Biochemical Modifications Induced in Human Blood by Oxygenation-Ozonation

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ABSTRACT: Some biochemical effects determined on human blood after addition of a gas mixture composed of oxygen (~96%) and ozone (~4%) have been evaluated. Ozone was used in a mild concentration ranging between 0.21 and 1.68 mM. Within few minutes after rapid mixing of the equal gas-liquid volumes, the ozone was consumed because by instantaneously reacting with biomolecules, generating reactive oxygen species (particularly hydrogen peroxide) having very short lifetime and lipid oxidation products. The following results are oxygen-ozone dose dependent: (1) The $pO_2$ values have risen from about 40 up to 400 mmHg. (2) By testing the highest ozone concentration, the total antioxidant capacity of blood decreased within 1 min from 1.35 to 0.91 mM but regained its normal values within 20 min owing to the rapid reduction of oxidized antioxidants operated by erythrocytes. (3) Similarly, intraerythrocytic reduced glutathione after ozonation decreased from the initial value of 5.71 to 4.56 μmol/g Hb. (4) Both hemolysis and methemoglobin showed a negligible increase. © 2006 Wiley Periodicals, Inc. J Biochem Mol Toxicol 20:133–138, 2006; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.20124

KEYWORDS: Ozone; Peroxidation; Reactive Oxygen Species; Lipid Oxidation Products; Antioxidants; Hydrogen Peroxide; Reduced Glutathione

INTRODUCTION

Ozone ($O_3$) is a very reactive gas and is considered one of the worst pollutants in the troposphere [1]. Its toxicity for the respiratory tract is well proven [2–4], and it is due to the continuous contact of the gas with the bronchial mucosa. It is protected only by a thin liquid film with insufficient amount of antioxidants. These findings have established that ozone is toxic and should never be breathed. However, this does not need to be extended to blood exposed to a single, small dose of ozone because this fluid tissue contains a number of hydro-liposoluble antioxidants and protective enzymes [5,6]. We would like to report our data showing that, under appropriate conditions, the blood’s antioxidant system can control moderate ozone dosages ranging from 0.21 to 1.68 mM. The nature of some mediators generated during the reaction occurring between blood and ozone has been elucidated.

MATERIALS AND METHODS

Ozone Generation and Measurement

Ozone was generated from medical-grade oxygen ($O_2$) using electrical corona arc discharge, by the $O_3$ generator (model Ozonosan PM 100 K, Hansler GmbH, Iffezheim, Germany), which allows the gas flow rate and $O_3$ concentration to be controlled in real time by photometric determination, as recommended by the Standardisation Committee of the International Ozone Association. The ozone flow-rate was kept constant at 3 L/min in all the experiments. Tygon polymer tubing and single-use silicon-treated polypropylene syringes (ozone resistant) were used throughout the reaction to ensure containment of $O_3$ and consistency in concentrations.

Collection of Human Blood and Plasma Samples

Blood samples were taken from healthy, nonsmokers, male blood donors in the morning at the Blood Bank of Siena Polyclinic. The donors were aged between 19 and 43 years, and they were informed of the purpose of the study. Either calcieparin (20 U/mL blood) or sodium citrate at 3.8% solution (1 mL per 9 mL blood)
was used as an anticoagulant, and blood samples were tested within 1 h. In experiments comparing the behavior of plasma to the blood, the plasma samples were obtained from the corresponding blood samples. Each sample was divided into the necessary aliquots (generally 5 mL each): an aliquot was used for assessing baseline values, while equal volumes (5 mL) of either pure medical-grade oxygen or of a gas mixture composed of oxygen-ozone were added to the second, third, and fourth aliquots, respectively.

**Gas Delivery to Biological Samples**

A predetermined volume of a gas mixture composed of O2 (~96%) and O3 (~4%), at various concentrations, was collected with a syringe and immediately introduced into a second syringe containing the samples via a multidirectional stopcock. We always used a blood or plasma/gas volume (5 mL) in a 1:1 ratio. We have previously determined [7,8] that a rapid rotation of the syringe along its longitudinal axis (about 80 cycles/min) for 1 min achieved complete mixing of the liquid-gas phases with minimal foaming and that, within this period of time, ozone reacted completely with substrates, implying that cell samples receiving ozone reacted with the ozone dose completely. The pO2 reached a value of about 400 mmHg, while blood pCO2 and pH values did not change. The concentrations of ozone ranged between 20 and 80 μg/mL per mL of the sample corresponding to 0.21–1.68 mM, respectively. In order to obtain reproducible results, it needs to be emphasized that O3 is a very reactive gas so that extremely rapid and precise handling is required. The final gas pressure remained at normal atmospheric pressure. It is worth emphasizing that O2 represents at least 96% of the O2–O3 gas mixture and that the O2 control is relevant.

