, delaying the onset of these adaptive metabolic changes, whereas it inhibited cell proliferation. In conclusion, α-tocopherol could abolish damage-induced "stress" signaling by desynchronizing mitochondrial adaptive responses, including mitochondria biogenesis, and consequently NAD/NADH redox, which seems to regulate gastric mucosal cell proliferation.

Introduction

In animals, reactive oxygen species (ROS) may influence cell proliferation, cell death (either apoptosis or necrosis), and the expression of genes and may be involved in the activation of several signaling pathways, activating cell signaling cascades, such as those involving mitogen-activated protein kinases [1]. Most of these ROS are produced at a low level by normal aerobic metabolism, mainly through mitochondrial metabolism, constantly repairing the ROS-induced cell damage [2].

There is evidence indicating mitochondrial function as a target for inducing gastric damage. Indeed, aspirin alone or combined with sodium bicarbonate promotes gastric erosions and induces disturbances in oxidative and biosynthetic functions [3], and salicylic acid and its derivatives readily elicit uncoupling of oxidative phosphorylation, in vitro [4]. In this context, indomethacin induces gastric mucosa ulcerations associated with increased myeloperoxidase activity and expression of the proinflammatory TNF-α, NF-κB, and JNK MAP kinase, where mitigation of TNF-α may ameliorate indomethacin-mediated gastropathy [5]. Despite the mechanisms of chemically induced tissue injury being still poorly understood, it is accepted that rapid epithelial repair after injury is a highly ATP-dependent process. However, little is known about the metabolic pathways required for this restitution [6]. It is known that high concentrations of ethanol are capable of damaging the gastric mucosa in vivo [7], promoting epithelial exfoliation, episodes of ischemia–reperfusion, augmented ROS generation, and alterations in cell redox state [8]. In addition,

Abbreviations: CD, conjugated diene; Cox, cytochrome *c* oxidase; IDH, isocitrate dehydrogenase; LP, lipid peroxidation; MDH, malate dehydrogenase; ROS, reactive oxygen species; SDH, succinate dehydrogenase; RCR, respiratory control ratio; TCA, tricarboxylic acid

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50% ethanol decreases levels of endogenous antioxidants and viability of isolated gastric mucosa cells, which is probably associated with mitochondrial disturbances [9].

Taking advantage of the aforementioned, we have developed a model of chronic mucosal injury induced by continuous ethanol ingestion, which is characterized by marked alterations in plasma membranes from gastric mucosa [10]. Increased oxygen free radicals and lipid peroxidative levels were also detected, but a further increased lipid peroxidation (LP) was distinctly separated from cell necrosis and rather linked to a coincident increased rate of cell proliferation [11]. Moreover, glucose oxidation was greatly enhanced in the injured mucosa, as well as oxygen consumption and acid secretion; a coupling between secretory and metabolic effects induced by ethanol was found. These effects were accompanied by a stimulated Ca^{2+} uptake by mucosal minces, and increased in vivo Ca^{2+} levels in cytosolic and mitochondrial fractions were also noticed. In addition, enhanced glucose and oxygen consumption was associated with higher ATP and $NADP^+$ availability, whereas the cytosolic NAD/NADH ratio was significantly reduced at the onset of gastritis [12]. In this context, acid secretion has been proven to be dependent on mucosal oxidative metabolism, which provides ATP for driving H^+-K^+-ATPase activity [13,14]. Acid secretion increases the levels of glycolytic intermediates including pyruvate, lactate, and fructose 1,6-diphosphate, as well as those of 6-phosphogluconate and ribulose 5-phosphate, also increasing most tricarboxylic acid (TCA) cycle intermediates. Interestingly, the NAD/NADH ratio is decreased in the cytoplasm, but enhanced in mitochondria [15]; thereby acid secretion seems to be dependent on TCA cycle activity and glycolysis, indicating a major role for mitochondria in the metabolism of gastric mucosa [16]. Therefore, redox-dependent processes influence most cellular functions, such as differentiation, proliferation, and apoptosis. Mitochondria are at the center of these processes, as mitochondria both generate ROS that drive redox-sensitive events and respond to ROS-mediated changes in the cellular redox state. Moreover, mitochondrial function and these redox modifications have an impact on metabolism, mitochondrial biogenesis, receptor-mediated signaling, and apoptotic pathways [17].

We have found an important role for ROS and LP in controlling cell proliferation and apoptosis rates during ethanol-induced gastritis in rats [18]. In fact, in vivo administration of the antioxidant α-tocopherol, a form of vitamin E, inhibited the compensatory cell proliferation that follows chronic rat gastric mucosa damage [18]. Here, α-tocopherol enhanced endogenous antioxidants while affecting translocation of active cyclin D1 within the nucleus, leading to a predominance of apoptotic events in ethanol-induced gastric damage [18].

Therefore, this study was undertaken to investigate the possible role of changes in cell redox state and adaptive mitochondrial changes in the modifications of gastric mucosa oxidant status, as well as the effects of oral administration of α-tocopherol in these adjusting mechanisms, which we think are essential for the compensatory hyperplasia that characterizes our experimental model of ethanol-induced gastric damage in rats.

Materials and methods

Animal model

The present model of chronic ethanol-induced mucosal injury in male Wistar rats (230–270 g body weight) has been reported previously in detail [10]. Briefly, animals fed a diet of lab chow and water ad libitum were subjected to overnight fasting with free access to water and received one intragastric gavage of 1 ml of saline solution or 1 ml of 50% ethanol (gastritis induction). Thereafter, rats were housed in individual cages with free access to food and water or 10% ethanol in water (gastritis group); treatment was continued for 5 days, and on the 5th day ethanol was withdrawn (*T*0). Experimental groups were: (1) gastritis, animals treated intragastrically with 1 ml of vegetable oil (vehicle) one to four times, and (2) plus vitamin E, animals receiving a daily intragastric administration of 100 IU/kg body wt of the α-tocopherol form of vitamin E (Sigma Chemical Co., St. Louis, MO, USA) under the same treatment schedule. In addition, animals not treated with ethanol but receiving α-tocopherol were run simultaneously. Animals were killed by decapitation after an overdose with sodium pentobarbital. All procedures were done according to the *Federal Regulations for Animal Care and Experimentation* (Ministry of Agriculture, SAGARPA).

Glucose oxidation

The rate of glucose oxidation by gastric mucosa samples, through pentose phosphate and glycolysis–TCA pathways, was estimated comparatively by measuring $^{14}CO_2$ production from 10 mmol/L glucose containing either D-[1-^{14}C]glucose or D-[6-^{14}C]glucose as previously described, in detail [12].

