

Effect of CO₂ on LPS-induced cytokine responses in rat alveolar macrophages

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Lang, Carol J., Ping Dong, Emma K. Hosszu, and Ian R. Doyle. Effect of CO₂ on LPS-induced cytokine responses in rat alveolar macrophages. *Am J Physiol Lung Cell Mol Physiol* 289: L96–L103, 2005. First published March 18, 2005; doi:10.1152/ajplung.00394.2004.—Alveolar macrophages (AM) may be exposed to a range of CO₂ and pH levels depending on their location in the alveoli and the health of the lung. Cytokines produced by AM contribute to inflammation in acute lung injury (ALI). Current ventilatory practices for the management of ALI favor low tidal volumes, which can give rise to increases in CO₂ and changes in pH of the alveolar microenvironment. Here we examined the effect of CO₂ on cytokine release from LPS-stimulated rat AM. AM were incubated for 1–4 h under different atmospheric gas mixtures ranging from 2.5–20% CO₂. To distinguish between effects of pH and CO₂, the culture media were also buffered to pH 7.2 with NaHCO₃. Cell metabolic activity, but not cell viability, decreased and increased significantly after 4 h at 20 and 2.5% CO₂, respectively. Increasing CO₂ decreased TNF- α secretion but had no effect on lysate TNF- α . Buffering the media abated the effects of CO₂ on TNF- α secretion. CO₂ increased cytokine-induced neutrophil chemoattractant factor-1 secretion only when the pH was buffered to 7.2. Effects of CO₂ on cytokine responses were reversible. In conclusion, the effects of CO₂ on cytokine lysate levels and/or secretion in AM are cytokine specific and, depending on both the cytokine and the immediate microenvironment, may be beneficial or detrimental to ALI.

inflammation; hypercapnia; acute lung injury

ACUTE LUNG INJURY (ALI) is an inflammatory condition that can arise from multiple clinical situations (32). However, sepsis and pneumonia are among the most common predisposing causes (3). In pneumonia, initiation of the inflammation occurs directly within the lung parenchyma (28). In sepsis, ALI arises indirectly from the “spillover” of systemic inflammatory mediators into the pulmonary environment (34). ALI is often initiated by lipopolysaccharide (LPS) released from the cell walls of gram-negative bacteria (5). Activation of alveolar macrophages (AM) by LPS requires precise sequences of events involving LPS binding proteins, CD14 receptors, the secreted glycoprotein MD2, Toll-like receptors (TLR), and nuclear translocation factor- κ B (NF- κ B) (9, 14, 22, 24). AM respond to LPS by secreting a pulse of cytokines that activate local inflammation. In sepsis, tumor necrosis factor- α (TNF- α) and interleukin-1 β (IL-1 β) are released during the first 30–90 min after exposure to LPS (3) and in turn activate a second level of inflammatory mediators including other cytokines, lipid mediators, and reactive oxygen species, as well as up-regulating cell adhesion molecules that result in the migration of inflammatory cells into the lung (12).

ALI often requires mechanical ventilation, where current strategies favor low tidal volume and high end-expiratory volume. Because ventilatory-induced stretch exacerbates ALI (18), the improved morbidity and mortality, which have been demonstrated using such protective strategies, may be due to reduced mechanical trauma (7). However, such strategies can also be accompanied by elevations in CO₂ (hypercapnia). Although hypercapnia can be controlled by increasing respiratory rate (33), there is evidence to suggest that hypercapnia may be beneficial in the prevention of, and recovery from, lung injury (6, 15, 16, 18, 19, 30–32). However, the beneficial effect of hypercapnia is currently a topic of hot debate, since in the major study that demonstrated benefits of reduced lung stretch (33), CO₂ levels did not differ between low and high ventilation. Moreover, at the cellular level, Lang et al. (20) determined that cell injury, mediated by nitric oxide activity, was enhanced in fetal rat alveolar type II epithelial cells exposed to hypercapnia. Understanding the influence of CO₂ and pH on the inflammatory functions of AM is important to our understanding of the inflammatory responses in ALI, or other inflammatory lung diseases, and may contribute to the current debate concerning the role of hypercapnia in ventilatory strategies.

In the lung, the pH of the epithelial lining fluid (ELF) is normally pH 6.7–6.9 (26). However, the acid-base status of the ELF varies widely in health and disease, and AM may be exposed to a range of pH and CO₂ levels depending on their location in the alveoli. For example, in cystic fibrosis and asthma, the pH of the ELF may fall below 6.5 (8, 13), and in tumors or abscesses, the pH can be <6.0 (25). High CO₂ can lead to hypercapnic acidosis of the ELF and may also influence AM cytokine release. TNF- α and IL-1 β are among the best characterized of the cytokines and can be regulated as archetypal acute inflammatory cytokines. Cytokine-induced neutrophil chemoattractant factor-1 (CINC-1), a member of the IL-8 family (23), is a potent neutrophil chemotactic and activating factor (29). Therefore, in this descriptive study, we investigate the influence of CO₂ and buffering pH on LPS-stimulated synthesis and/or secretion of TNF- α , IL-1 β , and CINC-1 proteins in cultured rat AM.

METHODS

Isolation of AM

All experiments were performed under the Flinders University Animal Ethics Committee, approval no. 385/04, and in compliance with Principles of Animal Care publication number 86-23 of the National Institutes of Health and the Australian Code of Practice

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for the Care and Use of Animals for Scientific Purposes (6th edition).

