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Reinterpreting the best biomarker of oxidative stress: The 8-iso-prostaglandin $F_{2\alpha}$ /prostaglandin $F_{2\alpha}$ ratio shows complex origins of lipid peroxidation biomarkers in animal models

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Abstract

Oxidative stress is elevated in numerous environmental exposures and diseases. Millions of dollars have been spent to try to ameliorate this damaging process using anti-oxidant therapies. Currently, the best accepted biomarker of oxidative stress is the lipid oxidation product 8-isoprostaglandin $F_{2\alpha}$ (8-iso-PGF $_{2\alpha}$), which has been measured in over a thousand human and animal studies. 8-iso-PGF $_{2\alpha}$ generation has been exclusively attributed to nonenzymatic chemical lipid peroxidation (CLP). However, 8-iso-PGF $_{2\alpha}$ can also be produced enzymatically by the prostaglandin-endoperoxide synthases (PGHS) *in vivo*. When failing to account for PGHS-dependent generation, 8-iso-PGF $_{2\alpha}$ cannot be interpreted as a selective biomarker of oxidative stress.

We investigated the formation of 8-iso-PGF $_{2\alpha}$ in rats exposed to carbon tetrachloride (CCl $_4$) or lipopolysaccharide (LPS) using the 8-iso-PGF $_{2\alpha}$ /PGF $_{2\alpha}$ ratio to quantitatively determine the source(s) of 8-iso-PGF $_{2\alpha}$. Upon exposure to a 120 mg/kg dose of CCl $_4$, the contribution of CLP accounted for only $55.6 \pm 19.4\%$ of measured 8-iso-PGF $_{2\alpha}$, whereas in the 1200 mg/kg dose, CLP was the predominant source of 8-iso-PGF $_{2\alpha}$ ($86.6 \pm 8.0\%$ of total). In contrast to CCl $_4$, exposure to 0.5 mg/kg LPS was characterized by a significant increase in both the contribution of PGHS (59.5 ± 7.0) and CLP ($40.5 \pm 14.0\%$).

In conclusion, significant generation of 8-iso-PGF $_{2\alpha}$ occurs through enzymatic as well as chemical lipid peroxidation. The distribution of the contribution is dependent on the exposure

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agent as well as the dose. The 8-iso-PGF_{2α}/PGF_{2α} ratio accurately determines the source of 8-iso-PGF_{2α} and provides an absolute measure of oxidative stress *in vivo*.

Graphical Abstract

Keywords

8-iso-PGF_{2α} / PGF_{2α} ratio; F₂-isoprostanes; oxidative stress; inflammation; biomarkers; lipid peroxidation

Introduction

The impact of oxidative stress on human health has been investigated in thousands of studies. [1–3]. Oxidative stress is characterized as the excessive generation of endogenous or exogenous free radicals. Currently, the best method to measure oxidative stress *in vivo* is to detect changes in the levels of oxidation products of endogenous molecules such as DNA, proteins, or lipids [4]. The markers most commonly used to measure oxidative stress *in vivo* are the arachidonic acid oxidation products, the F₂-isoprostanes. [5, 6]. There are many F₂-isoprostanes, but the most often measured is 8-iso-prostaglandin F_{2α} (8-iso-PGF_{2α}). The excessive generation of 8-iso-PGF_{2α} has been attributed solely to increases in nonenzymatic or chemical lipid peroxidation [6, 7]. This notion was challenged by numerous researchers using a variety of *in vitro* and *in vivo* model systems [8–12]. It is now recognized that there are two mechanisms that generate 8-iso-PGF_{2α}, and both may contribute to elevated levels of this biomarker during disease and exposure. [13]. The two documented mechanisms for 8-iso-PGF_{2α} formation are chemical lipid peroxidation and prostaglandin-endoperoxide synthases (PGHS)-mediated enzymatic lipid peroxidation. In chemical lipid peroxidation, 8-iso-PGF_{2α} is formed by an initiating radical on a small molecule, and the substrate is either a free polyunsaturated fatty acid in solution or an esterified fatty acid to phospholipids, triglycerides, or cholesterol. This results in neither stereo- nor regio-selective oxidation of the parent lipid [14–16]. 8-iso-PGF_{2α} is formed through PGHS mediated enzymatic lipid peroxidation by an initiating tyrosyl radical located at the PGHS enzyme active site. The substrate is exclusively the free poly-unsaturated fatty acid which is spatially constrained within this active site, resulting in both stereo- and regio-selective oxidation [17, 18]. Unfortunately, no method to distinguish between chemical and enzymatic lipid peroxidation *in vivo* has existed until recently. Distinguishing the contribution of each pathway *in vivo* is crucial for correct interpretation of the biomarker and any subsequent conclusions.

