Ambient pCO_2 modulates intracellular pH, intracellular oxidant generation, and interleukin-8 secretion in human neutrophils

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Abstract: Although neutrophils are a critical component of the inflammatory process, their functional regulation is incompletely understood. Of note, although pCO_2 varies physiologically and pathologically in the neutrophilic milieu, its affect on neutrophil biological processes is unresolved. We demonstrate here that neutrophils respond to hypo- and hypercarbia, (0.04% and 10%) by increasing and decreasing, respectively, intracellular oxidant production (basally and in response to opsonized *Escherichia coli* and phorbol esters). Further, hypo- and hypercarbia increase and decrease, respectively, the release of IL-8 from LPS-stimulated cells; both effects are attenuated by the carbonic anhydrase inhibitor, acetazolamide. Anion exchange did not restore pH_i under hypocarbic conditions, however partial restoration of pH_i under hypercarbic conditions was achieved by Na^+/H^+ exchange and vacuolar ATPases. Abrogation of pCO_2-induced changes in pH_i prevented hypocarbia-induced generation of reactive oxidant species. These observations suggest that CO_2 modifies neutrophil activity significantly by altering pH_i. *J. Leukoc. Biol.* 71: 603–610; 2002.

Key Words: *hypocarbia · hypercarbia · acetazolamide · HCO_3^-*

INTRODUCTION

The neutrophil is the dominant cell in the acute inflammatory response and is a critical component of the host's reaction to invading microorganisms [1]. Specific aspects of this response include the generation of toxic oxygen radical species and the production of immunomodulatory molecules such as interleukin (IL)-8 [2, 3]. However, the manner in which such processes are regulated is undefined. Paradoxically, although central to the inflammatory response, neutrophils are implicated in the pathogenesis of diseases, such as emphysema [4], adult respiratory distress syndrome [5], and cystic fibrosis [6], where they appear to be regulated inappropriately. Given the remarkable, functional changes accompanying neutrophilic activation and the requirement of these cells to function in a milieu whose composition and character deviate substantially from the physiological norm, the generally appropriate regulation of their activities is remarkable. However, our understanding of these regulatory processes is incomplete.

Neutrophilic inflammation may be modulated not only by activation of cell-surface receptors and signal-transduction cascades but also by aspects of the prevailing milieu [7, 8]. These latter influences include temperature, ionic, and gaseous composition [9–11]. The neutrophil is likely to be exposed to variable CO_2 tensions under physiological and pathological conditions, and this may contribute significantly to the regulation of its cellular function. Although homeostatic mechanisms exist to regulate systemic CO_2 tensions tightly, altered CO_2 levels are noted acutely and chronically in vivo. In disorders of respiratory gas exchange, hypercarbia may result, for example, in patients with chronic obstructive pulmonary disease [12]. In enclosed, poorly perfused spaces, such as abscess cavities, metabolic activity may also elevate pCO_2 [13]. In contrast, during the hyperventilatory phase of conditions, such as pulmonary embolic disease, bronchopulmonary dysplasia, and asthma, as well as during mechanical ventilation in patients with respiratory failure, systemic hypocarbia may be manifest [14–17]. Cells in the thin surface liquid of the (primarily proximal) airways may be exposed to lower mean CO_2 tensions than systemic values, as a result of the contact of the thin-surface liquid layer with respiratory gases, whose composition at some phases of the respiratory cycle approximates those of the atmosphere [18]. The same is likely to be true in internal cavities during surgical procedures [19, 20].

Although there is evidence in support of a role for CO_2 in the modulation of activities of mammalian cells, little available data characterize its effects on neutrophil function. Of note, in human neutrophils, in vitro, hypocarbic alkalization of the culture media has been correlated with alterations in adhesion-molecule expression and attachment to endothelial cells, although the mechanism of these effects was not defined [21]. Changes in pCO_2 have been associated with alterations in effector function in other mammalian cells. Exposure to air versus 100% CO_2 increases lipopolysaccharide (LPS)-induced

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tumor necrosis factor α (TNF-α) and IL-1 production from peritoneal macrophages [22].