Isolation of gastric mucosa mitochondria and measurement of oxygen consumption

The stomach was removed and dissected along the lower curvature and the gastric mucosa was totally excised. Mitochondria isolation was performed through an established protocol for liver samples [19]: whole gastric mucosa was homogenized (1/5 w/v) with a buffer solution (pH 7.4) containing 225 mmol/L sucrose, 10 mmol/L Tris–HCl, 0.3 mmol/L EGTA, and 0.2% fatty acid-free bovine serum albumin (BSA). The mitochondrial pellet was obtained by differential centrifugation, according to the method described by Hogeboon and Schneider [20]. Mitochondrial respiration and ADP phosphorylation were recorded polarographically with a Clark-type electrode (YSI, Yellow Springs, OH, USA) in 3 ml of medium containing 225 mmol/L sucrose, 10 mmol/L Tris–HCl, 10 mmol/L KCl, 7 mmol/L $MgCl_2$, 0.4 mmol/L EDTA, 7.5 mmol/L $KHPO_4$, and 0.05% BSA, plus 10 mmol/L sodium succinate or 10/1 mmol/L sodium glutamate/malate, as substrates (State 4). Mitochondrial State 3 was initiated by adding of 250 μmol/L ADP [21].

Determination of mitochondrial enzyme activities

In isolated mitochondria from our experimental groups, the following enzymatic activities were measured: cytochrome *c* oxidase (Cox; EC 1.9.3.1) was determined by the method described by Rafael [22] and succinate dehydrogenase (SDH; EC 1.2.1.16) according to the technique of King [23]. The activity of two mitochondrial matrix enzymes, malate dehydrogenase (MDH; EC 1.1.1.37) and isocitrate dehydrogenase (IDH; EC 1.1.1.42), was assessed by methods reported elsewhere [24,25].

Assays for lipid content, lipid peroxidation, and protein carbonyl groups

Mitochondrial levels of ROS were estimated through the method described by Viarengo et al. [26], using the fluorescence signal generated by ROS reacting with 2′,7′-dichlorodihydrofluorescein diacetate (Molecular Probes). Mitochondrial membrane LP-related CDs were determined as previously described [19], whereas protein carbonyl content in plasma membranes, an index of oxidative damage, was estimated according to Levine et al. [27]. In extracts of total lipids, obtained from isolated mitochondrial membranes, the amount of total phospholipids was quantified according to the method of García-Saínz and Fain [28] and

cholesterol was determined by the colorimetric method reported by Abell et al. [29].

Determination of citrulline, nitrites, lactate, and pyruvate from gastric mucosal samples

We prepared perchloric acid extracts (200 mg of tissue, w.w. in 3 ml of 8% of perchloric acid w/v) from homogenates obtained from gastric mucosa samples of our experimental groups. In these extracts, nitrites were quantified by the Griess reaction [30], and citrulline was quantified as described by Ceriotti [31]; both metabolites were used as an index of NO production. In addition, through enzymatic methods [32], lactate and pyruvate were determined in neutralized acid extracts and used to calculate the cytoplasmic redox state [32].

Western blot analysis of cytosolic and mitochondrial Bax/Bcl2

Equal amounts of gastric mucosa cytosol and mitochondria proteins were separated by SDS–PAGE on a 12% (w/v) polyacrylamide gel, followed by blotting onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After blotting, nonspecific sites were blocked with Tris-buffered saline–Tween buffer containing 5% fat-free milk, and nitrocellulose membranes were incubated with either monoclonal anti-Bcl2 (0.2 μg/ml mouse monoclonal IgG) or monoclonal anti-Bax (0.2 μg/ml, mouse monoclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 2 h at room temperature, washed, and incubated with secondary horseradish peroxidase-conjugated anti-mouse (0.2 μg/ml) for 2 h at room temperature. Membranes were incubated with ECL chemiluminescence reagents and exposed to photographic film, and a densitometry analysis was done with Quantity One software 4.6.0 (Bio-Rad).

Calculation of mitochondrial recovery and protein amount

Mitochondrial recovery (yield) was calculated using a marker enzyme, namely Cox. From here, the organelle yield was calculated from the ratio between the specific activity of Cox in the homogenate and that in the subcellular fraction [33].

Statistics

All results are expressed as the mean ± SEM; significance of the differences among groups was assessed by two-way ANOVA and, in the case of significance, by a post hoc Newman–Keuls analysis.

Results

Effect of α-tocopherol on cytoplasmic NAD/NADH redox state in gastric mucosa from animals subjected to gastritis

The levels of lactate and pyruvate were estimated in acid extracts obtained from gastric mucosa of our experimental groups (Fig. 1). In controls, the lactate/pyruvate ratio was 20.7 ± 2.4 and, from here, a calculated NAD/NADH ratio of 462 ± 47 was obtained (Fig. 1). At the onset of gastritis, there was a significant increase in the lactate/pyruvate ratio and a consequent decrease in the NAD/NADH ratio, compared with controls. During the recovery period, these ratios were normalized and at 72 h after ethanol withdrawal, an enhanced NAD/NADH was noted, which returned within the normal range thereafter (Fig. 1). In contrast, mucosa samples from those animals also treated with α-tocopherol had higher lactate levels, without modifying significantly the pyruvate content (Fig. 1). This led to an increased lactate/pyruvate ratio and a consequent diminished NAD/NADH ratio, along the 96 h of the recovery period (Fig. 1), indicating a more reduced cytoplasmic redox state in gastric mucosa from animals treated with α-tocopherol.

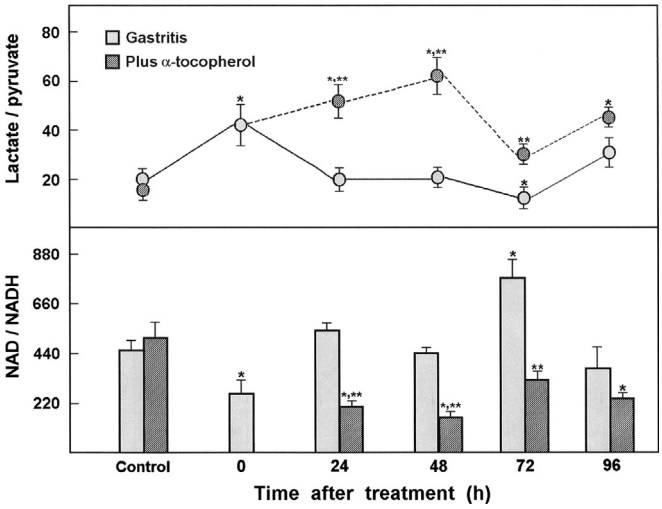

Fig. 1. Lactate/pyruvate ratio and the calculated cytoplasmic NAD/NADH ratio in gastric mucosa from animals with gastritis and treated with vitamin E. Results are the means ± SE of five independent observations (animals) per experimental point. The cytoplasmic NAD/NADH ratio was calculated from the K_{eq} for the lactate dehydrogenase reaction, as described under Materials and methods. Statistical significance: $^{*}p < 0.01$ vs control group and $^{**}p < 0.01$ against the group with gastritis.