The method to distinguish 8-iso-PGF_{2α} generation by PGHS-mediated enzymatic lipid peroxidation from chemical lipid peroxidation is described previously by van 't Erve *et al.*

(13) and is based on measuring the 8-iso-PGF_{2α} / PGF_{2α} ratio. The 8-iso-PGF_{2α} / PGF_{2α} ratio can quantitatively distinguish the contribution of both chemical and enzymatic lipid peroxidation to the total measured 8-iso-PGF_{2α} level. 8-iso-PGF_{2α} and PGF_{2α} are both produced by either pathway; however, the proportion in which they are made is distinct. Chemical lipid peroxidation generates racemic mixtures of F₂-isoprostanes [15] with 8-iso-PGF_{2α} and PGF_{2α} produced in a 1:1 ratio [13]. In contrast, PGHS-mediated enzymatic lipid peroxidation preferentially generates PGF_{2α}, and the ratio between 8-iso-PGF_{2α} and PGF_{2α} is 0.008:1 and 0.004:1 for PGHS-1 and -2, respectively [13].

In this manuscript, the 8-iso-PGF_{2α} / PGF_{2α} ratio was validated in male Fisher 344 rats exposed to carbon tetrachloride (CCl₄) [4] or lipopolysaccharide (LPS) [19], both known inducers of 8-iso-PGF_{2α} formation in animal models. The mechanism of 8-iso-PGF_{2α} formation in these exposures was expected by us to be primarily from chemical lipid peroxidation for CCl₄ and PGHS-mediated enzymatic lipid peroxidation for LPS. In reality, both treatments showed simultaneous significant increases in chemical lipid peroxidation as well as PGHS-mediated enzymatic lipid peroxidation; the relative contribution of each pathway depended on the dose and the specific treatment. Additional evidence for PGHS-dependent contribution to the total 8-iso-PGF_{2α} levels was obtained by using the pharmacological PGHS inhibitors indomethacin and meclofenamic acid. Samples that were analyzed for this study consisted of newly generated samples as well as those from the BOSS II study [4], which have been in continuous storage at -80 °C.

The results in this manuscript demonstrate two sources of 8-iso-PGF_{2α} formation *in vivo*. The sources can be distinguished by measuring the 8-iso-PGF_{2α} / PGF_{2α} ratio in plasma. The results presented here further challenge the long held notion that 8-iso-PGF_{2α} is solely a product of chemical lipid peroxidation *in vivo* and is a selective marker for oxidative stress. Quantifying the contributions of both pathways provides vital mechanistic insights into diseases and environmental exposures. These mechanistic insights are imperative to correctly interpret experimental findings based on this biomarker of oxidative stress.

Materials and Methods

Materials

8-iso-PGF_{2α}, PGF_{2α}, 8-iso-PGF_{2α}-d₄, and PGF_{2α}-d₄ were purchased from Cayman (Ann Arbor, MI, USA). Indomethacin, meclofenamic acid, carbon tetrachloride, methanol, ethanol, and acetic acid were purchased from Sigma Aldrich (St. Louis, MO, USA). LPS (E. coli, O111:B4) was purchased from EMD Millipore (Billerica, MA, USA).

Animals and treatment protocol

Male Fisher 344 rats (260–280 g) were obtained from Charles River Laboratories (Raleigh, NC, USA). Rats were group-housed in a temperature-controlled room at 23–25 °C with a 12/24 hour light/dark cycle and allowed free access to food and water. The studies adhered to the National Institutes of Health guidelines for the care and handling of experimental animals. All animal studies were approved by the NIEHS Institutional Review Board. Prior to exposure, animals were fasted for 12 hours prior to injections. On the day of study,

animals (n=6 per treatment group) were injected IP with carbon tetrachloride (120 mg/kg or 1200 mg/kg) dissolved in canola oil or LPS (0.5 mg/kg or 1.5 mg/kg) in isotonic saline. After 2 or 7 hours, animals were anesthetized and blood was drawn through the dorsal aorta with a single draw vacutainer needle (21 gauge) into an open vacutainer blood collection tube containing heparin. Containers were kept on ice until processing. Animals pretreated with indomethacin or meclofenamic acid prior to challenge with LPS or carbon tetrachloride were injected subcutaneously with 5 mg/kg indomethacin 24, 12 and 2 hours prior to challenge or with 5 mg/kg meclofenamic acid 12 and 2 hours prior to challenge [20].