These diverse effects on cellular function are rational because the cell membrane is readily permeable to CO_2, and in turn, alterations in CO_2 may alter intracellular pH (pH_i), which has the capacity to modulate biological processes. Indeed, many substances known to affect neutrophil function elevate pH_i specifically, suggesting that the change in CO_2/pH may act to modulate effector processes. Together, these studies suggest that CO_2 may be a molecular species with significant, bioregulatory properties. Surprisingly, studies to test this hypothesis in neutrophils are lacking, representing a significant gap in our knowledge of the cell's biology. In particular, it is not clear to what extent the neutrophil pH homeostatic mechanisms compensate for potential pCO_2-induced alterations in pH_i or to what extent such changes depend on extracellular pH. The aim of these experiments was to characterize the effects of hyper- and hypocarbia on intracellular oxidant generation and IL-8 release and to correlate these changes with alterations in pH_i. We demonstrate that slow alterations in the pCO_2 of the culture medium are associated with changes in pH_i for which the cell compensates poorly. Subsequent changes in oxidant and IL-8 production by neutrophils were also observed.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma Chemical Co. (Poole, Dorset, UK) unless otherwise stated.

Isolation and culture of neutrophils

Neutrophils were isolated from heparinized, peripheral venous blood of normal, healthy donors. Blood was layered onto a cell-separation medium (Ficoll-Paque, research grade, Amersham Pharmacia Biotech, Uppsala, Sweden) and was centrifuged for 30 min at 1250 rpm. The neutrophil-rich layer was mixed with 3% dextran-saline solution in the presence of autologous plasma to sediment red blood cells. Remaining erythrocytes were then subjected to brief hypotonic lysis (0.2% saline) twice, after which the cells were washed twice, counted, and resuspended in RPMI 1640 (1×10^6/ml) containing 25 mM $NaHCO_3$ and were supplemented freshly with L-glutamine 0.3 g/L and buffered with Bufferall, according to the manufacturer's instructions. The neutrophil population was at least 95% pure by morphological assessment of cytospin preparations. The initial, external pH is kept constant by the use of Bufferall, which has greater buffering capacity than phosphate- and carbonate-based buffering systems. Each experiment was carried out in conjunction with cells that were loaded with the fluorescent probe, carboxy-SNARF (Molecular Probes Europe, Leiden, Holland), and were incubated without stimulation. These cells maintained a very constant pH (pH_i did not deviate by more than 0.01 of a pH unit during a 120-min time course). Therefore, it is unlikely that the external pH changed during this time. All experiments were performed within 5 h of isolation of neutrophils.

Measurement of pH_i

pH_i was determined using SNARF, and pH_i in loaded cells was measured using flow cytometry (FACScan, Becton Dickinson, Mountain View, CA), as described previously [23]. Briefly, an in situ calibration was performed on the isolated neutrophils. After loading with SNARF, the cells were washed twice in phosphate-buffered saline (PBS) and were resuspended in HEPES-buffered solutions containing 135 mM KCl and 10 μM nigericin at various pHs between 6.2 and 7.8. The K^+ ionophore nigericin equilibrates H^+ concentration across the outer cell membrane when internal and external K^+ concentrations approximate one another. Once stabilized, the ratio of the emitted fluorescence in the red and blue regions of the spectrum was measured, and a calibration curve was generated. Ratio-metric measurements reduce errors as a result of photobleaching, cell thickness, and instrument stability as well as leakage and nonuniform loading of the indicator. A calibration curve was obtained for pH 6.2–7.8. Differences in starting pH were observed for the different experiments and occurred as a result of subtle pH variations in the calibration buffers used. However, individual experiments were carried out in duplicate under the specific conditions (differing CO_2 tensions in the presence/absence of inhibitors) for each calibration carried out.

At each time point, a minimum of 5000 cells was analyzed. All experiments were carried out on a 1 ml cell suspension at 1×10^6/ml contained in polystyrene flow tubes (Becton Dickinson, Mountain View, CA), and the apertures of the tubes were sealed with parafilm while still in the incubator to prevent loss of CO_2 and were transferred immediately for pH_i analysis. Analysis took 1–2 s from the piercing of the parafilm seal.

The roles of a number of mechanisms of pH_i regulation described were also assessed. These processes include passive proton conductance [via $ZnCl_2$-sensitive reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-associated channels], Na^+-independent and Na^+-dependent HCO_3^-/Cl^- exchangers [sensitive to 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS)], proton-translocating ATPases (sensitive to bafilomycin A_1), and Na^+/H^+ exchange (sensitive to amiloride). In addition, because neutrophils contain carbonic anhydrase, we tested the effect of its inhibitor acetazolamide on pCO_2 effects on pH_i.