Effect of α-tocopherol on gastric mucosa glucose oxidation in animals subjected to gastritis

Fig. 2 shows glucose oxidation by isolated gastric mucosal minces, as $^{14}CO_2$ production from 10 mmol/L glucose containing either D-[1-^{14}C]glucose (Fig. 2A) or D-[6-^{14}C]glucose (Fig. 2B). Minces of gastric mucosa obtained from rats with gastritis had a significantly increased oxidation of D-[1-^{14}C]glucose at early times after completion of gastritis induction (24–48 h), whereas D-[6-^{14}C]glucose oxidation was higher mainly at 48 to 72 h after ethanol withdrawal (Fig. 2B). The enhancement of glucose oxidation by these preparations was ascribed mainly to pathways using glucose radiolabeled in C-6 (glycolysis and TCA cycle activities), because the ratio of D-[1-^{14}C]/D-[6-^{14}C]glucose (5.0 ± 0.6; in controls) was significantly decreased in these animals (Fig. 2C). Treatment with α-tocopherol in rats with gastritis promoted a drastic decrease in the oxidation of D-[1-^{14}C]glucose and largely blocked the gastritis-induced enhancement of D-[6-^{14}C]glucose oxidation, lowering the D-[1-^{14}C]/D-[6-^{14}C]glucose ratio (Fig. 2C), compared with control animals and with those with gastritis (Fig. 2).

Oxygen consumption by gastric mucosal minces from animals with gastritis and treated with α-tocopherol

Oxygen consumption by minces of gastric mucosa is an established metric assay to probe mitochondrial activity. At *T*0, samples of gastric mucosa obtained from animals with gastritis showed a progressive increase in oxygen consumption (Fig. 3), using glucose as substrate (10 mmol/L), which was maximum at 48 h after ethanol withdrawal and rapidly declined thereafter, reaching values significantly lower than seen in control preparations. The time course of oxygen consumption coincided with the capacity of oxidizing D-[6-^{14}C]glucose by minces of gastric mucosa taken from animals with gastritis (Figs. 2A and B). In addition, α-tocopherol administration blocked the increased oxygen

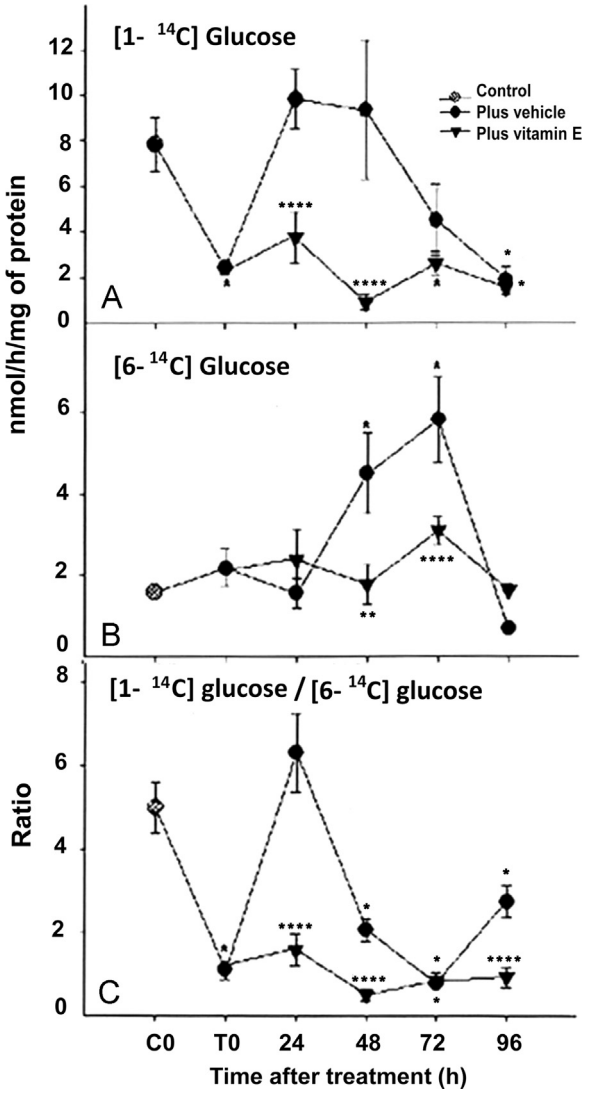

Fig. 2. Oxidation of [1-^{14}C]glucose and [6-^{14}C]glucose by gastric mucosa from animals with gastritis and treated with vitamin E. Results are expressed as the means ± SE of five independent observations (animals) per experimental point. Experimental protocol was described in detail in [28]. Co, control animals. Statistical significance as indicated in Fig. 1.

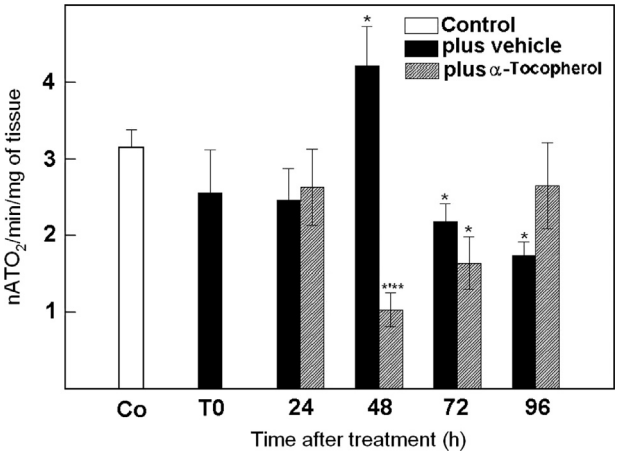

Fig. 3. Oxygen consumption by minces of gastric mucosa from animals with gastritis and treated with α-tocopherol. Results are expressed as the means+SE of five individual observations per point. The oxygen consumption was monitored in the presence of 10 mmol/L glucose, as substrate. Co, control values. Statistical significance as indicated in Fig. 1.

Table 1
Oxygen consumption in State 3 (ADP-stimulated) and the respiratory control ratio (RCR) by isolated mitochondria from gastric mucosa of animals with gastritis and treated with α-tocopherol.

Group	With succinate		With glutamate/malate	
	State 3	RCR	State 3	RCR
Control	26 ± 4	2.13 ± 0.28	18 ± 3	3.35 ± 0.51
Gastritis				
Time 0	67 ± 9*	2.34 ± 0.31	35 ± 5*	3.05 ± 0.37
24 h	79 ± 11*	2.16 ± 0.30	42 ± 6*	3.30 ± 0.43
48 h	30 ± 4	1.88 ± 0.16	16 ± 2	3.80 ± 0.29
72 h	22 ± 2	2.31 ± 0.28	12 ± 2	3.09 ± 0.34
96 h	27 ± 4	1.81 ± 0.22	16 ± 3	3.94 ± 0.58
Plus vitamin E				
24 h	64 ± 14*	2.28 ± 0.43	36 ± 7*	3.66 ± 0.63
48 h	18 ± 2**	3.17 ± 0.28*,**	11 ± 2	4.69 ± 0.52*,**
72 h	66 ± 14*,**	2.19 ± 0.40	41 ± 8*,**	3.72 ± 0.78
96 h	11 ± 2*,**	2.03 ± 0.25	8 ± 2*,**	3.54 ± 0.44

Results are expressed as the mean ± SE for at least five individual observations per group. State 3 using succinate or glutamate/malate as substrate in the presence of ADP is expressed as $nATO_2$ min^{-1} mg^{-1} of protein.

* $p < 0.01$ vs the control group.

