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Ozone exposure effect on systemic prostaglandin F_{2α} in rat plasma and urine may not reveal pulmonary damage through inflammation

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Abstract

The acute ozone induced lung injury model has been widely used to explore injury and repair processes induced by oxidant overload. The current study evaluated acute ozone exposure effects on prostaglandin F_{2α} (PGF_{2α}) in male Fischer rat plasma and urine with the hypothesis that ozone may induce an inflammatory response in the body that can be measured by the induction of PGF_{2α}. That might then lead to the identification of potential marker for acute lung injury through systemic inflammation. The time and dose-dependent effects of ozone exposure on the plasma and urinary levels of a major PGF_{2α} metabolite 15-keto-dihydro-PGF_{2α} were determined using a radioimmunoassay. No statistically significant differences in the PGF_{2α} metabolite were found between the control and the experimental groups at either ozone exposure dose (2 ppm and 5 ppm) or any time point (2h, 7h and 16 h) post exposure for plasma and at 7 different post exposure time points (between 2–80 h) for urine. It is concluded that acute ozone exposure does not cause changes in plasma and urinary PGF_{2α}, and therefore their measurement in plasma and urine may not be used to reveal pulmonary inflammation and damage by ozone.

Keywords

Ozone; prostaglandins; cyclooxygenases; oxidative stress; inflammation; lipids

Introduction

Ozone is a highly reactive molecule that can directly induce acute lung injury in both humans and laboratory animals [1–3]. Oxidative stress and inflammation are likely the major mechanism causing cell and tissue injury through ozonide and hydroperoxide formation with release of inflammatory mediators and neutrophil recruitment [3–5]. A potential role of inflammation and oxidative stress is well documented in the pathology of cancer [6, 7],

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cardiovascular [8–11], rheumatic [12], lung [3, 13] and Alzheimer's diseases [14, 15]. Oxidation of lipids through non-enzymatic free radicals and enzymatic reactions (such as cyclooxygenases, lipoxygenases etc.) leads to rapid formation of several bioactive eicosanoids; that give rise to oxidative stress, which may subsequently lead to inflammatory responses. It is also well recognized that the initiation of oxidative stress and inflammation are concurrent biological processes. These processes involve cross-talk between cells on the availability of specific biomolecules and catalytic inducers in the oxidative/inflammatory microenvironment. A notable example of such is the *in vivo* formation of both F₂-isoprostanes and primary prostaglandins explicitly by two separate biochemical mechanisms: 1) free radicals, and 2) cyclooxygenases acting on arachidonic acid. These are recognized biomarkers of oxidative stress and inflammation, respectively. We have shown earlier in several experimental and clinical studies that both F₂-isoprostanes and prostaglandins (PGs) are released systemically following various experimental challenges (e.g. carbon tetrachloride (CCl₄), lipopolysaccharide (LPS) in disease models and also in studies of healthy or diseased conditions in humans [8, 9, 16, 17]. In this connection, we have previously described an experimental model of oxidative stress induced by oral administration of CCl₄ to rats, and the subsequent formation of F₂-isoprostanes, as a classical model of oxidative stress [18, 19]. It was found that CCl₄-induced oxidative stress in the rats further activates inflammatory cyclooxygenases and subsequently induces the formation of prostaglandin F_{2α} (PGF_{2α}) [18, 19]. Comparable to that model, ozone is believed to be an inducer of both oxidative stress and inflammation leading to cell death. Previous oxidative stress biomarker studies have demonstrated no effect of ozone inhalation exposure on rat plasma and urine oxidative products of lipids, proteins, DNA and plasma antioxidants [20]. Since we were not able to measure oxidative stress and the exact molecular mechanisms of ozone-induced lung injury are not fully understood, we hypothesized that ozone inhalation exposure may induce prostaglandin-endoperoxide synthases (PGHS)-mediated enzymatic lipid peroxidation that can be measured by the systemic inflammatory prostaglandin F_{2α} which might then lead to the identification of potential marker for acute lung injury primarily through systemic inflammation *in vivo*.

Materials and methods

Three hundred and forty four Male Fisher rats (260–280g) were acquired from Charles River Laboratories (Raleigh, NC, USA) for the experiments. Animals were housed three to a solid-bottom polycarbonate cage with a filter top. Animal rooms were maintained at 20–25 °C with 35–70% relative humidity with altering 12-h light and dark cycles. The rats had free access to deionized, reverse-osmosis-treated water and received autoclaved NIH 31 rodent chow (Zeigler Bros, Gardners, PA, USA) *ad libitum*. Rats were fasted overnight and then placed in the ozone exposure chambers for all experiments. Control rats received an equal exposure to air. Rats were anesthetized with Nebutal (0.1 ml/100 g body weight), and 5 ml of blood were removed from the dorsal aorta proximal to its bifurcation into the common iliac arteries. The studies followed the Guidelines for the Care and Handling of Experimental Animals of the National Institute of Health, USA. All animal studies were approved by the National Institute of Environmental Health Sciences and Environmental Protection Agency Review Board, USA.

