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Nitric Oxide Protection of Rat Liver from Lipid Peroxidation, Collagen Accumulation, and Liver Damage Induced by Carbon Tetrachloride

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ABSTRACT. The aim of this work was to determine if the inhibition or stimulation of NO synthesis modulates liver damage induced by the chronic administration of CCl_4. CCl_4 was administered three times a week for 8 weeks to male Wistar rats treated simultaneously with N_ω-nitro-L-arginine methyl ester (L-NAME, 100 mg/kg, p.o., twice a day), aminoguanidine (AG, 4 g/L in the drinking water), or L-arginine (500 mg/kg, p.o., twice a day); appropriate controls were performed. Serum NO_2^- + NO_3^- increased in the groups treated with CCl_4 and/or L-arginine, but the effect was prevented by either L-NAME or AG. In the liver, lipid peroxidation and collagen content increased, while glycogen content decreased in the CCl_4-treated group ($P < 0.05$); L-NAME and AG accentuated these effects. Serum enzyme activities of alanine aminotransferase (ALT), alkaline phosphatase, and γ-glutamyl transpeptidase (γ-GTP) and bilirubin content increased about 2-, 3-, 2-, and 6-fold, respectively, after CCl_4 intoxication ($P < 0.05$); L-NAME or AG cotreatment further increased the enzyme activities ($P < 0.05$). L-Arginine treatment protected the liver partially from the elevation of collagen, bilirubins, and alkaline phosphatase and from glycogen depletion induced by CCl_4 intoxication ($P < 0.05$), but showed no significant effect on ALT, γ-GTP, or lipid peroxidation. These results suggest that NO protects the liver against oxidative injury, because NO inhibition by L-NAME or AG increased lipid peroxidation and the other markers of liver injury studied herein. BIOCHEM PHARMACOL **56**;6:773–779, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. free radicals; extracellular matrix; fibrosis; hepatoprotectors; liver damage; cirrhosis

Evidence indicates that reactive oxygen intermediates are pathogenic in several types of liver injury [1]. Reactive oxygen intermediates are substances with one or more unpaired electrons, and include superoxide anion, hydrogen peroxide, and hydroxyl radical. These substances have been linked to membrane and DNA damage, lipid peroxidation reactions, and the induction of hepatocyte killing [2–4].

Some evidence suggests that NO may act as an antioxidant and may interact with superoxide anion and other radicals to produce less toxic species [5, 6]. In contrast, other evidence suggests that NO may interact with reactive oxygen intermediates to form more toxic species. The reaction of NO with a superoxide anion can produce the peroxynitrite anion, which can decompose to generate a strong oxidant with reactivity similar to that of a hydroxyl radical [7]. Peroxynitrite can induce sulfhydryl oxidation [8] and lipid peroxidation [9].

Liver diseases constitute a major problem of worldwide proportions. In Africa and Asia, their main causes are viral and parasitic infections. In Europe and America, alcohol abuse is the major cause of liver diseases. CCl_4 is a well known hepatotoxin that is widely used to study the induction of toxic liver injury in a range of laboratory animals. Damage by CCl_4 is regarded as the analogue of liver damage caused by a variety of hepatotoxins in humans. Injury in both animals and humans is similar in mechanism and has long been recognized as dependent upon the cleavage of the carbon-to-chlorine bond and peroxidative decomposition of cytoplasmic membrane lipids [1]. CCl_4-induced cirrhosis shares several characteristics with human cirrhosis of different etiologies; thus, it is an adequate model of human cirrhosis [10].

The aim of the present work was to study the effect of NO modulation (by using NO synthesis inhibitors or precursors) on chronic liver damage induced by CCl_4 administration. It was observed that endogenous NO protected the liver from lipid peroxidation, fibrosis, and damage.