** $p < 0.01$ vs the group with gastritis.

consumption by the rat gastric mucosa (Fig. 3). Addition of KCN to the incubating medium inhibited oxygen consumption by minces of gastric mucosa in the range of 85–91%, without significant differences among experimental groups (data not shown); this would suggest that almost 90% of the consumed oxygen was performed by mitochondria and that other oxygen-consuming reactions (i.e., MAO activity) were not affected by the treatments. Because the stimulatory effect of chronic gastric mucosa damage on glucose oxidative metabolism predominated on the catabolic pathways (glycolysis and TCA cycle) over glucose-using anabolic pathways (i.e., pentose phosphate shunt), we next examined the mitochondrial oxidative capacity in our experimental groups.

Substrate oxidation in isolated mitochondria from animals with gastritis and treated with α-tocopherol

Mitochondrial function was assessed in isolated organelles from our experimental groups. The RCR obtained in isolated mitochondria was quite similar to that previously obtained in rat gastric mucosa [34]. At the onset of gastritis (*T*0) increased mitochondrial oxidation of succinate was found in either the absence (State 4) or the presence (State 3) of 250 μM ADP, which did not significantly modify the ADP/O ratio (not shown). These effects remained significantly higher at 24 h after ethanol withdrawal, but returned to within control values thereafter, except for the mitochondrial respiratory control ratio (State 3/State 4), which was unchanged (Table 1). Treatment with α-tocopherol of rats subjected to gastritis elicited a similar pattern of succinate oxidation and ADP phosphorylation during the recovery period; however, after 72 h of α-tocopherol administration, we found a significant increase in mitochondrial State 3, when oxidizing succinate (Table 1). Moreover, a significantly increased mitochondrial respiratory control was found at 48 h after α-tocopherol administration (Table 1). When glutamate/malate was used as substrate for mitochondrial site I, despite the RCR being higher in all the mitochondrial preparations, the effects of either gastritis or α-tocopherol administration were less marked, but still statistically significant (Table 1). Administration of α-tocopherol did not induce significant changes in the mitochondrial parameters in control stomachs (data not shown).

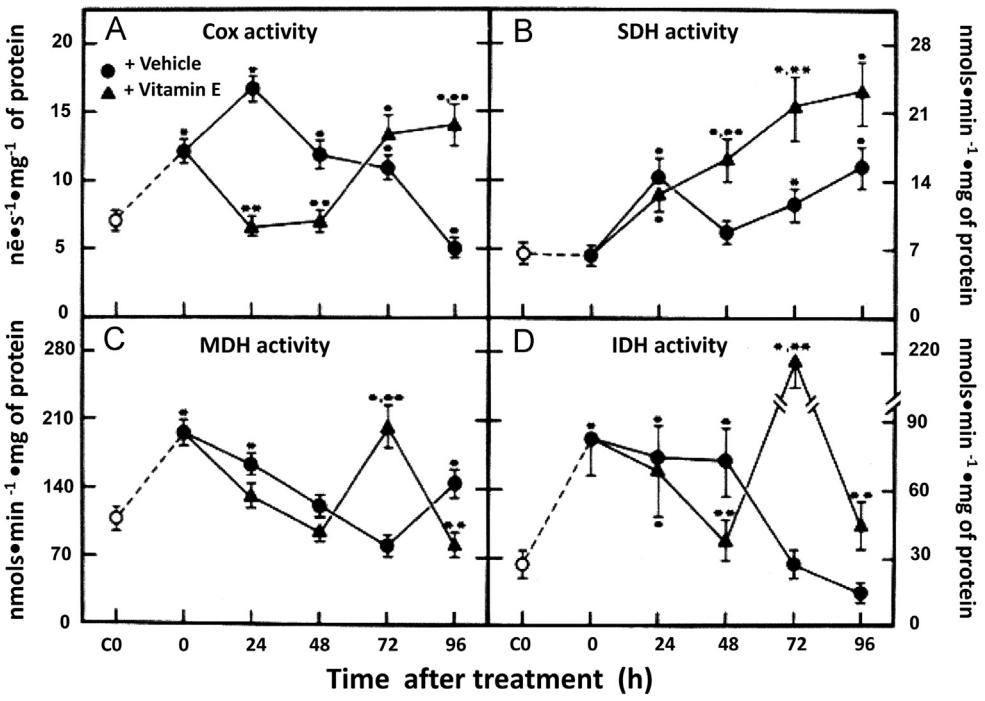

Fig. 4. Activities of mitochondrial enzymes in gastric mucosa from animals with gastritis and treated with α-tocopherol. (A) Cytochrome *c* oxidase, Cox; (B) succinate dehydrogenase, SDH; (C) malate dehydrogenase, MDH; (D) isocitrate dehydrogenase, IDH. Results are expressed as the means ± SE of five independent observations per experimental point. Co, control values. Statistical significance as indicated in Fig. 1.

Mitochondrial enzyme activities in isolated preparations from animals with gastritis and treated with α-tocopherol

The activities of both membrane-attached enzymes, namely Cox and SDH (electron transport chain), were increased in isolated mitochondria from injured gastric mucosa, peaking at 24 h after ethanol withdrawal and remaining higher up to 72–96 h of the recovery period (Figs. 4A and B). α-Tocopherol administration readily modified patterns of mitochondrial enzymatic activities, eliciting a "mirror-image" regarding Cox activity (Fig. 4A), but promoting a linear increase in SDH activity (Fig. 4B). Regarding the "soluble" mitochondrial MDH and IDH activities, that of MDH remained higher for up to 24 h after ethanol withdrawal and gradually returned to control values (Fig. 4C), whereas IDH activity was enhanced by gastric mucosa damage during the first 48 h of the recovery period (Fig. 4D). α-Tocopherol also modified these enzyme activities because, for both mitochondrial enzymes, the vitamin promoted maximum peaks of enzyme activity at 72 h (Figs. 4C and D), suggesting a delay in gastritis-induced changes in mitochondrial enzyme activities.

Changes in oxidative parameters and in the lipid composition of isolated mitochondria from gastric mucosa

Increased oxygen consumption could either be used for ATP generation or lead to increased mitochondrial ROS production. However, the amount of dichlorodihydrofluorescein-detectable generation of ROS in mitochondrial membranes was not modified at the onset of gastritis (*T*0) and remained quite similar to control values thereafter (Fig. 5A). As for conjugated dienes, they did not significantly change compared with control mitochondria, except for a significant decrease in CDs at 24 h after ethanol withdrawal (Fig. 5B). Unexpectedly, administration of α-tocopherol to animals subjected to gastritis promoted a significant increase in mitochondrial ROS exclusively at 3 days of the recovery period (Fig. 5A) and completely blocked the gastritis-induced diminution of CDs (Fig. 5B). The level of mitochondrial carbonyl groups (Fig. 5C) was

