0026-895X/94/020391-07$03.00/0

MOLECULAR PHARMACOLOGY, 46:391–397, 1994.

Nitric Oxide Production during Endotoxic Shock in Carbon Tetrachloride–treated Rats

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Received March 18, 1994; Accepted May 16, 1994

SUMMARY

Earlier studies showed that hepatotoxicant-treated experimental animals were more susceptible than controls to the lethal effects of bacterial endotoxin. The exact mechanisms of this effect were not understood. In this paper we showed that nitric oxide (·NO) was produced in whole blood and in liver tissues of rats that had been treated with a nonlethal dose of CCl_4 (1.3 g/kg) followed by a low dose of lipopolysaccharide (LPS) (100 μg/kg). EPR spectroscopy was used in this study to detect nitrosyl-protein complexes. Hemoglobin-nitrosyl complexes were detected in both whole blood and liver. By performing analyses of EPR spectra obtained from hepatocytes exposed to ·NO, we were able to identify EPR signals attributable to nitrosyl-cytochrome P420 in rat liver. We found that nitrosyl complex formation in red blood cells and liver was inhibited by treatment with N^G-monomethyl-L-arginine, suggesting enzymatic biosynthesis of ·NO. A small but significant inhibition of nitrosyl complex formation by gadolinium trichloride pretreatment was found in the liver, suggesting that Kupffer cells were also involved in ·NO biosynthesis, because this treatment decreased Kupffer cells. There was a synergistic effect of CCl_4 and LPS on the serum levels of the hepatic enzymes aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and sorbitol dehydrogenase, which are indices of parenchymal cell damage. N^G-Monomethyl-L-arginine treatment increased these hepatic enzyme activities, suggesting a protective role for ·NO. EPR resonances at $g \sim 2.48$, 2.29, and 1.91, due to low-spin cytochromes P450/P420 (Fe^{3+}), were decreased in the livers of LPS-induced rats that had been previously treated with CCl_4, indicating cytochrome P450/P420 destruction or at least a change in the valence state of the cytochrome P450/P420 heme groups to Fe^{2+} in the presence of ·NO. Because nitrosyl-cytochrome P450 is not stable, the concomitant detection of nitrosyl-cytochrome P420 (Fe^{2+}) could account, at least in part, for the decrease of the ferric low-spin heme groups. Our novel observations of hepatic nitrosyl species suggest that ·NO plays an important role during hepatic injury caused by CCl_4 in hosts exposed to endotoxin.

It is widely accepted that the hepatotoxicity of CCl_4 results from the metabolism of CCl_4 by cytochrome P-450-dependent monooxygenases to the trichloromethyl free radical (1, 2). This free radical and related reactive species may cause cellular damage by initiating lipid peroxidation or covalently binding to protein, ultimately leading to cell death. In addition, free radical scavengers exhibit a protective effect against CCl_4-induced liver injury (3).

Earlier studies reported that sublethally CCl_4-treated rabbits were 120 times more susceptible than normal rabbits to the lethal effect of bacterial endotoxin (LPS) (4). Moreover, endotoxin has been implicated as a causative factor in the development of hepatic necrosis in experimental animals exposed to CCl_4 (5, 6), halothane (7), or galactosamine (8). Endotoxin absorbed from the gut becomes involved in hepatotoxicity by its interaction with peritoneal and splenic macrophages and Kupffer cells (6, 9). These cells, when stimulated, produce reactive mediators including oxygen free radicals, tumor necrosis factor, and leukotrienes, thereby causing cellular damage (10–12). Kupffer cells, in response to LPS, metabolize L-arginine to produce citrulline and nitrite/nitrate, which are end products of ·NO (13). Therefore, it is plausible that ·NO plays an important role during hepatic necrosis caused by toxicants. To date, *in vivo* ·NO production and its role in experimental animals exposed to hepatotoxicants have not yet been demonstrated.

Upon CCl_4 administration, Kupffer cells increase in number (14) and peripheral monocytes/macrophages (15) are recruited to the liver. Both Kupffer cells and hepatocytes respond to cytokines and LPS by expressing an inducible isoform of NOS (16, 17). These findings suggest that ·NO may be produced by the liver when rats are administered CCl_4. In this paper, we present data showing that, upon immunologic activation with

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ABBREVIATIONS: LPS, lipopolysaccharide; L-NMA, N^G-monomethyl-L-arginine; NOS, nitric oxide synthase; AST, aspartate aminotransferase; ALT, alanine aminotransferase; SDH, sorbitol dehydrogenase; LDH, lactate dehydrogenase; ·NO, nitric oxide; RBC, red blood cell.

