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Ozone induces synthesis of systemic prostacyclin by cyclooxygenase-2 dependent mechanism in vivo

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ABSTRACT

Under certain pathological conditions, e.g., infectious or neoplastic diseases, application of ozone exerts therapeutic effects. However, pharmacological mechanisms are not understood. Since an interaction with the arachidonic acid metabolism is suggested we investigated the effect of intraperitoneal insufflation of ozone on prostanoid system in vivo. Upon ozone application (4 mg/kg) to rats we observed an approximate 3-fold increase in excretion rate of 6-keto-prostaglandin (PG) $F_{1\alpha}$ and of 2,3-dinor-6-keto-$PGF_{1\alpha}$, the measurable stable products of prostacyclin. In plasma and vessel tissue 6-keto-$PGF_{1\alpha}$ concentration was also significantly increased. In contrast, excretion rates for PGE_2 and thromboxane (TX) B_2 did not change. F2-isoprostanes, regarded as endogenous indicators of oxidative stress, were also unaffected by ozone application. Oxygen insufflation used as control was without any effect on prostanoid levels. Ozone caused increase in 6-keto-$PGF_{1\alpha}$ by arterial but not by venous vessel tissues with peak activity 6–9 h following insufflation. The increase in PGI_2 synthesis was dependent on cyclooxygenase (COX)-2 activity, demonstrated by its sensitivity towards COX-2 inhibition, and by enhanced COX-2 mRNA and protein expression in vessels. Ozone exerted no rise in excretion rate of prostacyclin metabolites in COX-$2^{-/-}$ but in COX-$1^{-/-}$ mice. Enzymatic activity and mRNA expression of vascular PGI_2 synthase (PGIS) was unaffected by ozone treatment. In summary our study shows for the first time that ozone insufflation causes enhanced expression of COX-2 in the vessel system leading to exclusive elevation of systemic PGI_2 levels. We assume that PGI_2 stimulation may contribute to the beneficial effects of ozone treatment.

1. Introduction

Ozone in the stratosphere protects man and animals from actinic skin tumors [1], but it is also known to cause radicals to be formed in biological systems. Ozone is primarily a non-radical molecule, but by ozonolyses it forms secondary radicals, hydroxides, aldehydes and different ozonides [2]. The formation of reactive oxygen species (ROS) and lipid oxidation products induced by ozone inhalation is assumed to be responsible for pulmonary toxicity [3,4], albeit the exact mechanisms are still not known. Recently endogenous ozone derived from leukocyte oxidation of water has been reported [5,6]. It is suggested that biologically produced ozone not only contribute to kill invading microorganisms but also to amplify inflammatory responses [5]. Apart from the lung, there is also some basic preclinical and clinical research [7], known by other forms of ozone applications on the blood system [8], intestinal tract, and on different local anatomical regions such as the spine and joints of man and animals [9]. Standing out of the large array of application methods is the relatively safe insufflation of large amounts of ozone into the abdominal cavity of rodents [10,11] and rabbits [12], also called O_3/O_2-pneumoperitoneum which in contrast to inhalation do not cause organ damage. Our recent studies using intraabdominal insufflated ozone have demonstrated therapeutic effects in various disease models, such as reduced lethality in peritonitis models [11,13] and complete remission of rabbit squamous cell carcinomas [12]. Despite these promising observations, signaling pathways triggered by ozone treatment have not been identified. Earlier studies on lung exposure to ozone have indicated that arachidonic acid metabolism represent a target for ozone action, however, conflicting data were obtained depending on way of ozone application and experimental model [14–17]. In the present study we investigated systemic effects of abdominally insufflated ozone on prostanoid metabolism. The prostanoids PGE_2, PGD_2, $PGF_{2\alpha}$, PGI_2 and thromboxane TXA_2 comprise a family of lipid mediators derived via the COX pathway of arachidonic acid [18].

Abbreviations: BW, body weight; COX, cyclooxygenase; PG, prostaglandin; PGIS, prostacyclin synthase; TX, thromboxane; ROS, reactive oxygen species.

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COX, which exists in two isoforms, the constitutive COX-1 and the inducible type COX-2, converts arachidonic acid to the intermediate PGH_2 which is subsequently metabolized to various prostanoids depending on the type of synthase co-expressed [19]. Prostanoids are short-living metabolites which are known to modulate inflammatory, hemodynamic and neoplastic processes within the body. In part, prostanoids or stable analogues of them were clinically used, e.g., PGI_2 for therapy of pulmonary arterial hypertension. Of note, isoprostanes which are prostaglandin-like compounds are produced by free radical peroxidation of arachidonic acid [20] and often used as quantitative biomarkers of oxidative stress [21]. Our study gives evidence that intraabdominal insufflated ozone triggers in a COX-2 dependent manner the specific formation of PGI_2 but not the synthesis of other types of prostanoids or of isoprostanes.

2. Materials and methods

2.1. Animals

Healthy (as given by FELASA recommendation) adult C57BL6 mice, Wistar rats and New Zealand rabbits were obtained from Charles River (Sulzfeld, Germany). Breeder pairs of COX-1 and COX-2 knockout mice were kindly provided by R. Langenbach (Nashville, Tennessee). Genotypes of the mice were determined by PCR analysis using specific oligonucleotides to the respective COX locus and the Neo cassette as described in [22]. The animals were maintained in individually ventilated cages in a temperature controlled (21 °C) room with 12 h light–12 h dark cycle. They were given standard diet and water ad libitum. The study was performed with permission of the regional animal welfare committee in Giessen (RP Giessen; Germany) and animal experimentation was according the International Guiding Principles for Biomedical Research Involving Animals.

2.2. Generation and application of ozonized oxygen

Ozonized oxygen was generated from medical oxygen by an ozone gas processor (Ozonosan, PTN 60, Dr. Hänsler GmbH, Iffezheim, Germany; Medozon ip Herrmann GmbH, Kleinwallstadt, Germany). Ozone concentrations were monitored and intraabdominal pressure was measured by the generator in mbar during and after ozone insufflations. The gas mixture (2.5%v O_3/97.5%v O_2) was insufflated by injection (80 ml/kg BW at a concentration of 50 μg O_3/ml resulting in a dose of 4 mg/kg BW, called ozone) into the right lower abdomen of anaesthetized mouse (forene), rat (forene) and rabbit (metodomidin/propofol/antisedan). The dose was based on our earlier observations from studies in laboratory animals [13]. As a control gas we used pure oxygen (called control). For collection of 24 h urine samples, animals were kept in metabolism cages and daily excretion of prostanoids was calculated as pg/h. Urine samples were kept at 4 °C during collection. To obtain plasma samples blood was collected in a tube containing EDTA, 10 μM indomethacin and 20 μM butylated hydroxytoluene.