Measurement of intracellular oxidant production

Global intracellular oxidant production was assessed by flow cytometry using the fluorescent probes 123-dihydrorhodamine (123-DHR, OrpegenPharma, Heidelberg, Germany) or DHCCH (Molecular Probes Europe). After periods of stimulation with medium alone, phorbol 12-myristate 13 acetate (PMA; 0.5 μM; Molecular Probes Europe) or human immunoglobulin G (IgG)-opsonized *Escherichia coli*, pathogen:phagocyte ratio of 16:1 (OrpegenPharma for Becton Dickinson, Cowley, Oxford, UK) under various experimental conditions, 1 ml cell suspension (1×10^6/ml 37°C), was loaded with 123-DHR or DHCCH for 10 min. Two dyes were assessed to ensure that changes in fluorescence did not reflect unforeseen sensitivity of the dyes to pH_i, although this has not been demonstrated in either case. Precisely at the end of each probe-loading period, an aliquot of sample was treated with a fixative solution containing 10% formaldehyde and sodium azide. Using flow cytometry, neutrophils were gated, the intracellular dyes were stimulated with argon laser light (488 nm), and the emitted fluorescence at 520 nm in at least 10,000 cells was measured. Intracellular oxidant generation was expressed in the arbitrary units of mean channel fluorescence. In all cases, the background fluorescence of cells under each appropriate experimental condition was subtracted from the value shown.

To establish if the buffering capacity of blood would ameliorate any effects of alterations in pCO_2 in neutrophils, intracellular oxidant generation was also assessed in a whole-blood assay. Briefly, 100 μl aliquots of lithium-heparinized whole blood from the same volunteers were incubated for fixed periods with normal saline or the stimulants as described above. The oxidant-sensitive probe was then added, and cells were incubated for 10 min at 37°C. Red cells were then lysed, and white cells were fixed partially [fluorescein-activated cell sorter (FACS) lysing solution, Becton Dickinson, Cowley, Oxford, UK] for 20 min prior to two washing steps in PBS. Finally, in separate experiments, samples were exposed to a permeabilizing solution containing propidium iodide (PI; Becton Dickinson, Cowley, Oxford, UK) to stain DNA. The samples were then analyzed by flow cytometry. At least 10,000–15,000 cells were processed, and neutrophils were differentiated on the basis of size and granularity, as indicated by low-angle, forward-scattering and right-angle, side-scattering properties of argon laser light (488 nm). Mean emitted fluorescence intensity expressed on a four-decade logarithmic scale at 520 nm with constant photomultiplier gain values was quantified as a measure of intracellular oxidant generation. Separate experiments were performed to analyze emitted fluorescence of PI at 520 nm and therefore, to allow the differentiation of viable cells or bacterial clumps on the basis of their DNA content. Only cells with the DNA-binding characteristics of viable mammalian cells were included in the analysis.

Measurement of LPS-induced neutrophil IL-8 release

Neutrophils were cultured at 1.0×10^6/ml in RPMI 1640, supplemented with 10% autologous serum in 10%, 5%, or 0.04 CO_2 for 2 h in the presence of LPS

(*E. coli* 0111:B4; 10 μg/ml). The cells were then sedimented, and the supernatants were stored at −80°C until analysis. Cell supernatant IL-8 was determined in triplicate using a quantitative sandwich enzyme immunoassay technique (R&D Systems, Abingdon, Oxon, UK). No IL-8 was detected in any serum-supplemented media in the absence of neutrophils.

Analysis and presentation of data

All data are presented as mean ± SD of mean. Statistical analysis was performed using GraphPad Prism software on a PC. Linear regression analysis was used for calibration and calculation of pH_i as well as IL-8 measurements. Gaussian distribution of pH_i and IL-8 were tested using the Kolmogorov Smirnov test. Over time, pH_i and IL-8 release were analyzed using repeated measures of analysis of variance (ANOVA) with Bonferroni's post-hoc correction. Differences over time between groups were compared using two-way ANOVA. A P value of <0.05 was used to indicate statistical significance.

The studies were approved by the institutional ethical committee.