LPS, ·NO was produced in whole blood and livers of rats that had been previously treated with CCl_4. Nitrosyl complexes, as adducts of ·NO binding to iron-containing proteins, were measured with EPR spectroscopy. Nitrosyl complex formation was inhibited by the specific NOS inhibitor L-NMA. Similarly, $GdCl_3$ pretreatment inhibited nitrosyl complex formation in the liver, suggesting that Kupffer cells were also involved in ·NO biosynthesis, because $GdCl_3$ selectively kills Kupffer cells *in vivo* (18). Hepatic enzyme activities in serum were measured as indicators of liver damage. The EPR spectra of the low-spin ferric cytochromes P450/P420 in livers of treated rats were also determined.

Experimental Procedures

Materials. L-NMA was obtained from Calbiochem. LPS from *Escherichia coli* 026:B6, carbon tetrachloride, and EDTA were obtained from Sigma. Gadolinium trichloride hexahydrate and ·NO gas (98.5%) were obtained from Aldrich.

Induction of ·NO synthesis *in vivo*. Male Sprague-Dawley rats (300–350 g) were treated intraperitoneally with a sublethal dose of CCl_4 (1.3 g/kg, using olive oil as a vehicle at 1:2 volume). After 20 hr, these rats were injected intravenously with a low dose of LPS (100 μg/kg), and the animals were sacrificed 4 hr later. Injection schedules for the treatment groups were as follows: $GdCl_3$ (20 mg/kg) was injected intravenously 24 hr before CCl_4 administration (18), and L-NMA (50 and 100 mg/kg) was injected intraperitoneally 2 hr after LPS administration. Whole blood was collected from the vena cava and the livers were removed and frozen immediately. RBCs were obtained from EDTA-treated whole blood without further washing.

Hepatocyte preparation. Hepatocytes were isolated from CD male rats by the procedure of Moldeus *et al.* (19). The viability of hepatocytes was determined to be >95% by the trypan blue exclusion test. Cells were resuspended in Fry's buffer. Hepatocytes (final concentration, 1.4 × 10^7 cells/ml) were mixed with an aliquot of ·NO-saturated deionized water, which was prepared according to the published procedure (20). Hepatocytes were incubated with ·NO for 1 hr at 37°.

EPR spectra of nitrosyl complexes. For experiments with cells (RBCs or hepatocytes), identical sample volumes were prepared by pipetting the samples into EPR quartz tubes (2 mm i.d. × 3 mm o.d.) to 2.5-cm depth. EPR quartz tubes were forced into the liver to fill in the tubes to a height of >2.5 cm. The samples were frozen in liquid nitrogen. EPR spectra were obtained with a Bruker ESP300 spectrometer using a finger-tip liquid nitrogen Dewar flask. The spectrometer was operated at 9.77 GHz with a 100-kHz modulation frequency. The EPR signal from Cr^{3+} in MgO was used as the g value marker (g = 1.9800 ± 0.0006) (21). The data were transferred to an IBM PC computer, where base-line correction and double integration were performed using software written in this laboratory. The data for relative integrated areas are indicated as mean ± standard error.

Biochemical assays. Serum activitites of AST, ALT, SDH, and LDH were measured according to published procedures (22–25).

Statistics. Analyses of variance statistical methods were applied, and pairwise comparisons were made using Fisher's least significant difference test. Data were considered statistically significant at the $p < 0.05$ level.

Results

An EPR spectrum of RBCs (as well as whole blood; data not shown) obtained from rats that had been treated with CCl_4 (1.3 g/kg) for 20 hr and then with LPS (100 μg/kg) and sacrificed 4 hr later exhibited signals of hemoglobin nitrosyl complexes indicative of ·NO production (Fig. 1A). This spectrum is the same as those observed in the whole blood of rats injected with a high dose of LPS (26). In our experiments, there were no