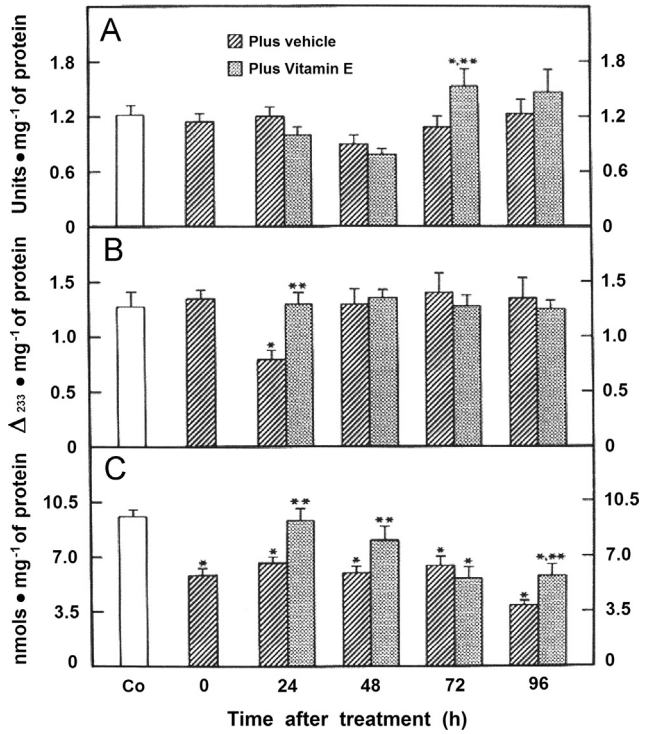

Fig. 5. The oxidative stress determined by ROS, conjugated dienes, and protein carbonyl contents in mitochondria isolated from gastric mucosa of animals with gastritis and treated with α-tocopherol. (A) ROS content (as units of fluorescence), (B) conjugated diene content, and (C) protein carbonyl content in mitochondria isolated from gastric mucosa of animals with gastritis and those treated with vitamin E. Results are expressed as the means ± SE of independent observations per experimental point. Statistical significance as indicated in Fig. 1.

decreased in animals undergoing gastritis. Intriguingly, α-tocopherol actually avoided this gastritis-induced decrease in mitochondrial oxidized proteins (Fig. 5C). These parameters were not significantly

Table 2
Phospholipid composition of mitochondrial membranes of gastric mucosa in animals subjected to ethanol-induced gastric damage and treated with vitamin E.

Group	T. P_i-lipids	Cardiolipin	Cholesterol	Cardiolipin (%)	Cholesterol/T. P_i-lipids
Control	149 ± 15	71 ± 4	17 ± 2	48 ± 6	0.11 ± 0.01
Gastritis					
Time 0	233 ± 30*	36 ± 5*	38 ± 6*	16 ± 2*	0.16 ± 0.02
24 h	181 ± 29	31 ± 4*	35 ± 6*	17 ± 2*	0.19 ± 0.02*
48 h	119 ± 19	18 ± 3*	54 ± 21*	15 ± 2*	0.45 ± 0.12*
72 h	160 ± 20	46 ± 7*	23 ± 3	29 ± 5	0.14 ± 0.02
96 h	197 ± 28	48 ± 7*	32 ± 5*	24 ± 4*	0.16 ± 0.03
Plus vitamin E					
24 h	137 ± 18	59 ± 9**	27 ± 4	43 ± 5**	0.20 ± 0.03
48 h	101 ± 13	21 ± 2*	24 ± 4**	21 ± 3*	0.24 ± 0.02*
72 h	278 ± 38*,**	52 ± 5*	25 ± 4	19 ± 2*	0.09 ± 0.01
96 h	308 ± 47*	49 ± 7*	50 ± 7*	16 ± 3*	0.16 ± 0.02

Results are expressed as the means ± SE for at least five individual observations per group. Levels for total phospholipids (T. P_i-lipids), cardiolipin, and cholesterol are expressed as nmol mg^{-1} of protein.

* $p < 0.01$ vs the control group.
** $p < 0.01$ vs the group with gastritis.

modified by the administration of α-tocopherol in animals without gastritis (data not shown). In this context, the total amount of phospholipids and cholesterol was increased in mitochondrial preparations at the onset of gastritis (*T*0), whereas α-tocopherol treatment enhanced total phospholipid content only at later times posttreatment (72 and 96 h); similarly, the level of mitochondrial cholesterol was also increased at the latest time tested (Table 2). As to the specific mitochondrial phospholipid, namely cardiolipin, it was gradually decreased in mitochondria preparations from *T*0 up to 72 h after ethanol withdrawal and increased thereafter, without reaching the control value; moreover, the percentage of cardiolipin with respect to total phospholipids was decreased at the onset of gastritis and increased gradually thereafter (Table 2). The gastritis-induced decline in cardiolipin level was early avoided by treatment with α-tocopherol (24 h), but lowered cardiolipin content was also noted thereafter (Table 2). Control animals receiving α-tocopherol did not show any difference in lipid composition (data not shown).

Distribution of Bax in cytosol and mitochondria of gastric mucosa in animals with gastritis and treated with α-tocopherol

In association with the changes in lipid composition and oxidative events, there were variations in the mitochondrial content of pro- and antiapoptotic proteins, namely Bax and Bcl2 (Fig. 6). Western blot analysis revealed an increase in the cytosolic Bax/Bcl2 ratio at the onset of gastritis (*T*0) and 24 h after ethanol withdrawal (Fig. 6A), due to an augmented cytosolic Bax protein, which returned to the control value, whereas Bcl2 expression was enhanced starting at 48 h after ethanol withdrawal. In contrast, α-tocopherol readily reduced the cytosolic Bax/Bcl2 ratio at all times assessed (Fig. 6A). In mitochondria, we found the opposite pattern: in animals with gastritis, this ratio was increased at later times (72 and 96 h; Fig. 6B) owing to an enhanced Bax expression, whereas α-tocopherol treatment avoided this shift on the mitochondrial Bax/Bcl2 ratio by blunting Bax expression (Fig. 6B).

Total Cox activity and calculated amount of gastric mucosa mitochondria in animals with gastritis and treated with α-tocopherol

Total activity for this enzyme per gram of gastric mucosa showed minor changes by the treatments tested. Indeed, gastric mucosal Cox activity was significantly decreased at 72 h after ethanol withdrawal, and this effect remained unchanged by α-tocopherol administration (Fig. 7). By knowing both total Cox activity per gram of gastric mucosa and the mitochondrial specific activity for this enzyme, it was possible to calculate the amount of mitochondrial protein in the gastric mucosa (mg/g of mucosa; Ref. [33]). With this approach, we found that gastric mucosa mitochondria content was lowered by chronic damage, remaining similarly low for up to 72 h and recovered at 96 h of ethanol withdrawal (Fig. 7). In contrast, those rats with gastritis and receiving α-tocopherol showed similar amounts of mitochondrial protein compared with controls, which declined thereafter (Fig. 7). Thus, α-tocopherol elicited a delay and a mirror image in the restoration of gastric mucosa content of mitochondria.

Content of nitrites and citrulline in the gastric mucosa obtained from animals with gastritis and treated with α-tocopherol

Cytosolic levels of nitrites and of citrulline, reflecting bioavailability of NO, were also measured in our experimental groups. In animals subjected to gastritis, citrulline content was drastically increased at *T*0 and remained higher 24 h after ethanol withdrawal, declining abruptly thereafter and presenting a second smaller peak (96 h; Fig. 8). α-Tocopherol administration presented a similar pattern of changes in gastric mucosa citrulline and nitrite levels, but clearly delayed by 48 h (Fig. 8), strongly suggesting that both compounds were formed by the same reaction.

