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Carbon dioxide directly suppresses spontaneous migration, chemotaxis, and free radical production of human neutrophils

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Abstract
Background Carbon dioxide (CO_2) insufflation during laparoscopy has been shown to dampen the systemic stress response to surgery. This is related to a suppression of peritoneal macrophage functions. *In vivo* data suggest that CO_2 can also affect neutrophils (polymorphonuclear cells, PMNs), the most abundant cell type in the inflamed peritoneal cavity. Nonetheless, the direct effects of CO_2 on PMNs have not yet been investigated.
Method PMNs were isolated from peripheral blood of healthy volunteers and incubated with (1) CO_2 (100% CO_2, pH 6.2), (2) hypoxic control (95% helium/5% CO_2, pH 7.4), and (3) control (95% air/5% CO_2, pH 7.4). Spontaneous and IL-8-induced migrations (chemokinesis and chemotaxis) during 2 h of exposure to different gases were measured with a transwell chamber system. The release of reactive oxygen species (ROS, luminometry) was determined after 15-min and 2-h exposures. In other sets of experiments, PMNs were exposed for 2 h or 4 h and kept under normal conditions for 18 h with lipopolysaccharide (LPS) stimulation thereafter. Final viability and apoptosis were assessed with fluorometry.
Results Exposure to 100% CO_2 completely blocked spontaneous and IL-8 induced migration of PMNs ($p < 0.001$ vs. controls). Neutrophil migration was slightly diminished in the hypoxic control group. PMA-stimulated ROS production was reduced even after short exposure to 100% CO_2 ($p < 0.05$). We observed a slight increase of caspase-3/7 activity after exposure to 100% CO_2 and/or hypoxia; however, total viability was not affected.
Conclusions CO_2 incubation directly and temporarily suppresses the proinflammatory functions of PMNs; this is caused only partially by the concomitant hypoxia. This effect will contribute to the dampened inflammatory response to laparoscopic surgery. Further studies are needed to investigate whether the temporary suppression of neutrophil functions could affect the clearance of bacterial contaminations.

Keywords Carbon dioxide · Immune system · Pneumoperitoneum

The insufflation of CO_2 in the abdominal cavity during laparoscopy reduces the systemic stress responses to abdominal surgery [1–6]. Peritoneal macrophages are known to initiate the inflammatory response in the peritoneal cavity. Therefore, to understand the mechanisms involved in the immunomodulation by CO_2, several authors have focused their research on the effects of CO_2 on the local macrophage population [1–5, 7, 8]. Nonetheless, many types of immunocompetent cells contribute to the postoperative inflammatory response. Polymorphonuclear cells (PMNs) represent the main immunocompetent cell type of the inflamed peritoneal cavity and they play a central role in host defense against invading microorganisms [3, 6, 7, 12–19]. Although *in vivo* data has demonstrated a significant effect of CO_2 pneumoperitoneum on the accumulation and function of PMNs, the mechanisms of this effect remain unknown [2, 13].

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To investigate potential direct effects of CO_2 on neutrophils, we assessed the direct impact of CO_2 on PMN functions, migration, ROS production, viability and apoptosis, *in vitro*.

Materials and methods

Cell preparation

Peripheral heparinized blood samples (20 ml) were collected from six healthy donors. PMNs were isolated using 6% HES (Plasmasteril®, Fresenius Kabi Deutschland, Bad Homburg, Germany) sedimentation followed by density gradient centrifugation with Ficoll-Paque (Amercham Biosciences, Uppsala, Sweden). Erythrocytes were eliminated by hypotonic lysis. After washing, PMNs were suspended in RPMI 1640 (PAA Laboratories, Pasching, Austria) supplemented with penicillin (PAA Laboratories) and fetal calf serum (FCS; PAA Laboratories). Purity and viability were checked using Coulter counter and trypan blue dye exclusion, respectively.