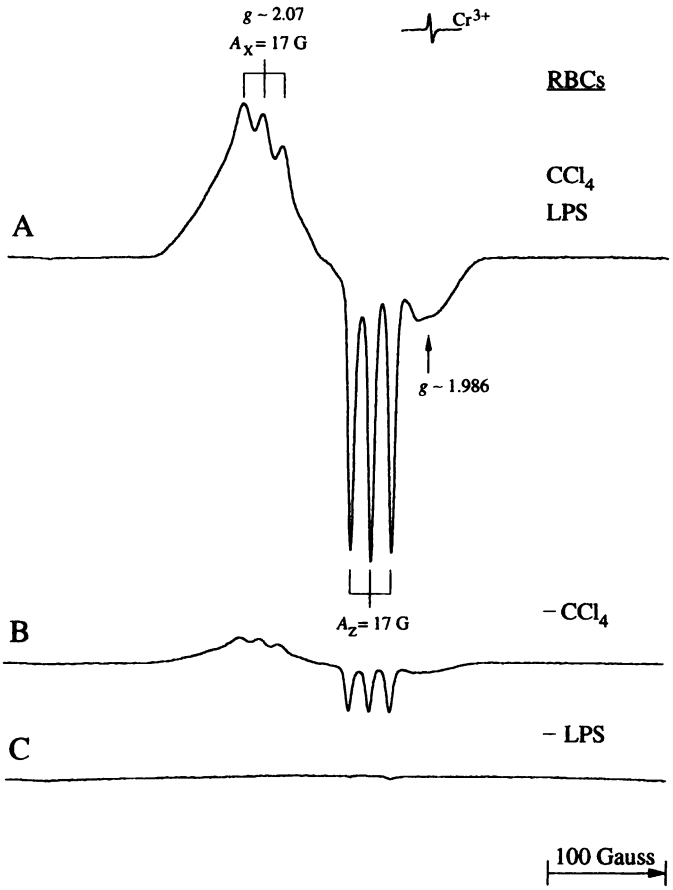

Fig. 1. EPR spectra (measured at 77°K) of RBCs from rats as follows. A, Rats were injected with CCl_4 (1.3 g/kg) and then, 20 hr later, with LPS (100 μg/kg); blood was obtained 4 hr after LPS injection. B, CCl_4 was replaced with olive oil. C, LPS was replaced with saline. The singlet signal is the marker from Cr^{3+} in MgO (g = 1.9800) (21). Spectrometer conditions were as follows: modulation amplitude, 4 G, microwave power, 10 mW; time constant, 1.3 sec; scan rate, 49 G/min.

detectable nitrosyl-hemoglobin signals during the first 2 hr after LPS injection (data not shown). This increase of nitrosyl-hemoglobin with time is consistent with inducible ·NO production. The EPR spectrum showed two distinctive sets of three lines due to hyperfine coupling constants (A_x and A_z) of 17 G at g_x = 2.07 and g_z = 2.013, which are characteristic of hemoglobin nitrosyl complexes at pH < 7 (27). In addition, the observed EPR peak at g = 1.986 was indicative of another species, identified as the hemoglobin hexacoordinate nitrosyl complex, with magnetic parameters of g_x = 2.08, g_y = 2.023, and g_z = 1.986 (26, 28). An EPR spectrum of RBCs from rats treated with LPS alone exhibited a much weaker signal for hemoglobin nitrosyl complexes (Fig. 1B). There were no detectable signals in RBCs of rats treated with CCl_4 alone (Fig. 1C). Even a lethal dose of CCl_4 (4 g/kg) gave only a weak signal (data not shown).

Similar observations were made in the liver tissues of LPS-induced rats that had been previously treated with CCl_4 (Fig. 2A). Under these conditions, there were no detectable signals in controls when CCl_4 or LPS was replaced with the corresponding vehicles (Figs. 2, B and C). There were also no detectable signals in whole blood or liver tissues from rats that had been pretreated with LPS (100 μg/kg) for 20 hr, injected with CCl_4 (1.3 g/kg), and sacrificed 4 hr later (data not shown).

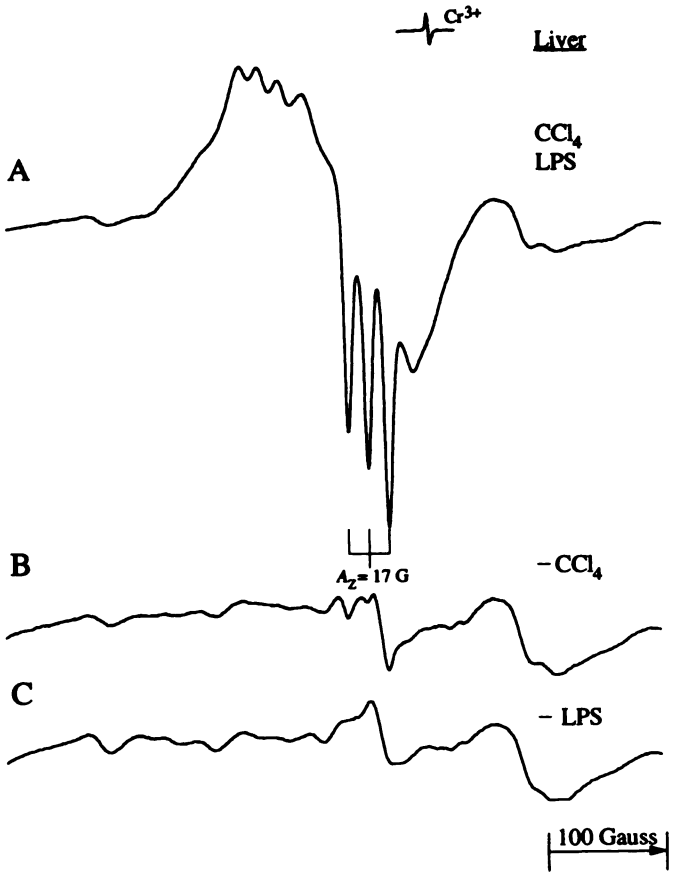

Fig. 2. EPR spectra (measured at 77°K) of liver tissues from rats as follows. A, Rats were injected with CCl_4 (1.3 g/kg) and then, 20 hr later, with LPS (100 μg/kg); livers were obtained 4 hr after LPS injection. B, CCl_4 was replaced with olive oil. C, LPS was replaced with saline. The singlet signal is the marker from Cr^{3+} in MgO (g = 1.9800) (21). Spectrometer conditions were as follows: modulation amplitude, 8 G, microwave power, 10 mW; time constant, 1.3 sec; scan rate, 49 G/min.

