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J Clin Invest. 1992;**90**(6):2502-2507. <https://doi.org/10.1172/JCI116143>.

Research Article

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Formation of Novel Non-cyclooxygenase-derived Prostanoids (F₂-Isoprostanes) in Carbon Tetrachloride Hepatotoxicity

An Animal Model of Lipid Peroxidation

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Abstract

These studies examine the *in vivo* formation of a unique series of PGF₂-like compounds (F₂-isoprostanes) derived from free radical-catalyzed nonenzymatic peroxidation of arachidonic acid. We have previously shown that levels of these compounds increase up to 50-fold in rats administered CCl₄. To understand further the formation of these compounds *in vivo*, we carried out a series of experiments assessing factors influencing their generation. After CCl₄ (2 ml/kg) was administered to rats, plasma F₂-isoprostanes increased 55-fold by 4 h. Levels declined thereafter, but at 24 h, they were still elevated 21-fold, indicating continued lipid peroxidation. Pretreatment of rats with isonicotinic acid hydrazide and phenobarbital to induce cytochrome P-450 enhanced the production of F₂-isoprostanes after CCl₄ administration eightfold and fivefold, respectively, whereas inhibition of the cytochrome P-450 system with SKF-525A and 4-methylpyrazole decreased formation of F₂-isoprostanes after CCl₄ by 55 and 82%, respectively. Further, the glutathione-depleting agents buthionine sulfoximine and phorone augmented the F₂-isoprostane response to CCl₄ by 22- and 11-fold, respectively. F₂-isoprostanes are formed *in situ* esterified to lipids and, in addition to increases in levels of free F₂-isoprostanes in the circulation, levels of F₂-isoprostanes esterified to lipids in various organs and plasma also increase sharply during CCl₄ poisoning. The measurement of F₂-isoprostanes may facilitate investigation of the role of lipid peroxidation in human diseases. (*J. Clin. Invest.* 1992. 90:2502-2507.) **Key words:** peroxidation • free radical • prostaglandin • eicosanoid • oxidation

Introduction

Tissue damage resulting from free radical injury is postulated to play a role in a variety of disease processes (1). Despite the large amount of research done to date, however, the association of free radicals with disease has often been indirect and circumstantial. A major reason for this is the inadequacy of techniques used to quantitate free radicals or the effects they produce in

biological systems (2). Measures of lipid peroxidation are often employed to implicate free radicals in pathophysiological processes. Free radicals attack unsaturated lipids in biological systems, resulting in the formation of a variety of products. Commonly used methods to quantitate lipid peroxidation include measurements of short-chain alkanes, malondialdehyde, and conjugated dienes. Each of these, however, suffers from inherent problems especially when used *in vivo*. For example, the measurement of short-chain alkanes is a cumbersome procedure and molar yields are affected by their metabolism and by oxygen tension (3). The measurement of malondialdehyde, although relatively simple to perform, is affected by metabolism of the compound as well as *ex vivo* generation of it. Further, compounds not arising from lipid peroxidation interfere with the measurement of conjugated dienes (2).

Recently, we described the formation of a series of PGF₂-like compounds, termed F₂-isoprostanes, produced *in vivo* in humans by a non-cyclooxygenase free radical-catalyzed mechanism involving peroxidation of arachidonic acid (4). (The term isoprostane has been preliminarily sanctioned by the ad hoc Committee on Eicosanoid Nomenclature constituted by the Joint Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry and International Union of Biochemistry. Particulars for the identification of individual compounds are under deliberation.) Formation of these compounds initially involves the formation of positional peroxy radical isomers of arachidonic acid, which undergo endocyclization to PGG₂-like compounds and are subsequently reduced to PGF₂-like compounds (Fig. 1). Circulating levels of these compounds increase dramatically in animal models of lipid peroxidation. In an effort to understand further the formation of these compounds *in vivo* and to explore the use of measuring F₂-isoprostanes as an index of endogenous lipid peroxidation, we carried out a series of studies assessing factors influencing the generation of these compounds in a much-studied animal model of lipid peroxidation involving the administration of CCl₄ to rats.