Plasma sample preparation

Whole blood was centrifuged no more than 30 min after collection (2000 rpm for 10 min at 4 °C) to remove RBCs. Plasma was isolated and stored neat at -80 °C. Prior to analysis, samples were thawed, spiked with internal standards, and kept on ice during sample preparation. 700 µL of rat plasma was subjected to solid phase extraction using 3 mL HLB SPE columns (Waters Corp, Billerica, MA). SPE columns were prewashed with 2 mL of methanol and water. Plasma aliquots were acidified prior to deposition on the column with 0.1 % vol/vol of 1 % acetic acid / 50 % methanol. After loading, samples were washed with 2 mL of a 5 % methanol solution. F₂-isoprostanes and prostaglandins were eluted from the column with 2 mL of methanol. Prior to evaporation, six µL of glycerol were added to each sample. Samples were evaporated in a centrifugal vacuum evaporator maintained at 40 °C and reconstituted with 50 µL of a 33 % ethanol / 0.5 % acetic acid solution.

LC/MS/MS oxylipid analysis

Oxylipid levels were determined in extracted plasma samples by liquid chromatography/tandem mass spectroscopy (LC/MS/MS) as previously described [21, 22]. Online liquid chromatography and electrospray ionization tandem mass spectrometry of extracted plasma samples were performed on an Agilent 1200 Series capillary HPLC with an AB Sciex API 3000 mass spectrometer equipped with a TurboIonSpray source or UltiMate 3000 RS HPLC system and Quantiva mass spectrometer (Thermo Fisher Scientific). Separations were achieved using a Halo C18 column (2.7 µm, 100 × 2.1 mm, Advanced Materials Technology, Wilmington, DE, USA), which was held at 50°C. The flow rate was 400 µL/min. Mobile phase A was 0.1% of acetic acid in 85:15 water:acetonitrile. Mobile phase B was 0.1% of acetic acid in acetonitrile. Gradient elution was used, and the mobile phase percent B was varied as follows: 20% B at 0 min, ramp from 0 to 5 min to 40% B, and ramp from 5 to 7 min to 55% B. The injection volume was 10 µL. Samples were analyzed in triplicate. All analytes were monitored simultaneously as negative ions in a multiple reaction monitoring experiment. Analytes were monitored as parent ion-product ion, mass / charge pairs with specific retention times and quantified against standard curves of analytes purchased from Cayman Chemical.

Data analysis, statistics, and calculation

MS data analysis—Peaks identified as 8-iso-PGF_{2α} or PGF_{2α} were integrated and normalized to their individual deuterated standards: 8-iso-PGF_{2α}-d₄ and PGF_{2α}-d₄. The

normalized peak areas were converted to concentrations using quantitation curves generated together with each batch of samples.

Calculations—The 8-iso-PGF_{2α} / PGF_{2α} ratio and subsequent calculations of the contribution of both CLP and PGHS to the total 8-iso-PGF_{2α} level were performed using a custom interface for the R package “Constrained Linear Mixed Effects (CLME)” [23, 24]. This program is freely available and instructions to download and use the program are provided in the supplementary materials. The calculations are derived from results in van 't Erve *et al.* [13] (Equation 1). A step-by-step process is described in scheme 1. Results are expressed as the mean ± SE for each variable and each sample.

$$\frac{\text{CLP}}{\text{PGHS}} = \frac{-0.996 * (8\text{-iso-PGF}_{2\alpha}/\text{PGF}_{2\alpha}) + 0.004}{0.507 * (8\text{-iso-PGF}_{2\alpha}/\text{F}_{2\alpha}) - 0.493} \quad \text{Equation 1:}$$

Comparisons between Treatment Groups—The first set of statistical analyses performed compared several endpoints across the treatment groups. Each comparison involved two or three treatment groups and was tested for each of the four endpoints, namely, 8-iso-PGF_{2α}, PGF_{2α}, and the absolute concentrations of PGHS and CLP. Computations were performed using the CLME package (version 2.0.4) for the Statistical software R. This nonparametric residual bootstrap methodology used in CLME is robust to non-normality and unequal variances between treatment groups.

In total, there were 25 comparisons to be made among treatment groups. The CLME software does not automatically adjust for multiple testing but produces a global *p*-value for each test, and the custom interface includes an option to conduct a Bonferroni correction. The program and associated tutorial can be found at: <http://www.niehs.nih.gov/research/resources/software/tox-pharm/tools/pgf/index.cfm>

Comparisons within Treatment Groups—Another set of statistical analyses performed was to investigate whether there was a significant difference between CLP and PGHS with a treatment group. These analyses were performed using a paired Sign Test. This is a robust, nonparametric procedure with minimal assumptions on the data.