Chemokinesis and chemotaxis assay

PMNs were resuspended to the final concentration of 2×10^6/ml. Cell migration was assayed in a 10-mm transwell chamber system (Nunc, Roskilde, Denmark) with 3.0-μm pore polycarbonate filters. PMN suspension (500 μl) was added to the upper compartment, and 500 μl of RPMI medium with/without IL-8 (10 μg/ml) were added to the lower. Cells were incubated at 37°C for 2 h in three different gases: (1) CO_2 (100% CO_2,), (2) hypoxic control (95% helium/5% CO_2), and (3) control (95% air/5% CO_2). In separate vials the pH was measured during and after the experiment as described in detail earlier [10].

Following incubation, the filters were fixed (methanol) and stained (Giemsa staining). After gentle rinsing with water, the cells on the upper surface of the filters were removed. Stained cells that had migrated to their lower surfaces were counted microscopically. Each experiment was performed three times and numbers of migrated cells were calculated as percent (100% = migrated PMN number in control condition).

Reactive oxygen species (ROS) assay

PMNs were resuspended in 190 μl/well RPMI 1640 supplemented with 10% FCS, 10 mM HEPES, and 12 mM lucigenin (Sigma-Aldrich Chemie, Taufkirchen, Germany). Just after exposure to the three different gases for 15 min or 2 h, PMNs were stimulated by 50 μM PMA (phorbol 12-myristate 13-acetate; Sigma-Aldrich Chemie, Taufkirchen, Germany). Chemiluminescence activity was monitored continuously for 30 min (37°C) (Microlumat, Berthold, Germany). Each experiment was duplicated and specific activities were calculated as percent (100% = specific activity in control condition).

Viability and apoptosis assay

PMNs were resuspended to the final concentration of 1×10^6/ml and prepared for 96-well plates. Cell viability was evaluated by trypan blue staining and commercial fluorometric assay kits (CellTiter-Blue® cell viability assay, Promega, Madison, WI). Apoptosis was determined by a fluorometric assay kit (Apo-ONE® homogeneous caspase-3/7 assay, Promega, Madison, WI). Briefly, cells were incubated in the three gases for 2 h or 4 h. PMNs were further incubated under normal conditions (95% air/5% CO_2, pH 7.4) for 18 h with 10 μg/ml LPS. During the last 1.5 h of normal incubation, profluorescent substrates were added. For the viability assay, redox dye (resazurin) that can be converted to fluorescent end product (resorufin) by viable cells was applied. For the apoptosis assay, caspase substrate Z-DEVD-R110 that can be exchanged to fluorescent Rhodamine 110 by caspase-3/7 enzymes was mixed with the cells. Finally, the fluorescence was measured by fluorometer. Each experiment was performed three times and data were calculated as percent (100% = fluorescence in control).

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) followed by Tukey-Kramer's multiple comparison's test. Data are given as percent, with the control group (standard cell culture conditions) set as 100%. The results were expressed as mean ± SEM. $p < 0.05$ was considered significant.

Results

The purity of the PMN suspension was greater than 95% and the original viability was greater than 98% in all samples. There was a drop in pH within minutes of incubation in 100% CO_2 to levels around pH 6.2. The pH remained stable at this level throughout the incubation period. There was no change in pH in the standard or hypoxic control group.

Chemokinesis and chemotaxis assay

A complete inhibition of chemokinesis as well as IL-8-induced chemotaxis of PMNs was found during incubation in 100% CO_2 compared to control. Migrated cells in 100% CO_2 were 0.6 ± 0.3% in chemokinesis and 0.2 ± 0.1% in IL-8-induced chemotaxis ($p < 0.001$). Hypoxia showed a slight inhibition of PMN migration, 55.5 ± 18.4% ($p < 0.05$) in chemokinesis and 71.1 ± 14.7% ($p > 0.05$) in IL-8-induced chemotaxis (Fig. 1).