Methods

Animals. Male Sprague-Dawley and Fischer 344 rats (200–300 g) were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). They were given food and water *ad libitum* and were housed in American Association for Laboratory Animal Care-approved facilities with alternating 12-h light and dark cycles. For all experiments, rats were fasted overnight and experiments were begun between 7 and 10 a.m. Rats remaining in experiments > 6 h were fed after that time. Rats were anesthetized with pentobarbital and blood was removed from the aortic bifurcation. In some experiments, livers or other tissues were obtained immediately after exsanguination and flash frozen in liquid nitrogen, after which time they were stored at –70°C.

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Received for publication 21 April 1992 and in revised form 9 July 1992.

J. Clin. Invest.

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0021-9738/92/12/2502/06 \$2.00

Volume 90, December 1992, 2502–2507

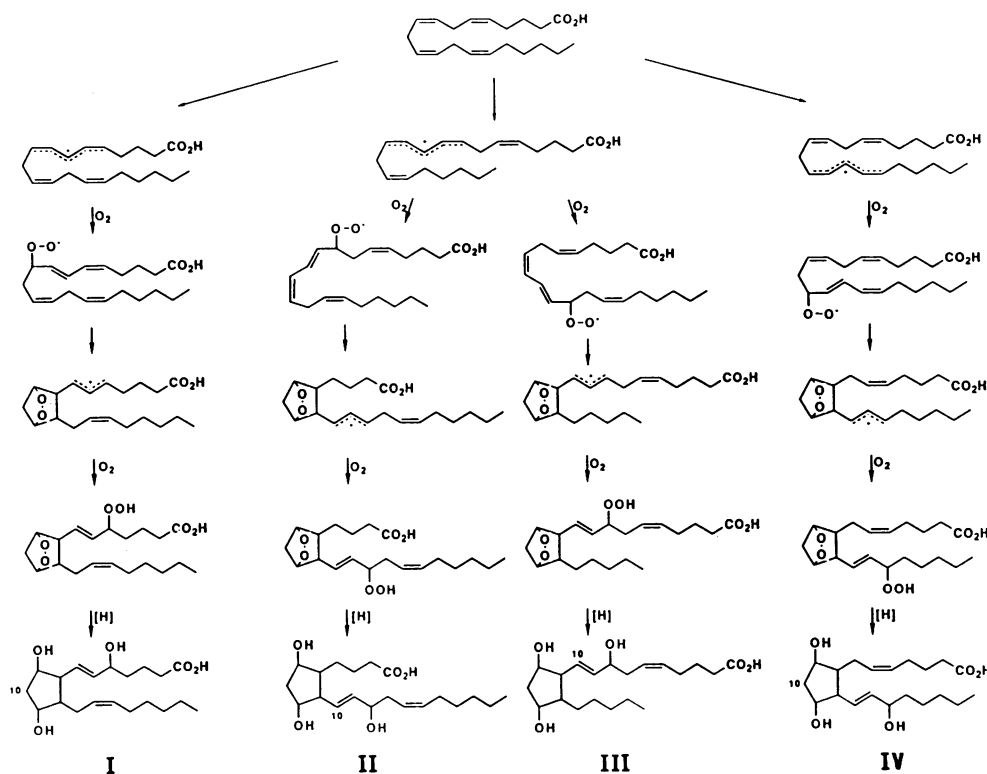


Figure 1. Proposed mechanism for the formation of F_2 -isoprostanes. For simplicity, stereochemistry is not specified. Four regioisomers (I-IV) can be formed, each comprised of eight racemic diastereomers. Reprinted with permission from reference 5 (copyright Academic Press).

Administration of CCl_4 . Depending on the experiment, CCl_4 was administered in dosages of 1, 2, or 3 ml/kg orogastrically diluted 1:1 with corn oil. Control animals were administered corn oil only. Animals were killed at the time points indicated in the text and figures.