For each treatment group, the difference between the absolute concentrations of CLP and PGHS was computed and the hypothesis that the median differed from zero tested. As there were 20 treatment groups in total, we applied a Bonferroni correction by multiplying each *p*-value by 20.

Results

Generation of 8-iso-PGF_{2α} during carbon tetrachloride exposure

The generation of 8-iso-PGF_{2α} as observed during exposure to carbon tetrachloride has been attributed solely to chemical lipid peroxidation initiated by the trichloromethylperoxyl radical. However, experiments with pharmacological inhibitors of PGHS and results showing that PGHS forms 8-iso-PGF_{2α} reveal that it is plausible that the PGHS pathway

contributes to the generation of 8-iso-PGF_{2α} *in vivo*. To determine the contribution of enzymatic lipid peroxidation by PGHS and chemical lipid peroxidation to the total measured free 8-iso-PGF_{2α} levels, we applied the 8-iso-PGF_{2α} / PGF_{2α} ratio approach [13]. In this approach, when the 8-iso-PGF_{2α} / PGF_{2α} ratio approaches 0.004, free 8-iso-PGF_{2α} is generated through enzymatic lipid peroxidation by PGHS due to the preference of PGHS to make PGF_{2α}, whereas when the ratio between the compounds approaches 1, the source is chemical lipid peroxidation, which produces equal amounts of 8-iso-PGF_{2α} and PGF_{2α}. For these studies, fresh as well as stored samples were tested for the presence of free 8-iso-PGF_{2α} and PGF_{2α} in non-saponified plasma. No statistical difference was found between stored and fresh samples with identical exposures (Figure S1 and supporting text).

To study the contribution of enzymatic and chemical lipid peroxidation during carbon tetrachloride exposure, free 8-iso-PGF_{2α} and PGF_{2α} were measured in non-saponified plasma of male Fischer 344 rats. The rats were exposed to bolus doses of vehicle control (canola oil) or carbon tetrachloride in canola oil (120 mg/kg and 1200 mg/kg) intraperitoneally. A dose-dependent increase in the concentration of both free 8-iso-PGF_{2α} and PGF_{2α} in the plasma was observed 7 hours after exposure (Figure 1A). For PGF_{2α}, a significant increase in concentration was observed only for the 1200 mg/kg dose (Figure 1A). The increasing ratio of 8-iso-PGF_{2α} / PGF_{2α} indicates a greater contribution of chemical lipid peroxidation (CLP) with an increasing dose of carbon tetrachloride (Figure 1B). The contribution by PGHS and CLP can be quantitatively distinguished using the 8-iso-PGF_{2α} / PGF_{2α} ratio and Equation 1 as described in van 't Erve *et al.* [13]. A statistically significant increase in the contribution of CLP to the measured total 8-iso-PGF_{2α} levels was observed at both doses (Figure 1C, Table S1). Surprisingly, a statistically significant increase over control was also observed in the contribution of PGHS to 8-iso-PGF_{2α} upon carbon tetrachloride exposure. The contribution of PGHS was nearly equal to that of CLP at the 120 mg/kg dose and did not further increase with the higher 1200 mg/kg carbon tetrachloride dose (Figure 2C, Table S1).

Upon 24-hour pretreatment with indomethacin, a significant decrease was observed in the levels of free 8-iso-PGF_{2α} and PGF_{2α} in both exposure groups compared to non-indomethacin-treated animals (Figure 1D). No significant increase in the levels of 8-iso-PGF_{2α} and PGF_{2α} was observed between the vehicle control and 120 mg/kg dose group with exposure to carbon tetrachloride in indomethacin-pretreated animals. With the 1200 mg/kg group, significant increases in the 8-iso-PGF_{2α} and PGF_{2α} levels compared to the vehicle control and 120 mg/kg groups were observed. The 8-iso-PGF_{2α} / PGF_{2α} ratio shows that indomethacin inhibited most PGHS-dependent enzymatic lipid peroxidation since the ratio shifted towards 1 for the control as well as exposed groups (Figure 1E). After distinguishing the contribution of the two pathways using the 8-iso-PGF_{2α} / PGF_{2α} ratio, a significant increase in the contribution of CLP to the total 8-iso-PGF_{2α} levels was observed only at the 1200 mg/kg dose group (Figure 1F, Table S1). Also, in both dose groups (indomethacin-treated) there was no increase in the contribution of PGHS to the total 8-iso-PGF_{2α}. Similar patterns with the PGHS inhibitor meclofenamic acid were observed (Figure S2 A–C). In addition, quantifying the sources of PGF_{2α} is also possible with the 8-iso-PGF_{2α} / PGF_{2α} ratio (Figure S3). PGF_{2α} is generated mostly enzymatically by PGHS but

can also be generated in significant amounts through chemical lipid peroxidation by a higher dose of CCl_4 .