Reactive oxygen species assay

Spontaneous ROS production was not different between each group (data not shown). However, a significant reduction of PMA-stimulated ROS production was detected in 100% CO_2 groups compared to control: 67.6 ± 5.9% after a 15-min exposure and 74.3 ± 7.5% after a 2-h exposure ($p < 0.05$). Hypoxia also showed a 82.8 ± 6.1% of inhibition only after the 2-h exposure ($p > 0.05$) (Fig. 2).

Viability and apoptosis assay

Trypan blue staining showed more than 98% viability of PMNs throughout the experiments in all settings (data not shown). The fluorometric assay also showed no difference in viability among each group, regardless of incubation time ($p > 0.05$) (Fig. 3). However, caspase-3/7 activity increased in the 100% CO_2 group: after a 2-h exposure it was 120.8 ± 18.1% ($p > 0.05$) and after a 4-h exposure it was 146.7 ± 11.0% ($p < 0.05$). Hypoxia alone also

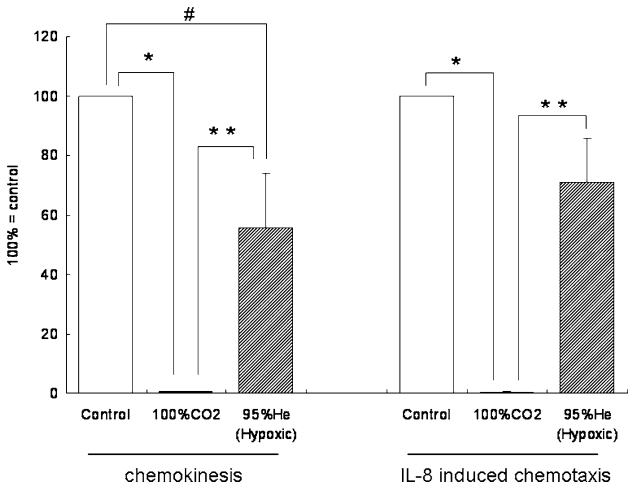

Fig. 1 Migrated PMN numbers during exposure to different gases. PMNs were incubated in three different gas conditions for 2 h. The number of each migrated cell was calculated as percent (100% = number of migrated PMNs in each control condition). Results are expressed as % ± SEM. $^{*}p < 0.001$ versus control, $^{**}p < 0.001$ versus hypoxic control, $^{\#}p < 0.05$ versus control. Spontaneous migration (chemokinesis without IL-8) and IL-8-induced migration (chemotaxis) of PMNs were completely inhibited in 100% CO_2, whereas they are slightly reduced in 95% He (hypoxic control). 254 × 190 mm (96 × 96 DPI)

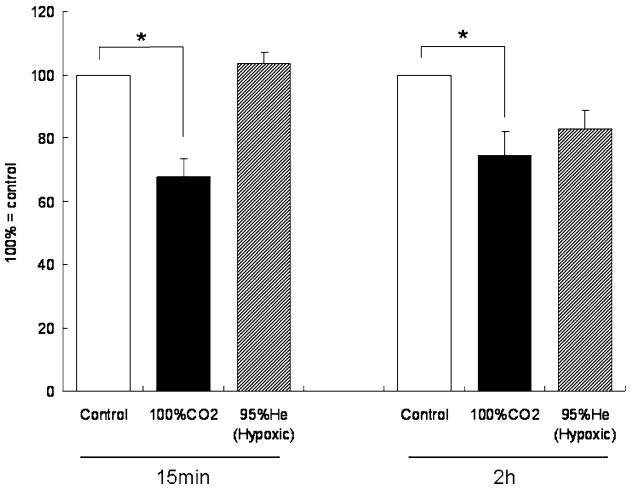

Fig. 2 ROS production from PMN exposed to different gases. PMNs were exposed to three different gas conditions for 15 min or 2 h. Thereafter, they were stimulated by 50 μM PMA (phorbol 12-myristate 13-acetate). Chemiluminescence activity was monitored continuously for 30 min (37°C). Specific activities (RLU = relative lux units) were calculated as percent (100% = specific activity in control condition). Results are expressed as % ± SEM. $^{*}p < 0.05$ versus control. A 15-min exposure to 100% CO_2 had the same level of inhibition as a 2-h exposure. 254 × 190 mm (96 × 96 DPI)