2.3. Determination of prostanoids and F2-isoprostanes

Prostanoids and F2 isoprostanes were determined by GC–MS/MS analysis as described by us earlier [23]. Briefly, extracts from plasma or urine samples were spiked with 1 ng deuterated internal standards. The methoxime was obtained through reaction with an O-methylhydroxylamine hydrochloride–acetate buffer. After acidification to pH 2.5, prostanoid derivatives were extracted, dried by evaporation, and the pentafluorobenzylesters were formed. Samples were purified by thin-layer chromatography using ethylacetate:hexan (w/w, 9:1) as a solvent, and three broad zones were eluted. After withdrawal of the organic layers, trimethylsilyl ethers were prepared by reaction with bis(trimethylsilyl)-trifluoroacetamide and thereafter subjected to GC–MC/MS. A Finnigan MAT TSQ700 GC–MS/MS equipped with a Varian 3400 gas chromatograph (Thermo Fisher Scientific, Waltham, MA) was used and GC–MS/MS parameters were as described [24]. Sample method for F2 isoprostanes was similar as described above. As F2-isoprostanes have no keto function, the methoximation step could be omitted. In the TLC purification step, a broad zone (RF 0.03–0.30) was scraped of the TLC plate [25]. This zone contained all regioisomers of F2 isoprostanes.

2.4. Determination of COX activity in vessel tissue

By surgical procedure samples of aorta abdominalis and vena cava caudalis were prepared, weighted, washed in PBS for 30 s, and immediately given in prewarmed Krebs solution. Reaction was started by addition of 10 μM arachidonic acid (Sigma–Aldrich, Steinheim, Germany) or vehicle for 30 min at 37 °C and stopped by addition of 4% formic acid. Samples were immediately kept on ice, centrifuged for 2 min at 10,000 × *g* and resulting supernatants were stored at −80 °C until prostanoid analysis.

2.5. Assay of PGIS activity

Enzymatic activity of PGIS was determined as described by us [26] with the following modifications. Whole tissue homogenates (50 mg) in PBS were mixed with 10 μM PGH_2 (CPS Chemie + -Service, Aachen, Germany) or vehicle for 1 min at 22 °C. The reaction was stopped by the addition of 1 mM $FeCl_2$ and 4% formic acid. After centrifugation supernatants were stored at −80 °C for analysis of 6-keto-PGF1α.

2.6. Application of cyclooxygenase inhibitors

Rats were treated for 3 days with naproxen (20 mg/kg) (Sigma–Aldrich, Steinheim, Germany), rofecoxib (10 mg/kg) (MSD, Haar, Germany), SC-560 (10 mg/kg) (Calbiochem, Darmstadt, Germany) or vehicle twice a day by s.c. injection. All substances were dissolved in dimethylsulfoxide (vehicle). Used concentrations have been shown to block activity of COX-1 (SC-560), COX-2 (rofecoxib) or both enzymes (naproxen) [27–29]. On the third day ozone (4 mg/kg BW) was insufflated and 6 h later the animals were deeply anaesthetized (ketamine, xylazine) and tissue samples from abdominal artery were prepared before euthanasia.

2.7. Quantitative Real Time PCR

By surgical procedure samples of aorta abdominalis were prepared and total RNA was isolated using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA concentration was quantified by absorption at 260 nm using a NanoDrop spectrophotometer (Peqlab Biotechnologie, Erlangen, Germany). 0.5 μg RNA was reverse transcribed with superscript II RNase and random hexamers (Invitrogen, Karlsruhe, Germany), and 1/40 volume of the resulting cDNA samples were used as templates for real time PCR by using SYBR Green supermix reaction (Applied Biosystems, Darmstadt, Germany) procedure with the 7500 Fast System (Applied Biosystems, Darmstadt, Germany). All reactions were run in triplicate to minimize experimental error. The following primer pairs were used: for COX-1, GTG GCT ATT TCC TGC AGC TC and CAG TGC CTC AAC CCC ATA GT; for COX-2, GCA GTT GTT CCA GAC AAG CA and AAG GGG ATG CCA GTG ATA GA; for PGIS, GGT GAC CTG TTG CCA CCC GGC and GCT GCC CAG GTC CAA CGG AGG; and for β-actin, TCC ATC ATG

AAG TGT GAC GT and GAG CAA TGA TCT TGA TCT TCA T. As internal control we used 18S-rRNA [30]. PCR was initiated at 95 °C for 15 min followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C for annealing and extension. The expression of mRNA was assessed relative to that of β-actin and relative quantitative level of samples was determined by the standard $2^{(-ddCt)}$ method and expressed as change (fold) relative to expression levels under control. Samples of PCR were run in agarose gels and visualized by ethidium bromide staining.

2.8. Immunofluorescence

By surgical procedure tissue samples of aorta abdominalis were embedded in Tissue-Tek OCT compound (Weckert Labortechnik, Kitzingen, Germany) and snap-frozen at −100 °C in isopentane cooled in liquid nitrogen. Frozen tissue was cut at 5 μm, thaw mounted on SuperFrost glass slides (Menzel GmbH, Braunschweig, Germany), air dried and fixed in acetone for 10 min at 4 °C. The slides were subsequently incubated overnight at 4 °C with anti COX-2 antibody (Cayman Chemicals, Ann Arbor, Michigan) in a dilution of 1:100. Following washing anti-rabbit antibodies labeled with Texas red rhodamine (Dianova, Hamburg, Germany) were used for visualization.

2.9. Statistics

Data were expressed as mean ± S.E.M. Differences were analyzed by one-way ANOVA test or unpaired Student's *t*-test as appropriate by using Prism software. Values of $p < 0.05$ were considered significant.

3. Results

In all experiments using bolus or repeated ozone insufflations no case of morbidity (e.g., significant weight loss, visible signs of pain) or mortality was observed. Blood analysis before and after treatment revealed that the number of white blood cells and of platelets remained unchanged, whereas hematocrit was slightly decreased (Table 1).

