CO_2 Is a Natural Inhibitor of the Generation of Reactive Oxygen Species by Phagocytes

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The generation of reactive oxygen species in leukocytes from 73 healthy donors is studied using the luminol- and lucigenin-dependent chemiluminiscent methods and the nitroblue tetrazolium test. The results suggest CO_2 is a potent inhibitor of generation of reactive oxygen species in leukocytes under pressure close to that in the blood (37.5 mm Hg) and under high pressure (146 mm Hg).

Key Words: carbon dioxide; reactive oxygen species; phagocytes; antioxidants

Earlier experiments on animals (dogs, calves) [3] and on cardiological patients [4] have revealed a phenomenon of stimulated generation of reactive oxygen species (ROS), including superoxide anion radical (SAR), hydroxyl radical, singlet oxigen, and hydrogen peroxide, by phagocytes (granulo- and monocytes) during their passage through the vascular bed of the lungs. Studying the mechanisms of this phenomenon, Kogan and co-workers found [9] that CO_2 blown through a leukocyte suspension in slightly buffered 0.9% NaCl physiological saline considerably inhibited the generation of ROS in these cells. However, since a sharp drop of the pH under these conditions (from 7.35 to 5.56) may by itself inhibit the generation of ROS, the study was continued under conditions of a "mild" pH shift and with an additional correction for the effect of the pH on the generation of ROS by leukocytes. A study of the effect of CO_2 on the generation of ROS by leukocytes is of biological interest since CO_2 is constantly being formed in all cells and tissues.

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MATERIALS AND METHODS

Seventy three healthy donors were examined. Eight to ten ml of venous heparinized blood was collected from each donor and the leukocyte mass was isolated by centrifugation at 60 g for 7-10 min and/or sedimentation at 4°C for 1.5-2 h, followed by addition centrifugation at 200 g for 7-10 min. The leukocyte mass was freed from erythrocytes by osmolysis [4], washed and recentrifuged at 1000 rpm for 5 min. The resultant leukocyte mass was incubated for 30 sec with 0.008% Triton ethanol-water solution to facilitate the penetration of superoxide dismutase (SOD) into the leukocytes. The leukocyte mass was resuspended in a strongly buffered physiological saline (buffer mixture) containing 0.9% NaCl isotonic solution and phosphate-alkaline buffer solution (7.5 ml + 2.5 ml, pH 7.35). The leukocyte mass consisted of phagocytes (granulo- and monocytes) and lymphocytes. The presence of lymphocytes did not interfere with the determination of either the basal or the stimulated chemiluminecsence (CL) indexes of ROS production by phagocytes, since these cells do not exhibit luminiscence at rest [2,3,7] and do not phagocysize after stimulation with particles. The concentration of leukocytes was determined and

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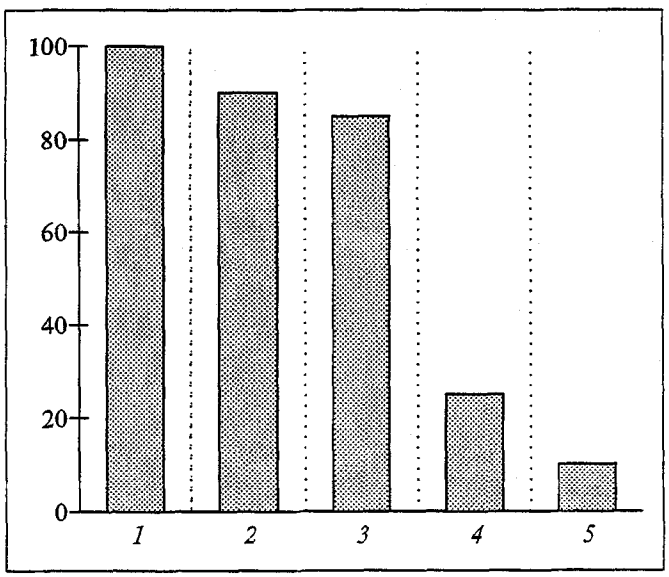

Fig. 1. Changes in basal luminol-dependent CL indexes (CLIIb) of the generation of ROS by leukocytes in 5.1% (37.5 mm Hg) and 20% (146 mm Hg) CO_2. Here and in Fig. 2: *1*) initial pH 7.35 (control 1); *2*) final pH 7.32 for experiments in 5.1% CO_2 (control 2, *a*); *3*) final pH 7.30 for experiments in 20% CO_2 (control 2, *b*); *4*) 5.1% CO_2 (37.5 mm Hg) + final pH 7.32 (experiment I); *5*) 20% CO_2 (146 mm Hg) + final pH 7.30 (experiment II).

