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The effect of timing of pneumoperitoneum on the inflammatory response

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Abstract
Background: We examined the effects of an identical period of pneumoperitoneum applied at three different time points after lipopolysaccharide (LPS) challenge. Two different insufflation gases were also compared.
Methods: Male rats (n = 70) were injected intravenously with 1 mg/kg of LPS (time 0). The time relationship between a 1.5-h period of insufflation and initial LPS stimulation was the experimental variable. All rats were killed 6 h after injection. CO_2 and helium insufflation were investigated. Ten control rats received LPS only. Serum interleukin-6 (IL-6) levels were determined by enzyme-linked immunosorbent assay (ELISA). Hepatic expression of α_2-macroglobulin, β-fibrinogen, and metallothionein were measured by Northern blot analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA).
Results: Expression of α_2-macroglobulin mRNA was lower in CO_2 groups compared to the control group ($p < 0.05$ at time 120 and 270). β-Fibrinogen message was diminished in CO_2 0 and 120 groups compared to control. Serum levels of IL-6 and expression of metallothionein mRNA did not show significant differences between groups.
Conclusions: These findings suggest that CO_2 pneumoperitoneum downregulates the inflammatory response to LPS challenge. Start time of CO_2 insufflation does not appear to alter hepatic expression of acute phase genes. The mechanism of α_2-macroglobulin downregulation does not appear to be due to IL-6.

Key words: Laparoscopy — Pneumoperitoneum — Inflammatory response — Acute-phase proteins — Lipopolysaccharide — Endotoxemia

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The widespread adoption of laparoscopic surgery since the early 1990s was driven by improved postoperative outcomes, including reduced pain, shorter hospitalization, and faster recovery of normal activity. The benefits of laparoscopy have been attributed to decreased wound trauma as a result of smaller incisions. However, other factors, such as the physiologic effects of pneumoperitoneum, may play an additional role. Identifying mechanisms by which carbon dioxide preserves immune function or exerts an antiinflammatory effect would further justify the use of laparoscopy in a wide range of patients.

The increased use of laparoscopy in septic surgical patients has stimulated interest in delineating the effect of pneumoperitoneum on the immune and inflammatory responses. Previous work has demonstrated that the cell-mediated immune response was better preserved in mice receiving pneumoperitoneum compared to extraperitoneal incisions or laparotomy [8]. Further work examined the modification of the inflammatory response in rodents challenged with bacterial lipopolysaccharide (LPS). Insufflation with CO_2 decreased the LPS-induced expression of the acute phase gene α_2-macroglobulin in comparison with control animals [1]. Studies comparing the effect of insufflation with CO_2 or He on a rodent model of abdominal sepsis, cecal ligation, and double puncture indicated that the expression of α_2-macroglobulin and β-fibrinogen was diminished following CO_2 laparoscopy as opposed to He or the open procedure [9]. Thus, these studies support the hypothesis that CO_2 affects the inflammatory process.

To further delineate possible mechanisms in the modulation of the inflammatory process by CO_2, we examined the timing of the administration of insufflation in a rodent model of endotoxemia. Injection of LPS results in a robust inflammatory response, which has been characterized for different systems, such as circu-

lating cytokines, infiltration of polymorphonuclear lymphocytes, and the acute-phase response. In our experimental approach, the time between LPS injection and sample collection was maintained constant. Rats were also insufflated for the same time period. The relationship between insufflation and initial LPS stimulation was the experimental variable. Hepatic expression of two acute phase genes (α_2-macroglobulin and β-fibrinogen) and the oxygen/metal scavenger metallothionein was analyzed. Our results suggest that CO_2, but not helium, pneumoperitoneum established at any time within the first 6 h of an endotoxin insult decreases the expression of α_2-macroglobulin.