RESULTS

The effect of alterations in ambient pCO_2 on cytosolic pH (pH_i) in human neutrophils, role of carbonic anhydrase, and mechanisms of proton translocation

Alterations in ambient pCO_2 were associated with significant changes in pH_i (**Fig. 1**). As demonstrated previously, hypercarbia led to cytosolic acidification, and hypocarbia caused alkalinization of the neutrophilic cytosol [24, 25]. Within 2 min, pH_i had dropped in cells exposed to 10% CO_2 (6.98±0.01 vs. 6.85±0.01) but had increased in cells incubated in 0.04% CO_2 (6.98±0.01 vs. 7.02±0.01). Our data supplement those published previously in that we subjected neutrophils to more gradual changes in pCO_2. By altering the pCO_2 at the liquid/air interface of the culture medium, its CO_2 content and thus, the pCO_2 at the cell membrane changed slowly. This was reflected by slow alterations in pH_i. Unexpectedly, the cell's physiological compensation was absent or incomplete, and pH_i did not return to normal over the course of the study period in response to 0.04% (7.26±0.01, 130 min) or 10% pCO_2 (6.86±0.01, 130 min). However, pH_i did return to normal over time when cells were returned to 5% CO_2 (unpublished results). This result was surprising and unexpected. Although differences in pH_i appear small quantitatively, they represent an 80–100% difference in intracellular (H^+) concentrations. pH_i remained stable in 5% CO_2-exposed cells throughout the period of all the experimental protocols. To ensure that SNARF did not leak from the cells during the incubation in 0.04 or 10% CO_2, fluorescence measurements were carried out on the medium and revealed that SNARF had not leaked from the cells during this period. In addition, the pH of the strongly buffered media was not altered significantly by alterations in pCO_2. pH_i was altered significantly when cells were subjected to the various stimuli used in the studies, and values were always more acidic under hypercarbic conditions and more alkaline under hypocarbic conditions (unpublished results). Under hypocarbic conditions, pH_i was more alkaline in resting cells and cells activated with PMA or *E. coli*. Inhibition of carbonic anhydrase attenuated pCO_2-induced alterations in pH_i (Fig. 1; 0.04% CO_2 vs. 0.04% CO_2+acetazolamide, 7.09±0.005 vs. 7.00±0.02, 20 min; 10% CO_2 vs. 10% CO_2+acetazolamide, 6.78±0.01 vs. 6.87±0.01, 20 min).

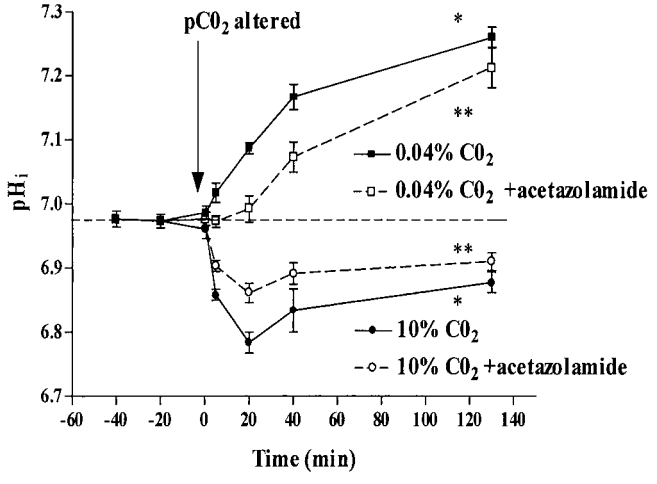

Fig. 1. The effect of pCO_2 on pH_i in normal neutrophils and its modulation by acetazolamide. pH_i was measured in neutrophils from normal human donors ($n=10$) using flow cytometry with the fluorescent probe C-SNARF. Cells were equilibrated initially in 5% CO_2 and exposed to 5%, 0.04%, or 10% pCO_2 subsequently, and resultant changes in pH_i were monitored. (pH_i was assessed in at least 10,000 cells from a 1 ml suspension at 1×10^6/ml). pH_i in cells exposed to 5% CO_2 was stable and is represented as a horizontal, dotted line. Alterations in pCO_2 led to monomorphic patterns of pH_i adjustment. Changes in pH_i under hypo- and hypercarbic conditions were attenuated significantly in the presence of acetazolamide (1 mM). *, $P < 0.05$ versus 5% CO_2; **, $P < 0.05$ acetazolamide treated versus untreated.