Time-dependent alterations of generation of 8-iso-PGF_{2α} during carbon tetrachloride exposure

To investigate the kinetics of free 8-iso-PGF_{2α} and PGF_{2α} formation, rats were exposed to carbon tetrachloride for various times. Three time points were investigated, namely 0, 2, and 7 hours after injection. After injection of 1200 mg/kg carbon tetrachloride, a sharp increase in both 8-iso-PGF_{2α} and PGF_{2α} was observed, followed by a slow decline at 7 hours, which was still significantly above control levels (Figure 2A). Throughout the time course, the increases in 8-iso-PGF_{2α} and PGF_{2α} concentrations were very similar. However, there was basal formation of ~1 ng/mL PGF_{2α}, which caused an offset between the two curves due to additional PGF_{2α} formation by PGHS. The 8-iso-PGF_{2α}/PGF_{2α} ratio increased but did not become 1, indicating that both enzymatic and chemical lipid peroxidation were contributing simultaneously to the total 8-iso-PGF_{2α} level. After the sources of 8-iso-PGF_{2α} were quantitatively distinguished using the 8-iso-PGF_{2α}/PGF_{2α} ratio, a large increase was found in the contribution of the CLP 2 hours after exposure, which persisted at the 7-hour time point although quantitatively lower as compared to the 2-hour time point (Figure 2C, Table S1). There was a smaller concomitant increase in the contribution of PGHS at the 2-hour time point, and this increase remained the same for 7 hours.

When animals were pretreated with indomethacin and exposed to 1200 mg/kg carbon tetrachloride, the increases from baseline and the actual plasma concentrations for both free 8-iso-PGF_{2α} and PGF_{2α} were identical (Figure 2D), which resulted in the 8-iso-PGF_{2α}/PGF_{2α} ratio shifting towards 1 throughout all time points (Figure 2E). After quantitating the contribution of PGHS and CLP, we verified that CLP was the major contributor to free 8-iso-PGF_{2α} formation upon exposure to carbon tetrachloride (Figure 2F, Table S1). At the 7-hour time point, indomethacin had a significant effect on the CLP contribution, possibly through inhibition of NADPH-oxidase activation [25]; this enzyme would not yet be activated at the 2-hour time point. In animals pretreated with indomethacin, the contribution of PGHS to the 8-iso-PGF_{2α} levels was significantly decreased and did not change from the control during the time course (Figure 2F, Table S1). The contribution of CLP in indomethacin-pretreated animals mimicked that of nonpretreated animals upon exposure to carbon tetrachloride. Pretreatment with indomethacin in the absence of carbon tetrachloride exposure resulted in complete inhibition of both 8-iso-PGF_{2α} and PGF_{2α} at both time points investigated (Figure S4), further indicating that the increase in PGF_{2α} as well as 8-iso-PGF_{2α} is through CLP.

Generation of 8-iso-PGF_{2α} during LPS exposure

Exposure to lipopolysaccharides (LPS) derived from *E. coli* has been shown to induce free 8-iso-PGF_{2α} [19]. This induction is reported to be through increased chemical lipid peroxidation from superoxide or peroxynitrite generated by the activation of NADPH-oxidase and nitric oxide synthases [26, 27]. In addition, LPS also induces PGHS-2, which can be a source of 8-iso-PGF_{2α} [28, 29]. Here we investigated the contribution of PGHS to 8-iso-PGF_{2α} in LPS-treated rats. Male Fisher 344 rats were exposed to 0.5 and 1.5 mg/kg of LPS for 2 hours; the levels of free 8-iso-PGF_{2α} and PGF_{2α} were subsequently measured in

the non-saponified plasma. After 2 hours of exposure, a small but significant increase in the 8-iso-PGF_{2α} level was observed (Figure 3A). However, simultaneously, a larger increase in the level of PGF_{2α} was seen (Figure 3A). This is shown in the 8-iso-PGF_{2α}/PGF_{2α} ratio, which barely increased upon exposure to LPS (Figure 3B). When the sources of 8-iso-PGF_{2α} were distinguished using the 8-iso-PGF_{2α}/PGF_{2α} ratio, it was found that the majority of 8-iso-PGF_{2α} was formed through generation by PGHS (Figure 3C, Table S1). A smaller increase in the contribution of CLP was also observed together with the increase in PGHS.