**Biochemical Determinations**

(a) **Chemiluminescence measurements:** The generation of reactive oxygen species (ROS) can be evaluated by a technique which utilizes chemiluminescence. Luminol (cyclic hydrazide-5-amino-2,3-dihydro-1,4-phthalazinedione) has been used to increase the sensitivity of the chemiluminescence technique [9] because it acts as a substrate which reacts with ROS to produce nitrogen and an excited aminophthalate ion, which emits light as it relaxes to the ground state. Measurements were carried out by a luminometer (model M2010, Lumac Biocounter) at 22°C and pH 7.3 by using luminol dissolved in phosphate-buffered saline (3.44 × 10⁻⁵ M final concentration) in polystyrene cuvettes after the addition of either ozonated saline or fresh human plasma anticoagulated with sodium citrate, at different times after exposure to either oxygen alone or oxygen-ozone at two concentrations. The resulting light output was recorded as chemiluminescent signals for one period of 10 s and are reported in Figure 1.

(b) Blood gas and blood electrolytes were determined with an IL-1620 blood gas analyzer (Instrument Laboratory, Lexington, MA, USA) and an ABL505 radiometer.

(c) Hemocytometric determinations were made with a standard blood analyzer.

(d) The hemoglobin determination was carried out using 20 μL of original blood, and on the basis of the hematocrit value; the respective volume of plasma was collected after the ozonation. Samples were mixed with 5 mL of the cyanide-methemoglobin reagent (Sclavo hemoglobin test kit). Optical density, read at 540 nm, was converted to hemoglobin according to a standard curve and is referred to as a percentage of total hemoglobin.

(e) Hydrogen peroxide (H2O2) was measured by using the enzymatic method described by Green and Hill [10]. 3-Amino-1:2:4-triazole (3.AT) from Sigma Chemical Co. was used as a catalase inhibitor at a final concentration of 20 mM. When testing catalase (Sigma, code 1345) with ozonated blood, the enzyme was used at a concentration of 20 U/mL. Regrettably, EURTech service cannot specify whether thymol, if any, was present or not in the catalase preparation.

(f) Total antioxidant status was determined in plasma samples according to Rice-Evans and Miller [11] and expressed in mM of plasma.

(g) Protein thiol groups (PTG) were measured in plasma according to Hu [12] using procedure 1 with 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) dissolved in absolute methanol. Values are expressed as mM of plasma.

(h) **Determination of thiobarbituric acid-reactive substances:** In order to evaluate the relevance of lipid peroxidation, thiobarbituric acid-reactive substances (TBARS) were assessed and reported as μM according to Pompella et al.[13]. In order to evaluate the lifetime of TBARS in the ozonated plasma, samples were incubated in sterile dishes at 37°C in a humidified atmosphere (95% air- 5% CO₂) for 8 h.

(i) Total reduced glutathione (GSH) and oxidized glutathione (GSSG) content of erythrocytes was promptly determined on the cell pellet after acid precipitation of proteins with 5% (w/v) trichloroacetic acid (TCA) according to the Tietze method [14].
Statistical Analyses

Results were expressed as the mean ± the standard deviation, and the data were analyzed using the paired Student’s t-test. $p$ values less than 0.05 ($*$) and 0.01 ($**$) were considered significant. When enough blood was available, either the oxygenated sample or the treated ones (O$_2$–O$_3$) were compared to the untreated sample (control).

RESULTS

At first, we examined the chemiluminescence reaction and hydrogen peroxide production in both physiological saline and fresh human plasma exposed to oxygen alone, or to the gas mixture oxygen–ozone, with ozone concentrations fixed at 40 and 80 μg/mL of gas per mL of saline or plasma. Determinations, started after 1 min of a rapid mixing of the liquid/gas phases, continued up to 60 min. Although exposure to oxygen (control) is ineffective, ozone generates hydrogen peroxide and causes the chemiluminescent reaction in both saline and plasma (Figure 1). However, while in the former solution, there is a consistent and prolonged increase, in the ozonated plasma both chemiluminescence and hydrogen peroxide increased immediately but decayed very rapidly with a half-life of less than 2 min. Addition of 3.AT, a catalase inhibitor, briefly prolonged the lifetime of hydrogen peroxide (Figure 1 right, bottom panel) suggesting that normal plasma contained both antioxidant compounds and traces of enzymes that are able to effectively quench the formation of ROS. Indeed, addition of catalase before ozonation totally inhibited the response. We also tried to measure a transitory increase in hydrogen peroxide in the ozonated blood, but even the isolation of plasma 1 min after the ozonation did not allow the detection of hydrogen peroxide suggesting that its reduction to water was much faster in blood than in plasma.