MATERIALS AND METHODS

Materials

L-NAME†, L-arginine, anthrone, picric acid, thiobarbituric acid, hydroxyproline, chloramine-T, ethylene glycol, bo-

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† *Abbreviations:* AG, aminoguanidine; ALT, alanine aminotransferase; cAMP, cyclic AMP; cGMP, cyclic GMP; γ-GTP, γ-glutamyl transpeptidase; and L-NAME, N_ω-nitro-L-arginine methyl ester.

vine serum albumin, *p*-dimethylaminobenzaldehyde, and activated charcoal were obtained from the Sigma Chemical Co. Citric acid, sodium acetate, sodium hydroxide, glacial acetic acid, hydrochloric acid, sodium thiosulfate, sodium chloride, toluene, sulfuric acid, iodine, ethanol, xylene, potassium hydroxide, formaldehyde, and trichloroacetic acid were obtained from J. T. Baker.

Animal Treatments and Liver Damage Induction

Male Wistar rats weighing around 80 g at the beginning of treatments were used. They had free access to food (Standard Purina chow diet) and water. Chronic liver damage was produced by i.p. administration of CCl_4 (0.4 g/kg of body weight) dissolved in mineral oil three times a week for 8 weeks.

Eight groups of ten animals each were analyzed. In group 1, CCl_4 was administered for 8 weeks. Animals in group 2 received L-NAME (100 mg/kg., p.o., twice a day) plus CCl_4. The rats in group 3 received AG (4 g/L) in the drinking water, in addition to CCl_4. Group 4 consisted of animals receiving L-arginine (500 mg/kg, p.o., twice a day) throughout the CCl_4 treatment. Groups 5, 6, 7, and 8 were appropriate controls of L-NAME, AG, L-arginine, and vehicles, respectively. Seventy-two hours after the end of the treatments, rats were anesthetized lightly with ether, blood was collected by cardiac puncture, and the liver was removed rapidly.

Nitrite + Nitrate Determination in Serum

Serum NO_2^- + NO_3^- levels were determined based on the Gries reaction [11]. Briefly, 400 μL of serum was centrifuged at 2000 *g* with a micropore filter (ultrafree MC microcentrifuge device, UFC 3; Millipore) to remove substances larger than 10 kDa. After passing the samples through a copper-plated cadmium column for nitrate reduction, nitrite was measured by the absorbance at 540 nm after mixing with a reagent consisting of 0.2% naphthylethylenediamine dihydrochloride, 0.4% procaine in 6% trichloroacetic acid. The efficiency of cadmium in the conversion of nitrate to nitrite was verified by measuring both nitrate and nitrite standards before and after sample measurement. The value obtained expresses the total amount of serum NO end products, namely NO_2^- + NO_3^-.

Determination of Hepatic Lipid Peroxidation and Glycogen Content

Lipid peroxidation was determined by measuring malondialdehyde (MDA) in liver homogenates using the thiobarbituric acid method according to Ohkawa *et al.* [12]. Liver pieces were separated for glycogen quantification with the anthrone reagent [13]. Protein determinations were performed according to the method described by Bradford [14].

Serum Enzyme Activities and Bilirubins

Serum was obtained for the following determinations: the activities of ALT [15], alkaline phosphatase [16], and γ-GTP [17], and for bilirubin content (Kit Merck–México).

Collagen Quantification

Collagen concentration was determined by measuring hydroxyproline content in fresh liver samples, after digestion with acid [18]. The procedure was as follows:

MATERIALS. Sodium acetate/citric acid buffer, pH 6.0: 1 L of the buffer contained 50 g of citric acid, 120 g of sodium acetate (trihydrate), 34 g of sodium hydroxide, and 15 mL of glacial acetic acid. The buffer was kept cold under a layer of toluene to avoid bacterial growth.

Chloramine-T solution: 0.141 g of chloramine-T was mixed with 2 mL of distilled water, 3.0 mL of peroxide-free methyl cellosolve, and 5 mL of the sodium acetate/citric acid buffer. This solution was prepared fresh before use.

Sodium thiosulfate: the solution was 2.0 M in distilled water and was stable for several weeks at room temperature.