Male Sprague-Dawley rats (mass range: 200–260 g) were obtained from the Institute of Medical and Veterinary Science, Gilles Plains, Adelaide, Australia. Rats were deeply anesthetized via intraperitoneal injection of methohexital sodium (100 mg/kg body wt; Eli Lilly, Sydney, Australia), the tracheae were cannulated, and the lungs were perfused with buffered saline solution A (150 mM NaCl, 5 mM KCl, 2.5 mM sodium phosphate, 10 mM HEPES, 0.2 mM EGTA containing 0.1% glucose, pH 7.4) at 10 ml/min via the pulmonary artery. The lungs were excised, degassed for 1 min, and lavaged with six separate 10-ml volumes of saline, each volume being instilled and withdrawn three times. The lavage fluid was centrifuged at 1,000 g at room temperature for 10 min, and the pellet was resuspended in culture media [DMEM containing 23.8 mM NaHCO₃, 10% FBS (vol/vol), and 1% (wt/vol) penicillin and streptomycin] at a density of 1.5×10^5 cells/ml. AM were plated at a density of 0.75×10^5 cells/cm² in 24-well culture plates (Nalge Nunc International, Rochester, NY) precoated at 37°C for 6 h with 5 µg/cm² rat IgG (Sigma-Aldrich, St. Louis, MO) in saline and incubated in a humidified atmosphere (pH 7.4, 95% O₂, 5% CO₂) at 37°C for 16 h. Nonadhered cells were removed by washing the wells with culture media.

Experimental Protocols

Cells were plated in triplicate per assay, and each protocol was repeated on five or six separate populations of isolated cells, i.e., on five or six occasions or days. The adhered cells were subjected to one of three protocols as follows.

Protocol I: gas mixture treatment. Cells were incubated for 1–4 h in fresh culture medium in a humidified atmosphere containing either 2.5, 5, 10, or 20% CO₂, with normal oxygen (21%) and the balance comprising N₂, with or without inclusion of 10 µg/ml of LPS (*Salmonella abortus equi*, Sigma-Aldrich). Under these conditions, the pH of the media was 7.2 at 2.5% CO₂, 6.8 at 5% CO₂, 6.4 at 10% CO₂, and 5.8 at 20% CO₂.

Protocol II: buffering medium pH. AM were incubated as described for protocol I. However, in this case, the DMEM was buffered to maintain a pH of 7.2 with increasing concentrations of NaHCO₃ (i.e., 7.1, 14.3, 28.6, or 56.1 mM NaHCO₃ during incubations with 2.5, 5, 10, and 20% CO₂ gas mixtures, respectively).

Protocol III: switching gas mixture. A schematic of the experimental design for protocol III is given in Table 1. AM were incubated, as described for protocol I, for a 4-h incubation. The culture media were then collected for cytokine secretion analysis (labeled 1st 4-h incubation in Figs. 2 and 6). Fresh DMEM was then added to the cells, and the cells were incubated for a further 4 h, using the same (i.e., 2.5% then 2.5% or 20% then 20%) or a different (i.e., 2.5% then 20% or

20% then 2.5%) gas mixture. The culture media were then collected for cytokine secretion analysis (labeled 2nd 4-h incubation in Figs. 2 and 6). Note that cell lysate samples could only be collected at the end of both 4-h incubation periods (Fig. 4). A similar set of experiments was also performed where the media were not changed after the first 4-h incubation period.

Collection of Culture Media and Cell Lysates

After incubation for 1, 2, 3, 4, or 8 h, the culture media were harvested and centrifuged at ~8,000 g at 4°C for 10 min. The cells were immediately washed with 1 ml of ice-cold saline, and 0.5 ml of ice-cold 50 mM Tris and 0.3 M saline containing 1% (wt/vol) Triton X-100 (pH 7.6) was added to each well. The lysates were centrifuged at ~14,000 g at 4°C for 15 min, and both the lysates and the culture medium were stored at –20°C for batch analysis.

Cell Viability

Nonadhered cells were removed by being washed with PBS containing 2 mM MgCl₂ and counted using a hemocytometer (Improved Neubauer, B.S. 748; Weber Scientific International, Teddington, UK). In addition, adhered cells were stained with a 20% (vol/vol) methanol solution of 0.2% (wt/vol) crystal violet for 15 min. If a cell membrane is intact, the cell retains the crystal violet dye. The cells were then washed with water, dried, and solubilized with SDS (1% wt/vol), and the absorbance was read at 540 nm on a Dynatech MR5000 plate reader (Baxter Diagnostics, Billingham, UK). Cell numbers were calculated relative to a standard curve of optical density for cell numbers ranging from 0 to 30,000 cells/well of a 96-well plate and used to calculate the data presented in Table 3.

Cell Metabolic Activity

Cell metabolic activity was assessed using a colorimetric assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) and phenazine methosulfate (PMS)] for dehydrogenase enzymatic activity according to the manufacturer's protocol (Promega, Madison, WI). Briefly, AM cells were plated at a density of 1.5×10^4 cells/well in 96-well plates and cultured in DMEM for 24 h. Twenty microliters of MTS and PMS were added to each well, and the cells were then incubated in the dark for 4 h with different gas mixtures as described for protocols I and II. In metabolically active cells, dehydrogenase enzymes convert MTS into a soluble formazan product. The quantity of formazan product was measured by absorbance at 490 nm (Dynatech MR5000 plate reader).