Discussion

Other than the study by Jorgensen et al. [3], in which the coupling of oxidative phosphorylation was measured in mitochondria isolated from the gastric mucosa, the present is the first study determining mitochondrial parameters and their relationships with signaling processes involved in cell proliferation and death in the rat gastric mucosa. Therefore, the present study was aimed at discerning whether changes in oxidative metabolism, linked to the cell NAD/NADH redox potential and in which mitochondria might play a major role, participate in the mechanisms underlying ethanol-induced rat gastric mucosa damage and its subsequent recovery. Moreover, because lipoperoxidative events [11,18], and their relationships with oxidative glucose metabolism [12], play a role in the compensatory gastric mucosal proliferation [11,18], we also evaluated the effects of administering α-tocopherol.

Here, we extended our study to the possible role of mitochondrial function in the metabolic adjustment required to drive a compensatory cell proliferation, as well as to assess mitochondrial changes as a putative target for the in vivo action of α-tocopherol on a complex signaling pathway mediated by ROS. The major energy source required to maintain the function of the gastrointestinal mucosa (i.e., acid secretion) is dependent on glycolysis and the TCA cycle [15,35], whereas the pentose phosphate pathway provides precursors for DNA and RNA synthesis and reducing potential in the form of NADPH for other biosynthetic pathways [36]. The latter is especially relevant considering that the rate of oxidative metabolic pathways can largely regulate the task of restitution of an injured gastric mucosa, because alterations in oxidative phosphorylation resulting from mitochondrial dysfunction seem to be involved in tumorigenesis [17].

Mitochondrial adaptations occur in response to cellular energy demand or supply, whereas mitochondrial transformations are part of a greater program of cell metamorphosis. However, little is known of the signals and downstream pathways that govern mitochondrial adaptations and transformations. At the onset of ethanol-induced gastritis in rats, glucose oxidation and oxygen consumption were enhanced in the injured gastric mucosa, associated with higher ATP and $NADP^+$ availability, despite the cytosolic NAD/NADH being significantly decreased by chronic ethanol administration [12].

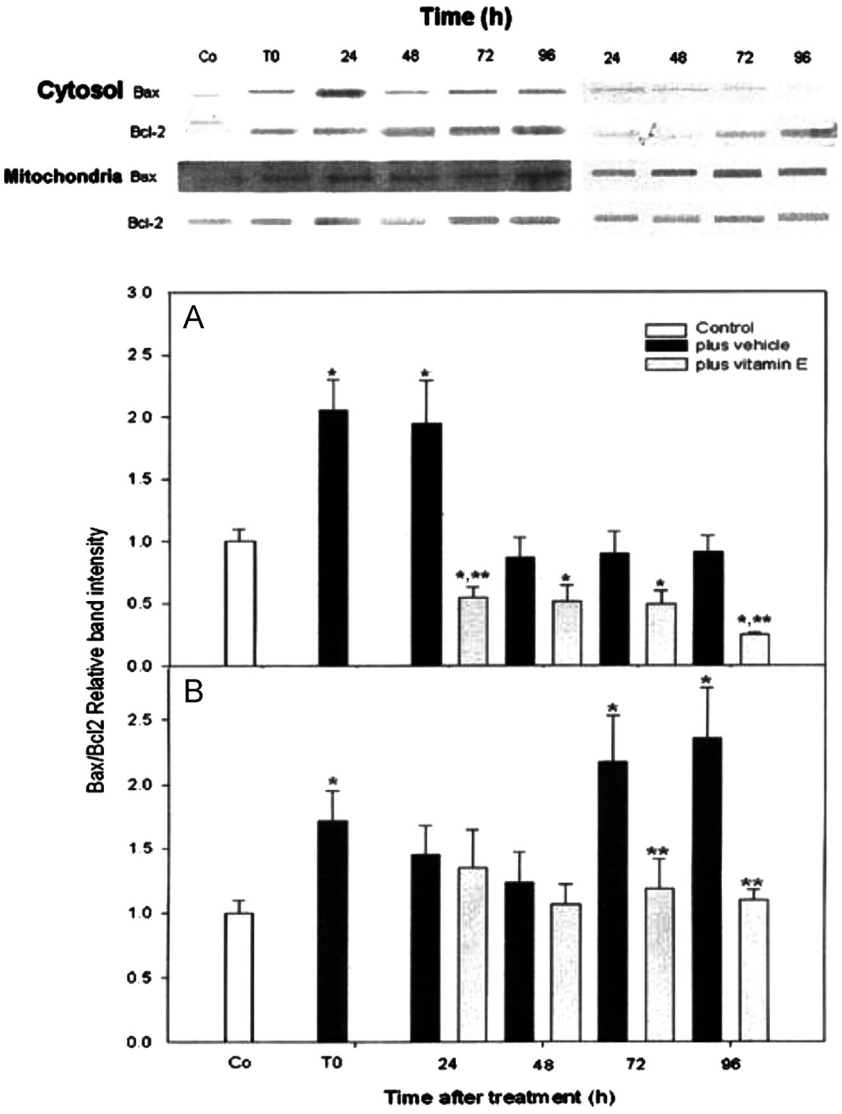

Fig. 6. Bax/Bcl2 ratio calculated from Western analysis of the gastric mucosa proteins from the experimental groups. Densitometry analyses for (A) cytosolic and (B) mitochondrial Bax/Bcl2 ratios in gastric mucosa from animals with gastritis and treated with vitamin E. Results are expressed as the means ± SE of independent observations per experimental point. Statistical significance as indicated in Fig. 1.

We found a sustained increase in glucose oxidation by the injured mucosa, through the pentose phosphate shunt as well as the catabolic rate of glucose, which suggested an accelerated glucose catabolism through the coordinated participation of the glycolytic and TCA cycle pathways (Fig. 2). This stimulated glucose utilization was practically abolished by in vivo treatment with α-tocopherol to rats with gastritis (Fig. 2). This finding constitutes one of the hallmarks of this study, because we demonstrated that administration of α-tocopherol, in addition to being a powerful antioxidant, exhibits some regulation on complex cell pathways, such as cell proliferation and the increased intermediary metabolism of glucose.

These results agree with the evidence that suggests that NAD (including NAD^+ and NADH) and NADP, and its reduced form (NADPH), are mediators of various biological processes, including energy metabolism, mitochondrial functions, Ca^{2+} homeostasis, generation of oxidative stress, gene expression, immunological functions, aging, and cell death [37]. For instance, NAD mediates energy metabolism and mitochondrial functions, whereas NAD(P)H is involved in the generation of ROS, as well as participating in cellular antioxidation systems. Moreover, NAD and NADP modulate multiple key factors in cell death, such as mitochondrial permeability transition, energy state, poly(ADP-ribose) polymerase-1, and apoptosis-inducing factor, thus influencing factors such as oxidative stress and mitochondrial activities [37].

Under our conditions, the effects of the treatments on the cell NAD/NADH redox state were mainly ascribed to fluctuations in lactate levels, whereas those of pyruvate were not significant (data not shown). A minor accumulation of lactate in adequately oxygenated cells and tissues could initiate wound healing, including vasculogenesis and collagen deposition. Lactate enhances a remarkable set of actions, probably mediated by ROS formation, such as increased levels of VEGF and TGF-β [38], endothelial cell mobility, increased collagen synthesis and deposition, and cell proliferation [39]. Lactate exerts its effects by reducing ADP-ribosylation; this occurs when excessive lactate accumulation reduces the pool of NAD^+ by converting it to NADH by the action of lactate dehydrogenase. Indeed, addition of NAD^+ reverses the effect of lactate on collagen deposition in cell cultures, and inhibitors of lactate dehydrogenase activity abolish the effects of lactate on vascularization [40].