When animals were pretreated with the PGHS inhibitor indomethacin, a large decrease in the PGF_{2α} concentration of the control group was observed, but no increase in 8-iso-PGF_{2α} or PGF_{2α} concentration upon exposure to LPS with either dose (Figure 3D). The 8-iso-PGF_{2α}/PGF_{2α} ratio does not change significantly with indomethacin pretreatment. The error bars are very large, so no true 8-iso-PGF_{2α} / PGF_{2α} ratio can be determined (Figure 3E). Quantifying the contribution of both pathways showed that indomethacin significantly inhibited the contribution of PGHS as well as CLP (Figure 3F, Table S1). In addition, quantifying the sources of PGF_{2α} is also possible with the 8-iso-PGF_{2α} / PGF_{2α} ratio (Figure S3). We determined that PGF_{2α} was generated nearly exclusively through PGHS, and its formation was completely inhibited with indomethacin treatment as expected.

Discussion

Here we describe our novel quantitative findings that distinction of chemical from enzymatic lipid peroxidation is important to accurately measure oxidative stress. We found a complex distribution of lipid peroxidation mechanisms in distinct animal models. The quantitative measurement of the mechanistic sources of 8-iso-PGF_{2α} was possible by using the 8-iso-PGF_{2α} / PGF_{2α} ratio. Two *in vivo* models, carbon tetrachloride and lipopolysaccharide exposure to male Fisher 344 rats, were used primarily to initiate chemical lipid peroxidation and enzymatic lipid peroxidation, respectively. Both of these models have previously shown increases in the level of free 8-iso-PGF_{2α}; however, numerous studies concluded that this increase was only due to nonenzymatic or chemical lipid peroxidation [4, 19, 30–32]. Several investigators have made observations of the possible contribution of PGHS to the free 8-iso-PGF_{2α} pool measured in various biological systems [8–12, 33–35]; however, this potential additional source is commonly ignored during the interpretation of this biomarker of oxidative stress in humans.

Exposure of rats to carbon tetrachloride resulted in a dose-dependent increase in the level of 8-iso-PGF_{2α} as well as PGF_{2α} in non-saponified plasma. The increases in the levels of both compounds are nearly identical, leading to the conclusion that chemical lipid peroxidation is a major source of 8-iso-PGF_{2α}. However, an increase in 8-iso-PGF_{2α} from enzymatic lipid peroxidation by PGHS is also observed. This increase in the PGHS contribution is independent of carbon tetrachloride dose and is significantly inhibited by pretreatment with the PGHS inhibitors indomethacin and meclofenamic acid. This inhibition of ≈30 % of 8-iso-PGF_{2α} is consistent with previous reports [12]. Increases in inflammation have been noted in previous studies of carbon tetrachloride exposure [30]. Literature data showed an increase in the PGF_{2α} metabolite 15-keto-13,14-dihydro-PGF_{2α} occurring concomitantly

with an increase in 8-iso-PGF_{2α} upon carbon tetrachloride exposure; this metabolite is considered a marker of increased PGHS-2 activity [30]. The induction of PGHS-2 is most likely due to the extensive liver necrosis observed with exposure to the large doses of carbon tetrachloride used; this excessive necrosis will in turn trigger an inflammatory response inducing PGHS-2.

From our experiments, exposure to carbon tetrachloride primarily initiates an increase in chemical lipid peroxidation. However, not all models of disease or exposure will produce 8-iso-PGF_{2α} predominantly through this pathway, as has been widely assumed. Exposure to lipopolysaccharide generates a strong inflammatory response. This leads to an increase in free 8-iso-PGF_{2α} [19], which was proposed to arise through the activation of NADPH-oxidases and nitric oxide synthases, specifically generating superoxide and nitric oxide, leading to the formation of peroxynitrite, which can initiate lipid peroxidation [27]. However, we find an even bigger increase in PGF_{2α}, which is indicative of a significant contribution by PGHS to measured free 8-iso-PGF_{2α} levels upon LPS exposure. This was confirmed by experiments using an inhibitor of PGHS activity, indomethacin. Even when the major pathway of 8-iso-PGF_{2α} is enzymatic lipid peroxidation by PGHS, there is a significant simultaneous rise in the contribution of chemical lipid peroxidation. This increase in chemical lipid peroxidation has been proposed to be due to activation/induction of NADPH-oxidases and nitric oxide synthases [31], but the total inhibition of 8-iso-PGF_{2α} by indomethacin as observed here is not totally consistent with this proposal. An alternate hypothesis is that alkoyl and peroxy radicals generated through the decomposition of PGHS-formed hydroperoxides, such as PGG₂ or HpETE's, catalyzed by heme or heme-containing proteins are the origin of chemical lipid peroxidation upon LPS exposure [36, 37]. A limitation of the 8-iso-PGF_{2α} / PGF_{2α} ratio is that it cannot distinguish between chemical lipid peroxidation mediated by, e.g., superoxide, peroxynitrite-derived radicals, alkoxyl radicals, or the trichloromethylperoxy radical during carbon tetrachloride exposure. The specific source of chemical lipid peroxidation can, at this point, be further distinguished only by employing enzyme inhibitors or specific radical scavengers.