Table 1. Procedure for protocol III: gas-switching experiments

Plate No.	1	2	3	4	5	6	7	8
	DAY 1				DAY 1			
	Isolate and culture AM in plates overnight				Isolate and culture AM in plates overnight			
	DAY 2				DAY 2			
	Add fresh media to AM cultures				Add fresh media to AM cultures			
Incubate 4 h at:	2.5% CO ₂	2.5% CO ₂	20% CO ₂	20% CO ₂	2.5% CO ₂	2.5% CO ₂	20% CO ₂	20% CO ₂
	Collect media for analysis				-			
	Sample label: 1st incubation				-			
	Cytokine secretion only				-			
	Add fresh media to cells				-			
	Change gas mixture or leave same				Change gas mixture or leave same			
Incubate 4 h at:	2.5% CO ₂	20% CO ₂	2.5% CO ₂	20% CO ₂	2.5% CO ₂	20% CO ₂	2.5% CO ₂	20% CO ₂
	Collect media and lyse cells to collect cell lysates				Collect media and lyse cells to collect cell lysates			
	Sample label: 2nd incubation Cytokine secretion (media) and production (lysates)				Sample label: accumulative secretion (media) and production (lysates)			

AM, alveolar macrophages.

Table 2. Viability and metabolic activity of isolated rat AM incubated for 4 h at different CO₂

%CO ₂	Normal Media			Buffered Media		
	pH	Cell viability	Metabolic activity [‡]	pH	Cell viability	Metabolic activity
2.5	7.2	0.11 (0.041)	0.40 (0.067) [†]	7.2	0.11 (0.007)	0.42 (0.032)
5	6.8	0.11 (0.023)	0.35 (0.039)	7.2	0.11 (0.022)	0.38 (0.037)
10	6.4	0.10 (0.026)	0.29 (0.039)	7.2	0.11 (0.019)	0.35 (0.057)
20	5.8	0.08 (0.019)	0.23 (0.046)*	7.2	0.11 (0.022)	0.36 (0.043)

Cell viability ($n = 5$) and metabolic activity ($n = 3-4$) data are presented as means (SD) absorbance (optical density measured at 490 nm of crystal violet and formazan product, respectively). %CO₂, concentration of carbon dioxide (percent). Normal media contained 7.1 mM NaHCO₃ in all cases. Buffered media contained 7.1, 14.3, 28.6, or 56.1 mM NaHCO₃ at 2.5, 5, 10, and 20% CO₂ gas mixtures, respectively. CO₂ ($F = 7.743$, $P = 0.001$) and buffering ($F = 10.552$, $P = 0.004$) significantly influenced AM metabolic activity using a 2-way ANOVA for CO₂ and buffering (CO₂/buffering interaction, $P = 0.128$). [‡]Significant influence of CO₂ on AM metabolic activity in normal media (1-way ANOVA, $F = 9.213$, $P = 0.002$). *Significant difference from 5% CO₂ (Dunnett's test, * $P = 0.02$; [†] $P = 0.001$).

Cytokine Measurement

For each experimental protocol, cytokine content was analyzed on samples collected from as many of the five to six separate experiments as possible. Supernatant and lysate TNF- α (Pharmingen Opt EIA, San Diego, CA) and supernatant IL-1 β (R&D Systems, Minneapolis, MN) were measured using ELISA as per the manufacturer's protocols. For measurement of CINC-1, media samples were lyophilized and concentrated threefold by resuspension in appropriate volumes of assay diluent (PBS + 10% FBS). Lysate samples were unable to be concentrated for CINC-1 analysis due to limited sample volumes. CINC-1 was measured using an inhibition ELISA previously developed in our laboratory. Briefly, samples were incubated with a rabbit anti-rat CINC-1, NH₂-terminal specific antibody (Assay Designs, Ann Arbor, MI) overnight at 4°C before being transferred to a vinyl ELISA plate (Costar, Cambridge, MA) coated with 50 ng/ml of CINC-1 (Peptide Institute, Osaka, Japan). Free antibody was captured and detected with horseradish peroxidase-conjugated sheep anti-rabbit IgG (Chemicon, Melbourne, Australia). Color development using a 3,3',5,5'-tetramethylbenzidine substrate reagent set (Pharmingen) was measured at 450 nm using a Dynatech plate reader (Dynatech Laboratories, Chantilly, VA).

Statistical Analysis

In all tables and figures, n refers to the total number of separate experiments (i.e., different populations of freshly isolated AM) in which we measured cell viability, metabolic activity, or cytokine amount. Statistical analyses were conducted using SPSS. All results are expressed as means (SD). Effects of CO₂ on cell viability, metabolic activity, and cytokine production and secretion were analyzed using one- and two-way ANOVA with Dunnett's post hoc tests (using 5% CO₂ as the control). Bonferroni post hoc tests were used to assess differences between incubation times.

RESULTS

Cell Viability and Metabolic Activity

Our method of AM isolation typically afforded $8.0 \pm 0.5 \times 10^6$ cells (means \pm SE; $n = 12$) of $>90\%$ purity. The %CO₂ under which the cells were cultured had no effect on the adherence of the AM (data not shown). With the use of crystal violet staining, neither CO₂ nor buffering significantly influenced cell viability (Table 2). However, CO₂ and buffering did influence cell metabolic activity, as measured by the MTS/PMS assay. Compared with that at 5% CO₂, which is considered a normal physiological CO₂ level, post hoc testing showed that the cut off for a significant difference in metabolic activity is 20% CO₂. At 20% CO₂, metabolic activity was $\sim 60\%$ of that at 5% CO₂. Conversely, at 2.5% CO₂, metabolic activity was $\sim 114\%$ of that at 5% CO₂. Buffering the culture media abated the CO₂-induced alterations in metabolic activity (Table 2).