Ehrlich's reagent: 0.5 g of *p*-dimethylaminobenzaldehyde was dissolved in 9.0 mL of absolute ethanol and 1.0 mL of 12 N HCl.

METHODS. Fresh liver samples (100 mg) were placed in ampules, 2 mL of 6 N HCl was added, and then the samples were sealed and hydrolyzed at 100° for 24 hr. Next, the samples were evaporated at 50° for 24 hr and resuspended in 3 mL of sodium acetate/citric acid buffer, pH 6.0; 0.5 g of activated charcoal was added, the mixture was stirred vigorously, and then it was centrifuged at 5000 *g* for 10 min. Hydroxyproline oxidation was initiated by adding 1 mL of chloramine T (0.05 M).

The mixture was kept for 20 min at room temperature, and the reaction was stopped by the addition of 2 M sodium thiosulfate and 1 N sodium hydroxide. The aqueous layer was transferred to test tubes. The oxidation product from hydroxyproline was converted to pyrrole by boiling the samples. The pyrrole-containing samples were incubated with Ehrlich's reagent for 30 min, and the absorbance was read at 560 nm. Recovery of known amounts of standards was carried out on similar liver samples to provide quantification.

Statistical Analysis

For statistical analysis, ANOVA with the Tukey test [19] was used to compare the groups. In all cases, a difference was considered significant when P was ≤ 0.05.

RESULTS

The NO end products NO_2^- + NO_3^- measured in serum increased nearly three-fold in the animals treated chroni-

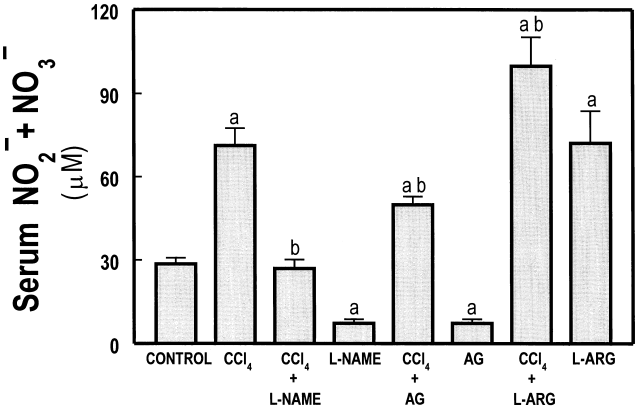

FIG. 1. NO end products expressed as $NO_2^- + NO_3^-$ concentration in serum from rats treated chronically with CCl_4 and with L-NAME, AG, and L-arginine (L-ARG) alone or in combination with CCl_4. Each bar represents the mean ± SEM; all determinations were performed in duplicate assays with samples obtained from ten different animals. Key: (a) significantly different from the control, $P < 0.05$; and (b) significantly different from the CCl_4-treated group, $P < 0.05$.

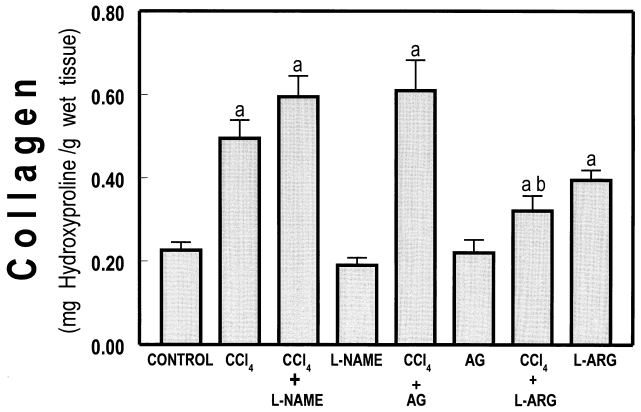

FIG. 3. Collagen content determined in livers from rats intoxicated with CCl_4 and treated with L-NAME, AG, or L-arginine (L-ARG). Each bar represents the mean ± SEM; all determinations were performed in duplicate assays with samples obtained from ten different animals. Key: (a) significantly different from the control, $P < 0.05$; and (b) significantly different from the CCl_4-treated group, $P < 0.05$.

cally with CCl_4 or L-arginine; animals receiving both CCl_4 and L-arginine showed higher values of $NO_2^- + NO_3^-$ than those receiving either of these drugs alone. Both NO synthesis inhibitors (L-NAME or AG) prevented this increase. AG or L-NAME treatments also reduced the basal levels of serum $NO_2^- + NO_3^-$ concentrations (Fig. 1).