ATP synthesis could be very active owing to a generalized increase in the TCA cycle acids, augmenting O_2 consumption during acid secretion [41], and mitochondrial activity would be the major site of activation [16]. Under our conditions, in addition to using metabolic

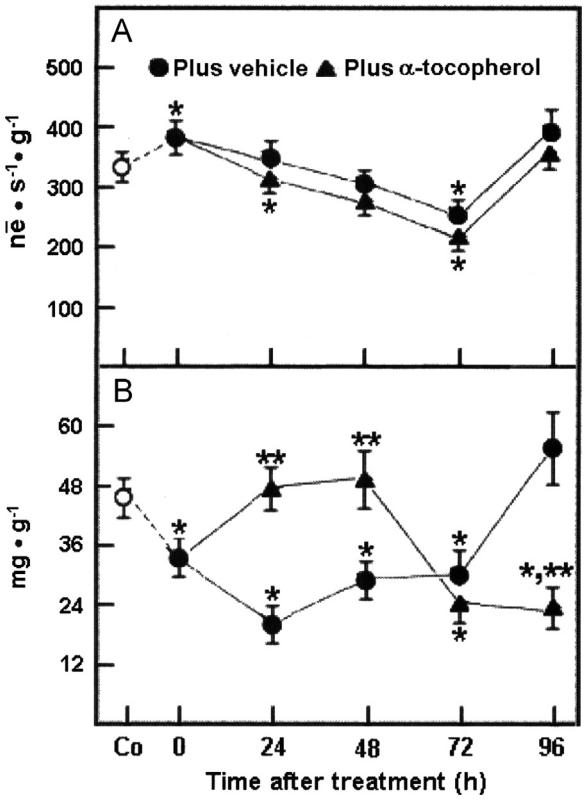

Fig. 7. Activity of cytochrome *c* and calculation of the amount of mitochondria protein in gastric mucosa from animals with gastritis and treated with α-tocopherol. Results are expressed as the means ± SE of five independent observations per experimental point. (A) Cox activity ($n\bar{e}\ s^{-1}\ g^{-1}$) in gastric mucosa damaged and treated with α-tocopherol. (B) Mitochondrial protein ($mg\ g^{-1}$ of gastric mucosa), as calculated from total activity per gram of mucosa in the homogenate and divided by the specific activity in mitochondrial fractions. Statistical significance as indicated in Fig. 1.

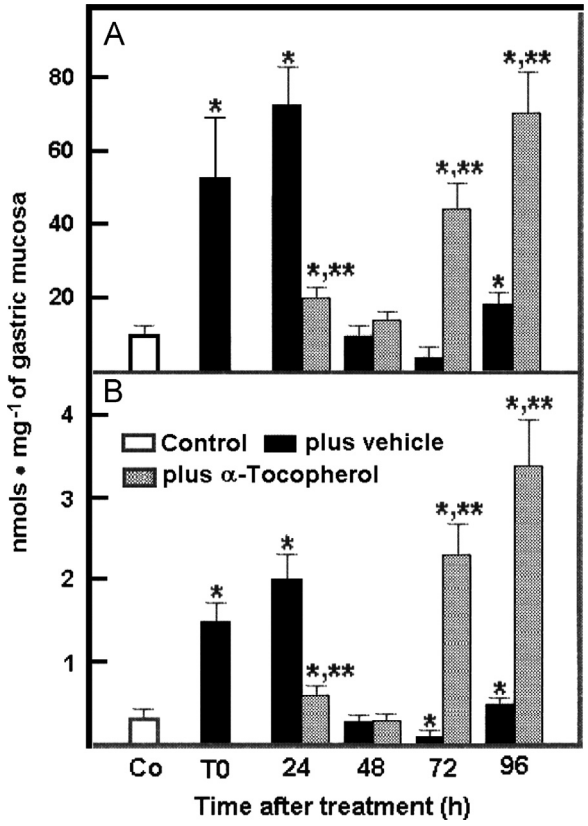

Fig. 8. Citrulline and nitrite content in gastric mucosa from animals with gastritis and treated with α-tocopherol. Results are expressed as the means ± SE of five independent observations per experimental point for (A) citrulline or (B) nitrite content, as a reflection of the levels of NO in isolated gastric mucosa by animals with gastritis and those treated with α-tocopherol. Statistical significance as indicated in Fig. 1.

energy (ATP) for controlling gastric acid secretion, augmented glucose utilization is clearly related to driving injured mucosa to proliferate [10,11]. The mitochondrial oxidative phosphorylation system plays a key role in energy production, the generation of free radicals, and apoptosis, which when deregulated leads to uncontrolled apoptosis [36]. In parietal cells, electron transport through the mitochondrial cytochrome chain participates in acid secretion [42], but present data indicate that mitochondria seem also to be deeply involved in the restitution of damaged gastric mucosal tissue. During oxidative phosphorylation, leakage of electrons frequently produces mitochondrial superoxide anions that are rapidly reduced to H_2O_2 by manganese superoxide dismutase. In our model, mitochondrial enzymes (involved in the TCA cycle or in the electron transport chain) increased early after ethanol withdrawal (Fig. 5), suggesting that mitochondria function more efficiently, avoiding production of CDs in this group (Fig. 5). Surprisingly, α-tocopherol, an antioxidant, indeed increased oxidative changes in mitochondrial proteins (72 h; Fig. 5), which might initiate an effective apoptosis in gastric mucosa from animals with gastritis, even in the presence of increased endogenous antioxidants [18].

A low production of ROS in the presence of higher mitochondrial electron transport and oxidative metabolism could occur and, in fact, contributes to the longevity of yeast [43]. Moreover, it is generally believed that mitochondrial superoxide anion production is directly proportional to ATP production, which is regulated mainly by oxygen and energy substrate, such as glucose availability [44]. This increased mitochondrial efficiency might be explained as a result of mitochondrial biogenesis. Here, total mitochondria protein per gram of gastric mucosa was decreased at the onset of gastritis and progressively recovered thereafter (Fig. 8), temporally coinciding with restitution of mucosal epithelium [10,18]. Interestingly, animals with gastritis and treated with α-tocopherol presented normal mitochondrial protein content at early times after ethanol withdrawal (Fig. 8), and these organelles did not readily show increased oxidative metabolism (Fig. 4). A possible explanation for this apparent discrepancy could be that ethanol-injured gastric mucosa occurred with a drastic loss of the gastric surface epithelium [8] and with a reduced mitochondria content (Fig. 8). After ethanol withdrawal, cell proliferation was strongly increased and restitution of gastric mucosa was accelerated [10,18]; because mitochondrial biogenesis takes longer than other subcellular structures in a proliferating tissue [44], it might be suggested that the content of mitochondrial protein was reduced at early times of gastric hyperplasia by a "dilution" effect, when calculated in comparison with the bulk of gastric mucosa proteins. Indeed, administration of α-tocopherol inhibited early cell proliferation, maintaining mitochondrial protein within the normal range, which decreased at the later times of the recovery period (Fig. 6), when a small fraction of gastric mucosa began to proliferate [18], which could be associated with a dilution effect of mitochondrial protein by active biogenesis. Moreover, the changes in lipid composition and oxidant status could be responsible for the altered mitochondrial function. Increased mitochondrial cholesterol/phospholipids ratio could also be related to an early mitochondrial biogenesis (Table 1). In fact, changes in mitochondria content in parietal cells are an adaptive process to cope with cellular activity of acid secretion [45].