adjusted to a standard value. The generation of ROS was determined by three methods: luminol-dependent CL (total generation of ROS by leukocytes) [3,4,8], lucigenin-dependent (mainly generation of SAR) [2,12], and by the nitroblue tetrazolium test (NBT) (generation of SAR only) [5,11]. For the determination of the basal ROS generation by leukocytes by the luminol-dependent CL method the incubation medium consisted of 0.2 ml of a leukocyte suspension containing a standard concentration of leukocytes (2.5×10^3 cells/μl, in all 5×10^5 cells per sample) in the buffer mixture (pH 7.35) and 20 ml saturated isoosmolar luminol aqueous solution (10^{-4} M), pH 7.35. When the basal generation of ROS was measured using the lucigenin-dependent method, the incubation medium contained the same ingredients except luminol, which was replaced with 20 μl 10^{-4} M lucigenin. For determination of the stimulated generation of ROS, phagocytosis-initiating particles were added to the system: 0.2 ml of a suspension of opsonized zymozan (500 mg zymozan + 50 ml buffer mixture) [10] or 0.1 ml 1% SiO_2 suspended in the buffer mixture. CL was recorded at 37°C using an LKB-Wallac 1251 luminometer (Sweden). CL was recorded until it reached the first maximum, which corresponded to the basal CL, and then zymozan or SiO_2 was added and the second maximum of CL corresponding to the stimulated CL was recorded. After this, the CL intensity indexes (CLII) of single phagocyte (granulo- and monocyte) - the basal (CLIIb) and stimulated (CLIIs) - were calculated by dividing the maximal CL of the total suspension by the number of phagocytes and multiplying on 10^6. When ROS was measured by the NBT test [5,11], the incubation medium contained 0.3 ml leukocyte suspension in the buffer mixture (5000 cells/μl, 1.5×10^6 cells per sample), 0.3 ml opsonized zymozan, 250 μg SOD in 0.2 ml buffer mixrute, and 0.3 ml 0.2% NBT aqueous solution. In the control samples albumin was substituted for SOD. Insoluble formazans resulting from the reaction of SAR with NBT were extracted with a dimethyl sulfoxide (DMSO): chloroform mixture (2:1) at 55-59°C and measured spectrophotometrically (λ=560 nm); the concentration was expressed in optical density units. The NBT test was used for determination of the zymozan-stimulated generation of SAR only. Since

TABLE 1. CO_2-Induced Changes in Lucigenin-Dependent CL Indexes of ROS Production by Leukocytes (Basal and Stimulated) ($n=5$)

Experimental conditions	CLIIs, mV/sec× ×10^6 leukocytes	IC CO_2	CLIIs, mV/sec× ×10^6 leukocytes	IC CO_2
Control 1 (initial pH 7.35)	70.9±8.9 (100%)		178.1±27.1 (100%)	
Control 2, *a* (final pH 7.32)	56.1±6.2 (79.1%)		165.2±25.7 (92.8%)	
Control 2, *b* (final pH 7.30)	44.9±5.9 (63.3%)		153.4±29.7 (69.3%)	
Experiment I (5.1% CO2, 37.5 mm Hg + final pH 7.32)	40.3±5.5* (59.7%)	1.39	106.3±13.8* (56.7%)	1.55
Experiment II (20% CO2, 146 mm Hg + final pH 7.30)	20.0±5.1*,** (28.2%)	2.25	84.6±14.7* (47.5%)	1.81

Note. IC CO_2 – CO_2 inhibition coefficient of ROS generation: ratio of CLII in control 2 (*a*, *b*) to CCII in experiment. *$p<0.05$; **$p<0.001$ vis-a-vis control.

TABLE 2. Effect of CO_2 on ROS Production by Leukocytes according to Optical Density of Formazans in the NBT Reaction

№ of exp.	Incubation of leukocytes in buffer mixture (pH)	*n*	Concentration of formazans in NBT test, optical density units (λ=560 nm)	IC CO_2
1.	7.35	15	0.205±0.013	
2.	7.25	6	0.226±0.010	
3.	7.20	8	0.221±0.017	
4.	7.35, in 5.1% CO_2 (P=37.5 mm Hg)	7	0.065±0.009*	3.15
5.	7.35, in 20% CO_2 (P=146 mm Hg)	8	0.040±0.006**	5.13

Note. *$p<0.001$ vis-a-vis No. 1; **$p<0.05$ between experiments. IC CO_2 – CO_2 inhibition coefficient of SAR generation by leukocytes: ratio of concentration of formazans No. 1 to concentration of formazams No. 4 or No. 5.

pH shifts (7.3-7.2) were found not to affect the formation of SAR, there was no need to make any correction for such changes (Table 2).