Figure 2 shows the degree of liver lipid peroxidation. The chronic administration of CCl_4 increased lipid peroxidation two-fold; L-NAME or AG cotreatment further increased it ($P < 0.05$). Administration of L-arginine to normal or CCl_4-treated rats did not produce any effect. Neither L-NAME nor AG by itself modified the degree of liver lipid peroxidation.

Liver collagen content increased about two-fold after CCl_4 treatment; L-NAME or AG cotreatment did not further increase it significantly. However, L-arginine prevented partially but significantly the fibrotic process induced by CCl_4, while L-arginine administered to normal rats increased the collagen content of the liver significantly (Fig. 3).

As can be seen in Fig. 4, hepatic glycogen content was reduced to 30% of its original value by CCl_4 intoxication; L-NAME or AG cotreatment decreased it to 15% of control values, whereas L-arginine preserved it partially but significantly. Neither L-NAME, AG, nor L-arginine by themselves modified glycogen content significantly.

Figure 5 shows the serum enzyme activities of ALT (upper panel), alkaline phosphatase (middle panel), and γ-GTP (lower panel). The three serum enzyme activities increased significantly after CCl_4 intoxication. This effect was accentuated by L-NAME or AG cotreatment ($P <$

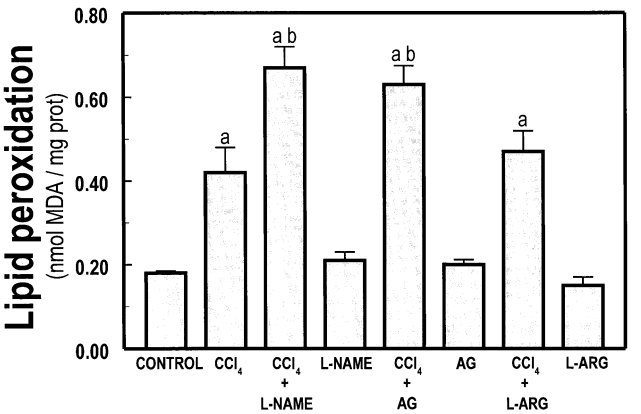

FIG. 2. Effect of L-NAME, AG, and L-arginine (L-ARG) on CCl_4-induced MDA formation in liver homogenates. Each bar represents the mean ± SEM; all determinations were performed in duplicate assays with samples obtained from ten different animals. Key: (a) significantly different from the control, $P < 0.05$; and (b) significantly different from the CCl_4-treated group, $P < 0.05$.

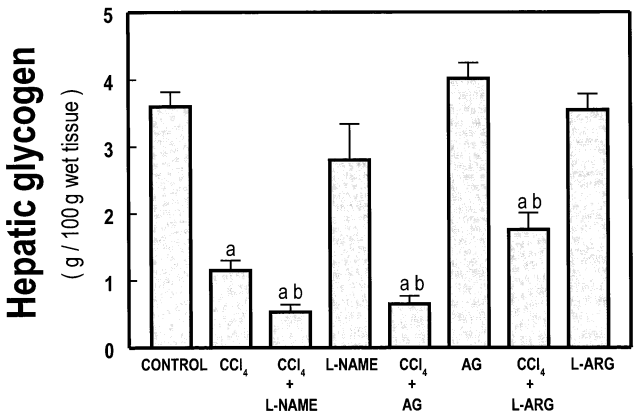

FIG. 4. Glycogen content determined in livers from rats intoxicated with CCl_4 and treated with L-NAME, AG, or L-arginine (L-ARG). Each bar represents the mean ± SEM; all determinations were performed in duplicate assays with samples obtained from ten different animals. Key: (a) significantly different from the control, $P < 0.05$; and (b) significantly different from the CCl_4-treated group, $P < 0.05$.