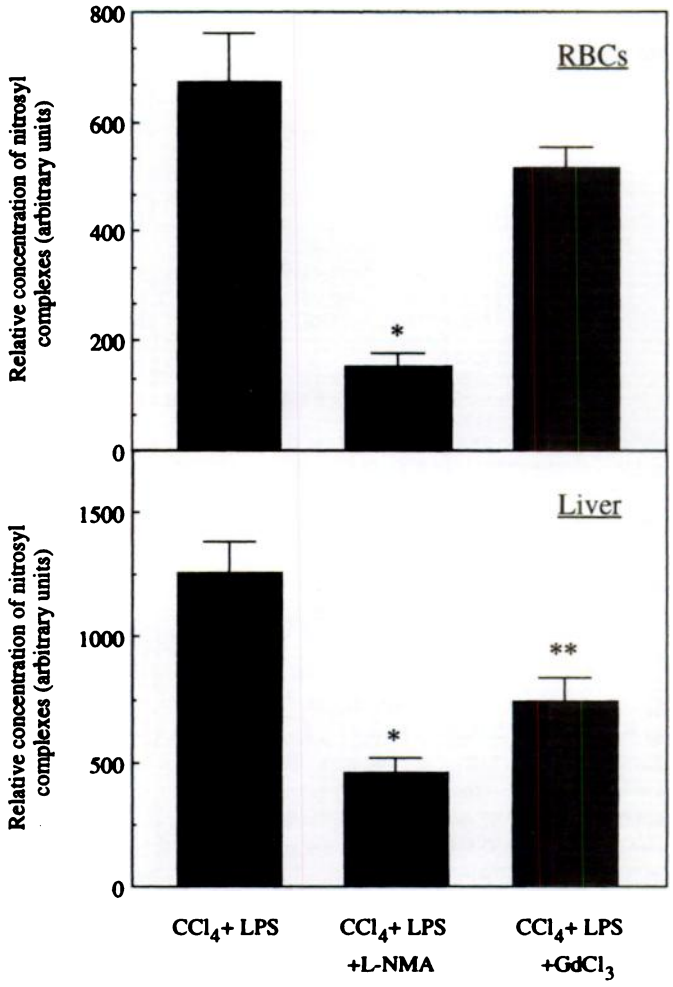

Fig. 3. Effects of gadolinium trichloride and L-NMA on the relative concentrations of nitrosyl complexes in RBCs (A) and livers (B). Experimental CCl_4 plus LPS treatment of rats (n = 8), treatment with $GdCl_3$ (20 mg/kg) (n = 10), and treatment with L-NMA (50 mg/kg) (n = 6) are described in Experimental Procedures. Data were obtained by double integration of EPR spectra measured under the conditions described in the legends to Figs. 1 and 2. Data are represented as mean ± standard error. L-NMA significantly (*, $p < 0.0005$) inhibited total nitrosyl complex formation in whole blood and livers of CCl_4- plus LPS-treated rats. Gadolinium trichloride significantly (**, $p < 0.005$) inhibited nitrosyl complex formation in livers of CCl_4- plus LPS-treated rats.

Relative concentrations of nitrosyl complexes in RBCs and livers were determined by performing double integration of EPR spectra. When CCl_4- plus LPS-treated rats were treated with L-NMA 2 hr after LPS injection, nitrosyl complex formation was inhibited both in RBCs and in the livers (Fig. 3). The sensitivity of the observed nitrosyl complex formation to L-NMA treatment showed that there was enzymatic biosynthesis of ·NO by NOS. Pretreatment with $GdCl_3$ killed at least 50% of Kupffer cells (11), and consistently there was a small but significant inhibition of nitrosyl complex production observed in liver ($p < 0.005$) (Fig. 3). This inhibition by $GdCl_3$ indicated that Kupffer cells or Kupffer cell products were involved in the ·NO production in liver. The uninhibitable portion of the nitrosyl complex signals in the livers was likely due to nitrosyl complexes formed in hepatocytes and other non-Kupffer cell sources. In contrast to the observation in liver, we found that $GdCl_3$ pretreatment caused only a slight, nonsignificant, inhibition in RBCs (Fig. 3).