The increase in 8-iso-PGF_{2α} from exposure to LPS can be completely blunted by pretreatment with indomethacin. This inhibitor was chosen for its effect on PGHS, but also has some inhibitory capacity towards NADPH-oxidases [25]. This broader inhibition can provide a possible explanation for the simultaneous inhibition of both PGHS and chemical lipid peroxidation. It would also explain the significant decreases in chemical lipid peroxidation upon carbon tetrachloride exposure after pretreatment with indomethacin (Figure 1F). A second explanation is that activation of PGHS is required prior to activation of NADPH-oxidase. The apparent increased CLP seen in Figures 1F and 3F for the control rats pretreated with indomethacin compared to the non-pretreated is most likely a method artifact due to high sensitivity of the 8-iso-PGF_{2α} / PGF_{2α} ratio to fluctuations when both compounds are present near the detection limit.

In this work, we have measured free 8-iso-PGF_{2α} and PGF_{2α} in plasma as these are the chemical structure and specimen frequently measured in human populations. The use of the 8-iso-PGF_{2α} / PGF_{2α} ratio to quantitate the distribution of enzymatic vs. chemical lipid peroxidation has yet to be validated in other biological specimens such as urine, cerebral

spinal fluid, exhaled breath condensate, or tissues. Due to the different distribution of distinct chemical structures of F₂-isprostanes and prostaglandins (free, esterified, glucuronidated, *etc.*) in each biological specimen, it is not known if the 8-iso-PGF_{2α} / PGF_{2α} ratio approach is applicable or even necessary in these type of specimens for correct biomarker interpretation.

In conclusion, the two models studied here show that there are two distinct forms of lipid peroxidation which can both induce the classic lipid peroxidation marker 8-iso-PGF_{2α}. These two forms of lipid peroxidation (chemical and enzymatic) are simultaneously induced to different extents in chemical exposure and inflammation. The classical interpretation that the measured increases in the biomarker of oxidative stress 8-iso-PGF_{2α} are due solely to alterations in chemical lipid peroxidation should no longer be accepted. The sources of 8-iso-PGF_{2α} must be distinguished in order to accurately and correctly interpret this biomarker of oxidative stress *in vivo*. Measuring the 8-iso-PGF_{2α} / PGF_{2α} ratio accomplishes this quantitatively.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

8-iso-PGF_{2α}	8-iso-prostaglandin F _{2α}
PGF_{2α}	prostaglandin F _{2α}
PGHS	prostaglandin-endoperoxide synthases, cyclooxygenases
CLP	chemical lipid peroxidation

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Highlights:

- The F₂-isoprostane, 8-iso-PGF_{2α}, is generated through two distinct pathways simultaneously in disease and exposure animal models
- Increases in free 8-iso-PGF_{2α} concentration in plasma have different origins in distinct animal models of oxidative stress, namely carbon tetrachloride and LPS exposure
- The contribution of each pathway can be quantitatively determined using the 8-iso-PGF_{2α}/PGF_{2α} ratio

Figure 1: Exposure to carbon tetrachloride increases the levels of 8-iso-PGF_{2α} generated mainly by chemical lipid peroxidation (CLP) in non-saponified plasma.