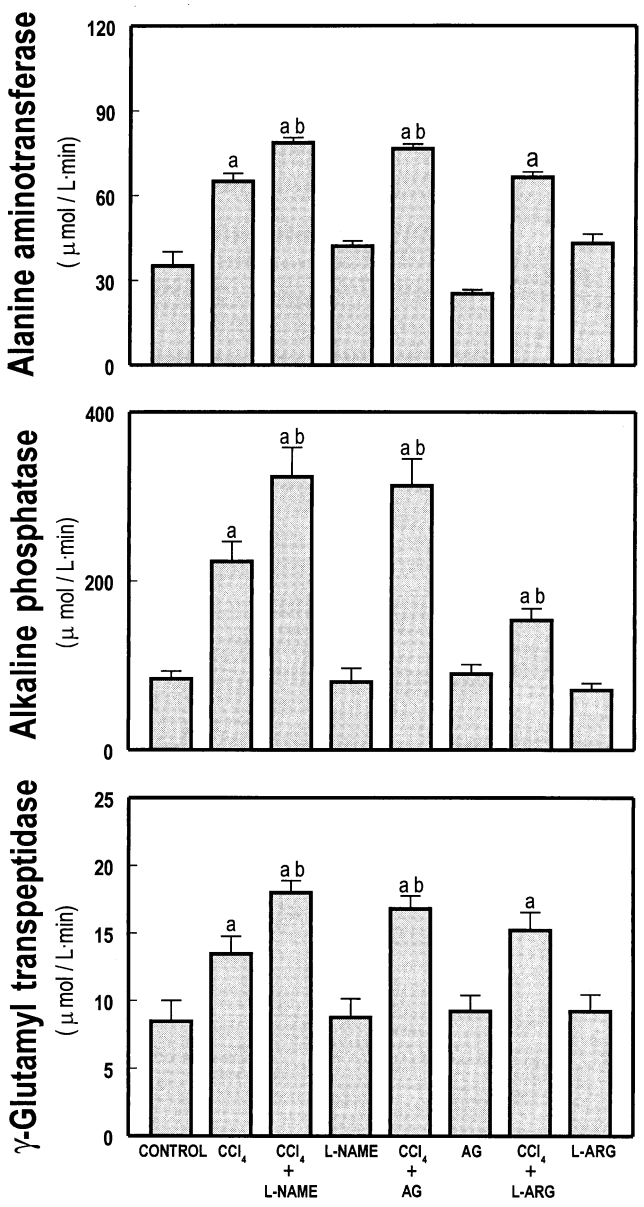

FIG. 5. Enzyme activities of ALT (upper panel), alkaline phosphatase (middle panel) and γ-GTP (lower panel) determined in serum from rats intoxicated with CCl_4 and treated with L-NAME, AG, or L-arginine (L-ARG). Each bar represents the mean ± SEM; all determinations were performed in duplicate assays with samples obtained from ten different animals. Key: (a) significantly different from the control, $P < 0.05$; and (b) significantly different from the CCl_4-treated group, $P < 0.05$.

0.05), while L-arginine preserved partially ($P < 0.05$) alkaline phosphatase activity but showed no effect on the elevated ALT and γ-GTP activity. Neither L-NAME, AG, nor L-arginine by themselves produced any significant effect on these enzyme activities.

CCl_4 treatment increased the concentration of bilirubins about six-fold. Cotreatment with L-NAME or AG did not modify this value, while L-arginine cotreatment partially prevented ($P < 0.05$) the increased concentration of bilirubins (Fig. 6).