Administration of other agents. In selected experiments, animals were pretreated with agents before the administration of CCl_4 . These included isonicotinic acid hydrazide (INH),¹ which was given in drinking water (1 mg/ml) for 10 d and stopped 24 h before CCl_4 administration, phenobarbital (45 mg/kg i.p.) administered daily for 5 d with the last dose 24 h before CCl_4 administration, buthionine sulfoximine (BSO) (4 mmol/kg i.p.) administered 3 and 1.5 h before CCl_4 , phorone (250 mg/kg i.p.) administered 1 h before CCl_4 , SKF-525A (150 mg/kg i.p.) given 2 h before CCl_4 administration, or 4-methylpyrazole (100 mg/kg i.p.) given 30 min before CCl_4 . In other experiments, thioacetamide (5 mmol/kg i.p.) was given to Sprague Dawley rats and acetaminophen (750 mg/kg orogastrically) was administered to Fischer 344 rats.

Serum glutamic pyruvic transaminase (SGPT). SGPT was measured in fresh plasma using a commercial kit (Sigma Chemical Co., St. Louis, MO) employing a spectrophotometric assay.

F_2 -isoprostanes. Free F_2 -isoprostanes were measured in fresh plasma after purification and derivatization employing gas chromatography/negative-ion chemical ionization mass spectrometry as described (5). In certain experiments, lipid extracts from plasma and various organs were analyzed for acylated F_2 -isoprostanes. In these experiments, lipids were extracted and subsequently subjected to base hydrolysis to liberate acylated fatty acid derivatives as described (5, 6). Free F_2 -isoprostanes were then quantified as above.

Assessment of the half-life of 8-epi-PGF_{2α} in the circulation of the rat. For these experiments, 8-epi-PGF_{2α} (a generous gift of Gordon Bundy of The Upjohn Co., Kalamazoo, MI) was infused for 30 min at a dose of 2 μg/kg per min into the jugular vein of euvoletic male Sprague-Dawley rats prepared as described (7). Two of the animals

underwent portacaval shunting and hepatic artery ligation before infusion of the prostaglandin as described below. Blood was removed from the femoral artery (0.5 ml) at selected times after the infusion was stopped and the concentration of 8-epi-PGF_{2α} was quantified as for F_2 -isoprostanes. From this, the disappearance half-life of 8-epi-PGF_{2α} in the circulation was determined.

Hepatic artery ligation and portacaval shunting. End-to-side portacaval anastomoses were performed on rats under pentobarbital anesthesia (65 mg/kg i.p.) by a modification of the technique described by Lee and Fisher (8). After construction of the shunt and just before infusion of 8-epi-PGF_{2α}, the hepatic artery was ligated.

Materials. CCl_4 was purchased from Fisher Scientific Co. Allied Corp. (Pittsburgh, PA). INH, BSO, acetaminophen, thioacetamide, and phorone were purchased from Aldrich Chem. Co. (Milwaukee, WI). Phenobarbital was purchased from Mallinckrodt Inc. (Paris, KY).

Statistical methods. Statistical evaluation was performed using Student's *t* test.

Results

Previously we showed that levels of F_2 -isoprostanes derived from free radical-catalyzed lipid peroxidation increased sharply (up to 50-fold) in rats administered CCl_4 (4). The formation of these compounds was not inhibited by indomethacin, indicating that the cyclooxygenase enzyme is not involved in their formation. To further explore the rate of formation of these compounds, we performed a time course study in which we examined the appearance of free F_2 -isoprostanes in the circulation of rats after administration of CCl_4 (2 ml/kg). We compared the rate of formation of these compounds with the extent of liver damage as assessed by increases in SGPT. As shown in Fig. 2, there was a rapid increase in circulating levels of F_2 -isoprostanes and they reached a maximum 4 h after administration of CCl_4 . Levels declined with time but were still

1. Abbreviations used in this paper: BSO, buthionine sulfoximine; INH, isonicotinic acid hydrazide; SGPT, serum glutamic pyruvic transaminase.

markedly elevated at 24 h (21-fold) and even 48 h (10-fold), suggesting that lipid peroxidation continues for ≥ 2 d after CCl_4 administration. Unlike the increase in isoprostane levels, however, SGPT increased at a much slower rate after treatment and continued to increase after 24 h.