Hepatotoxicity was assessed by the serum activities of AST, ALT, LDH, and SDH (Table 1). The activities of all hepatic enzymes were elevated in sera of rats treated with either LPS or CCl_4 alone, whereas these levels were markedly elevated in sera of CCl_4- plus LPS-treated rats (Table 1). The levels of hepatic enzyme activities found in sera of CCl_4- plus LPS-treated rats were much greater than the sum of activities found for CCl_4-treated and LPS-treated rats, indicating that synergistic hepatic damage had occurred. L-NMA treatment in the CCl_4- plus LPS-treated rats caused an even greater increase, in a dose-dependent manner (Table 1). There was no significant difference in the extent of inhibition of nitrosyl complex formation with 50 mg/kg or 100 mg/kg L-NMA (data not shown). The elevated levels of hepatic enzyme activities due to L-NMA treatment suggested that ·NO may have a protective role in liver. When pretreatment with $GdCl_3$ was performed before CCl_4 and LPS injections, the activities of all hepatic enzymes were not significantly changed from those of CCl_4- plus LPS-treated rats (Table 1). Although the previous study showed a protective effect of $GdCl_3$ treatment in CCl_4-treated rats (11), the partial protection or lack of protection by $GdCl_3$ treatment in our study may be due to the additional hepatic damage from oxygen radicals produced by peripheral macrophages and Kupffer cells stimulated by LPS (12, 29).

Because hepatocytes represent the majority of liver cells and

TABLE 1

Hepatic enzyme levels in the sera of CCl_4-, LPS-, and CCl_4- plus LPS-treated rats and the effects of $GdCl_3$ and L-NMA

Values are represented as mean ± standard error (n = 5/group).

Treatment	AST	ALT	LDH	SDH
	unit/liter	*units/liter*	*units/liter*	*units/liter*
Control	58 ± 3	34 ± 2	273 ± 41	8 ± 1
LPS	125 ± 12[a]	47 ± 3	479 ± 122	12 ± 1
CCl_4	255 ± 39[a]	98 ± 34[a]	332 ± 44	158 ± 40[a]
CCl_4 + LPS	690 ± 71[b]	237 ± 52[b]	3,670 ± 610[b]	319 ± 26[b]
CCl_4 + LPS + 50 mg/kg L-NMA[c]	940 ± 150	620 ± 140[d]	4,820 ± 580	361 ± 76
CCl_4 + LPS + 100 mg/kg L-NMA	1,150 ± 290	1,070 ± 470[d]	10,100 ± 2,700[d]	760 ± 160[e]
CCl_4 + LPS + 20 mg/kg $GdCl_3$[f]	840 ± 160	159 ± 35	4,800 ± 1,000	420 ± 98

[a] $p < 0.05$ versus control.
[b] $p < 0.0005$ versus control or LPS and $p < 0.05$ versus CCl_4.
[c] L-NMA was injected intraperitoneally 2 hr after LPS treatment.
[d] $p < 0.01$ versus CCl_4 plus LPS.
[e] $p < 0.05$ versus CCl_4 plus LPS or CCl_4 plus LPS plus 50 mg/kg L-NMA.
[f] $GdCl_3$ was injected intravenously 24 hr before CCl_4 administration.

blood is present in the liver, we digitally combined EPR spectra of nitrosyl complexes from hepatocytes and whole blood to characterize the *in vivo* EPR spectrum of CCl_4- plus LPS-treated rat liver (Fig. 4). An EPR spectrum of hepatocytes exposed to 10 μM $^{\cdot}NO$ is shown in Fig. 4A. This spectrum was the same as that reported previously for the nitrosyl-cytochrome P420 complex, which exhibits magnetic parameters of $g_x = 2.09$, $g_y = 2.029$, $g_z = 2.008$, and $A_z = 17$ G (30). The EPR spectrum shown in Fig. 4B was obtained from whole blood of CCl_4- plus LPS-treated rats. Spectral addition (1:1) of the EPR spectra of Fig. 4, A and B, resulted in the composite spectrum shown in Fig. 4F. We obtained an absolute EPR lineshape (Fig. 4E) by subtracting the background spectrum (Fig. 4C) from the experimental spectrum (Fig. 4D). The spectrum from a CCl_4- plus LPS-treated rat liver (Fig. 4E) was well matched with the composite (Fig. 4F) of the nitrosyl complexes of cytochrome P420 and hemoglobin. It is noted that the peak at $g \sim 2.11$ from nitrosyl-cytochrome P420 (Fig. 4A) contributes to the simulated spectrum (Fig. 4F), fitting to the shoulder at $g \sim 2.11$ observed in the liver (Fig. 4E). A nonheme nitrosyl complex had previously been reported for hepatocytes exposed to cytokines and LPS (31). In our study, it would not be possible to determine small contributions from nonheme nitrosyl components, because the nonheme nitrosyl peak ($g \sim 2.04$) overlaps one of the peaks of nitrosyl-cytochrome P420 ($g \sim 2.05$). In any case, a small contribution of nonheme nitrosyl complexes cannot be excluded in the CCl_4- plus LPS-treated rat livers.