An interesting finding emerged when we evaluated the variation of TAS concentration in plasma after ozonation and rapid (1 min) mixing of the liquid–gas phases of either fresh blood or the respective plasma withdrawn from five donors: Figure 2 shows that, after ozonation of plasma with a medium (40 μg/mL) and a high (80 μg/mL) ozone concentration, TAS levels progressively decreased at first and then remained stable after 20 min. The decrease is ozone dose dependent and varies between 46% and 63%. On the other hand, TAS levels in blood decrease (from 11% to 33%, respectively) also in the first minute after ozonation but then recover and return to the original value within 20 min, irrespective of the ozone concentration indicating the capacity of blood to regenerate the oxidized antioxidants.

FIGURE 1. Kinetics of the chemiluminescent signals and of the production of H$_2$O$_2$ after exposing either saline (left panel) or fresh human plasma (right panel) to oxygen alone or to two concentrations of ozone (40 and 80 μg/mL per mL of solvent). The bottom panels show the effect of catalase inhibitors present in both saline and plasma.

Furthermore, ozonation of blood performed within a mild range of ozone concentrations (10–80 μg/mL) caused a partial oxidation (~20%) of intracellular GSH. These results, determined immediately after 1 min ozonation, are shown in Table 1 with a concomitant increase in GSSG. However, if after 1 min ozonation, blood is incubated for 20 min at 37°C, GSH values are practically similar to the control sample suggesting again a prompt intracellular reconstitution of GSH (data not shown).

The ozonation of blood implies lipid peroxidation [15] and partial oxidation of thiol groups [16] mostly present in albumin. Figure 3 shows the significant variations of TAS, PTG, TBARS, and hemolysis values determined after 1 min ozonation with two ozone concentrations (40 and 80 μg/mL, respectively, in comparison to untreated and oxygenated samples). Peroxidation of lipids by ozone most likely occurs in lipids bounded to albumin and chylomicrons, and it does not seem to affect the erythrocytic membrane [17]. Throughout a decade, we have performed many determinations for

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FIGURE 2. Kinetics of total antioxidant (TAS) levels in human blood and plasma samples of five normal donors. Plasma was separated from the same blood samples, and both were exposed to either oxygen (control) or oxygen–ozone with ozone concentrations of 40 and 80 μg/mL per mL of sample for 1 min. TAS levels were rapidly reconstituted only in blood samples. n = 5; **p < 0.01.

assessing the degree of hemolysis, which represents the simplest and most reliable test for ascertaining erythrocyte damage. Hemolysis becomes consistent only when the ozone concentration rises above 90 μg/mL of gas per mL blood. Interestingly, donors with a high TAS value (160–185 mM) did not show any hemolysis even after using an ozone concentration of 90 μg/mL gas per mL blood. However, when the ozone concentration rises up to 200 μg/mL, the hemolysis increased up to prohibitive values of 6 ± 1.8% and 12.6 ± 8.4% in blood anticoagulated with either citrate or heparin, respectively, suggesting an enhancing effect of plasmatic Ca++. It appears therefore that the antioxidant system of blood, represented by a number of hydro-liposoluble compounds, is responsible for the lack of hemolysis. Moreover, within our range of ozone concentrations, we have determined a transitory increase in methemoglobin of less than 1%, no loss of K+ and only a negligible increase in LDH in the plasma. We have recently reported [18] that there is no variation in the activity of crucial erythrocytic enzymes such as SOD, GSH-Px, GSH-Rd, and G6PD.

Lipid oxidation products (LOPs) are known to be constituted by a very heterogeneous group of potentially cytotoxic compounds such as alkenals, malondialdehyde, and isoprostanes [1,15,19], which represent useful markers of ozone activity. In contrast with the short-lived hydrogen peroxide, we have found that LOPs are rather stable in vitro because their levels remain practically constant during 9 h of incubation at 37°C. On the other hand, we have previously observed that, after reinfusion of ozonated blood in the donor, the plasma levels of LOPs decreased rapidly with a half-life of about 4 min [20].