It could be argued that elevated levels of F_2 -isoprostanes at 24 or 48 h after CCl_4 administration do not represent ongoing lipid peroxidation but instead represent decreased clearance of these compounds because of impaired metabolism by a damaged liver, as the liver is known to be important in the metabolism of prostanoids (9). One of the F_2 -isoprostanes expected to be formed in abundance is 8-epi-PGF $_{2\alpha}$ (4). We obtained this compound in pure form and thus could explore the role of the liver in the clearance of F_2 -isoprostanes by examining the clearance of 8-epi-PGF $_{2\alpha}$ from plasma after its intravenous infusion into rats. The rate of 8-epi-PGF $_{2\alpha}$ disappearance was examined in two groups of animals, normal rats and rats made anhepatic by the creation of a portacaval shunt and ligation of the hepatic artery. Experiments were carried out for ≤ 120 min as anhepatic rats become hypoglycemic after this time. After an infusion of 8-epi-PGF $_{2\alpha}$ lasting 30 min, the elimination half-life of the compound was 15 and 17 min in two normal animals and 20 and 23 min in two anhepatic animals. This indicates that extrahepatic tissues can accomplish rapid clearance of F_2 -isoprostanes from circulation as has been shown for many other eicosanoids (9). Therefore, it is unlikely that diminished clearance from hepatic damage could account for elevated plasma levels of isoprostanes after CCl_4 .

As shown in Fig. 2, the formation of isoprostanes precedes the appearance of biochemical markers of hepatic necrosis. This suggests that the formation of isoprostanes is not simply a nonspecific manifestation of cell death. To confirm this, F_2 -isoprostanes were measured in two models of chemically induced liver damage in which lipid peroxidation has not been causally implicated (10). As shown in Table I, the administration of thioacetamide to rats resulted in liver injury, as measured by SGPT release, which far exceeded the increased levels of plasma F_2 -isoprostanes at 24 h. Further, as shown in Table II, the administration of acetaminophen to Fischer 344 rats was associated with only small increases in plasma F_2 -isoprostanes despite marked increases in SGPT. Although increases in F_2 -isoprostane levels using both agents were small, it should be pointed out that the increases were statistically significant ($P < 0.05$ for thioacetamide at 6 and 24 h compared with time 0

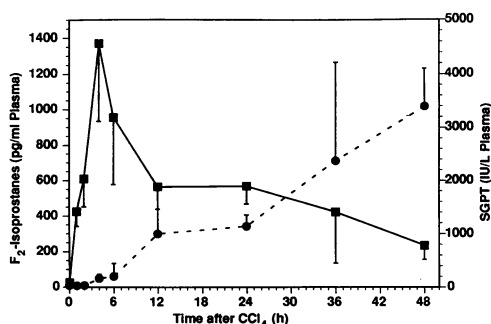


Figure 2. Levels of free F_2 -isoprostanes (■) and (●) SGPT in plasma of rats. Levels were measured at various times after the administration of CCl_4 (2 ml/kg) orogastrically. All time points represent a mean of four animals. The vertical line indicates 1 SD.

Table I. Effect of Thioacetamide on Plasma F_2 -Isoprostane Levels and Serum SGPT in Rats

Time	SGPT	F_2 -isoprostanes
h	IU/liter	pg/ml
0	34±9.6	20±3
6	47±11.7	24±2*
24	372±181*	57±31*

Sprague-Dawley rats were administered thioacetamide (5 mmol/kg i.p.) and then killed at times noted. Blood was removed from the aortic bifurcation and measured for F_2 -isoprostanes and SGPT. Results are based on four animals per time point. * $P < 0.05$ vs. time 0.

and $P < 0.05$ at 24 h for acetaminophen treatment). Nonetheless, these studies imply that hepatic necrosis per se is not associated with marked increases in circulating F_2 -isoprostane levels and that CCl_4 induces a form of liver injury different from thioacetamide and acetaminophen.