Materials and methods

Animals

The Johns Hopkins University Animal Care and Use Committee approved the following experimental protocol. Sprague-Dawley male rats (275–300 g) were maintained in central animal facilities, with water and chow ad libitum. Rats were fasted for 12 h prior to the beginning of the experiment. Prior to LPS injection, all animals had induction of anesthesia in an isoflurane chamber. Control animals were recovered after injection and remained unmanipulated until being killed. Animals in the insufflation groups were placed on blow-by isoflurane anesthesia for the duration of the intervention. They were allowed to recover during the remainder of the experiment, until being killed. Insufflation was achieved by introducing a 20-gauge angiocatheter into the peritoneal cavity through the midline of the epigastrium and then attaching an automatic insufflator (Olympus) set to a maximum pressure of 4 mmHg. This pressure was chosen as it provides adequate distention of the abdomen to allow for laparoscopic instrument manipulation, but has been shown to only reduce portal venous blood flow by 8% in the rat model [12].

Experimental design

Rats were divided into seven groups (10 rats per group) (Fig. 1). All rats were injected with LPS 1 mg/kg (Sigma—purified *Escherichia coli* O26:B6) and killed with an excess of isoflurane at 6 h post injection. Control rats received no intervention other than injection. Group 0 was insufflated immediately after injection for 1.5 h. Group 120 was insufflated 2 h after LPS injection for 1.5 h. Group 270 was insufflated 4.5 h after injection for 1.5 h. The three insufflation groups were further divided into groups of CO_2 pneumoperitoneum and He pneumoperitoneum (10 rats each). After death, blood was collected by cardiac puncture and serum was isolated and kept frozen at −80°C until analysis. After cardiac puncture, liver tissue was immediately harvested, flash frozen in liquid nitrogen, and stored at −80°C.

RNA analysis

Frozen liver samples were homogenized in Trizol (Gibco) and RNA was isolated as indicated by the manufacturer's instructions. RNA was separated in agarose–formaldehyde gel (12 μg of RNA), transferred onto nylon membranes (GeneScreen Plus, NEN Research Products, Boston MA) by capillary action, and cross-linked with UV light. Blots were stained with methylene blue (0.03%) in 3 M NaOAc, pH 5.2, and scanned prior to probing. Samples were also analyzed by slot blotting (10 μg of sample RNA per slot). The following cDNA probes were prepared: α_2-macroglobulin (rat, full-length), β-fibrinogen (pig, fragment), metallothionein (pig, full-length), and the 28S rRNA subunit (human), by the random primer method [7] using radioactive ATP and CTP [5]. Membranes were hybridized with radiolabeled cDNA probes for 48 h, then membranes were washed in 1× SSC, 1% sodiumdodecylsulfate (SDS) for 10 min at 42°, followed by washing in 2× SSC, 0.1% SDS for 30 min at 42°C. The membranes were exposed to a phosphor screen (Molecular Dynamics) for up to 16 h and images were obtained via the Molecular Dynamics scanner and image acquisition software. Signal intensity was calculated in the following manner: For the Northern blot, images of the methylene blue stain were imported, and for each band a value was obtained. Intensity of each α_2-macroglobulin band was then acquired, and each band was normalized to its corresponding 18rRNA signal. Each slot blot was normalized to the signal of the corresponding well of the r28s membrane.

The resulting normalized signal intensities were imported into an Excel database (Microsoft). Values were removed for wells determined to have degraded mRNA. Averages were then calculated for each group, and all groups were compared by one-way analysis of variance (ANOVA) using SigmaStat 2.0 (Jandel Corporation).