Recent evidence supports the notion that NO plays a critical role in initiating and integrating signaling events underlying mitochondrial biogenesis in various tissues, that is, mitochondrial

proliferation and activation, as well as enhancement of coupled respiration and ATP concentration [46]. Treatment with NO donors also increases mitochondrial mass in various cell types, including brown adipocytes, U937, and HeLa cells [47], and mitochondrial biogenesis is mediated by the stimulation of guanylate cyclase, generation of cGMP, and activation of PGC-1α [48], a master regulator of mitochondrial biogenesis and oxidative phosphorylation, stimulating transcription factors that regulate expression of nuclear and mitochondrial genes [46]. Our data show an early increase in citrulline and nitrites (NO by-products) in animals with gastritis, which was delayed by 48 h after treatment with α-tocopherol (Fig. 8). This coincides with enhanced mitochondrial enzyme activities and changes in the lipid mitochondrial membrane composition, also in agreement with active mitochondrial biogenesis and reduced ROS production in the damaged gastric mucosa [49]. Moreover, dietary nitrate protects against diclofenac-induced gastric ulcers probably via enhanced nitrite-dependent intragastric NO formation and concomitant stimulation of mucus formation [50], which highlights the important role of NO in the metabolism of gastric mucosa cells and its controlled proliferation.

In the present model, we found an early increase in release of cytochrome *c* from mitochondria into cytosol, which did not correlate with active caspase-3 and -9. At later times of recovery, glucose metabolism was normalized, rate of cell proliferation was slowed down, and caspase activation occurred [18]. During apoptotic stimulation, cardiolipin avidly binds to cytochrome *c* on the mitochondria, resulting in an increase in its catalytic peroxidase activity. This complex selectively oxidizes cardiolipin, and cytochrome *c* is released into the cytosol through membrane pores, probably involving interactions with proapoptotic Bcl2 family members, such as Bax [48,51,52]. Here, decreased mitochondrial cardiolipin content coincided with cytochrome *c* release, whereas treatment with α-tocopherol showed an inverse pattern of cardiolipin level (Table 1; Ref. [18]). Cell death by apoptosis is characterized by the need for new gene expression, involved in the regulation of apoptosis including that of antiapoptotic protein Bcl2 and proapoptotic protein Bax, forming multimers that translocate to the outer mitochondrial membrane, where they form pores [53]. Our results demonstrate that α-tocopherol treatment desynchronizes proliferative and apoptotic events, apparently by delaying the mitochondrial apoptotic pathway (Bax/Bcl2 ratio), favoring the extrinsic pathway [18]. The aforementioned could be an example of the so-called retrograde regulation through mitochondrial signaling mechanisms. This event is a communication pathway from the mitochondria to the nucleus that is used to describe the cellular response to the changes in the functional state of the mitochondria [54], such as mitochondrial membrane potential and elevation of calcium levels [55].

Therefore, mitochondria can play a central role in the regulation of cell proliferation and apoptosis and in the modulation of metabolism; accordingly, defective organelles contribute to cell transformation and cancer, diabetes, and neurodegenerative diseases. In fact, regarding the dependency on the modulation of respiratory rate and on the production of hydrogen peroxide, it is accepted that the mitochondrial oxidative rate has to remain depressed for cell proliferation [56]. In response to stress signals, traffic of pro- and antiapoptotic mitochondrial proteins in the intermembrane is modulated by the redox condition determined by mitochondrial oxygen utilization and mitochondrial NO metabolism [56]. However, we are demonstrating that physiological compensatory hyperplasia of rat gastric mucosa occurs with increased mitochondrial metabolism, associated with collateral pathways for glucose oxidation, i.e., the pentose phosphate shunt. In this regard, gallic acid prevents nonsteroidal anti-inflammatory drug-induced activation of caspase-9, a marker for the mitochondrial pathway of apoptosis, and restores the mitochondrial transmembrane potential and dehydrogenase activities. Therefore, the inhibition of mitochondrial oxidative stress by gallic acid is associated with the inhibition of mitochondrial dysfunction (promoted by nonsteroidal anti-inflammatory drugs) and activation of apoptosis in gastric mucosal cells [57].

Concerning the possible mechanisms underlying the actions of α-tocopherol on cell proliferation and/or death, we have found that the inhibitory effects of α-tocopherol on partial hepatectomy-induced rat liver regeneration seemed to be due to a kind of modulation of cell signaling pathways, through regulating the rate of ROS generation. Indeed, α-tocopherol can exert a controlling role on the expression or activity of proteins acting in cell signaling cascades. Some of the remarkable actions of α-tocopherol on cell signaling, such as protecting the production of NO, prostaglandins, and mainly products from protein kinase activity, could influence the rate of cell proliferation beyond its antioxidant function [58]. Recently, we found that altered activation and translocation of STAT-1 and -3 proteins and inhibited retinoid metabolism seem to be involved in the α-tocopherol-induced inhibition of rat liver regeneration, inducing in the cell redox state during proliferation of liver cells [59]. This could be related to evidence showing that activated STAT-3 is capable of augmenting oxidative phosphorylation in mitochondria [60]. In this context, it has been recently reported that Trolox treatment increases glutathione- and mitofusin-dependent mitochondrial filamentation, increases expression of fully assembled mitochondrial complex I, elevates activity of mitochondrial enzymes, and increases cellular oxygen consumption, suggesting that antioxidants can be upstream regulators of mitochondrial mitofusin levels, morphology, and function in healthy human skin fibroblasts [61]. A similar scenario could be induced by α-tocopherol in mitochondria from the injured rat gastric mucosa, as shown in this study.

In conclusion, after installation of chronic gastric damage in rats treated with ethanol, the compensatory restitution of gastric surface epithelium is accompanied by recovery of functional integrity of plasma membranes [10], a selective and transient increase in lipoperoxidative events, triggering a reliable cell proliferation [10,19]. These events are preceded by an increased oxidative metabolism, which seems to be driven by changes in the cell NAD/NADH redox state and in mitochondrial function and probably during biogenesis of these organelles. Administration of α-tocopherol to these animals ameliorated mucosal inflammation and normalized gastric acid secretion (not shown). However, high α-tocopherol dosing induced several consequences in mitochondrial structure and function, probably abolishing a damage-induced "stress" signaling, which leads to a desynchronized mitochondrial adaptive response, including delayed mitochondrial biogenesis in the rat gastric mucosa. Hence, data suggest that α-tocopherol can act as a potent inhibitor of gastric mucosa cell proliferation.

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