For a study of the effect of CO_2 on the generation of ROS (after A. Kh. Kogan) the above-described incubation systems were placed in a 6-liter miniature pressure chamber and the following gas mixtures were created by replacing air with CO_2 (pressure was controled with a manometer): 1) 5.1% CO_2 + 94.9% air (partial pressures (P) 37.5 and 692.5 mm Hg, respectively); 2) 20% CO_2 + 80% air (P=146 and 584 mm Hg, respectively); total pressure in the chamber was below the atmospheric pressure (730 mm Hg). This preserved a residual rarefaction (30 mm Hg) necessary for hermetic sealing of the chamber. The incubation was performed at 37°C and lasted 20 min for CL methods and 45-60 min in the NBT test. The initial pH of 7.35 dropped to 7.32 by the end of the experiment in 5.1% CO_2 and to 7.30 in 20% CO_2. Therefore, for CL measurements control samples were incubated in air (P=730 mm Hg) at pH 7.35 (control 1), 7.32, and 7.30 (controls 2, *a* and *b*). The effect of CO_2 on the generation of ROS by leukocytes was evaluated from the absolute values of CLIIb and CLIIs after incubation in the CO_2 atmosphere, from their percentage of control 1 (taken as 100%), and from the coefficient of CO_2 inhibition of the generation of ROS, which was calculated as the ratio between CLII at pH 7.32 or 7.30 (one of the final pH values, control 2, *a* or *b*) and CLII after incubation in CO_2 + final pH. When the effect of CO_2 on ROS generation was assayed using the NBT test, the coefficient of CO_2 inhibition was calculated in a simpler way: the concentration of formazans formed in air (optical density units) was divided by the concentration of formazans formed in the CO_2-containing gas mixtures.

RESULTS

The data obtained by three different methods (luminol- and lucigenin-dependent CL and NBT test) show that CO_2 in concentrations of 5.1% and 20% inhibits ROS production by leukocytes at rest (CLIIb) and during phagocytosis (CLIIs) of zymozan and SiO_2 particles. In 5.1% CO_2 CLIIb decreased 3.88-3.52-fold for luminol-dependent CL (two experimental series) and 1.39-1.45-fold for lucigenin-dependent CL; in 20% CO_2 CLIIb measured by the same methods decreased 9.38-10.03- and 2.25-22.19-fold, respectively (Fig. 1, Table 1). CLIIs decreased in a similar way but to a lesser extent: 3.24-1.55- and 7.95-1.81-fold, respectively. The zymosan-induced generation of SAR measured by the NBT test decreased 3.15- and 5.13-fold (in all cases $p<0.001$ or $p<0.05$). The differences in absolute values of ROS production are probably due

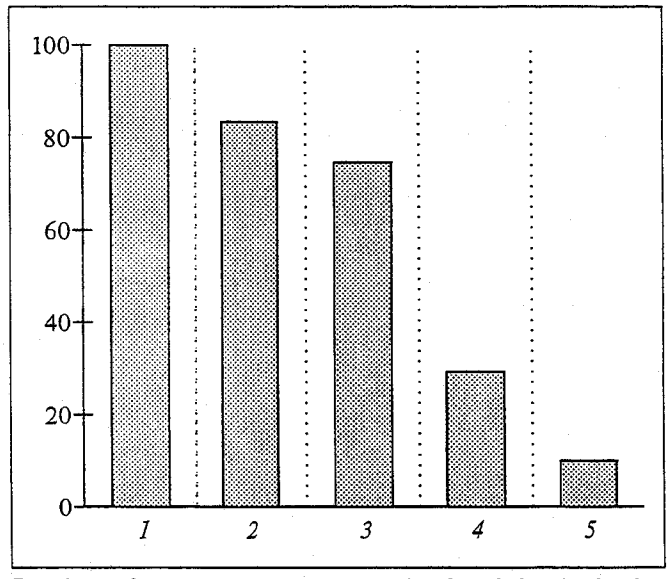

Fig. 2. Changes in zymosan-stimulated luminol-dependent CL indexes (CLIIb) of the generation of ROS by leukocytes in 5.1% (37.5 mm Hg) and 20% (146 mm Hg) CO_2.

to peculiarities of the methods used. Since our experiments were carried out in a pressure chamber where the effect of CO_2 proper (5.1% and 20%) on leukocytes was combined with a lower pO_2 (145.4 and 122.6 mm Hg instead of 159.6 mm Hg in air), the question arises as to whether the inhibition of ROS generation by leukocytes under these conditions is due to the lowered PO_2. The data obtained by us and similar findings of Babior *et al.*, which demonstrated that a drop of pO_2 to 104 mm Hg and lower by substituting N_2 for O_2 did not significantly alter the generation of ROS by leukocytes, suggest the opposite. Our findings are consistent with the data on inhibition of phagocytosis by CO_2 [6]. It may also be hypotesised that CO_2 stimulates cell growth in culture not only as a sourse of buffer bicarbonate [13], but also as an antioxidant.

Thus, the results suggest that CO_2 under a pressure close to that in the blood (37.5 mm Hg) and under a high pressure (146 mm Hg) is a potent inhibitor of the generation of ROS by leukocytes.

There results are of interest in light of the regular year-by-year increase of CO_2 mass in the atmosphere, leading to the hothouse effect reported by Kelling et al. [1].

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