3.1. Effect of single ozone application on synthesis of prostanoids and isoprostanes

Following single abdominal insufflation of ozone or control to rats, we analyzed amount of various prostanoids in 24 h urine samples by GC–MS/MS and determined urinary excretion. Compared to control we observed a significant rise in the excretion of prostacyclin metabolites within 24 h following ozone insufflation. Renal excretion rates for 6-keto-$PGF_{1\alpha}$, the hydration product of PGI_2, and for 2,3-dinor-6-keto-$PGF_{1\alpha}$, the metabolism product of 6-keto-$PGF_{1\alpha}$, increased approximately 3-fold and rates for both metabolites returned to basal levels on the following untreated days (Fig. 1A and B). Interestingly, excretion rate for PGE_2, the main systemic product of arachidonic acid remained unaffected by ozone treatment (Fig. 1C). In order to evaluate whether alterations in arachidonic acid metabolism are consequences due to oxidative stress caused by ozone insufflation, we determined F2-isoprostanes, which are formed in vivo from free radical-catalyzed peroxidation of arachidonic acid and are regarded as biomarkers for oxidative stress. Under our experimental setting we observed no significant change in daily excretion of F2-isoprostanes following ozone insufflation (Fig. 1D). Renal function expressed by urine electrolyte, creatinine concentration and urine volume was unaffected by the treatment (data not shown).

Regarding the rise in urinary concentration of prostacyclin metabolites we questioned whether plasma levels were also raised and whether the effect of ozone was limited to the species rat. In

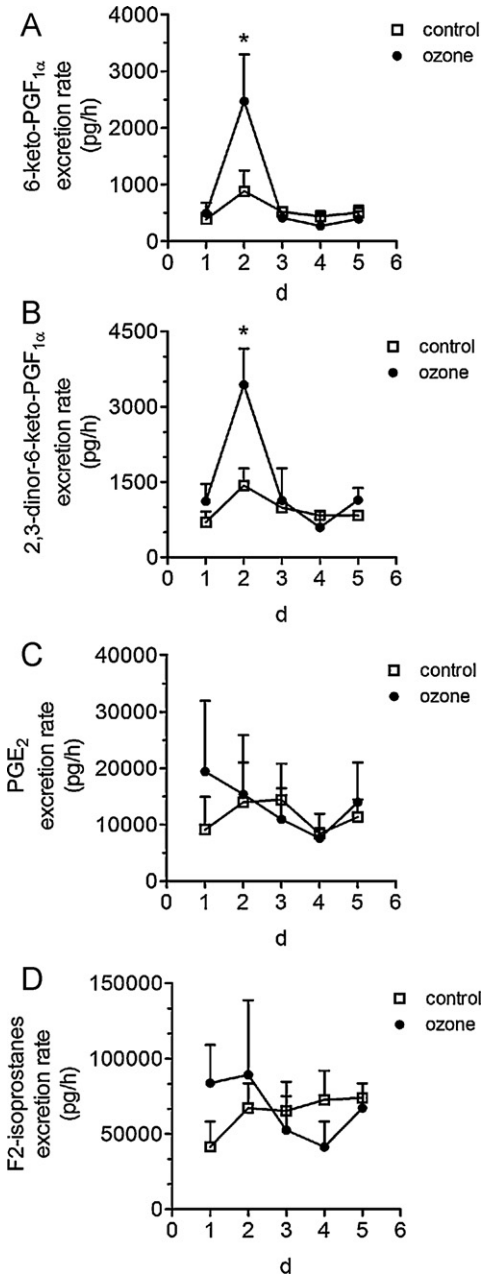

Fig. 1. Excretions rates for 6-keto-$PGF_{1\alpha}$ (A), 2,3-dinor-6-keto-$PGF_{1\alpha}$ (B), PGE_2 (C), and F2-isoprostanes (D) following single ozone insufflation in rats. Animals were kept in metabolism cages to collect 24 h urine samples under untreated conditions (from day 0 to day 1). On day 1 animals were treated with ozone or control at 10 a.m. and 24 h urine was collected every day until day 5. Prostanoid concentrations in urine samples were determined by GC–MS/MS and calculated as excretion rates (pg/h). Data represent means ± S.E.M.; $n = 6–8$; *, $p < 0.05$ versus control group.

Table 1
Number of white blood cells, platelet count and hematocrit in rat blood following ozone insufflation.

	Control	6 h after ozone application
White blood cells ($10^3/mm^3$)	14.76 ± 0.84	15.66 ± 1.29
Platelet count ($10^3/mm^3$)	286.8 ± 22.0	267.3 ± 64.8
Hematocrit (%)	44.56 ± 1.14	41.64 ± 1.12*

Rats were insufflated by ozone or control. Six h later heparinized plasma samples were taken and blood parameters determined. Data represent means ± S.E.M.; $n = 8$.
* $p < 0.05$ compared to control.

Table 2
Effect of ozone treatment on plasma concentrations of 6-keto-$PGF_{1\alpha}$, PGE_2, and F2-isoprostanes.

	Control (pg/ml)	24 h after ozone application (pg/ml)
PGE_2	937.1 ± 390.1	779.8 ± 339.5
6-Keto-$PGF_{1\alpha}$	47.2 ± 7.8	109.6 ± 22.3*
F2-isoprostanes	1225.0 ± 362.7	1471.0 ± 384.9

Rats were insufflated by ozone or control. Six h later heparinized plasma samples were taken and formed prostanoids were determined by GC–MS/MS. Data show means ± S.E.M.; $n = 8$.
* $p < 0.05$ compared to control.

Table 3
Excretion rates for 2,3-dinor-6-keto-$PGF_{1\alpha}$ in different species following ozone insufflation.

Species	Control	Ozone
	2,3-Dinor-6-keto-$PGF_{1\alpha}$ (pg/h)	
Rabbit	1560 ± 337	7064 ± 2407*
Rat	1118 ± 341	3438 ± 718*
Mouse	1202 ± 294	4349 ± 1514*

Animals were insufflated by ozone or control and 24 h urine samples were collected. Formed prostanoids were determined by GC/MS-MS. Data represent means ± S.E.M.; $n = 5–8$.
* $p < 0.05$ compared to control.

agreement to our urine data we observed significantly elevated 6-keto-$PGF_{1\alpha}$ concentrations in rat plasma samples, whereas amounts for PGE_2 and F2-isoprostanes were unaffected by ozone treatment (Table 2). In mice and rabbits ozone insufflation was also able to increase daily excretion of 2,3-dinor-6-keto-$PGF_{1\alpha}$ (Table 3).

3.2. Effect of multiple ozone applications on synthesis of prostanoids and isoprostanes

Next we questioned whether repetitive insufflation of ozone, once a day for five consecutive days, may result in continuous elevation of PGI_2 synthesis. We observed persistent high levels of the PGI_2 metabolites 6-keto-$PGF_{1\alpha}$ and 2,3-dinor-6-keto-$PGF_{1\alpha}$ in rats during ozone insufflation. Starting on day 6 ozone was omitted and excretion rates for prostacyclin metabolites decreased to control levels within 24 h (Fig. 2A and B). In contrast, no statistically significant differences between ozone treated and vehicle treated group were observed in the excretion rates for TXB_2, the inactive but stable hydrolysis product of TXA_2, for 2,3-dinor-TXB_2, the metabolism product of TXB_2, and also for PGE_2 and F2-isoprostanes (Fig. 2C–F).