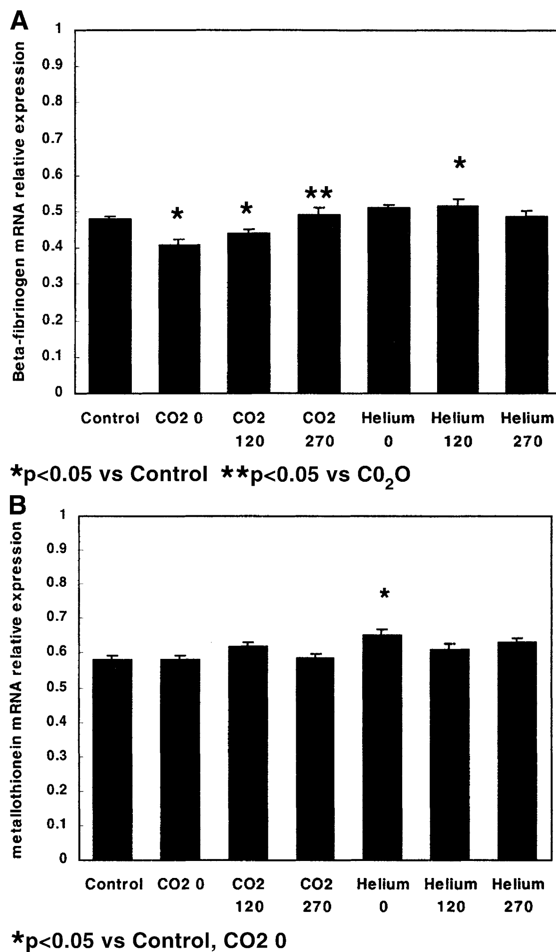

Fig. 1. Experimental design demonstrating the timing of the three insufflation groups. All rats are injected with lipopolysarcharide 1 mg/ml at time 0. Control animals receive no further intervention until being killed at 6 h. All insufflation groups receive 1.5 h of pneumoperitoneum with either CO_2 or He and are killed at 6 h. The experimental variable is the timing between injection and insufflation. Animals are insufflated at 0, 120, or 270 min.

ELISA

A commercial enzyme-linked immunosorbent assay (ELISA) (Biosource International, Camarillo, CA) was used to quantitate serum IL-6 levels. Statistics were performed as noted above.

Results

Liver samples were collected after death and total RNA was isolated. Analysis of steady-state mRNA levels by

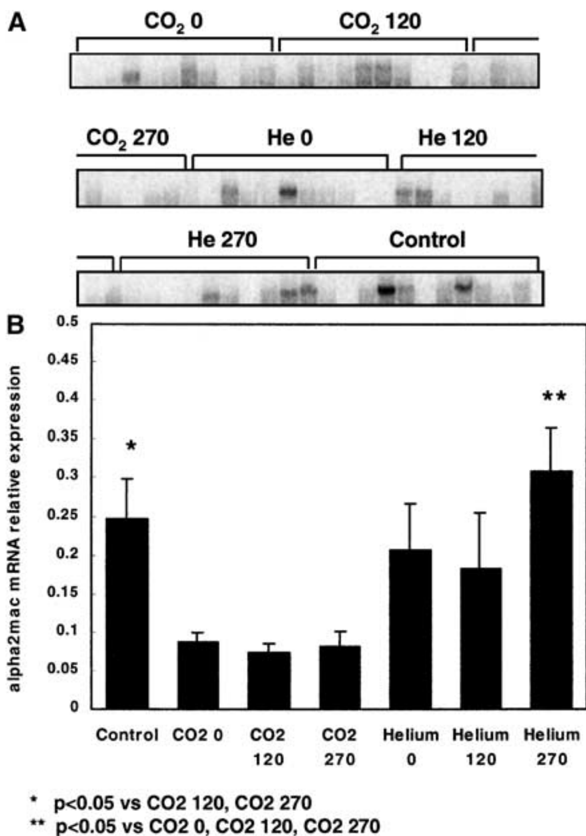

Fig. 2. (**a**) Autoradiograph of α_2-macroglobulin northern blot. Band intensities were measured and then normalized with methylene blue staining intensities of the 18s ribosomal band, (**b**) Normalized α_2-macroglobulin Northem blot values for each group were averaged and graphed as relative amounts of mRNA expression. Groups were compared with one-way ANOVA analysis. The control group was statistically different from CO_2 120 and CO_2 270. The He 270 group was statistically different from all CO_2 groups.