TNF- α and IL-1 β Secretion

Protocol I. There was no appreciable TNF- α secretion [35 pg/ml (SD 21) from all experimental groups] in the absence of LPS. LPS-stimulated secretion was influenced by incubation time and %CO₂ in a clear, dose-responsive manner (Fig. 1). Post hoc testing showed significant differences in TNF- α secretion between the 5% CO₂ group and the 2.5 and 20% CO₂ groups. After 4 h, TNF- α secretion increased by $\sim 30\%$ under 2.5% CO₂ but decreased by $\sim 20\%$ under 20% CO₂ (Table 3).

Fig. 1. Effect of CO₂ on LPS-stimulated TNF- α secretion from alveolar macrophages (AM) after 1- to 4-h incubation in protocols I and II. TNF- α was measured in culture media using an ELISA (Pharmingen Opt). $N = 5$ or 6. Protocol I: time [F -statistic (F) = 211.4, $P = 0.0$] and CO₂ ($F = 21.4$, $P = 0.0$) influenced TNF- α secretion (2-way ANOVA). Protocol II: time ($F = 2,037.5$, $P = 0.0$) and CO₂ ($F = 13.0$, $P = 0.0$) influenced TNF- α secretion (2-way ANOVA). Significant difference from 5% CO₂ group (Dunnett's post hoc tests, * $P < 0.05$, ** $P < 0.01$).

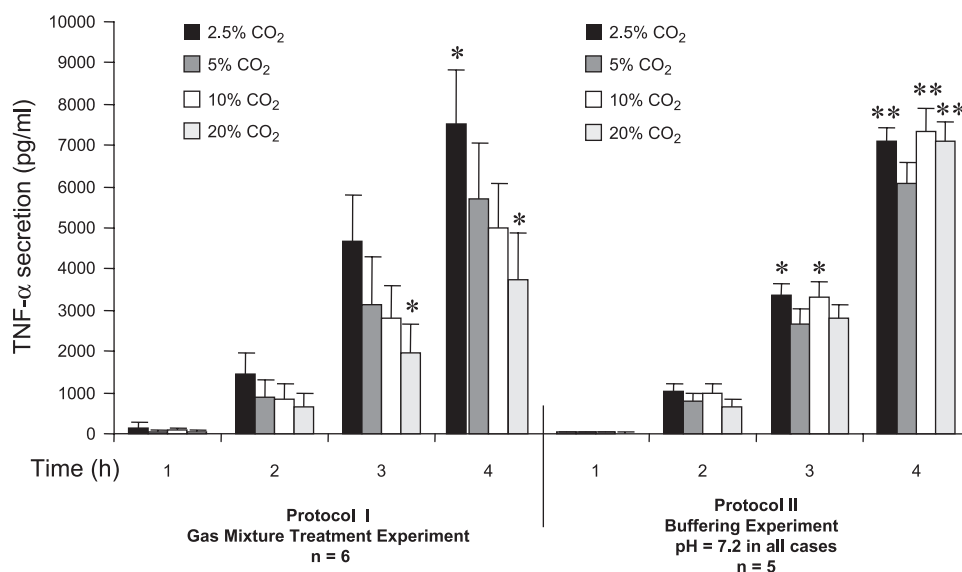


Table 3. Cytokine release expressed as pg $\times 10^{-3}$ per viable cell after 4-h incubations

%CO ₂	TNF- α Secreted [†]	Lysate TNF- α	CINC-1 Secreted
2.5	55.1 (9.80)*	18.6 (3.71)	37.3 (11.35)
5	41.8 (9.92)	17.2 (3.55)	27.7 (12.67)
10	36.6 (7.98)	18.2 (3.51)	34.4 (10.00)
20	27.3 (8.03)*	19.6 (4.63)	38.4 (5.83)
Protocol II: buffering experiment (pH = 7.2 in all cases)			
%CO ₂	TNF- α Secreted [†]	Lysate TNF- α [†]	CINC-1 Secreted [†]
2.5	52.2 (2.30)‡	19.5 (1.13)	38.8 (11.39)
5	44.4 (3.90)	17.7 (1.45)	40.2 (5.51)
10	53.9 (3.84)‡	21.6 (2.38)‡	57.0 (5.30)*
20	52.1 (3.42)‡	21.9 (1.69)‡	59.7 (1.93)*

Significant influence of CO₂ (1-way ANOVA, [†] $P < 0.01$). *Significant difference from 5% CO₂ (Dunnett's test; * $P < 0.05$; ‡ $P < 0.01$).

Protocol II. There was also a significant effect of incubation time and %CO₂ on LPS-stimulated TNF- α secretion in buffered media (Fig. 1). However, in media buffered to pH 7.2, the CO₂ effect was not dose responsive. Buffering the cell culture media abated the inhibitory effect of higher CO₂ concentrations on TNF- α secretion that we observed in protocol I (Fig. 1), such that after 4 h, TNF- α secretion was significantly higher at 2.5, 10, and 20% CO₂ gas mixtures than that observed at 5% CO₂ (Table 3 and Fig. 1).