Study Design

Study design

Ozone Inhalation Exposure—The rats were placed in individual stainless steel wire exposure cages (27.3 cm long × 14.6 cm wide × 7.75 cm tall). They were weighed just prior to inhalation exposure and again at 2h post-exposure. Rats were exposed to either HEPA-filtered room air (0 ppm) or 2 ppm and 5 ppm ozone, for 2 hours in whole body chambers under controlled flow, temperature and relative humidity. Ozone was generated from oxygen by a silent arc discharge generator (OREC, Phenix, AZ), and its entry into the Rochester style “Hinnners” chambers was controlled by mass flow controllers (Coastal Instruments Inc., Burgaw, North Carolina). The ozone concentrations were recorded using photometric ozone analyzers (API model 400, Teledyne Instruments; San Diego, California). The chamber and apparatus are operated at a slightly positive pressure to minimize dilution of the O₃ concentration throughout the exposure system. Ozone had no effect on environmental variables: air temperature and relative humidity in the chambers were maintained at ~23 °C and 46%, respectively and were monitored continuously throughout the study.

Animals from each of the two experimental dose groups (2 ppm and 5 ppm ozone) were exposed for 2 h, and specimen collection was performed at several time points (2 h, 7 h, and 16 hours after the ozone exposure for blood plasma collection). Each group consisted of 5 or 6 rats for each time point. Animal exposure treatment and biological sample preparation were described previously [20] and carried out at the US Environmental Protection Agency, Research Triangle Park (RTP), NC, USA. Exposures to ozone began no later than 8 am on a Monday, and urine was collected twice daily (7:00 am and 6:00 pm) beginning from Monday evening; the last collection was performed on Friday morning. Post exposure time points for urine sample collection were 8, 22, 32, 46, 56, 70, and 80 hours.

Whole blood and plasma sample preparation—Blood (5 ml) was drawn through single draw vacutainer needles of 21 G into open vacutainer blood collection tubes containing heparin. The contents of the tubes were mixed by gently inverting the tubes 2–3 times. For plasma specimens blood was centrifuged (2,000 rpm for 10 min at 4°C), and both the blood and plasma were frozen immediately on dry ice and stored at –70°C.

Urine sample preparation—Rats were housed individually in 4400 cm³ (20 cm diam.×14 cm high) plastic metabolism cages (Nalge Nunc, Rochester, NY) for seven days prior to exposure for acclimatization. Following exposures to ozone, the animals were returned to the metabolism cages, and urine was collected for 80 hours. The temperature of the urine collection tubes was maintained at 4°C by enclosing them in coils made from copper tubing through which cooled ethylene glycol was circulated. After collection, urine samples were centrifuged to remove extraneous debris (400×g, 15 min., 4 degrees C), and the supernatants were removed and stored at – 70°C.

Assay of 15-keto-dihydro-PGF_{2α}

The plasma samples from this study were analyzed for 15-keto-dihydro-PGF_{2α}, a major metabolite of primary PGF_{2α}, by a radioimmunoassay developed by Basu [21]. In brief, an

antibody was raised in rabbits by immunization with 15-keto-dihydro-PGF_{2α} coupled to BSA at the carboxylic acid by the 1,1'-carbonyldiimidazole method. The cross-reactivity of the antibody with PGF_{2α}, 15-keto-PGF_{2α}, PGE₂, 15-keto-13,14-dihydro-PGE₂, 8-iso-15-keto-13,14-dihydro-PGF_{2α}, 11β-PGF_{2α}, 9β-PGF_{2α}, TXB₂ and 8-iso-PGF_{3α} was 0.02, 0.43, <0.001, 0.5, 1.7, <0.001, <0.001, <0.001, and 0.01%, respectively. The detection limit of the assay was about 45 pmol/l. Measurements of urine samples were performed at the Uppsala University, Uppsala, Sweden. The urinary levels of 15-keto-dihydro-PGF_{2α} were adjusted for creatinine values, which were measured by a commercially available assay kit (ILTM Test by Monarch Instrument, Amherst, NH, USA).