Recent reports have shown that $^{\cdot}NO$ decreases cytochrome P450-mediated metabolism, and $^{\cdot}NO$ binds to both ferric and ferrous cytochrome P450 heme groups, as measured by optical spectrophotometry (32, 33). In our study, where high levels of $^{\cdot}NO$ were observed (Figs. 1 and 2), we investigated the status of liver cytochrome P450 by recording EPR spectra over a scan range of 1000 G (Fig. 5). The known EPR signals of low-spin ferric ion of both cytochrome P450 and cytochrome P420 showed similar resonance peaks at $g \sim 2.45$, 2.26, and 1.90 (34, 35); we observed these cytochrome species at $g \sim 2.48$, 2.29, and 1.91 resonances in normal rat hepatocytes and liver tissues (Fig. 5, A and C). When hepatocytes were mixed with 15 μM $^{\cdot}NO$, these ferric cytochrome P450/P420 peaks at $g \sim 2.48$ and 2.29 were markedly decreased in intensity, compared with the control (Fig. 5A), and concomitantly there was an increase of nitrosyl-cytochrome P420 resonances at $g \sim 2.0$–2.1 (Fig. 5B). The same observations were found in the liver of a CCl_4- plus

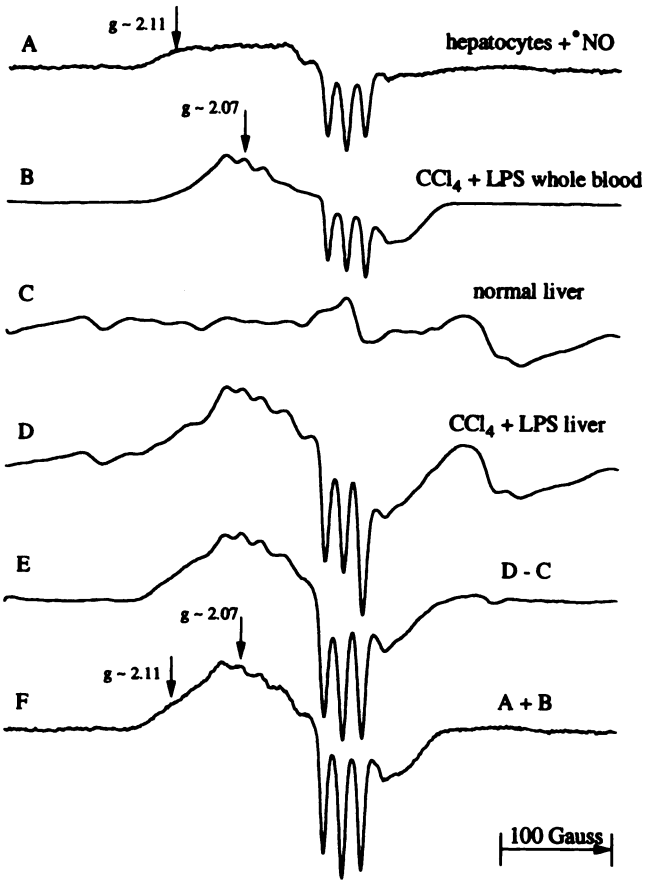

Fig. 4. EPR analyses to characterize *in vivo* signals observed in the liver. A, EPR spectrum obtained from a mixture of hepatocytes (1.4×10^7 cells/ml) exposed to $^{\cdot}NO$ (10 μM final concentration). The background signals from hepatocytes alone were subtracted from this spectrum. B, EPR spectrum obtained from whole blood of experimental CCl_4- plus LPS-treated rats. C, EPR spectrum obtained from normal rat liver. D, EPR spectrum obtained from the liver of an experimental CCl_4- plus LPS-treated rat. E, EPR spectrum obtained from the subtraction of the spectrum shown in C from the spectrum shown in D. F, EPR spectrum obtained from the addition (1:1) of the spectra shown in A and B. Spectrometer conditions for A were as follows: modulation amplitude, 2 G, microwave power, 10 mW; time constant, 0.66 sec; scan rate, 107 G/min. Spectrometer conditions for B-D were the same as described in the legend to Fig. 2.