Protocol III. The effect of CO₂ on LPS-stimulated TNF- α secretion in AM was reversible (Fig. 2). A similar pattern of LPS-stimulated TNF- α secretion was observed in both the nonaccumulative (1st and 2nd incubations) and accumulative gas-switching experiments (Fig. 2). It should be noted that in experiments where the culture media were changed after the first incubation, for the 2.5% CO₂ concentration there was a slight increase in the amount of TNF- α secreted in the second incubation period, compared with the first incubation period, even when gas mixture was kept the same. The amount of

IL-1 β measured in cell supernatants was negligible in all cases (data not shown).

Lysate TNF- α

Protocol I. There was relatively little TNF- α [107 pg/ml (SD 10) from all experimental groups] measured in cell lysates in the absence of LPS. In LPS-stimulated AM, lysate TNF- α was influenced by incubation time, but not CO₂, in normal media (Fig. 3).

Protocol II. Incubation time and CO₂, using a two-way ANOVA, did statistically influence lysate TNF- α levels in LPS-stimulated AM cultured in buffered media. However, the statistical influence of CO₂ on lysate TNF- α levels was not dose responsive, and lysate TNF- α was only significantly higher in the 10 and 20% CO₂ groups when calculated as pg/viable cell (Table 3) and not as pg/ml (Fig. 3). However, although the increases in lysate TNF- α between 5% CO₂ and 10 and 20% CO₂ groups are small (6–13%), they are consistent at 2-, 3-, and 4-h time points.

Protocol III. Protocol III supports observations made in protocol I, where there were no differences in lysate TNF- α levels between cells incubated in 2.5 or 20% CO₂ for both incubation periods in normal media. However, when cells were exposed to a change in CO₂ between the first and second incubation periods, there were small, but significant, changes measured in TNF- α in the cell lysates (Fig. 4).

CINC-1 Secretion

Protocol I. There was no appreciable CINC-1 secretion in the absence of LPS. LPS-stimulated secretion was not influenced by incubation time or CO₂.

Protocol II. CO₂, but not incubation time, influenced CINC-1 secretion in buffered media (Fig. 5). In buffered media, CINC-1 secretion was 35–40% higher in 10 and 20% CO₂ groups than in the 5% CO₂ groups.

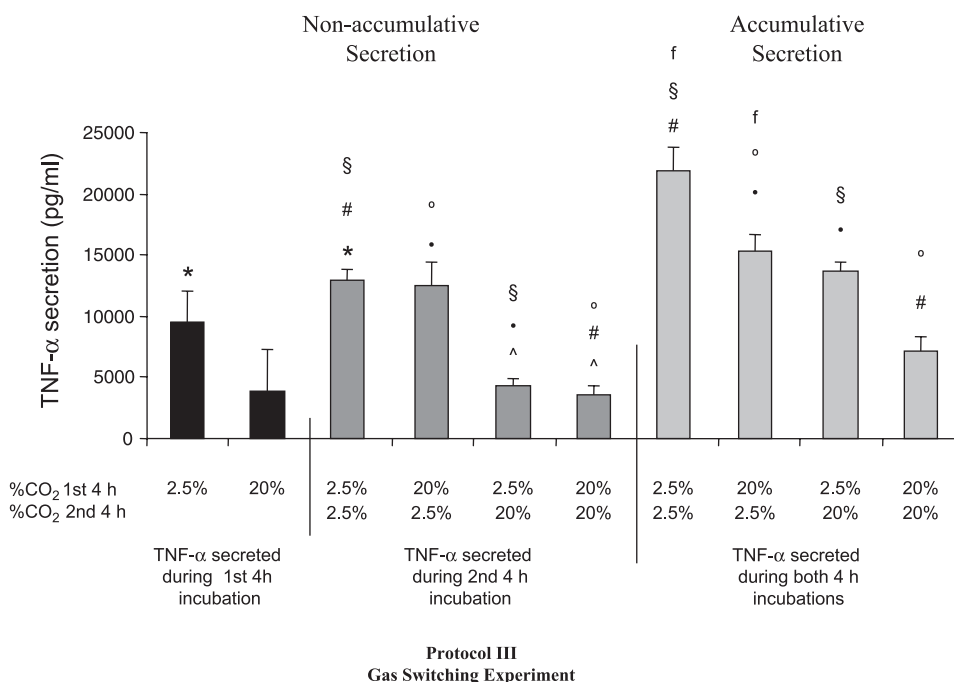
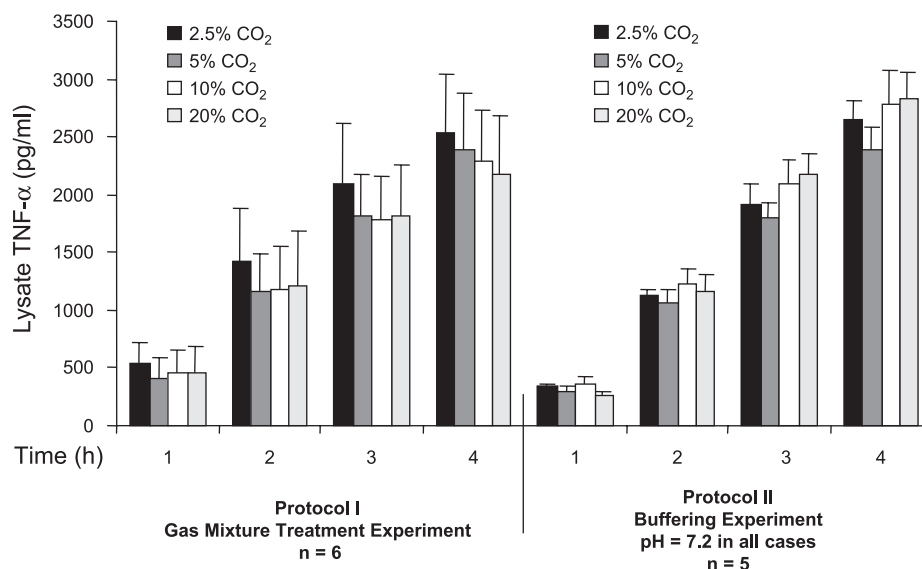