The presence of inhibitors of HCO_3^-/Cl^- exchange (DIDS, 0.5 mM) and passive proton conductance ($ZnCl_2$, 50 μM) did not alter pH_i in cells cultured in 5% CO_2 (5% CO_2 vs. 5% CO_2+DIDS vs. 5% CO_2+$ZnCl_2$, 7.02±0.01 vs. 7.02±0.00 vs. 7.03±0.01, respectively, at 20 min), suggesting that these pH regulatory mechanisms do not participate in maintenance of resting pH_i. However, singly and in combination, these inhibitors significantly abrogated the rise in pH_i in 0.04% CO_2-exposed cells (see **Fig. 2A**; 0.04% CO_2 vs. 0.04% CO_2+$ZnCl_2$ vs. 0.04% CO_2+DIDS, 7.23±0.01 vs. 7.14±0.00 vs. 7.12±0.01, respectively, at 30 min). In the presence of both, alkalization was inhibited fully (7.05±0.01, 30 min). The extent of alkalization was not altered by inhibition of proton-translocating ATPases (bafilomycin A_1, 100 nM) or by inhibiting Na^+/H^+ exchange (amiloride, 0.1 mM; unpublished results). This suggests that hypocarbic alkalization in neutrophils requires HCO_3^- influx as well as proton efflux through $ZnCl_2$-sensitive, passive proton conductance channels. The activity of the latter channels is initiated in association with activation of NADPH oxidase and the generation of intracellular oxidants, suggesting that hypocarbia may be associated with an enhanced respiratory burst in neutrophils. Under hypercarbic conditions, cells underwent cytosolic acidification from which they partially recovered (Fig. 2B; 6.92±0.02, 30 min). This recovery was impeded significantly in the presence of amiloride (0.1 mM; 6.87±0.00, 30 min) and bafilomycin A_1 (100 nM; 6.83±0.01, 30 min). It was not affected by DIDS or $ZnCl_2$ (not shown).