A: Exposure to carbon tetrachloride induces a statistically significant increase in free 8-iso-PGF_{2α}. A small increase in PGF_{2α} is seen concomitantly. This increase in PGF_{2α} differs significantly from control only at the 1200 mg/kg dose. **B:** The 8-iso-PGF_{2α} / PGF_{2α} ratio increases depending on the carbon tetrachloride dose. **C:** The sources of the total measured 8-iso-PGF_{2α} levels distinguished using the calculated 8-iso-PGF_{2α} / PGF_{2α} ratio and equation 1. A dose-dependent increase in 8-iso-PGF_{2α} from CLP is observed upon exposure with carbon tetrachloride with a significant but non-dose-dependent increase in the contribution of PGHS. **D:** Rats are pretreated with indomethacin, which inhibited PGHS activity significantly as seen in the decrease in PGF_{2α} levels. Increases in 8-iso-PGF_{2α} are observed similar to the non-pretreated animals upon carbon tetrachloride exposure. **E:** The 8-iso-PGF_{2α} / PGF_{2α} ratio does not change with increasing carbon tetrachloride dose after

pretreatment with indomethacin. **F:** After distinguishing the sources of 8-iso-PGF_{2α}, the increase in the contribution of 8-iso-PGF_{2α} from PGHS as well as CLP is significantly decreased from indomethacin-pretreated animals; however, the increase in CLP persists even with indomethacin pretreatment. No increase in the contribution of PGHS compared to control is observed after indomethacin pretreatment. Six animals were treated per group. * Different from control (p<0.05), ** different from control and other dose (p<0.05), § different from non-pretreated group (p<0.05), # different from other dose but not control (p<0.05). Bars display mean ± SE.

Figure 2: Carbon tetrachloride induces a time-dependent increase in 8-iso-PGF_{2α} generated predominantly by chemical lipid peroxidation in non-saponified plasma.

A: A time-dependent increase in 8-iso-PGF_{2α} and PGF_{2α} is observed upon exposure to 1200 mg/kg carbon tetrachloride; however, there is basal formation of ~1 ng/mL PGF_{2α} which causes an offset between the two curves due to continued PGF_{2α} formation by PGHS.

B: The 8-iso-PGF_{2α} / PGF_{2α} ratio increases over control at the 2-hour time point and remains increased at the 7-hour time point.

C: The sources of the 8-iso-PGF_{2α} levels from **Figure 2A** are distinguished using the calculated 8-iso-PGF_{2α} / PGF_{2α} ratio and equation 1. A time-dependent increase in the contribution of CLP is seen upon exposure. A significant increase in the contribution of PGHS is also observed; however, this does not change with time.

D: After pretreatment with indomethacin, a time-dependent increase in 8-iso-PGF_{2α} and PGF_{2α} is still observed upon exposure to 1200 mg/kg carbon tetrachloride; both 8-iso-PGF_{2α} and PGF_{2α} are produced at nearly equal levels at each time point investigated. **E:** The 8-iso-PGF_{2α} / PGF_{2α} ratio remains near 1 throughout the time course after pretreatment with indomethacin before carbon tetrachloride exposure. **F:** After pretreatment with indomethacin, the increase in the contribution of PGHS is significantly inhibited. The time-dependent increase in CLP is different for non pretreated rats only at the 7-hour time point.

Six animals were treated per time point. * Different from control (p<0.05), ** different from

control and other time point ($p<0.05$), § different from non-pretreated group ($p<0.05$), # different from other dose but not control ($p<0.05$). Points display mean $\pm$ SE.

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Figure 3: Exposure to LPS induces the contribution of PGHS to the generation of free 8-iso-PGF_{2α} in non-saponified plasma.

A: After exposure to LPS, the levels of 8-iso-PGF_{2α} significantly increase compared to control at both doses, but PGF_{2α} significantly increases only at the high dose. **B:** The 8-iso-PGF_{2α} / PGF_{2α} ratio increases only slightly upon exposure to LPS at both the 0.5 mg/kg and 1.5 mg/kg dose. **C:** When the sources of 8-iso-PGF_{2α} are distinguished, a significant contribution in both LPS doses is seen for PGHS as well as CLP at the 0.5 mg/kg dose. Only the contribution of PGHS is increased significantly at the 1.5 mg/kg dose. **D:** After pretreatment with indomethacin, both 8-iso-PGF_{2α} and PGF_{2α} are significantly inhibited. **E:** The 8-iso-PGF_{2α} / PGF_{2α} ratio does not change from control in both LPS doses. **F:** The contributions of both PGHS and CLP to the 8-iso-PGF_{2α} levels are significantly inhibited with indomethacin pretreatment. Six animals were treated per group. * Different from

control ($p<0.05$), different from non-pretreated group ($p<0.05$), § different from non-pretreated group ($p<0.05$). Bars display mean $\pm$ SE.

Scheme 1:

Separation of measured 8-iso-PGF_{2α} levels into the fractions formed by PGHS and CLP using the 8-iso-PGF_{2α} / PGF_{2α} ratio.