Fig. 2. Effect of CO₂ on LPS-stimulated TNF- α secretion from AM is reversible. In nonaccumulative gas-switching experiments, media were collected after a first 4-h incubation at either 2.5 or 20% CO₂ (1st incubation) and replaced with fresh media, which were then collected after a second 4-h incubation period at the same or different CO₂ (2nd incubation). In accumulative gas-switching experiments, AM were incubated in 2.5 or 20% CO₂ for a first 4-h incubation period and then kept under the same or opposite CO₂ for a second 4-h incubation period, and the media were collected at the end of both incubations (see Table 1). TNF- α levels were measured in culture media using ELISA (Pharmingen Opt EIA). $N = 4-6$. *Significant difference from first 4-h incubation (paired t -tests; $P < 0.05$); ^, #, ●, ○, §, and f, significant differences between pairs of symbols (paired t -tests; $P < 0.05$) within each experiment.

Fig. 3. Effect of CO₂ on lysate TNF- α levels in LPS-stimulated AM after 1- to 4-h incubation in protocols I and II. TNF- α was measured in cell lysates using an ELISA (Pharmingen Opt EIA). $N = 5$ or 6. Protocol I: time ($F = 100.9$, $P = 0.0$), but not CO₂ ($F = 1.813$, $P = 0.15$), influenced lysate TNF- α (2-way ANOVA). Protocol II: time ($F = 873.4$, $P = 0.0$) and CO₂ ($F = 9.7$, $P = 0.0$) influenced lysate TNF- α (2-way ANOVA). Post hoc comparisons showed no significant differences from 5% CO₂ at any time point (Dunnett's post hoc tests, P always > 0.06).



Protocol III. When cells were exposed to a change in CO₂ in the gas-switching experiments, there were no differences in CINC-1 secretion between experimental groups (Fig. 6). Note that levels of CINC-1 in cell lysates (both in the presence and absence of LPS) were too low for accurate measurement using our in-house assay.

DISCUSSION

The Model

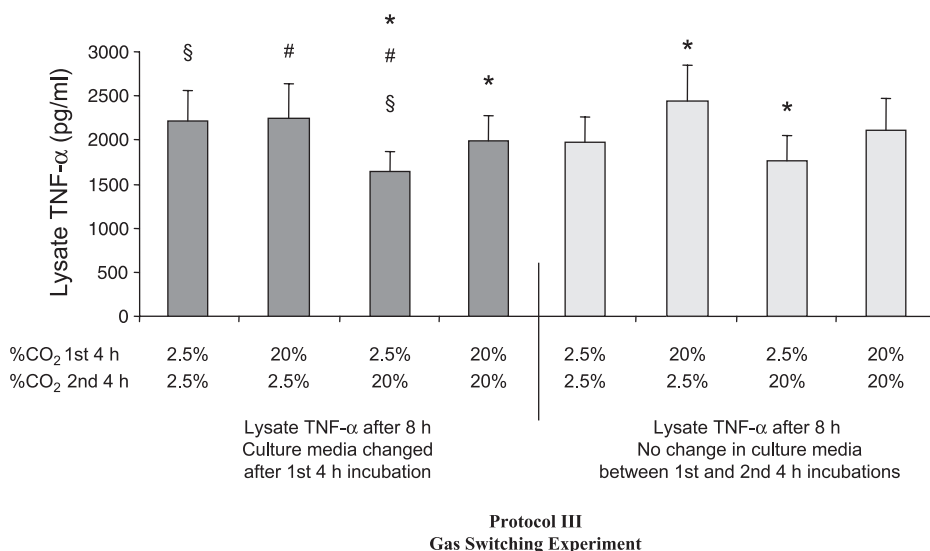
The aim of this study was to determine the impact of CO₂ on AM cytokine release at a cellular level, and although these experiments have arisen from current physiological debate surrounding the beneficial effects of hypercapnia in ALI, the impact of hypocapnia on AM function also remains to be determined. Hence, although they are not usually observed in ventilated patients in vivo, we have also included 2.5 and 20% CO₂ gas mixtures in this study. Concentrations of 2.5 and 20% CO₂ may be pharmacologically relevant. Moreover, AM would be exposed to a range of pH and CO₂ levels in vivo, depending

on their location in the alveoli and the injury state of different regions in the lung. For the buffering experiments, a pH of 7.2 was chosen since it was the pH recorded for culture media under our lowest gas mixture (2.5% CO₂). In hindsight, a pH of 6.8 (i.e., that at 5% CO₂) may have been more appropriate, from a physiological perspective, to use in protocols II and III, since in vivo, the pH of the ELF has been estimated at pH 6.7–6.9 (26). Nevertheless, neutralizing the pH of the culture media did significantly alter cytokine patterns in response to different CO₂ levels, suggesting that pH does contribute to the cytokine responses we observed here.