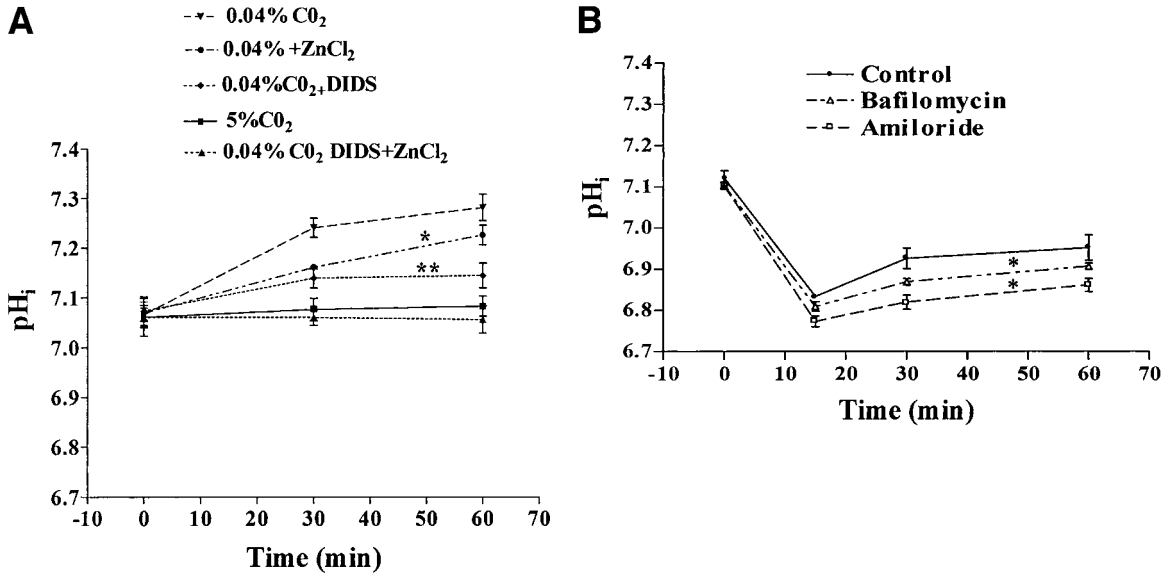

Fig. 2. Compensatory pH_i regulatory mechanisms during pCO_2-induced alterations in pH_i. (A) pH_i was measured in neutrophils (isolated from normal donors, $n=10$) exposed to 0.04% or 5% CO_2 in the presence or absence of DIDS (0.5 mM), $ZnCl_2$ (50 μM), or the two inhibitors in combination. In the absence of the inhibitors, pH_i was stable and failed to alter pH_i in cells exposed to 5% CO_2 but abrogated pH_i changes under hypocarbic conditions. *, $P < 0.05$ 0.04% CO_2 + $ZnCl_2$ versus 0.04% or 5% CO_2; **, $P < 0.05$ 0.04% CO_2 + DIDS versus 0.04% or 5% CO_2. (B) pH_i was measured in neutrophils (isolated from normal donors, $n=10$) exposed to 5% or 10% CO_2 in the presence or absence of bafilomycin A_1 (100 nm) or amiloride (0.1 mM). Neither compound affected pH_i in 5% CO_2-exposed cells (not shown). Both inhibited the incomplete recovery of pH_i in these cells. *, $P < 0.05$ versus control.

The effect of a reduction in ambient pCO_2 on intracellular oxidant generation in neutrophils

We measured intracellular oxidant generation because the activity of superoxide-generating NADPH oxidase is known to be pH-sensitive [26, 27], and we have demonstrated that hypocarbia alters pH_i. Similar qualitative results were obtained with oxidant-sensitive dye, and therefore, only results pertaining to cells loaded with 123-DHR are presented. The basal level of intracellular oxidant generation is low after 60 min in unstimulated, isolated neutrophils exposed to 5% CO_2 (see **Fig. 3A**) but is increased significantly following stimulation with *E. coli* (Fig. 3B) or PMA (Fig. 3C). However, oxidant generation was significantly higher under hypocarbic conditions in unstimulated and neutrophils stimulated with PMA