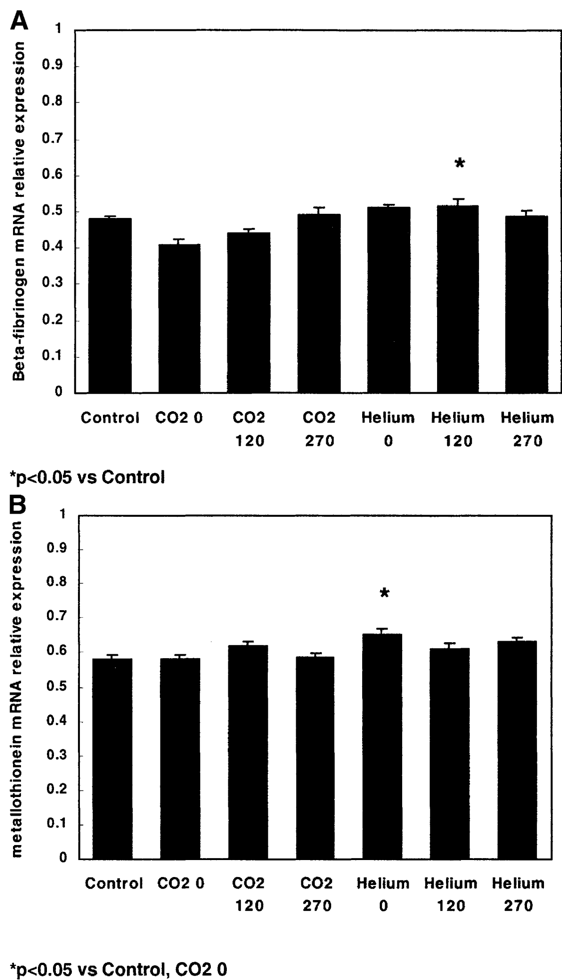

Fig. 3. Results of slot blotting of isolated hepatic RNA. Membranes were hybridized with probes for β-fibrinogen (**a**) and metallothionein (**b**). The signal intensity of each spot was acquired and normalized with the intensity of the matching location on a third membrane, probed for r28S. (**a**) β-fibrinogen results. The intensities of each group were averaged and graphed. Statistical analysis was Performed by one-way ANOVA. Statistical differences were found between the control group and He 120, as well as between CO_2 0 and CO_2 270. (**b**) Metallothionein results. Graphing of group averages revealed a statistical difference between He 0 and the control and CO_2 0.

Northern blotting was performed for the hepatic protein α_2-macroglobulin; slot blotting was performed for β-fibrinogen and metallothionein. For each protein, the three time points (0, 120, and 270 min) were compared to the control for both CO_2 insufflation and He insufflation groups.

Analysis of α_2-macroglobulin indicated a decrease in the expression of this gene (30–35%) in all CO_2 groups with respect to the control (Fig. 2). However, He insufflation did not significantly affect the levels of α_2-macroglobulin steady-state mRNA compared to the control group.

Expression of β-fibrinogen was decreased in the CO_2 0 and CO_2 120 groups compared to the control group, whereas the other groups did not show statistically significant differences in β-fibrinogen expression (Fig. 3a). When the expression of β-fibrinogen was compared among the different CO_2 groups, time 0 and time 270 were significantly different. Levels of metallothionein steady-state mRNA demonstrated a significant difference between group He 0 compared to CO_2 0 and the control (Fig. 3b).

Plasma levels of IL-6 were also measured in this experiment. No significant differences were observed among the group (Fig. 4).

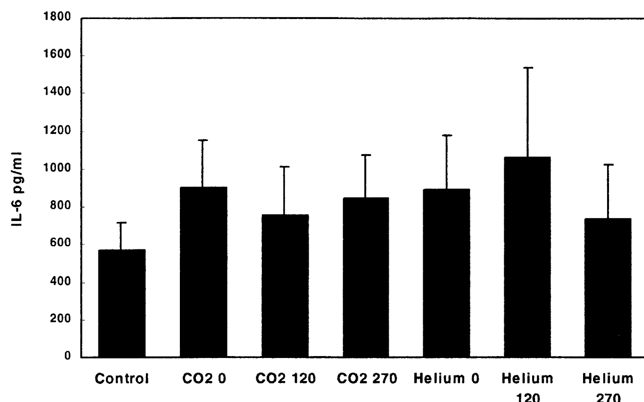

Fig. 4. Rat serum was assayed for IL-6 using a commercial kit. All groups displayed elevation of IL-6 levels. There was no statistical difference between groups by ANOVA.