Bronchoalveolar lavage fluid and lung histopathological analysis—The histopathological signs for lung injury and indicators of inflammation in bronchoalveolar lavage fluid are described in detail in two previous studies [20, 22]. Briefly bronchoalveolar lavage fluid (BALF) was obtained from all animals at all time points and doses used for the study. The whole lung of each animal was lavaged through injecting, suctioning and reinjecting three times of 30 ml/body weight 0.85% NaCl solution. This last lavage fluid was withdrawn and immediately placed on ice. Protein and enzyme assays were performed on the aliquots of the supernatant after cell separation by centrifugation (25). The cell pellet was resuspended in saline, separated into fractions and stained for cell counts as described previously (18,25). Lung specimens were stained with hematoxylin–eosin and examined post-exposure (18).

Statistical Analysis

Statistical comparisons were performed by analysis of variance, using pooled estimates of error. When the variance increased substantially with the mean, data were logarithmically transformed prior to analysis. Where multiple urinary samples were obtained from the same animals, both the assay variance within animals and the variance between animals were taken into account in the analysis. Groups of rats exposed to ozone were compared to controls at the same time point. Values of $p < 0.05$ were considered statistically significant. Statistical analyses were performed at NIEHS, RTP, NC, USA.

Results

Bronchoalveolar lavage fluid analysis and histopathological findings are described in detail for each time point and dose in a previous publication (18, 25). Briefly, signs of lung toxicity were observed after both high and low doses of ozone exposure. For example, at a dose of 2 ppm exposure, injury was marked by significant increases in BALF total protein, N-acetylglucosaminidase, and lavageable ciliated cells, whereas infiltration of neutrophils was observed only at the higher 5 ppm concentration. Severity of the abnormalities in the lung tissue in general depended on the time and dose exposure of ozone. Signs of toxicity and inflammation were localized to the bronchioles and terminal bronchioles, where loss of apical blebs of Clara cells, necrosis with denudation of epithelium lining, and accumulation of aggregates of exfoliated epithelial cells, polymorphonuclear (PMN) cells, macrophages, and cell debris in the lumen appeared to dominate (18).

In addition, groups of high dose (5 ppm) ozone-exposed rats showed histological changes that were often localized to the centriacinar region of the lungs, involving the terminal bronchioles, alveolar ducts, and proximal alveoli, sparing the larger airways. They were characterized by necrosis of the epithelium lining, presence of necrotic and intact exfoliated cells, and a few PMN cells, macrophages, and fibrin in the lumen (18,25).

Plasma levels of PGF_{2α} metabolite 15-keto-dihydro-PGF_{2α}

Blood samples were collected at three time points (2h, 7h and 16 h) after the rats were exposed to ozone, and PGF_{2α} metabolite levels were measured in plasma. The effect of ozone exposure on plasma concentration of 15-keto-dihydro-PGF_{2α} is shown in Table 1. Exposure to both doses (2 and 5 ppm) of ozone produced measurable levels of PGF_{2α} metabolite in plasma. However, changes were not significantly different from those of the controls at any time point of the experiment (2h, 7h and 16 h).

Urinary levels of PGF_{2α} metabolite 15-keto-dihydro-PGF_{2α}

Urinary samples were collected for an extended period of time (8, 22, 32, 46, 56, 70 and 80 h) after exposure to ozone, and PGF_{2α} metabolite levels were measured by radioimmunoassay. The effect of ozone exposure on the urinary concentration of 15-keto-dihydro-PGF_{2α} is presented in Table 2. Exposure to both doses (2 and 5 ppm) of ozone produced measurable levels of PGF_{2α} metabolite in urine. However, the increases were not significantly different from those of the controls at any time point of the experiment (8, 22, 32, 46, 56, 70 and 80 h).

Discussion

In the current study we used ozone exposure of rats to study inflammatory responses through the formation of inflammatory prostaglandins that might then lead to the identification of potential marker for acute lung injury through systemic inflammation in vivo.

To investigate time- and dose dependent effect we exposed rats to two different doses of ozone: lower dose of 2 ppm and higher dose of 5 ppm for 2 h. The lower dose was selected as an example of a possible human exposure (corresponding dose of 0.4 ppm) during vigorous human exercise whereas the higher dose illustrates a serious oxidant overload that may initiate severe oxidant injury and inflammation [2, 22]. Previous studies have reported the use of the same experimental model to identify gene expression profiles [22] and biomarkers of oxidative stress in rat plasma and urine (13, 15). We report here results that characterize lack of ozone mediated inflammatory response through measurement of plasma and urine concentrations of PGF_{2α} metabolite. PGF_{2α}, a potent primary prostaglandin is involved in acute and chronic inflammation [8]. 15-Keto-dihydro- PGF_{2α}, a major metabolite of PGF_{2α}, is increased during inflammatory response and can be used as indicator of in vivo lipid peroxidation through the cyclooxygenase (COX) pathway [8]. It is well known that the airway epithelium is the first line of defense and the primary site of inhaled ozone-induced lung injury [3, 22, 23]. To understand the degree of injury caused by the ozone exposure, sections of lungs and bronchoalveolar lavage fluids (BALF) were examined from all animals

