Biochemical Pharmacology 83 (2012) 506–513

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

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

Siegfried Schulz [b], Simone Ninke [a], Bernhard Watzer [c], Rolf Michael Nüsing [a,*]

[a] Institute of Clinical Pharmacology, Johann Wolfgang Goethe University, Theodor Stern Kai 7, 60590 Frankfurt, Germany
[b] Veterinary Service and Laboratory Animal Medicine, Philipps-University Marburg, 35033 Marburg, Germany
[c] Department of Pediatrics, Philipps-University Marburg, 35033 Marburg, Germany

ARTICLE INFO

Article history:
Received 29 September 2011
Accepted 23 November 2011
Available online 2 December 2011

Keywords:
Ozone
Insufflation
Cyclooxygenase
Isoprostanes
Prostanoids
Oxidative stress

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.

* Corresponding author. Tel.: +49 69 6301 7676; fax: +49 69 6301 7636.
E-mail address: r.m.nuesing@med.uni-frankfurt.de (R.M. Nüsing).

0006-2952/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2011.11.025

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