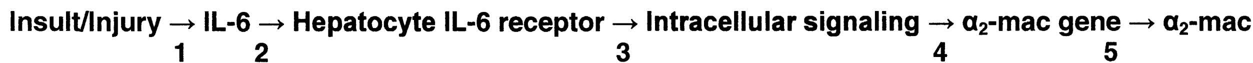

Fig. 5. Acute injury stimulates the production of the acute-phase protein α_2-macroglobulin via the cytokine mediator IL-6. The numbers represent potential sites of action where CO_2 may exert an anti-inflammatory effect. CO_2 acts after step 1.

Discussion

Disrupting the integrity of tissues through injury induces the body to act swiftly to repair itself. The response is both local, as seen with increased vascular permeability and recruitment of leukocytes, and systemic [10]. The local response can result in more local injury due to the release of proteolytic enzymes by leukocytes. The systemic responses can vary in intensity and include the following alterations: fever, neutrophilia, altered nutrition metabolism and hormone release, alterations of serum cations, stimulation of the adrenal–pituitary axis, immunosupression, and increased hepatic production of acute phase proteins [4, 10]. This entire process is known as the acute-phase response.

The acute-phase response is regulated via multiple soluble chemical mediators, including cytokines. Cytokines released at the site of injury are responsible for both the local and systemic priming of the acute-phase response. Although many different cell types can produce cytokines, the initial release of crucial proinflammatory cytokines is from activated macrophages at the site of injury. Interleukin-1 (IL-1), Tumor necrosis factor–α (TNF-α), and IL-6 in turn stimulate the cellular release of a secondary wave of cytokines, as well as exerting a direct effect on systemic target organs [3].

The liver is an important target of cytokine stimulation. Acute-phase proteins are regulated by a family of genes that are expressed in hepatocytes in response to inflammatory mediators. The production of acute-phase proteins increases by at least 25% during the acute-phase response. In contrast, other hepatic genes are downregulated during the acute-phase response. These are termed "negative" acute-phase proteins [7, 10]. Acute-phase proteins often have functions that assist the body in counteracting the insult that stimulated the inflammatory response, such as complement proteins, coagulation proteins, or proteinase inhibitors, which help keep the local inflammatory response from becoming too robust. Metal binding proteins, such as haptoglobin, hemopexin, and ceruloplasmin, are increased, as are the major acute-phase proteins, serum amyloid A, C-reactive protein, and serum amyloid P component. The negative acute-phase proteins include albumin, prealbumin, transferrin, and apolipoproteins AI and AII [14].

In this experiment, hepatic tissue collected 6 h after induction of endotoxemia is noted to have reduced levels of α_2-macroglobulin mRNA in all three groups of animals exposed to CO_2 pneumoperitoneum, although the time 0 insufflation group's difference did not reach statistical significance. Thus the effect of CO_2 on α_2-macroglobulin can occur up to 6 h after the establishment of endotoxemia. There was a similar trend in the β-fibrinogen group, although the blunting of expression was less marked and appeared to diminish over the time course of the experiment. Insufflation with helium did not reduce expression of either of these acute-phase proteins. Metallothionein's pattern of expression was affected by neither the timing of insufflation nor the insufflation gas used.