3.3. Time dependent effect of ozone on prostanoid synthesis by vessel tissue

The main systemic sources of prostacyclin are endothelial cells and smooth muscle cells of vessels. Therefore we studied the prostanoid formation by vessel samples from abdominal arteries and veins. Rats were treated with ozone or control and vessel tissue was prepared at different time points to determine its ability to form PGI_2, PGE_2, and F_2-isoprostanes from exogenously added arachidonic acid. Our experiments led to the following observations: First, arterial tissue samples produced higher amounts of

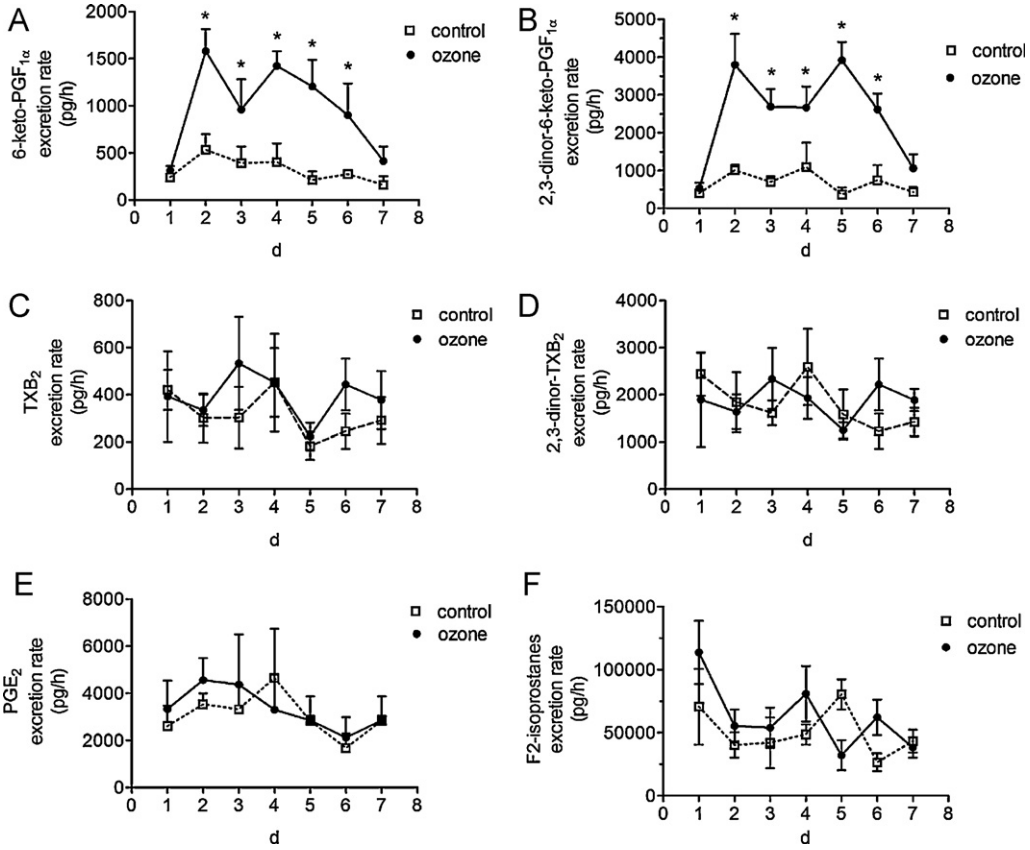

Fig. 2. Excretions rates for 6-keto-$PGF_{1\alpha}$ (A), 2,3-dinor-6-keto-$PGF_{1\alpha}$ (B), TXB_2 (C), 2,3-dinor-TXB_2 (D), PGE_2 (E), and F2-isoprostanes (F) following multiple ozone insufflation in rats. Animals were kept in metabolism cages for 1 day to collect 24 h urine samples under untreated conditions (from day 0 to day 1). From day 1 to day 5 animals were treated with ozone (4 mg/kg) or control at 10 a.m. and 24 h urine was collected until day 7. Prostanoid concentrations were determined by GC–MS/MS and calculated as excretion rates (pg/h). Data represent means ± S.E.M.; $n = 6–8$; *, $p < 0.05$.

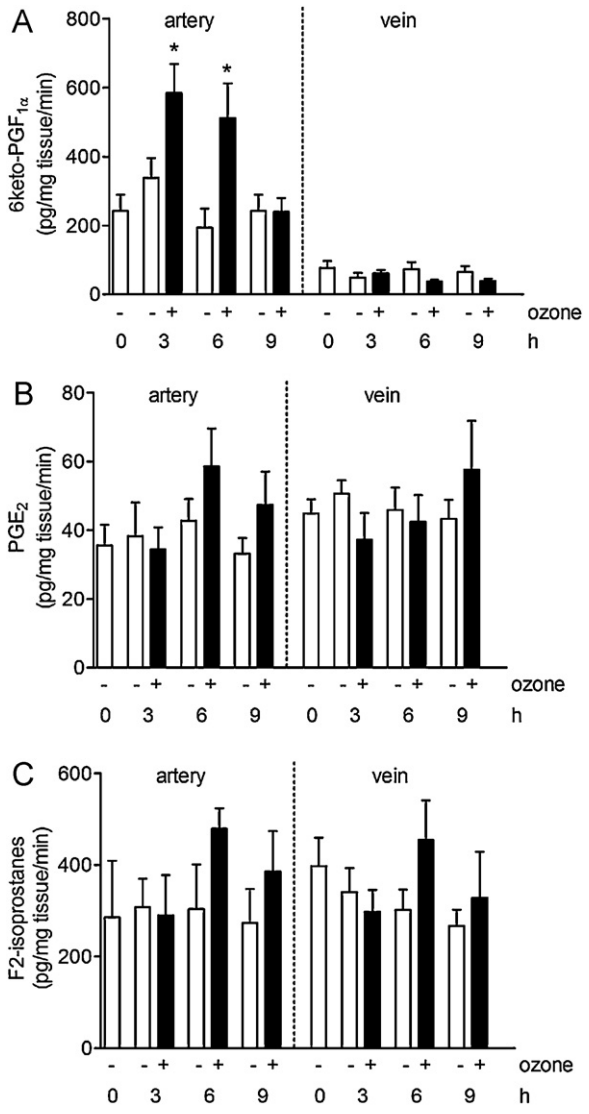

Fig. 3. Synthesis of 6-keto-$PGF_{1\alpha}$ (A), PGE_2 (B), and F2-isoprostanes (C) by isolated tissue samples of arteries and veins from abdomen of ozone treated rats. Rats were treated once with ozone and at the indicated time points animals were anaesthetized, abdominal arteries and veins were prepared, and vessel tissue samples were incubated with arachidonic acid. Synthesized prostanoids were determined by GC–MS/MS. Data show means ± S.E.M.; $n = 5$; *, $p < 0.05$.