The effect of various doses of CCl_4 on the circulating levels of F_2 -isoprostanes was then examined. For these experiments animals were killed at either 4 or 24 h. These time points were chosen because circulating F_2 -isoprostane levels are maximal at 4 h after CCl_4 and decline to a plateau level that is still elevated above baseline values by 24 h (Fig. 2). As shown in Fig. 3, increasing doses of CCl_4 administered orogastrically resulted in similar F_2 -isoprostane levels in the circulation of rats at 4 h but a dose-dependent increase in levels at 24 h. This difference at 24 h was statistically significant ($P < 0.05$) for the group of animals receiving 3 ml/kg CCl_4 compared with the other doses. In all animals examined at 24 h after receiving CCl_4 , SGPT levels were markedly elevated over baseline values (33±3 IU/liter for untreated control group vs. 2,700±640 IU/liter for the 24-h group receiving 1 ml/kg CCl_4 , 4,400±820 for the 24-h group receiving 2 ml/kg CCl_4 , and 5,000±710 for the 24-h group receiving 3 ml/kg; $P < 0.05$ for all 24-h groups compared with untreated animals).

We then assessed the role of the cytochrome P-450 system in CCl_4 -induced formation of F_2 -isoprostanes. CCl_4 is metabolized in vivo by the P-450 system to the trichloromethyl radical ($\text{CCl}_3\cdot$), which is the radical responsible for the lipid peroxidation occurring after administration of the agent (11). As shown in Fig. 4, pretreatment of rats with either phenobarbital, which

Table II. Effect of Acetaminophen on Plasma F_2 -Isoprostane Levels and Serum SGPT in Rats

Time	SGPT	F_2 -isoprostanes
h	IU/liter	pg/ml
0	46±20	24±3
6	53±14	27±15
24	1,350±500*	34±8*

Fischer 344 rats were administered acetaminophen 750 mg/kg orogastrically and were killed at times noted. Blood was removed from the aortic bifurcation and measured for F_2 -isoprostanes and SGPT. Results are based on four animals per time point. * $P < 0.05$ vs. time 0.

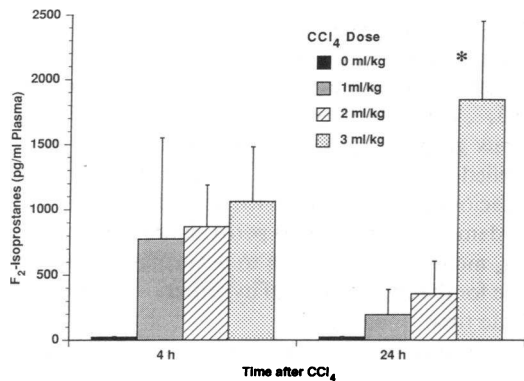


Figure 3. Effects of varying dosages of CCl₄ on circulating plasma F₂-isoprostane and SGPT levels in rats 4 and 24 h after administration. Results represent four animals per group. **P* < 0.05, 3 ml/kg CCl₄ vs. 1 or 2 ml/kg CCl₄ 24 h after administration.

increases total liver cytochrome P-450 content (12), or INH, which does not increase total cytochrome P-450 but selectively increases particular isoforms, including the IIE-1 isoform (12, 13), markedly increased levels of circulating F₂-isoprostanes both at 4 and 24 h.

The effect of inhibition of the cytochrome P-450 system on the formation of F₂-isoprostanes after CCl₄ administration was then examined. As shown in Fig. 5, pretreatment of rats with either SKF-525A (14) or 4-methylpyrazole (15) markedly decreased circulating F₂-isoprostane levels at 4 h.