DISCUSSION

In contrast with the data showing the offensive action of ozone on either washed erythrocytes suspended in a saline solution [21–23] or on cells in tissue culture [24], we have shown that appropriate ozone concentrations acting on blood for only a few minutes do not harm erythrocytes. The use of unphysiological conditions using saline-washed erythrocytes totally deprived of the protective plasma antioxidants have led to the conclusion that ozone is always toxic for blood. Leist et al. [25], Halliwell [26], and ourselves [27] have pointed out that oxidative stress can be more relevant in vitro than in vivo owing to a reduced level of antioxidants in tissue culture media. Thus, the aim of this paper is to show that the exposure of blood to appropriate doses of ozone is immediately followed by a number of reactions generating several messengers, which can exert important biological activities without damaging blood components. The description of these activities is

| TABLE 1. Variations of Erythrocytes GSH and of GSSG When Human Blood of Four Normal Donors Is Exposed for 1 min to Either Oxygen or Progressively Increasing Ozone Concentrations |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                                | Control | Oxygen | O₂ 20 μg/mL | O₂ 40 μg/mL | O₂ 60 μg/mL | O₂ 80 μg/mL |
| GSH (μmol/g Hb)                | 5.70 ± 0.24 | 5.50 ± 0.13 | 5.3 ± 0.06* | 5.0 ± 0.06* | 4.8 ± 0.11* | 4.56 ± 0.15* |
| GSSG (μmol/g Hb)               | 0.16 ± 0.01 | 0.18 ± 0.01* | 0.20 ± 0.01* | 0.23 ± 0.01* | 0.25 ± 0.01* | 0.28 ± 0.01* |

n = 4; *p < 0.05.
FIGURE 3. Effect of 1-min exposure to either oxygen or ozone (at concentrations of 40 and 80 µg/mL gas per mL of blood) on TAS, PTG, TBARS, and hemolysis of five samples of blood from normal donors. n = 5; *p < 0.05; **p < 0.01.

Beyond the scope of this paper, but they are extensively discussed elsewhere [28].

Ozone is not only 10-fold more soluble in water than oxygen, but once dissolved in the plasmatic water, it reacts instantaneously with biomolecules (uric and ascorbic acids, albumin-thiol groups, polyunsaturated fatty acids, etc.). As a consequence, in contrast with oxygen, ozone does not follow Henry’s law and the mixing of the gas with the liquid phase is within 1 min followed by the total consumption of ozone with the simultaneous generation of oxidized compounds, hydrogen peroxide (as a typical ROS), and a heterogeneous mixture of low molecular weight aldehydic end products (as LOPs), as follows:

\[ \text{Ozone} \rightarrow \text{Plasma} \rightarrow \text{ROS} \]
\[ + \text{LOPs} + \text{Oxidized antioxidants} \]

The reaction can be precisely controlled by knowing the TAS value of the plasma and the ozone dose (gas volume \(\times\) ozone concentration, µg/mL). Therefore, it is imperative to correctly calibrate the ozone dose to the blood’s antioxidant capacity: experimental data performed during the past decade [7,8,27] have indicated that the safe range of ozone concentration is within 10–80 µg/mL of gas per mL of blood (0.21–1.68 mM). An ozone concentration below 10 µg/mL is hardly effective because the ozone dose is instantly quenched by hydrosoluble antioxidants, whereas a concentration above 80 µg/mL may (depending upon the individual TAS value) overwhelms the antioxidant capacity, causes some erythrocytic damage, and generates an excess of LOPs.

As it can be observed in Figures 1–3 and Table 1, several reactions happened simultaneously during blood ozonation ex vivo and hydrogen peroxide generated during the first minute in the plasma established a very transient, yet effective, extra-intracellular gradient on blood cells [29,30]. Hydrogen peroxide is now widely recognized as a crucial signaling molecule [31–34], and its sudden rise in the cytoplasm is able to trigger a number of biochemical reactions without noxious effects because its concentration, being of only a few micromoles, is promptly reduced to water by GSH and specific enzymes. We have shown (Figure 2) that in blood the modest fall of TAS levels is rapidly corrected by the fast reduction in oxidized GSH and dehydroascorbate owing to well-defined recycling processes [35,36].

We believe that LOPs are responsible for a second wave of reactions happening during the infusion of ozonated blood into the donor when submicromolar concentrations of LOPs, distributed all over the body, interact with cell receptors in the bone marrow, liver, central nervous system, and endocrine glands. Dianzani [37] has suggested that LOPs, at submicromolar concentrations, can become useful stimulatory signals able to induce the important phenomenon of the adaptation to the oxidative stress and the upregulation of the hemeoxygenase-1 [20,27,38–40]. Obviously only a precisely and mild ozonation of blood yielding a moderate dose of LOPs must be performed for avoiding any toxic risk.

In conclusion, these data may help to put in the correct perspective the biological activity and lack of toxicity of judicious ozone doses added to blood ex vivo. We have come to consider ozone as a new medical drug, and it is based on many biological and clinical data [28]. Almost needless to say, depending upon the dose, ozone, as any other drug currently used by conventional medicine, can be biologically useful or toxic.

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