The mechanism by which the acute-phase response stimulates the eventual production of α_2-macroglobulin is mapped out in Fig. 5. IL-6 produced at the site of injury acts directly on the hepatocyte to promote expression of the α_2-macroglobulin gene. In this experiment, the measurement of plasma IL-6 revealed elevated levels of IL-6 in all groups, but with no statistical differences between groups. If we had found a decrease in the levels of circulating IL-6 in animals exposed to CO_2 insufflation, it would have suggested that the anti-inflammatory effect occurred early in the pathway, at step 1. Metallothionein is not a classic acute-phase protein, but in a state of endotoxemia its expression is increased as a result of IL-6 binding to the hepatocyte [11]. The absence of statistical differences in the elevation of metallothionein expression in this experiment further suggests that step 1 is not the site of the anti-inflammatory effect of CO_2. Expression of α_2-macroglobulin must be influenced by other factors in addition to IL-6.

Analysis of the kinetics of acute-phase gene expression products in cultured rat hepatocytes revealed a 3 h lag in the production of α_2-macroglobulin, although its time of maximum expression is not delayed [3]. This may explain why α_2-macroglobulin is more susceptible to the influence of CO_2insufflation, and why the effect appears to dissipate in the β-fibrinogen group. The delay in α_2-macroglobulin gene transcription may result from additional intracellular interactions that have not yet been described, and which may be susceptible to influence by other mediators stimulated by CO_2. This would involve intervention at sites 3, 4 or 5 in Fig. 5 and may include alterations in cell signaling pathways, gene promoters, gene transcription and mRNA stability, and protein synthesis and excretion.

Some data suggests that the metabolic properties of CO_2 are responsible for the observed antiinflammatory effects. West has shown that cultured murine peritoneal macrophages stimulated with LPS produced less TNF-α and IL-1 when exposed to CO_2 versus air or He [15]. West measured cytosolic acidification in cells exposed to CO_2 and also demonstrated that in an *in vivo* model the peri-peritoneal cells experienced a decrease in pH [16]. This acidification may, in turn, exert an effect on peritoneal macrophages. A co-stimulatory cytokine signal from these macrophages could be diminished as a result of CO_2 insufflation.

In this experiment, the lack of α_2-macroglobulin gene expression decrease in the He groups suggests that the effect of insufflation on the acute-phase response is linked to CO_2 exposure of the peritoneum, rather than just a mechanical effect related to the pressure of pneumoperitoneum. We have previously found that CO_2 insufflation in rats injected with LPS diminishes TNF-α release, but enhances secretion of IL-10. Helium insufflation also reduced TNF-α, although not as dramatically. Helium did not change IL-10 levels from the control value (unpublished data). The antiinflammatory cytokine, IL-10, thus has a potential role as the mediator released after peritoneal acidification. IL-10 could be directly affecting the hepatocytes, or it may diminish systemic secretion of a co-stimulatory signal required by hepatocytes. Alternatively, CO_2 exposure could cause an alteration in the cycle of inflammation. It is well documented that there is an initial wave of proinflammatory cytokines, followed by an antiinflammatory wave. Insufflation might trigger an earlier shift in the antiinflammatory wave.

The growing body of evidence that suggests the metabolic activity of CO_2 plays an important role in the systemic inflammatory response has been documented in clinical studies of patients undergoing laparoscopic surgery, as well as the animal models. Barle et al. found that during laparoscopic cholecystectomy, the total rate of protein synthesis decreased, although the rate of albumin synthesis remained constant [2]. Decreased protein synthesis could be the result of a reduction of mRNA transcription in the hepatocyte. Clinical researchers have also documented similar results in IL-6 levels during laparoscopic surgery. Sietses et al. examined patients undergoing laparoscopic surgery who were randomly assigned to CO_2, He, or abdominal wall lift groups. They saw no significant differences in IL-6 levels between the groups, but found decreased levels of the acute-phase reactant C-reactive protein in the CO_2 group only [13].

A potential antiinflammatory role for CO_2 insufflation makes laparoscopic surgery an attractive option for patients in septic states who need surgery, as well as patients needing oncologic resections who may have improved outcomes through prevention of a postoperative immunosuppressive state. Our experiment supports-the use of CO_2 insufflation in models of endotoxemia, and suggests that the benefit may occur at any point in the progression of the systemic insult.

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