A variety of substances are believed to protect against oxidant stress in vivo. Among these is glutathione (16). One might therefore predict that glutathione depletion would worsen lipid peroxidation and tissue damage from CCl₄ and thus increase circulating levels of F₂-isoprostanes. To test this hypothesis, rats were administered the glutathione-depleting agents BSO or phorone, and then were treated with CCl₄. As Fig. 6 shows, although no enhanced lipid peroxidation was apparent at 4 h, there was a marked increase in levels of circulating F₂-isoprostanes at 24 h in the BSO- and phorone-pretreated animals (*P* < 0.05 at 24 h for either agent compared with no pretreatment). SGPT levels were not significantly higher in BSO-pretreated animals in comparison to controls at 24 h but were higher (*P* < 0.05) at 24 h in the phorone-treated group (data not shown).

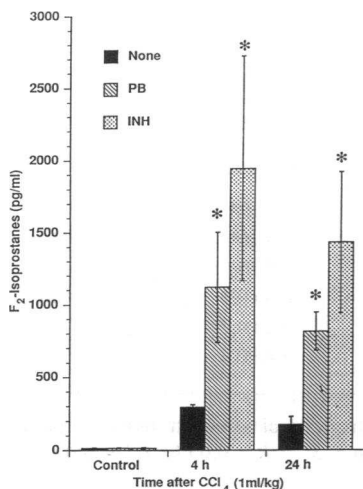


Figure 4. Effects of INH and phenobarbital (PB) pretreatment on circulating free F₂-isoprostane levels. Rats were killed at 4 and 24 h after administration of CCl₄ (1 ml/kg). *n* = 5 animals per group. **P* < 0.05 for pretreatment compared with no pretreatment.

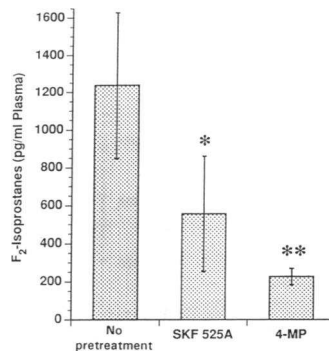


Figure 5. Effects of SKF-525A and 4-methylpyrazole (4-MP) pretreatment on F₂-isoprostane levels. F₂-isoprostanes were measured in plasma of rats 4 h after administration of CCl₄ (1 ml/kg). *n* = 5 animals per group. **P* < 0.05, pretreatment with SKF-525A vs. no pretreatment. ***P* < 0.005, pretreatment with 4-methylpyrazole vs. no pretreatment.

Recently, we discovered that F₂-isoprostanes are formed in situ on phospholipids by free radical-catalyzed peroxidation of esterified arachidonic acid and subsequently are released in free form, presumably by phospholipases (17). After administration of CCl₄, levels of F₂-isoprostanes esterified to lipids in the liver increase rapidly and precede the appearance of increased quantities of free F₂-isoprostanes in the circulation. This suggests that F₂-isoprostanes are initially formed esterified in lipids in the liver and free compounds are then released into the circulation. Since the liver is also the major organ involved in lipoprotein synthesis and secretion, it was of interest to examine whether increases in levels of F₂-isoprostanes esterified to plasma lipids also occur after administration of CCl₄. Fig. 7 shows the time course of appearance of increased levels of F₂-isoprostanes esterified in plasma lipids in CCl₄-treated rats in comparison with levels of free F₂-isoprostanes in plasma and levels of F₂-isoprostanes esterified in liver lipids. Measurable levels of F₂-isoprostanes esterified in plasma lipids were detected at baseline and increased after the administration of CCl₄ in parallel with increases in the levels of free compounds. These increases lagged behind the appearance of acylated F₂-isoprostanes in the liver. This suggests that the isoprostane-containing lipids in the plasma are initially formed in the liver and then released into the circulation.

Although the liver is the major target organ of CCl₄ toxicity, other tissues, such as the kidney, are also known to incur damage from CCl₄ (11). Therefore, it was of interest to examine the