PGI_2 metabolite 6-keto-$PGF_{1\alpha}$ compared to tissue samples from veins (Fig. 3A). Second, ozone treatment caused a significant increase in 6-keto-$PGF_{1\alpha}$ formation 3–6 h following ozone insufflation which returned back to basal levels at later time point (Fig. 3A). Third, the venous capability to produce PGI_2 was unaltered by ozone treatment (Fig. 3A). Fourth, the ability of abdominal artery or vein tissue to produce PGE_2 or F2-isoprostanes was unaffected by ozone (Fig. 3B and C).

Table 4
Prostanoid concentration in arterial vessel tissue of rats.

Prostanoid	Control	Ozone
	pg/mg tissue	
6-Keto-$PGF_{1\alpha}$	7.70 ± 0.76	16.59 ± 1.32*
PGE_2	1.63 ± 0.08	1.43 ± 0.06

Animals were insufflated by ozone or control and 6 h later arterial vessel tissue was prepared and weighted. Prostanoids were extracted from whole tissue and determined by GC–MS/MS. Data represent means ± S.E.M.; $n = 5$.
* $p < 0.05$ compared to control.

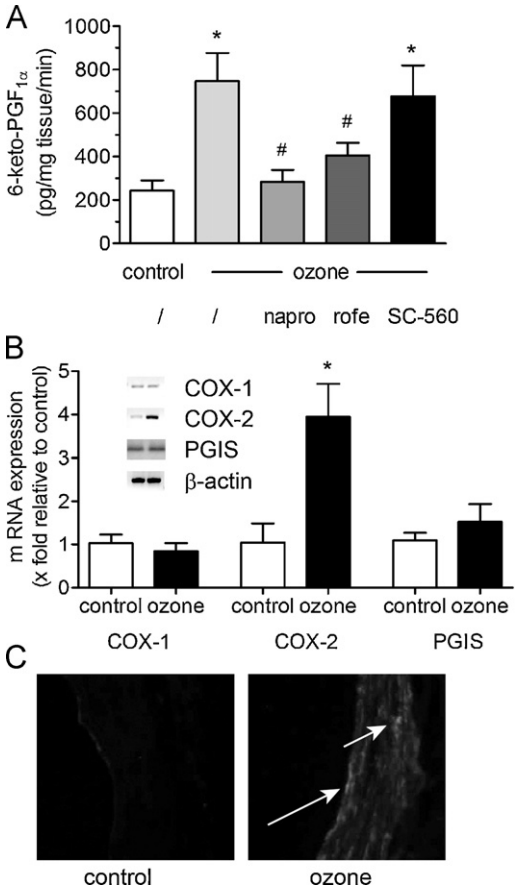

Fig. 4. Effect of ozone on activity (A), expression of mRNA (B) and protein (C) of COX-2. For activity measurements rats were treated for 3 days with naproxen (20 mg/kg), rofecoxib (10 mg/kg), SC-560 (10 mg/kg) or vehicle twice a day. Thereafter ozone was insufflated and after 6 h animals were anaesthetized and arteries were prepared from abdomen. Tissues were incubated with 10 μM arachidonic acid for 30 min at 37 °C. Formed prostanoids were determined by GC–MS/MS. Data show means ± S.E.M.; $n = 6$; *, $p < 0.05$ compared to control; #, $p < 0.05$ compared to ozone without inhibitor. PGIS, COX-1 and COX-2 mRNA expression were determined by quantitative real time PCR (data show means ± S.E.M.; $n = 4$; *, $p < 0.05$ compared to control). COX-2 protein expression was studied by immunofluorescence in abdominal vessels prepared from vehicle and ozone-treated rats (staining of endothelial cells is exemplarily marked by long arrow, staining of smooth muscle cells by short arrow).

Extraction of prostanoids from whole arterial vessel samples revealed that ozone application caused significant higher tissue content of 6-keto-$PGF_{1\alpha}$ compared to control, whereas PGE_2 concentration were similar in both groups (Table 4).

3.4. Role of COX-1 and COX-2 in ozone-induced prostacyclin synthesis

In order to investigate the cyclooxygenase type, COX-1 and/or COX-2, responsible for 6-keto-$PGF_{1\alpha}$ formation, we first treated rats for two days with COX unspecific and specific inhibitors, prepared arterial vessel tissue and determined its ability to produce 6-keto-$PGF_{1\alpha}$ from exogenously added arachidonic acid. Naproxen, known to inhibit both COX enzymes, and rofecoxib, a specific inhibitor for COX-2, significantly suppressed ozone induced formation of 6-keto-$PGF_{1\alpha}$ (Fig. 4A). Treatment with SC-560, a potent COX-1 selective inhibitor was without significant effect on synthesis of 6-keto-$PGF_{1\alpha}$. Second, we determined COX mRNA expression by quantitative real-time PCR and COX-2 protein expression in control- and ozone-treated arteries from rat. Whereas mRNA expression for COX-1 remained on a similar level independent from type of treatment, COX-2 mRNA expression was significantly induced by ozone insufflation (Fig. 4B). Weak antigenicity towards anti COX-2 antibodies was observed in

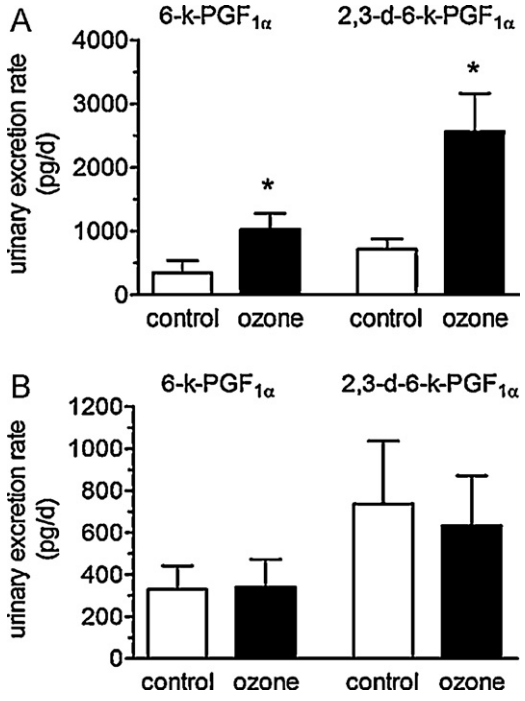

Fig. 5. Excretions rates for 6-keto-$PGF_{1\alpha}$ and 2,3-dinor-6-keto-$PGF_{1\alpha}$ in ozone-treated COX-$1^{-/-}$ (A) and COX-$2^{-/-}$ (B) mice. Animals were treated with ozone or control and 24 h urine samples were collected. Urinary prostanoid concentrations in 24 h urine were determined by GC–MS/MS and calculated as excretion rates (pg/h). Data represent means ± S.E.M.; $n = 6$; *, $p < 0.05$ versus control group.