Cell Viability and Metabolic Activity

High CO₂ reduced metabolic activity but did not significantly influence cell viability. Conversely, low CO₂ increased metabolic activity. These observations are plausible, since metabolic activity is likely to be affected by changes in pH, to a greater extent than, or at least prior to, detectable changes in cell viability. Both the 60% decrease in metabolic activity

Fig. 4. Lysate TNF- α in LPS-stimulated AM in gas-switching experiments. AM were incubated in 2.5 or 20% CO₂ for a first 4-h incubation period and then kept under the same or opposite CO₂ for a second 4-h incubation period, with or without a change in media. Cells were lysed at the end of the 8-h incubation period. TNF- α levels were measured in culture media using ELISA (Pharmingen Opt EIA). $N = 4$ –6. *, #, §, Significant difference between pairs of symbols (paired t -tests; $P < 0.05$) for the different experiments.



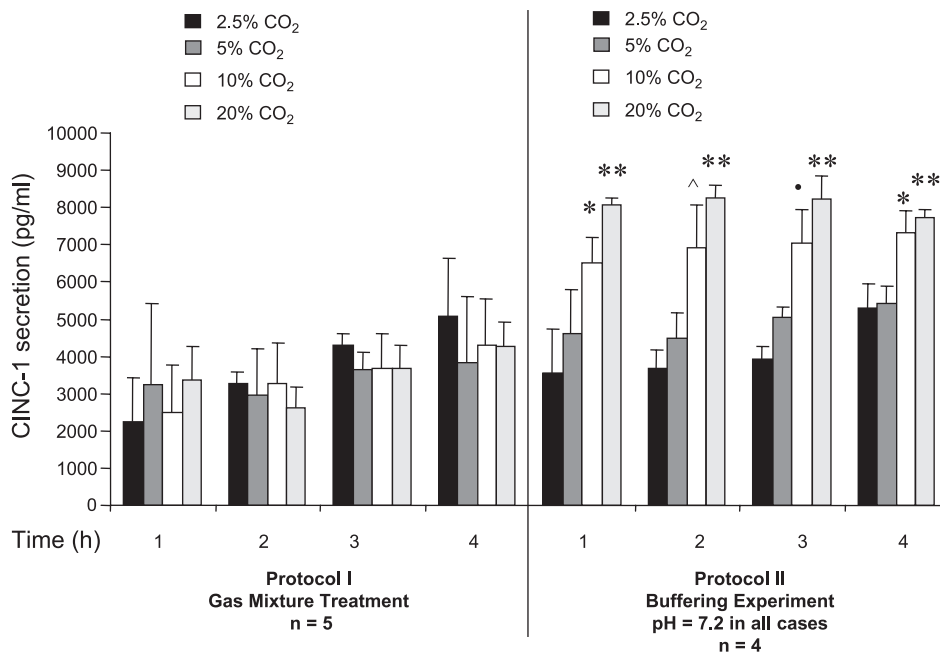


Fig. 5. Effect of CO₂ on LPS-stimulated cytokine-induced neutrophil chemoattractant factor-1 (CINC-1) secretion after 1- to 4-h incubation in protocols I and II. CINC-1 was measured in culture media using an in-house ELISA. Data are presented as pg/ml of CINC-1. $N = 4$ or 5. In protocol I, CINC-1 secretion was not influenced by CO₂ or time (2-way ANOVA). In protocol II, CINC-1 secretion was influenced by CO₂ ($F = 41.6$, $P = 0.0$), but not time ($F = 0.5$, $P = 0.7$) (2-way ANOVA). Significant difference from 5% CO₂ (Dunnett's post hoc test, * $P < 0.05$, ** $P < 0.01$, $\Delta P = 0.059$, $\bullet P = 0.078$).

observed at 20% CO₂ and the 14% increase in metabolic activity observed at 2.5% CO₂ were abated when the media were buffered to pH 7.2. This suggests that CO₂-mediated changes in pH may play a pivotal role in the alteration of cell metabolic activity. The data cannot be completely explained by CO₂-induced changes in metabolic activity, since a significant deterioration of metabolic activity was only apparent at 20% CO₂. Twenty percent CO₂ has also been observed as the cut-off level for significant deterioration in lung viability in vivo (17).

TNF- α and IL-1 β

We demonstrate that high CO₂ attenuates secretion of TNF- α from LPS-stimulated AM but has no effect on lysate TNF- α in AM incubated in normal medium. Conversely, low CO₂ enhanced both lysate and secreted TNF- α in

LPS-stimulated AM cultures. The increases we observed in TNF- α secretion at low CO₂ may be due, at least in part, to increases in cell metabolic rate, since metabolic activity also increased in AM incubated at low CO₂. Buffering the media abated the inhibitory effects of high CO₂ on TNF- α secretion, suggesting that this inhibitory response to CO₂ is modulated by extracellular pH (pH_e). Studies by Bidani et al. (4) and Heming et al. (10, 11) support this finding. The small increase in lysate TNF- α levels we observed in AM incubated in buffered media, at high CO₂, could be related to the retention of synthesized TNF- α , in a similar manner to that suggested by Bidani et al. (4) and Heming et al. (10). We found no appreciable IL-1 β in the cell culture media, regardless of the presence or absence of LPS, or CO₂. This finding is consistent with the observation that IL-1 β lacks the signal