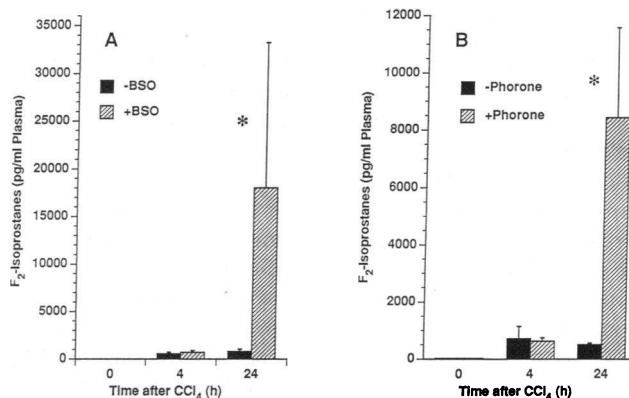


Figure 6. Effects of (A) BSO and (B) phorone pretreatment on F₂-isoprostane levels. F₂-isoprostanes were measured in the plasma of rats at 4 and 24 h after administration of CCl₄ (1 ml/kg). *n* = 4 animals per group. **P* < 0.05 pretreatment compared with no pretreatment. Phorone administered without CCl₄ did not elevate circulating F₂-isoprostane levels at 4 or 24 h (data not shown).

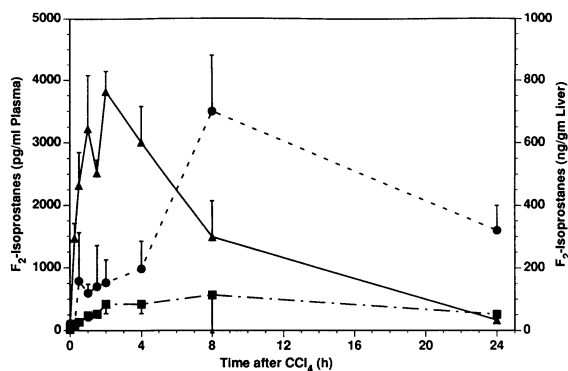


Figure 7. Levels of F₂-isoprostanes esterified in plasma lipids of rats at various times. The animals were administered CCl₄ (1 ml/kg). F₂-isoprostanes esterified in plasma lipids (—●—) were compared with free plasma levels (---■---) and levels esterified to lipids in liver (---▲---). Each time point represents the mean of results in four animals. The dose of CCl₄ used in this experiment is one-half of that used in the experiment shown in Fig. 1.

extent to which CCl₄ induces the formation of isoprostane-containing lipids in other organs. As anticipated, isoprostane-containing lipids measured 2 h after administration of CCl₄ were most abundant in the liver. However, substantial increases were also found in the kidney and lung as shown in Table III. Thus, it is possible that not only the liver but other tissues also contribute to free and lipid-associated levels of F₂-isoprostanes that are present in the circulation after CCl₄.

Discussion

Carbon tetrachloride induces lipid peroxidation through formation of the trichloromethyl (CCl₃·) radical (11). Previous studies have shown marked increases in the exhalation of short-chain alkanes in vivo by animals administered this agent (10, 18). The studies herein were undertaken to examine factors influencing levels of F₂-isoprostanes in the setting of CCl₄-induced lipid peroxidation in vivo. These compounds result from free radical-catalyzed peroxidation of arachidonic acid. We have previously shown markedly increased circulating levels of F₂-isoprostanes after the administration of CCl₄ (4). As an extension of this preliminary work, we now show that CCl₄ dramatically increases plasma F₂-isoprostane levels in a time-dependent manner. Interestingly, although plasma levels were maximal at 4 h, they were still significantly elevated above baseline levels at 24 and 48 h. This implies that lipid peroxidation was continuing even at this later time. CCl₄-induced lipid peroxidation destroys the cytochrome P-450 responsible for the generation of trichloromethyl radicals and has been shown, using in vitro systems, to inhibit its own metabolism (19). This would suggest that the destructive effect of CCl₄ should limit its metabolism to a brief period after its administration. Our data, however, would suggest otherwise. Our results show that lipid peroxidation begins rapidly in vivo after CCl₄ poisoning but continues for a far longer period of time than would be predicted from the in vitro studies. Further, the hepatic dysfunction resulting from CCl₄ injury does not appear to account, to a significant extent, for the elevated plasma levels of F₂-isoprostanes measured 24–48 h after poisoning since the elimination

half-life of the isoprostane 8-epi-PGF_{2α} was not markedly altered by devascularization of the liver.