sections of control artery. Following ozone application strong immunofluorescence was detected in endothelial cells and smooth muscle cells in sections of arterial vessel (Fig. 4C). Third, we analyzed activity and expression of PGIS, the enzyme secondary to COX in the synthesis of PGI_2. We observed no significant difference in vascular mRNA expression of PGIS between vehicle and ozone-treated rats (Fig. 4B). Moreover, PGIS activity remained unchanged (27.70 ± 4.16 ng 6-keto-$PGF_{1\alpha}$/min/50 mg protein, vehicle group; 36.59 ± 6.32 ng 6-keto-$PGF_{1\alpha}$/min/50 mg protein, ozone-treated group; $p > 0.05$). Fourth, we studied daily urinary excretion of PGI_2 metabolites in COX isoform deficient mice treated by ozone insufflation. Compared to control unaltered excretion rates for 6-keto-$PGF_{1\alpha}$, and 2,3-dinor-6-keto-$PGF_{1\alpha}$ were observed in COX-$2^{-/-}$ mice whereas ozone was able to raise these metabolites significantly in COX-$1^{-/-}$ mice (Fig. 5A and B).

4. Discussion

In the present study we disclose a yet undescribed action of ozone on endogenous prostanoid metabolism and give first evidence that ozone insufflation causes selective stimulation of prostacyclin synthesis in vivo by induction of vascular COX-2 expression.

In the body formed PGI_2 rapidly decomposes to 6-keto-$PGF_{1\alpha}$ which is further metabolized to the more stable 2,3-dinor-6-keto-$PGF_{1\alpha}$. Based on non-enzymatic and enzymatic metabolism and on the different half-lives of prostacyclin metabolites it is assumed that 6-keto-$PGF_{1\alpha}$ reflects predominantly renal formation of prostacyclin, whereas 2,3-dinor-6-keto-$PGF_{1\alpha}$ is a measure for systemic prostacyclin synthesis [31]. In our experiments ozone stimulated the formation of both prostacyclin metabolites, 6-keto-$PGF_{1\alpha}$ and 2,3-dinor-6-keto-$PGF_{1\alpha}$. This finding indicates that systemic (including renal) synthesis of PGI_2 is increased which is in line with our observation that rat plasma and vessel tissue levels of 6-keto-$PGF_{1\alpha}$ were also elevated after ozone insufflation. Interestingly only aortic vessels but not venous vessels were able to synthesize elevated PGI_2 amounts following ozone application. Compared to veins, arteries produced about 10 times more PGI_2. This observation is in agreement with previous reports indicating that endogenous PGI_2 production is generally greater in arteries than in veins [32,33]. Higher flows, pressures and shear stress in the arterial circulation are assumed to contribute to relatively higher PGI_2 production [32].

With regard to the effect of ozone on prostanoid system three points are of interest. First, ozone caused selective formation of prostacyclin, but not of other prostanoids. Under all experimental conditions biosynthesis of PGE_2, which is generally the main prostanoid formed from endogenous arachidonic acid, remained unaltered. Urinary TXB_2, regarded as a measure for renal TXA_2 synthesis, and urinary 2,3-dinor-TXB_2, reflecting systemic TXA_2 synthesis, were also not affected by ozone treatment. The main source of systemic TXA_2 is the platelet and TXA_2 is one of the most potent aggregatory and vasoconstrictory metabolite known, which is balanced under physiological conditions by prostacyclin [34]. This fact let us assume that ozone treatment shifts the vessel prostanoid system to a more anti-aggregatory and anti-vasoconstrictory status by selective increase of PGI_2 formation. Second, single insufflation of ozone results in a transient increase in 6-keto-$PGF_{1\alpha}$ going back to basal levels within 24 h, whereas multiple insufflations result in a sustained enhanced 6-keto-$PGF_{1\alpha}$ level, depending on frequency of insufflation. This may have clinical impact which allows specific and timely determined stimulation of the prostacyclin synthesis within the body. Clinical benefit is attributed to prostacyclin and stable prostacyclin metabolites [35], such as beraprost and treprostinil which are used in the treatment of primary pulmonary hypertension [36]. The use of ozone as an alternative prostacyclin supplier may be worth considering. Third, daily excretion rates for F2-isoprostanes remained unaffected by ozone. F2-isoprostanes are known to be elevated by ROS [37] and are assumed as a marker for oxidative stress in biological systems. The lack of measurable changes in F2-isoprostanes in plasma as well as in urinary excretion indicates that under our experimental conditions ozone do not cause systemic oxidative stress. We suggest that the way of ozone administration may be important as ozone is known as a strong oxidant agent and direct ozone exposure, e.g., by inhalation causes severe pulmonary damage initiated by the generation of ROS and lipid oxidation products [38]. This is reflected by increase in exhaled isoprostanes concentration [39]. Insufflated ozone may exhibit less oxidant potency at least in the blood system.

Rate limiting step of the arachidonic acid cascade is attributed to the COX step. Two types of cyclooxygenase do exist, COX-1 and COX-2 and both enzymes are known to be expressed in the vessel system, especially by the endothelium but also by smooth muscle cells and both enzymes contribute to vascular PGI_2 synthesis [19]. Our experiments give evidence that ozone induces time-dependently vascular expression of mRNA and protein for COX-2 but not for COX-1 and we observed increase in COX-2 protein expression in endothelial cells lining the vessels as well as in smooth muscle cells. Generation of vascular 6-keto-$PGF_{1\alpha}$ was inhibited by selective COX-2 inhibitor rofecoxib, but not by SC-560, known to specifically inhibit COX-1 enzyme activity. Our observation supports the finding that PGI_2 is the predominant COX-2 product of the vascular system [40]. Moreover, mice with targeted disruption of COX-2 gene do not produce higher PGI_2 levels following ozone insufflation. The important role of COX-2 for PGI2 synthesis is underlined by our observation that PGIS expression and activity remains unaltered in vascular tissue. Summarizing, these observations indicate that most likely COX-2 is responsible for increase in prostacyclin synthesis following ozone insufflation. In support of this hypothesis we observed on one side no alteration in 2,3-dinor-TXB_2 excretion, a

TXA_2 metabolite reflecting COX-1 dependent TXA_2 synthesis by thrombocytes which are the major source for systemic thromboxane, and on the other side the induction of PGI_2 synthesis in COX-$1^{-/-}$ mice by ozone. The mechanisms leading to induction of COX-2 remains elusive. One possible mechanism is given by the observation that oxidized LDLs induce COX-2 expression and PGI_2 production in vitro [41]. However, if ozone causes oxidized LDLs or other lipid peroxidation products under our experimental conditions has to be clarified.