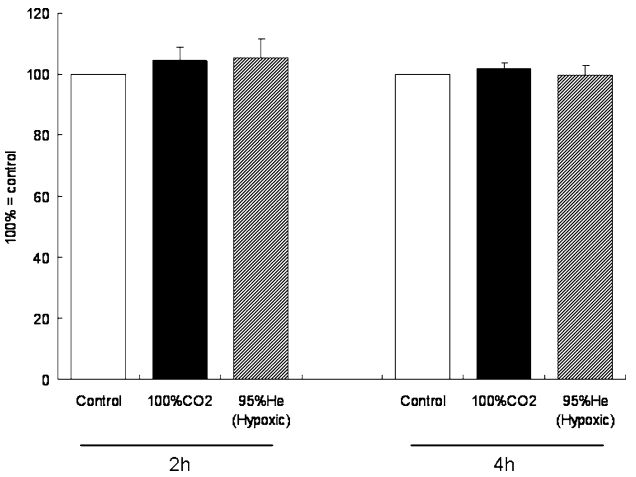

Fig. 3 Viability of PMN after exposure to different gases. PMNs were exposed to three different gas conditions for 2 h or 4 h and then further incubated in normal conditions for 18 h with 10 μg/ml LPS. Number of viable cells was measured by a fluorometric assay. Each number of viable cells was calculated as percent (100% = viable PMN number in each control condition). Results are expressed as %± SEM. No difference was detected among three groups. 254 × 190 mm (96 × 96 DPI)

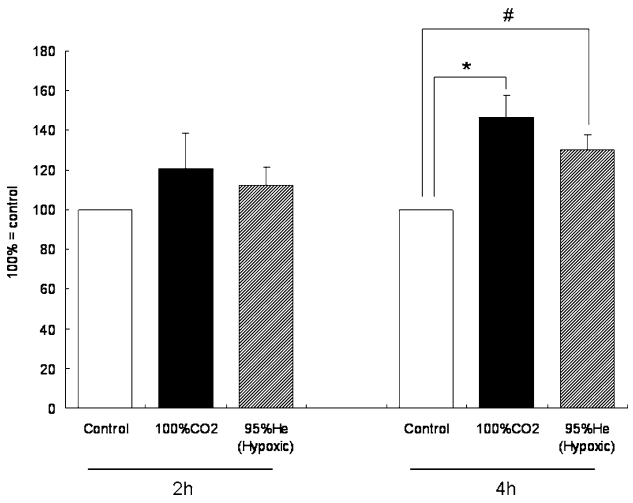

Fig. 4 Apoptosis of PMN after exposure to different gases. PMNs were exposed to three different gas conditions for 2 h or 4 h and further incubated in normal conditions for 18 h with 10 μg/ml LPS. Apoptotic cell number (caspase-3/7 activity) was assessed via fluorometric assay. Activity of caspase-3/7 in each group was calculated as percent (100% = caspase-3/7 activity in each control condition). Results are expressed as % ± SEM. $^{*,\#}p < 0.05$ versus control. Caspase-3/7 activity was increased in the 100% CO_2 group. It was less pronounced in the hypoxic group. 254 × 190 mm (96 × 96 DPI)

increased the caspase-3/7 activity after 4 h of exposure (130.4 ± 7.1%, $p < 0.05$) (Fig. 4).