and were reported in detail in previous studies [22, 24]. Because lung toxicity was observed after both high and low doses of ozone exposure (18) and was characterized mainly by necrosis of the epithelium lining, with presence of PMN cells and macrophages it was suggested that ozone exposure will cause changes in $\text{PGF}_{2\alpha}$ concentration both in plasma and urine. Furthermore injury markers in BALF to assess immediate toxicity and inflammation in the ozone exposed rats suggested that $\text{PGF}_{2\alpha}$ were sensitive to ozone exposure, a finding confirmed by other studies as well [22, 24].

Despite histopathological changes in BALF and lung tissue our data showed no difference in the plasma and urinary levels of $\text{PGF}_{2\alpha}$ metabolite 15-keto-dihydro- $\text{PGF}_{2\alpha}$ caused by inhalation ozone exposure of two distinctive doses 2 ppm and 5 ppm compared to the control groups (0 ppm). This finding was evident both for the three examined time points (2h, 7h and 16 h) for plasma samples and for an extended period of time (8–80 h) for urinary samples. In contrast, it has been well documented in animal models and human studies that exposure to ozone induces secretion of many different proinflammatory factors into the lung, including, IL-1, TNF- α , IL-6, and IL-8 [3]. However, there are no studies performed with ozon induction to the rats and $\text{PGF}_{2\alpha}$ formation and release. One earlier study mentioned that primary $\text{PGF}_{2\alpha}$ increased in plasma in ozon-sensitive humans [25]. However, the measurement of plasma $\text{PGF}_{2\alpha}$ is not suitable due to rapid metabolism to degraded metabolites and artifactual formation during blood sample collection. The current valid parameter of $\text{PGF}_{2\alpha}$ synthesis and release is the measurement of its primary metabolite in plasma, 15-keto-dihydro- $\text{PGF}_{2\alpha}$ but not $\text{PGF}_{2\alpha}$ itself in plasma [26]. The assessment of $\text{PGF}_{2\alpha}$ in oxidative stress or inflammation-related conditions has been associated with their induction above normal ranges [18]. Present literature reviews show that ozone induced oxidative stress causes inflammasome activation with the release of IL-1, other cytokines and proteases driving lung inflammation and changes in the chemical composition of bronchoalveolar lavage fluids for several days after exposure to inhalation of ozone [27, 28]. This is possibly due to the induction of the innate immunity of the body [3]. The extended time-points we have chosen in this study together with two different ozone doses were appropriate to possibly detect $\text{PGF}_{2\alpha}$ changes in plasma and/or urine caused by the ozone exposure. However, $\text{PGF}_{2\alpha}$ metabolite 15-keto-dihydro- $\text{PGF}_{2\alpha}$ concentration measured in plasma and urine was not changed in our rat model.

We have previously described, that CCl_4 -induced oxidative stress activates cyclooxygenases and significantly induces $\text{PGF}_{2\alpha}$ in vivo, in both peripheral plasma and urine [18, 24, 29]. CCl_4 also induced oxidative stress biomarker F_2 -isoprostanes in rat plasma in a time and dose dependent pattern (16,17). An earlier study with the same biological samples as used in the current study, showed no effect of ozone on plasma and urine F_2 -isoprostanes [30]. It was concluded that no available product of oxidation in biological fluid has yet met the required criteria of being a biomarker of ozone induced oxidative stress. From our current findings of absence of $\text{PGF}_{2\alpha}$ induction, it could be assumed that many interacting factors may perhaps be involved in the outcome. For example, the free radical mechanism of oxidative stress by ozone may not be relevant, or oxidative damage may have occurred but been repaired endogenously prior to its detection. Since we have already reported significant changes in non-enzymatic lipid peroxidation after CCl_4 administration (16, 17), perhaps ozone may not be as powerful oxidizing agent as CCl_4 . The current study showed no effect

of ozone on $\text{PGF}_{2\alpha}$ metabolite, which is more comprehensible since ozone has shown no influence on the oxidative stress biomarkers as previously concluded [30]. Although ozone can impair the phagocytic capacity of alveolar macrophages [31, 32] others report that neutrophil depletion inhibits airway hyperresponsiveness induced by ozone exposure [33, 34] and genetic control of susceptibility to ozone-induced lung inflammation [22, 35, 36].