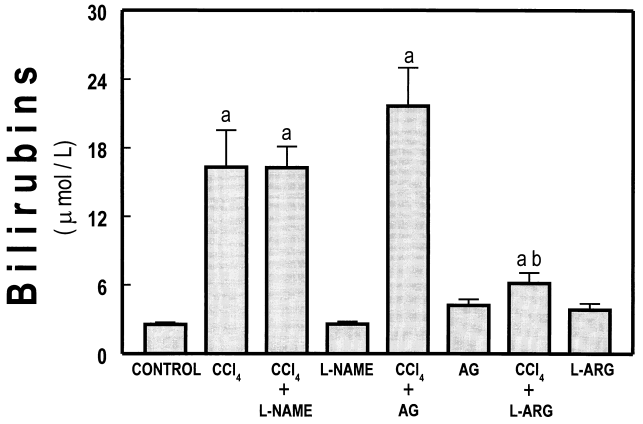

FIG. 6. Concentration of bilirubins determined in serum from rats intoxicated with CCl_4 and treated with L-NAME, AG, or L-arginine (L-ARG). Each bar represents the mean ± SEM; all determinations were performed in duplicate assays with samples obtained from ten different animals. Key: (a) significantly different from the control, $P < 0.05$; and (b) significantly different from the CCl_4-treated group, $P < 0.05$.

DISCUSSION

The present results indicate that endogenous NO plays a hepatoprotective role in chronic CCl_4 intoxication. This effect of NO could be attributed to its ability to interact with superoxide anion and other radicals to produce less toxic species [5, 6].

Rapid, extensive lipid peroxidation of the membrane structural lipids has been proposed as the basis of CCl_4 hepatocellular toxicity and has been reviewed extensively [20]. Accordingly, the degree of lipid peroxidation increased about two-fold in the CCl_4 group, while both NO synthesis inhibitors, L-NAME or AG, induced a further increase.

It has been postulated that prevention of tissue injury and inflammation by oxidative stress is due, in part, to the radical scavenging capability of metallothionein in tissues and cells [21], and it has been demonstrated that NO may play an important role in the induction of metallothionein [22]. However, other groups have shown that metallothionein does not scavenge the trichloromethyl radical *in vivo* [23].

It has been demonstrated previously, that a single dose of CCl_4 combined with a small dose of lipopolysacharide (LPS) produces, synergistically, liver damage and overproduction of NO [24]; both effects could be interpreted as a toxic effect of NO. However, because inhibition of NO synthesis with *N*-monomethylarginine increased liver damage in that model [24], it is more likely that NO is acting as a hepatoprotective compound and that liver damage is mediated by CCl_3-induced oxidative stress and lipid peroxidation [1], and by tumor necrosis factor-α release, which has been shown to correlate with liver damage [25].

Alkaline phosphatase is an ectoenzyme of the hepatocyte plasma membrane; an increase in serum alkaline phosphatase activity has been related to damage to the liver cell

membrane [26]. γ-GTP is an enzyme embedded in the hepatocyte plasma membrane, mainly in the canalicular domain; again, the liberation of this enzyme to serum indicates damage to the cell and thus injury to the liver. It is important to point out that serum γ-GTP activity is considered to be one of the best indicators of liver damage [27, 28]. ALT is a cytosolic enzyme of the hepatocyte, and an increase of this enzyme in the serum reflects an increase in plasma membrane permeability, which, in turn, is associated with cell death [27]. The three enzyme activities were increased significantly by CCl_4 administration; a further increase was observed in the groups treated with L-NAME or AG in addition to CCl_4, indicating that NO acts by protecting the liver.

NO has been implicated in a variety of cell insults. For example, NO inhibition aggravates gentamicin-induced renal failure, suggesting that endogenously released NO plays a protective role in gentamicin nephrotoxicity [29]. Also, the induction of NO synthesis minimizes reperfusion injury in a model of liver perfusion [30, 31], while inhibition of NO production increases dimethyl-nitrosamine-induced liver injury in rats [32]. Furthermore, NO plays a protective role against immunological liver injury [33] and protects the gastric mucosa from endothelin-1-induced gastric ulcers in rats [34]. The present results are in agreement with these reports, indicating that NO possesses important cytoprotective properties. However, there is evidence that NO inhibition protects against lipid peroxidation in cerebral ischemia [35]. It seems that NO plays an important role in neuronal toxicity, in particular [36, 37].