Prostacyclin plays a prominent role in vascular hemostasis, acting as a potent inhibitor of platelet aggregation and as a vasodilator [42,43]. As a major systemic product of COX-2 it is abundantly produced during cardiac ischemia/reperfusion offering cytoprotection [44]. The critical role of PGI_2 to vascular integrity has been highlighted through findings that certain COX-2 inhibitors depress prostacyclin generation predisposing patients to increased risk of thrombotic stroke and myocardial infarction [45]. Next to tonus regulation and cytoprotection it has been shown that PGI_2 exerts also anti-metastasing [46] and immune regulatory actions [47,48], however, detailed mechanistic steps underlying these effects are still not enlightened. In different animal models it has been shown that ozone exerts cytoprotective effects [10,49–52], and recently we demonstrated therapeutic efficacy of intraperitoneal ozone insufflation in models of peritonitis [13], sepsis [11], allograft rejection [53] and of neck and head cancer [12] in which lethality was significantly reduced. Whether prostacyclin and/or its metabolites had finally a regulatory function in protection from organ damage in these models, however, remains to be clarified. We hypothesize that one of the mechanisms underlying the beneficial effect of ozone treatment may be related to selective stimulation of systemic prostacyclin synthesis and that diseases depending on endogenous PGI_2 action, such as pulmonary hypertension may benefit from ozone treatment.

Conflict of interest

The authors declare no conflict of interest.

References

[1] Amron DM, Moy RL. Stratospheric ozone depletion and its relationship to skin cancer. J Dermatol Surg Oncol 1991;17:370–2.
[2] Pryor WA, Das B, Church DF. The ozonation of unsaturated fatty acids: aldehydes and hydrogen peroxide as products and possible mediators of ozone toxicity. Chem Res Toxicol 1991;4:341–8.
[3] Goldstein BD. The pulmonary and extrapulmonary effects of ozone. Ciba Found Symp 1978;295–319.
[4] Hassett C, Mustafa MG, Coulson WF, Elashoff RM. Splenomegaly in mice following exposure to ambient levels of ozone. Toxicol Lett 1985;26:139–44.
[5] Babior BM, Takeuchi C, Ruedi J, Gutierrez A, Wentworth Jr P. Investigating antibody-catalyzed ozone generation by human neutrophils. Proc Natl Acad Sci U S A 2003;100:3031–4.
[6] Wentworth P, Jones Jr LH, Wentworth AD, Zhu X, Larsen NA, Wilson IA, et al. Antibody catalysis of the oxidation of water. Science 2001;293:1806–11.
[7] Bocci V, Zanardi I, Travagli V. Ozone: a new therapeutic agent in vascular diseases. Am J Cardiovasc Drugs 2011;11:73–82.
[8] Bocci VA, Zanardi I, Travagli V. Ozone acting on human blood yields a hormetic dose–response relationship. J Transl Med 2011;9:66.
[9] F Bocci V.A.. Scientific and medical aspects of ozone therapy. State of the art. Arch Med Res 2006;37:425–35.
[10] Rodriguez ZZ, Guanche D, Alvarez RG, Rosales FH, Alonso Y, Schulz S. Preconditioning with ozone/oxygen mixture induces reversion of some indicators of oxidative stress and prevents organic damage in rats with fecal peritonitis. Inflamm Res 2010;58:371–5.
[11] Schulz S, Rodriguez ZZ, Mutters R, Menendez S, Bette M. Repetitive pneumoperitoneum with ozonized oxygen as a preventive in lethal polymicrobial sepsis in rats. Eur Surg Res 2003;35:26–34.
[12] Schulz S, Haussler U, Mandic R, Heverhagen JT, Neubauer A, Dunne AA, et al. Treatment with ozone/oxygen-pneumoperitoneum results in complete remission of rabbit squamous cell carcinomas. Int J Cancer 2008;122:2360–7.
[13] Bette M, Nusing RM, Mutters R, Zamora ZB, Menendez S, Schulz S. Efficiency of tazobactam/piperacillin in lethal peritonitis is enhanced after preconditioning of rats with O3/O2-pneumoperitoneum. Shock 2006;25:23–9.
[14] Fouke JM, DeLemos RA, Dunn MJ, McFadden Jr ER. Effects of ozone on cyclooxygenase metabolites in the baboon tracheobronchial tree. J Appl Physiol 1990;69:245–50.
[15] Fouke JM, Wolin AD, McFadden Jr ER. Effects of ozone on lung mechanics and cyclooxygenase metabolites in dogs. Prostaglandins 1991;42:343–53.
[16] Leikauf GD, Zhao Q, Zhou S, Santrock J. Ozonolysis products of membrane fatty acids activate eicosanoid metabolism in human airway epithelial cells. Am J Respir Cell Molecul Biol 1993;9:594–602.
[17] Miller PD, Ainsworth D, Lam HF, Amdur MO. Effect of ozone exposure on lung functions and plasma prostaglandin and thromboxane concentrations in guinea pigs. Toxicol Appl Pharmacol 1987;88:132–40.
[18] Smith W. Prostanoid biosynthesis and mechanism of action. Am J Physiol 1992;263:F181–91.
[19] Smith WL, DeWitt DL, Garavito RM. Cyclooxygenases: structural, cellular, and molecular biology. Annu Rev Biochem 2000;69:145–82.
[20] Morrow JD, Hill KE, Burk RF, Nammour TM, Badr KF, Roberts LJd. A series of prostaglandin F2-like compounds are produced in vivo in humans by a non-cyclooxygenase, free radical-catalyzed mechanism. Proc Natl Acad Sci U S A 1990;87:9383–7.
[21] Morrow JD, Roberts LJ. The isoprostanes: unique bioactive products of lipid peroxidation. Prog Lipid Res 1997;36:1–21.
[22] Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, et al. Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 1995;83:473–82.
[23] Klein T, Reutter F, Schweer H, Seyberth HW, Nüsing RM. Generation of the isoprostane 8-epi-prostaglandin F-2 alpha in vitro and in vivo via the cyclooxygenases. J Pharmacol Exp Ther 1997;282:1658–65.
[24] Schweer H, Watzer B, Seyberth HW. Determination of seven prostanoids in 1 ml of urine by gas chromatography-negative ion chemical ionization triple stage quadrupole mass spectrometry. J Chromatogr 1994;652: 221–7.
[25] Schweer H, Watzer B, Seyberth HW, Nusing RM. Improved quantification of 8-epi-prostaglandin F2 alpha and F2-isoprostanes by gas chromatography/triple-stage quadrupole mass spectrometry: partial cyclooxygenase-dependent formation of 8-epi- prostaglandin F2 alpha in humans. J Mass Spectrom 1997;32:1362–70.
[26] Nüsing R, Goerig M, Habenicht AJ, Ullrich V. Selective eicosanoid formation during HL-60 macrophage differentiation. Regulation of thromboxane synthase. Eur J Biochem 1993;212:371–6.
[27] Bachi A, Brambilla R, Fanelli R, Bianchi R, Zuccato E, Chiabrando C. Reduction of urinary 8-epi-prostaglandin F-2 alpha during cyclooxygenase inhibition in rats but not in man. Br J Pharmacol 1997;121:1770–4.
[28] Olliges A, Wimmer S, Nusing RM. Defects in mouse nephrogenesis induced by selective and non-selective cyclooxygenase-2 inhibitors. Br J Pharmacol 2011;163:927–36.
[29] Tanaka A, Araki H, Hase S, Komoike Y, Takeuchi K. Up-regulation of COX-2 by inhibition of COX-1 in the rat: a key to NSAID-induced gastric injury. Aliment Pharmacol Therap 2002;16(Suppl. 2):90–101.
[30] Schmittgen TD, Zakrajsek BA. Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. J Biochem Biophys Methods 2000;46:69–81.
[31] McAdam BF, Catella-Lawson F, Mardini IA, Kapoor S, Lawson JA, FitzGerald GA. Systemic biosynthesis of prostacyclin by cyclooxygenase (COX)-2: the human pharmacology of a selective inhibitor of COX-2. Proc Natl Acad Sci U S A 1999;96:272–7.
[32] Fann JI, Cahill PD, Mitchell RS, Miller DC. Regional variability of prostacyclin biosynthesis. Arteriosclerosis 1989;9:368–73.
[33] Johnson AR. Human pulmonary endothelial cells in culture. Activities of cells from arteries and cells from veins. J Clin Invest 1980;65:841–50.
[34] Flavahan NA. Balancing prostanoid activity in the human vascular system. Trends Pharmacol Sci 2007;28:106–10.
[35] Mubarak KK. A review of prostaglandin analogs in the management of patients with pulmonary arterial hypertension. Respir Med 2010;104:9–21.
[36] Safdar Z. Treatment of pulmonary arterial hypertension: the role of prostacyclin and prostaglandin analogs. Respir Med 2011;105:818–27.
[37] Roberts LJ, Morrow JD. Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. Free Radic Biol Med 2000;28:505–13.
[38] Pryor WA, Squadrito GL, Friedman M. The cascade mechanism to explain ozone toxicity: the role of lipid ozonation products. Free Radic Biol Med 1995;19:935–41.
[39] Montuschi P, Nightingale JA, Kharitonov SA, Barnes PJ. Ozone-induced increase in exhaled 8-isoprostane in healthy subjects is resistant to inhaled budesonide. Free Radic Biol Med 2002;33:1403–8.
[40] FitzGerald GA, Patrono C. The coxibs, selective inhibitors of cyclooxygenase-2. N Engl J Med 2001;345:433–42.
[41] Smith LH, Boutaud O, Breyer M, Morrow JD, Oates JA, Vaughan DE. Cyclooxygenase-2-dependent prostacyclin formation is regulated by low density lipoprotein cholesterol in vitro. Arterioscler Thromb Vasc Biol 2002;22: 983–8.
[42] Gryglewski RJ. Prostacyclin among prostanoids. Pharmacol Rep 2008;60:3–11.
[43] Iniguez MA, Cacheiro-Llaguno C, Cuesta N, Diaz-Munoz MD, Fresno M. Prostanoid function and cardiovascular disease. Arch Physiol Biochem 2008;114: 201–9.
[44] Sakai A, Yajima M, Nishio S. Cytoprotective effect of TRK-100, a prostacyclin analogue, against chemical injuries in cultured human vascular endothelial cells. Life Sci 1990;47:711–9.
[45] Fitzgerald GA. Coxibs and cardiovascular disease. N Engl J Med 2004;351: 1709–11.