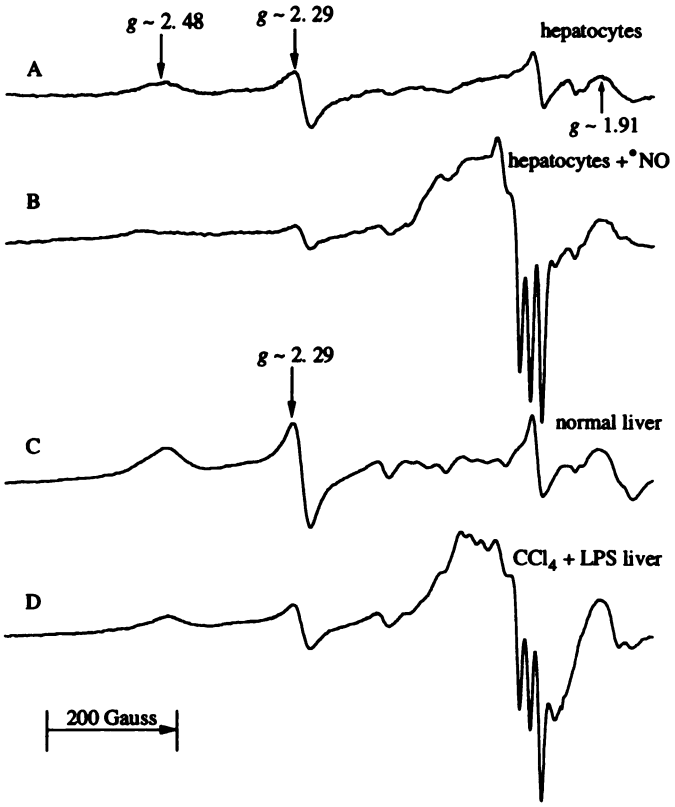

Fig. 5. Ferric cytochrome P450/P420 resonances in rat hepatocytes and liver at 77°K. A, EPR spectrum obtained from untreated rat hepatocytes. Resonances at $g \sim 2.48$, 2.29, and 1.91 are signals of low-spin ferric cytochromes P450 and P420. B, EPR spectrum obtained from hepatocytes exposed to ·NO (15 μM final concentration). C, EPR spectrum obtained from normal rat liver. D, EPR spectrum obtained from the liver of an experimental CCl_4- plus LPS-treated rat. Spectrometer conditions were as follows: modulation amplitude, 8 G; microwave power, 10 mW; time constant, 1.3 sec; scan rate, 89.5 G/min.

TABLE 2

Relative intensity of ferric low-spin heme groups ($g \sim 2.29$) in the livers of rats treated with CCl_4 or CCl_4 plus LPS, as described in Experimental Procedures

Values are represented as mean ± standard error (n = 4/group).

Treatment	Intensity
	% of control
Control	100 ± 2
CCl_4	74 ± 9
CCl_4 + LPS	45 ± 4*
CCl_4 + LPS + L-NMA	53 ± 6

*$p < 0.0005$ versus control.

LPS-treated rat (Fig. 5D), compared with normal liver (Fig. 5C). The decrease of ferric cytochromes P450/P420 correlating with an increase of nitrosyl-cytochrome P420 unambiguously showed the binding of ·NO to cytochromes P450/P420 *in vivo* (Fig. 4). The extent of decrease of the ferric cytochrome P450/P420 signal at $g \sim 2.29$, normalized to percentage of control, is shown in Table 2. Treatment with CCl_4 alone caused a small but not significant decrease in ferric cytochromes P450/P420, whereas a 45% decrease was found in the livers of CCl_4- plus LPS-treated rats. However, L-NMA treatment did not cause a significant change in the ferric cytochromes P450/P420 of the CCl_4- plus LPS-treated rat livers.

Discussion

It is generally accepted that a free radical mechanism is operative in CCl_4 hepatotoxicity (1, 2). It has been shown only recently that Kupffer cells are involved in CCl_4 toxicity, as evidenced by protection by $GdCl_3$ treatment, and this treatment does not alter the cytochrome P450-dependent metabolism of CCl_4 (11). Studies have shown that, when rats are treated with CCl_4, Kupffer cells increase in number during the first 24 hr (14) and tumor necrosis factor-α mRNA levels increase in Kupffer cells (36). Kupffer cells and Kupffer cell/hepatocyte co-cultures release ·NO upon immunologic activation by LPS (37), and Kupffer cell products are required for ·NO production in hepatocytes (38). These findings strongly imply that ·NO may form and play a role in the metabolism and removal of CCl_4.

In this paper we present data on ·NO production in rats that had been pretreated with a nonlethal dose of CCl_4, followed by a low dose of LPS to induce NOS. Nitrosyl-hemoprotein complexes indicative of ·NO production were measured by EPR at 77°K in RBCs and liver tissues (Figs. 1 and 2). With administration of the specific NOS inhibitor L-NMA, the observed nitrosyl signals were inhibited, indicating that the ·NO production was indeed from enzymatic metabolism of L-arginine (Fig. 3). Pretreatment of CCl_4- plus LPS-treated rats with $GdCl_3$ caused an insignificant inhibition in RBCs but a 40% inhibition in liver (Fig. 3), consistent with the report that Kupffer cell activity was diminished by 50% (11). This observation suggests that, upon activation by LPS, Kupffer cells and Kupffer cell products mediate ·NO production in the liver before ·NO transport to the circulating blood. This finding is in agreement with the previous report that multiple cytokines, which induce the amplified levels of ·NO in hepatocytes, are indeed produced by LPS-activated Kupffer cells (38).