Hock *et al.* [38] studied the inhibition of NO synthase by AG in acute endotoxemia and found that neither NO nor acute inflammatory cell accumulation is solely responsible for the depressed cardiovascular function after intravenous administration of LPS. Similarly, the present results obtained with L-NAME, AG, and L-arginine suggest that NO participates only partially in protecting the liver against CCl_4 intoxication. On the other hand, although L-NAME has shown beneficial effects against the toxicity of some substances, e.g. sulphur mustard, not mediated by NO synthesis inhibition [39], those effects are probably not responsible for the present observations since L-NAME afforded no protection against CCl_4 toxicity. On the contrary, it accentuated liver damage; in addition, AG, another NO synthesis inhibitor, produced effects similar to those of L-NAME.

Glycogen is the main source of liver energy; without it, the liver is not able to carry out energy-coupled hepatic functions. It has been reported that CCl_4-chronic liver damage induces alterations in hepatocyte membranes by increasing the cholesterol content, cholesterol/phospholipids, and cAMP content [4]. Increased levels of cAMP, in turn, produce a drop in glycogen content [4]. On the other hand, NO may modulate hepatic carbohydrate metabolism [40]. NO activates soluble guanyl cyclase. In turn, cGMP may stimulate the hydrolysis of cAMP by cGMP-stimulated cAMP phosphodiesterase, preventing glycogen breakdown [40]. In agreement with this explanation, in this work NO inhibition accentuated liver glycogen depletion, whereas L-arginine ameliorated it.

Hepatic fibrosis is an important feature of chronic liver disease. The replacement of normal hepatic parenchymal tissue by connective tissue compromises the functional capacity of the liver and disrupts the normal architecture relationships of the organ. An important consequence of liver fibrosis is the deposition of connective tissue around the hepatic sinusoids, so that vascular diffusion barriers are disrupted and sinusoidal blood flow tracts are narrowed. The resultant portal hypertension and impaired clearance of endogenous and exogenous metabolites contribute to hepatic dysfunction. Because collagen is the major component of the fibrotic tissue, we evaluated the effect of NO on the hepatic content of collagen in CCl_4-fibrotic rats. L-Arginine was capable of preventing partially, but significantly, the liver collagen increment induced by CCl_4 intoxication, while L-arginine administration to normal rats increased collagen content significantly. On one hand, there is evidence that stimulation of NO release results in reduced production of collagen in mesangial cells [41] and in cultured vascular smooth muscle cells [42]. On the other hand, L-arginine administration induces transforming growth factor-β expression, which, in turn, leads to extracellular matrix deposition, and fibrosis results [43]. The present findings are in agreement with a balance of both effects.

CCl_4 by itself induced a three-fold increase in serum $NO_2^- + NO_3^-$ concentrations; L-arginine induced a similar effect. In addition, the administration of both compounds simultaneously produced a further increase. This might explain the beneficial effects of L-arginine on fibrosis, which is associated with liver function in general and with bilirubins and carbohydrate metabolism in particular. On the other hand, L-arginine treatment failed to prevent lipid peroxidation and the liberation of enzymes to the serum. A possible explanation is that the further increase in NO production induced by L-arginine cotreatment was not enough to scavenge lipidic (hydrophobic) free radicals since NO is a hydrophilic compound with a very short life time. In this case, plasma membrane damage produced by lipid peroxidation will allow membrane (alkaline phosphatase and γ-GTP) and cytosolic (ALT) enzymes to be liberated to the serum.

In summary, our results suggest that endogenous NO protects the liver against damage induced by chronic CCl_4 intoxication probably acting by decreasing the toxicity of free radicals, by modulating hepatic carbohydrate metabolism, and by reducing collagen production.

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