In a different study we have shown that administration of 8-iso- $\text{PGF}_{2\alpha}$ (8-iso-prostaglandin $\text{F}_{2\alpha}$), a major bioactive F_2 -isoprostane, intravenously to rabbits may lead to a significantly increased inflammatory $\text{PGF}_{2\alpha}$ formation [37]. This shows that F_2 -isoprostane formation may induce $\text{PGF}_{2\alpha}$ metabolite formation and release as an effect of a free radical-induced inflammatory response. However, chronic exposure to ozone (2–3 ppm) twice weekly for 3 h causes repeated bouts of inflammation with progressive destruction of alveolar epithelial cells and emphysema within six weeks, resembling in part that found in COPD [38, 39]. In the current study we could not show any rise of $\text{PGF}_{2\alpha}$. Together with the earlier findings of a lack of increase of any oxidative stress biomarkers [30], we conclude that ozone administration at the two different doses applied with the biological samples collected at the given time points is not sufficient to produce any significant inflammatory response in vivo in the rats that can be detected by non-different $\text{PGF}_{2\alpha}$ metabolite levels in this study.

Even though validated LC/MS/MS is now regarded as a gold standard method for determination of eicosanoids our RIA method is validated extensively both by comparing many similar experimental studies for about 20 years.

In conclusion, ozone exposure did not induced inflammatory responses through the formation of inflammatory prostaglandins in the body and therefore measurement of $\text{PGF}_{2\alpha}$ concentration in plasma and urine may not be used as a marker for acute lung injury through systemic inflammation in vivo. The current negative results as a consequence of ozone inhalation exposure are of great significance, because they provide evidence that in complex systems, as the present in vivo investigation, the assays used may not provide meaningful data of ozone oxidation and inflammation in experimental and human studies.

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Abbreviations

$\text{PGF}_{2\alpha}$	prostaglandin $\text{F}_{2\alpha}$
8-iso-$\text{PGF}_{2\alpha}$	8-iso-prostaglandin $\text{F}_{2\alpha}$
CCL_4	carbon tetrachloride
LPS	lipopolysaccharide
ROS	reactive oxygen species
TXB_2	thromboxane B_2

COPD

chronic obstructive pulmonary disease

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Table 1

Effect of ozone exposure (control-0 ppm, 2 ppm and 5 ppm) on plasma levels of 15-keto-dihydro-PGF_{2α} (pg/ml) of male Fisher rats

Ozone Exposure	Time Point 2 hours (n)	Time Point 7 hours (n)	Time Point 16 hours (n)
Control 0 ppm	165 ± 12 (6)	178 ± 14 (6)	164 ± 8 (6)
2 ppm	178 ± 27 (6)	169 ± 19 (6)	171 ± 13 (6)
5 ppm	191 ± 18 (6)	182 ± 25 (6)	166 ± 17 (6)

Analysis of variance: The 9 groups do not differ significantly; values are Means ± SE; sample size is 6/group; samples were collected 2h, 7h and 16h after the ozone exposure. Control samples from non-exposed to ozone rats were collected at the same time points.

Table 2

Effect of ozone exposure (control-0 ppm, 2 ppm and 5 ppm) on urinary levels of 15-keto-dihydro-PGF_{2α} (nmol/mmol creatinine) of male Fisher rats.

Time points after the Ozone exposure	Control 0 ppm (n)	Ozone 2 ppm (n)	Ozone 5 ppm (n)
8 hours	0.51 ± 0.04 (3)	0.49 ± 0.07 (4)	0.53 ± 0.07 (6)
22 hours	1.04 ± 0.33 (6)	1.29 ± 0.42 (6)	0.47 ± 0.05 (6)
32 hours	0.45 ± 0.04 (6)	0.66 ± 0.12 (6)	0.47 ± 0.03 (6)
46 hours	1.35 ± 0.46 (6)	0.77 ± 0.22 (6)	0.65 ± 0.16 (6)
56 hours	0.51 ± 0.03 (6)	0.64 ± 0.08 (6)	0.64 ± 0.08 (6)
70 hours	0.86 ± 0.09 (6)	0.66 ± 0.08 (5)	1.01 ± 0.24 (6)
80 hours	0.60 ± 0.10 (6)	0.72 ± 0.14 (5)	0.58 ± 0.05 (6)

Analysis of variance: The 3 groups do not differ significantly; sample size is n= 5–6/group; Urine samples were collected 8h, 22h, 32 h., 46 h, 70 h and 80 h after the ozone exposure. Control samples from non-exposed to ozone rats were collected at the same time points.