Discussion

PMNs play an important role in the initiation, progression, and resolution of the inflammatory response to surgery [3, 6, 8, 12–19]. During the first several hours after the onset of bacterial contamination, there is a massive exudation of PMNs into the peritoneal cavity. PMNs phagocytose the bacteria via opsonization and kill them with oxygen products or proteolytic enzymes [14]. Lack of any one of these functions is associated with increased susceptibility of infection, so that activation of PMNs is essential to protect the host from microorganisms. On the other hand, excessive activation of PMNs after surgical trauma contributes to the subsequent immunosuppression, causes organ dysfunction complications, and can ultimately lead to death. Therefore, PMN functions are quite crucial factors in determining the clinical outcome of surgical patients [12].

Meanwhile, laparoscopic surgery has been shown to preserve the postoperative immune functions and CO_2 pneumoperitoneum is considered a major factor of this effect [1–9]. Sietses et al. [6] demonstrated that circulatory, systemic PMN functions were preserved after laparoscopic Nissen fundoplication compared with open surgery. Ure et al. [2] and Moehrlen et al. [13] showed a reduction in PMN recruitment into the peritoneal cavity after CO_2 pneumoperitoneum in experimental models.

In the current study, we focused on the direct effects of CO_2 on isolated human PMNs *in vitro*, without any other complex interactions between PMNs and other cells *in vivo* [13–15, 20–23].

The first step of PMN participation in the inflammatory response is migration to the local site of inflammation. Chemokinesis is a spontaneous random movement and chemotaxis is a directed cellular movement in proportion to chemical stimulation such as caused by IL-8, a prototypic intrinsic chemokine that is produced mainly by activated local macrophages. Normally, PMNs show strong chemotactic ability according to the concentration of IL-8 [16]. However, PMN mobility, both chemokinesis and chemotaxis, was almost completely abolished by incubation in 100% CO_2, even though hypoxia alone had only a slight effect.

A similar phenomenon was seen in ROS production. PMNs possess the most powerful system of ROS generation among all cell types, i.e., the NADPH oxidase, which they use to kill ingested microorganisms [17]. We found a significant reduction in ROS production by PMNs just after the exposure to 100% CO_2. Interestingly, only a short exposure (15 min) to 100% CO_2 resulted in a similar suppression compared with a 2-h exposure. Hypoxia alone inhibited ROS production only after a full 2-h exposure.

Induction of PMN apoptosis is a key event that initiates the resolution of inflammation and is necessary for the balance between proinflammatory and anti-inflammatory cell functions for the prevention of a surgery-induced immune dysfunction [12, 13, 17, 18]. The potent intracellular machinery responsible for this apoptosis is a family of proteases with cysteine at their active site that split their target proteins at specific C-terminal sites of aspartic acids known as caspases (cysteine-dependent aspartate-specific proteases) [13, 17]. Caspases-3/7 are key enzymes for apoptosis in mammalian cells [17]. The activities of these enzymes were significantly higher after 100% CO_2 exposure; however, cell viability was kept to greater than 98%. This is most likely related to the limited observation period in our experiments.

Incubation in 100% CO_2 acts via two effects on the local micromilieu, i.e., hypoxia ($pO_2 = 0$ mmHg) and acidification (pH 6.2) [5, 7–11, 24]. These effects occur quite rapidly in our *in vitro* setting and the pH change occurs rapidly *in vivo* [8–11, 24]. In previous studies from our group, we demonstrated that the effect of CO_2 on peritoneal macrophages is mainly driven by the pH change in the peritoneal cavity [10]. Similar to the effect of acidosis on the inflammatory response of macrophages, our data suggest that the acidification of the medium played a major

role in the modulation of PMN functions. Nonetheless, we also observed a similar but less pronounced effect of hypoxia, suggesting that the mechanism might not be specific.

In summary, we observed that exposure to 100% CO_2 directly altered neutrophil functions, i.e., blocked the mobility, reduced ROS production, and increased apoptosis. This observation helps to explain the beneficial effects observed during and following sterile operations *in vivo*, but raises the question of to which degree the dampened neutrophil activity could affect the clearance of bacterial contaminations.

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