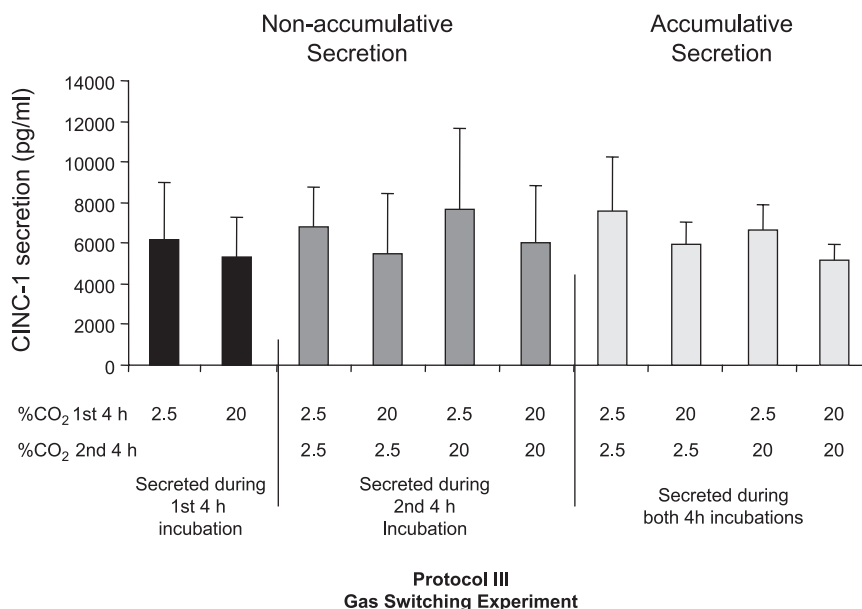


Fig. 6. CINC-1 secretion in gas-switching experiments (protocol III). AM were incubated as described in Fig. 2. CINC-1 levels were measured in cell lysates using an in-house ELISA. $N = 4-6$. There was no significant difference in CINC-1 secretion between groups for any of the experiments (paired t -tests; $P > 0.05$ in all cases).

sequence necessary to translocate the protein to the endoplasmic reticulum for secretion (1).

CINC-1

Incubation time did not influence CINC-1 secretion, suggesting that CINC-1 release is immediate and rapid after LPS-induced AM activation. We observed no effect of CO₂ on the release of CINC-1 from AM incubated in normal media, suggesting that neither CO₂ nor CO₂-induced changes in pH_e influence CINC-1 secretion in these cells. However, given the pH-mediated effects CO₂ had on AM metabolic activity and TNF- α secretion, as well as the intrinsic role CO₂ is known to play in cellular acid-base status (21), this conclusion seems unlikely. We suggest that CO₂ plays a role in regulating CINC-1 release independently of pH, since in buffered media, where the pH was kept constant, increases in CO₂ significantly enhanced CINC-1 secretion from AM. In AM incubated in normal media, the stimulatory effect of this non-pH-mediated mechanism may have been counteracted by decreases in metabolic rate. Nevertheless, from a physiological perspective, the ELF is thought to have little buffering capacity (26). Hence, normal in vivo patterns of CINC-1 secretion from AM in response to hypercapnia are more likely to mimic those of cells grown in normal culture media where high CO₂ has no effect on CINC-1 secretion. However, since increases in CINC-1 could lead to an accumulation of inflammatory cells into the lung, our observations in buffered media could explain, at least in part, the reduction in the protective effects of hypercapnic acidosis with buffering, previously observed in vivo (15).

Possible Mechanisms for CO₂-Mediated Cytokine Responses in AM

The effects of CO₂ on TNF- α secretion in AM were abated when the media were buffered, indicating that the effects of CO₂ on TNF- α are, at least in part, pH mediated. Both TNF- α and IL-1 β production in LPS-activated monocytes have been reported to increase in concert with a rise in intracellular pH (pH_i), mediated via the influx of Na⁺ by Na⁺/H⁺ ion exchangers (27). Similarly, hypoxia-induced decreases in pH_i are associated with enhanced activation of NF- κ B and increased expression of intracellular adhesion molecule (35). Hypercapnia, probably via changes in cellular acid-base status (2), increases nitric oxide production and the nitration of proteins (20). Furthermore, hypercapnic acidosis can attenuate LPS-stimulated NF- κ B activation and the lung's inflammatory response by the inhibition of I κ B- β degradation (32) and endogenous xanthine oxidase (30), respectively. Either one, or all, of these mechanisms may contribute to the effects of CO₂ described in this study. Further studies should identify more precise mechanisms of CO₂-induced responses in primary cultures of rat AM.

In conclusion, this study provides evidence for differential effects of CO₂ on TNF- α and CINC-1 in rat AM. Buffering experiments demonstrate that cellular acid-base status mediates the effects of CO₂ on cytokine secretion in AM, at least in part, by modulating metabolic activity. Because TNF- α is a key cytokine in the initiation of lung inflammation (3), it is possible that the 50% decrease we observed in TNF- α secretion in response to high CO₂ could lead to reductions in lung injury in ventilated patients. However, caution must be taken before

concluding that hypercapnia is beneficial in ALI, because the effects of CO₂ on inflammatory mediators are differential and may differ between lung cell types. In AM, the increase in CINC-1 in response to CO₂, albeit in the presence of high bicarbonate, may be cause for concern. Moreover, many other regulatory mechanisms and cell interactions occur in vivo that may modify the AM responses we observed here in vitro.

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