CCl_4 and LPS synergistically increased hepatic damage, which was measured as hepatic enzyme activities (Table 1). This effect is due to activation of Kupffer cells by LPS, resulting in the release of reactive oxygen species, which are responsible for this increased liver damage in CCl_4-pretreated rats (12, 29). An elevation of hepatic enzyme serum activities was observed due to L-NMA treatment of the CCl_4- plus LPS-treated rats, suggesting a protective role of ·NO in this study. These data are similar to those found in the studies of *Corynebacterum parvum*- plus LPS-treated mice (39). In contrast to that study, we found that the histology of liver slices showed the same centrilobular damage with L-NMA treatment of the CCl_4- plus LPS-treated rats (data not shown). In our study, we found that the extent of liver damage was more sensitive to L-NMA than $GdCl_3$ treatment (Table 1). Several lines of evidence show that ·NO exhibits a protective effect as a ligand with ferrous ions or heme compounds by preventing membrane lipid peroxidation (40), by protecting against cytotoxicity from reactive oxygen species (41), and by inhibiting the oxidation of low density lipoprotein (42). It is possible that ·NO may play a role in our experimental model of liver injury by inhibiting lipid peroxidation.

·NO causes both reversible and irreversible inhibition of cytochrome P450-mediated *O*-dealkylase activity (32). The reversible inhibition is proposed to involve the direct binding of ·NO, which is later released, whereas the irreversible inhibtion is due to destruction of the heme or apoprotein moieties of cytochrome P450. Perhaps ·NO could exhibit a protective role

in our study through the decrease of cytochrome P450-catalyzed production of the toxic trichloromethyl free radical. From previous *in vivo* spin-trapping studies (43), it was found that ~25% of the adducts of trichloromethyl radical present 1 hr after CCl_4 administration remained in the liver after 20 hr. Thus, the metabolic activitiy of cytochrome P450 can contribute to CCl_4 toxicity in our experimental protocol. It has also been shown that ·NO is involved in the suppression of cytochrome P450 activity and content in liver microsomes prepared from rats that had been injected with high-dose LPS (33). Therefore, ·NO can play a regulatory role in the liver through modulation of cytochrome P450. Inhibition of cytochrome P450 by direct binding of ·NO to the heme group would result in the formation of cytochrome P450-nitrosyl complex. This complex is not stable, and it decomposes with time to a nitrosyl species that has an EPR spectrum similar to that of the cytochrome P420-nitrosyl complex (30). The composite of the cytochrome P420- and hemoglobin-nitrosyl complexes gave a good match to the spectrum detected in CCl_4- plus LPS-treated rat liver (Fig. 4), demonstrating that the cytochrome P420-nitrosyl complex was formed. Concomitantly, the EPR resonances of ferric cytochromes P450/P420 were decreased in hepatocytes exposed to ·NO and in the CCl_4- plus LPS-treated rat livers, compared with controls (Fig. 5; Table 2). The detected cytochrome P420-nitrosyl species likely comes from ·NO binding to the ferric cytochromes P450/P420, and its autoreduction results in the paramagnetic ferrous nitrosyl complexes (Fig. 5). It is noted that NOS has protein sequences similar to those of cytochrome P450 (44, 45), and the EPR spectrum of purified brain NOS exhibits ferric heme groups in both high-spin and low-spin states (46). Therefore, the *in vivo* EPR signals measured at 77°K (Fig. 5D), where inducible NOS was active, were likely to be from a mixture of low-spin ferric heme groups from NOS and cytochrome P450.

In summary, we have shown that high levels of ·NO are produced in experimental CCl_4-treated rats sensitized by a nonlethal dose of endotoxin. Synergistically increased liver damage was observed under these conditions. Nitrosyl-cytochrome P420 was formed in the livers of CCl_4- plus LPS-treated rats, and the resonances of low-spin ferric heme groups were concomitantly decreased. Our study has demonstrated that, although the simultaneous presence of the hepatotoxicant CCl_4 and a very low dose of bacterial endotoxin increases hepatotoxicity, the formation of ·NO is not responsible for the increased toxicity and is in fact protective.

Acknowledgments

We would like to thank Dr. J. F. Mahler for his expert assistance with the histology of liver slices, Dr. K. T. Knecht for her advice on *in vivo* $GdCl_3$ treatment, Ms. Ann Lockhart for her advice on the statistical analyses, and Mr. R. E. Wilson for his technical assistance.

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Correction

The chemical definition of RM7, a cannabinoid receptor agonist (1), was incorrectly reported in the article by Felder *et al.* (Vol. 42, No. 5, pp. 838–845, 1992). This compound is also referred to as HU249 (2). The correct chemical name for RM7 is 1,3-methanodibenzofuran-9-ol,7-(1,1-dimethylheptyl)-1,2,3,4,4a,9b-hexahydro-2,2-dimethyl-4-methylene, [1R-(1α,3α,4aβ,9bβ)].

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