Hyperbaric oxygen treatment, which reduces metabolism of CCl₄ to CCl₃·, has been shown to reduce liver injury and mortality in rats given CCl₄ (19). This treatment was most effective if applied immediately after CCl₄ administration but remained efficacious even when applied at 24 h. The present results, which demonstrate that lipid peroxidation and, by implication, CCl₄ metabolism continue to occur past 24 h, provide a rationale for the effectiveness of hyperbaric oxygen treatment at this late time.

Our studies also imply that CCl₄ induction of F₂-isoprostane generation is clearly linked to lipid peroxidation rather than to hepatic necrosis. Poisoning of rats with either of the hepatotoxins, acetaminophen or thioacetamide, which are not associated with significant lipid peroxidation (10), does not markedly increase circulating levels of free F₂-isoprostanes.

It is known that a variety of mechanisms are present in vivo to protect living organisms from lipid peroxidation (16). One interesting observation to emerge from our work is that glutathione is an important component of this defense system. Animals pretreated with glutathione-depleting agents have exaggerated increases in free plasma F₂-isoprostanes after CCl₄ administration. Other reports have indicated that glutathione can protect against CCl₄ (20).

Induction of the cytochrome P-450 system also enhanced the formation of F₂-isoprostanes in vivo. This effect is probably indirect in that this enzyme system converts CCl₄ to CCl₃·, which catalyzes peroxidation of lipids and isoprostane formation. Other studies have shown increases in CCl₄ metabolism by liver microsomes of animals pretreated with agents such as INH and phenobarbital, which induce the P-450 system (12). Our data extend those observations by showing that pretreatment with either of these agents markedly augments CCl₄-induced generation of F₂-isoprostanes, indicating that enhanced CCl₄ metabolism is associated with increased lipid peroxidation in vivo. Further, inhibition of the cytochrome P-450 system with either SKF-525A or 4-methylpyrazole significantly inhibits CCl₄-induced generation of F₂-isoprostanes. It has previously been shown that administration of SKF-525A to rats reduces CCl₄-induced liver injury (14).

Table III. Levels of Free F-Isoprostanes Measured After Hydrolysis of Lipids from Various Organs of Untreated Control Rats and CCl₄-treated Rats

Tissue	F ₂ -isoprostane levels		Fold-increase
	Control	CCl ₄ treated	
	ng/g tissue	ng/g tissue	
Liver	6.1±0.7	460±94*	77
Kidney	1.2±0.4	19±6*	15
Lung	0.7±0.2	5.5±0.5*	7.9
Muscle	0.5±0.2	0.9±0.1	1.8
Heart	2.5±0.5	4.2±0.7*	1.7
Brain	1.0±0.1	1.6±0.3	1.6

CCl₄-treated rats were killed 2 h after oral administration of 1 ml/kg CCl₄. Results are mean±SD; n = 3. *P < 0.05 CCl₄ treated vs. control.

From the work reported herein, it should be recognized that circulating free F_2 -isoprostanes represent but one pool of F_2 -isoprostanes in vivo because these substances also are esterified in phospholipids both in tissues and in plasma. Although suggestive, it remains to be proven that F_2 -isoprostanes that exist acylated to plasma lipids are carried on lipoproteins.

Thus, taken together, these studies have defined a series of factors that influence the formation of F_2 -isoprostanes in an in vivo situation of exaggerated lipid peroxidation and provide a basis to explore the use of these compounds as markers of lipid peroxidation in human disease states.

Acknowledgments

We thank Drs. Diarmuid O'Shea, Richard Kim, and Grant Wilkinson for assistance with half-life determinations of 8-epi-PGF_{2α}. We also acknowledge the skillful technical assistance of Tanya Minton and William Zackert and the secretarial help of Amanda Simpson.

Supported by National Institutes of Health grants HL-02499, GM-42056, ES-07028, ES-02497, DK-43883, and DK-39201. J. D. Morrow is a Howard Hughes Medical Institute Physician Research Fellow.

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