[46] Tisdale MJ. Role of prostaglandins in metastatic dissemination of cancer. Minireview on cancer research. Exp Cell Biol 1983;51:250–6.
[47] Zhou W, Blackwell TS, Goleniewska K, O'Neal JF, Fitzgerald GA, Lucitt M, et al. Prostaglandin I2 analogs inhibit Th1 and Th2 effector cytokine production by CD4T cells. J Leukoc Biol 2007;81:809–17.
[48] Zhou W, Hashimoto K, Goleniewska K, O'Neal JF, Ji S, Blackwell TS, et al. Prostaglandin I2 analogs inhibit proinflammatory cytokine production and T cell stimulatory function of dendritic cells. J Immunol 2007;178:702–10.
[49] Guven A, Gundogdu G, Sadir S, Topal T, Erdogan E, Korkmaz A, et al. The efficacy of ozone therapy in experimental caustic esophageal burn. J Pediatr Surg 2008;43:1679–84.
[50] Madej P, Plewka A, Madej JA, Nowak M, Plewka D, Franik G, et al. Ozonotherapy in an induced septic shock. I. Effect of ozonotherapy on rat organs in evaluation of free radical reactions and selected enzymatic systems. Inflammation 2007;30:52–8.
[51] Madej P, Plewka A, Madej JA, Plewka D, Mroczka W, Wilk K, et al. Ozone therapy in induced endotoxemic shock. II. The effect of ozone therapy upon selected histochemical reactions in organs of rats in endotoxemic shock. Inflammation 2007;30:69–86.
[52] Zamora ZB, Borrego A, Lopez OY, Delgado R, Gonzalez R, Menendez S, et al. Effects of ozone oxidative preconditioning on TNF-alpha release and antioxidant-prooxidant intracellular balance in mice during endotoxic shock. Mediat Inflamm 2005;2005:16–22.
[53] Stadlbauer TH, Eisele A, Heidt MC, Tillmanns HH, Schulz S. Preconditioning with ozone abrogates acute rejection and prolongs cardiac allograft survival in rats. Transplant Proc 2008;40:974–7.