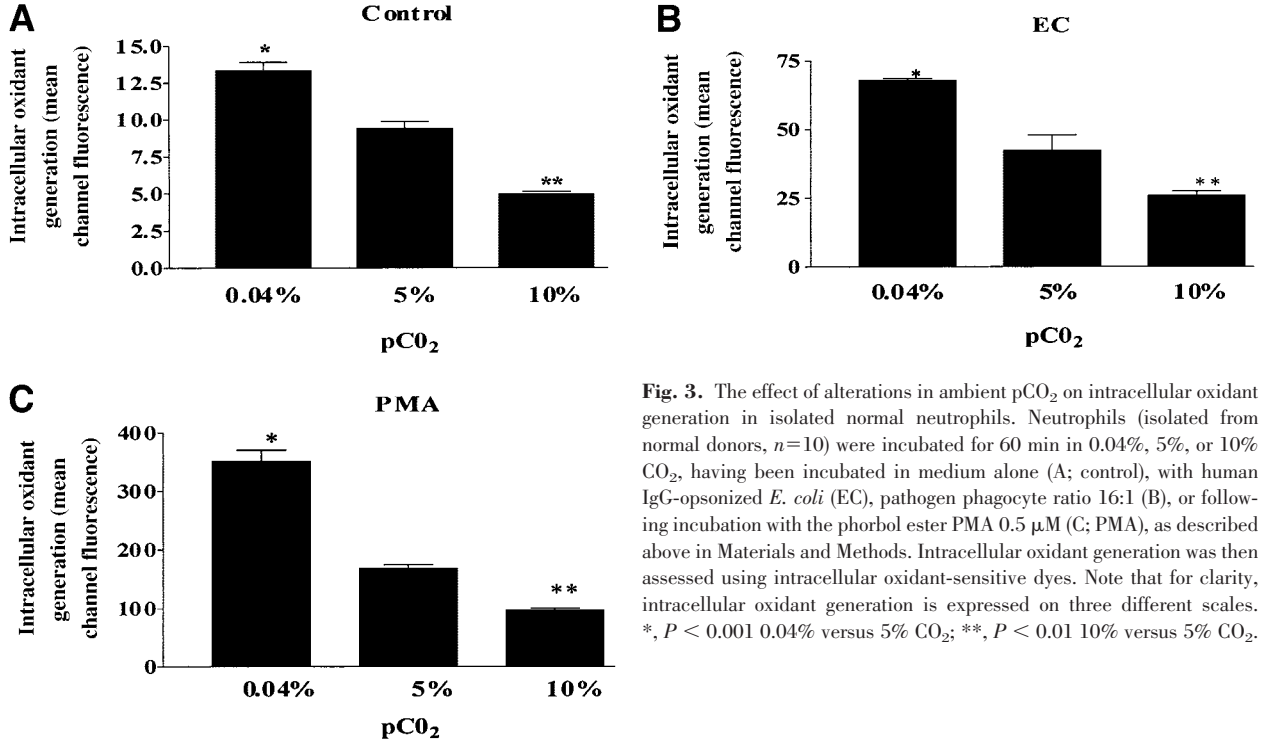

Fig. 3. The effect of alterations in ambient pCO_2 on intracellular oxidant generation in isolated normal neutrophils. Neutrophils (isolated from normal donors, $n=10$) were incubated for 60 min in 0.04%, 5%, or 10% CO_2, having been incubated in medium alone (A; control), with human IgG-opsonized *E. coli* (EC), pathogen phagocyte ratio 16:1 (B), or following incubation with the phorbol ester PMA 0.5 μM (C; PMA), as described above in Materials and Methods. Intracellular oxidant generation was then assessed using intracellular oxidant-sensitive dyes. Note that for clarity, intracellular oxidant generation is expressed on three different scales. *, $P < 0.001$ 0.04% versus 5% CO_2; **, $P < 0.01$ 10% versus 5% CO_2.

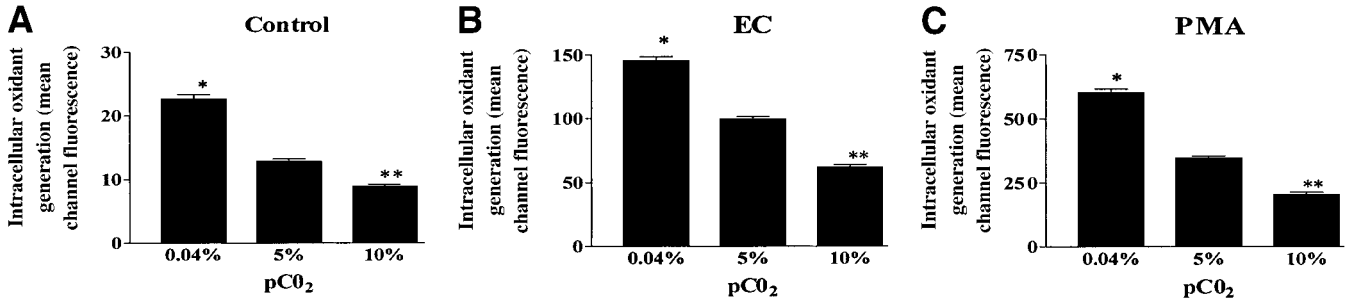

Fig. 4. The effect of alterations in ambient pCO_2 on intracellular oxidant generation in neutrophils from whole blood. Aliquots of whole blood (normal donors, $n=10$) were incubated alone (A; control), with human IgG-opsonized *E. coli*, pathogen phagocyte ratio 16:1 (B; EC), or with the phorbol ester PMA 0.5 μM (C; PMA) for 60 min as described above in Materials and Methods. Following loading with oxidant-sensitive dye, fixation, and red-cell lysis, intracellular oxidant generation was than assessed by flow cytometry with identification of neutrophils on the basis of characteristic light-scattering properties. Note that for clarity, intracellular oxidant generation is expressed on three different scales. *, $P < 0.001$ 0.04% versus 5% CO_2; **, $P < 0.01$ 10% versus 5% CO_2.

and opsonized *E. coli* and was suppressed under hypercarbic conditions in basal and stimulated states. A similar pattern of pCO_2 dependence was observed when assaying intracellular oxidant generation in a whole-blood assay (**Fig. 4, A–C**).

This is in agreement with other studies of the effect of DIDS on oxidant generation. The presence of PMA at the concentration used in these experiments was associated with no significant alteration in pH_i in 5% CO_2-exposed cells, reflecting a balance between proton extrusion and metabolic acid generation at this level of activation (unpublished results). However, pH_i was higher in 0.04% CO_2-exposed samples (unpublished results).

The effect of inhibition of HCO_3^-/Cl^- exchange and passive proton conductance on augmented, intracellular oxidant generation in neutrophils exposed to hypocarbia

Under our experimental conditions, we have established that hypocarbic alkalinization in neutrophils is abrogated in the presence of DIDS and $ZnCl_2$. We measured oxidant generation in the presence of both inhibitors to establish if prevention of the change in pH_i would prevent the elevation of oxidant production. $ZnCl_2$ or DIDS had no effect on intracellular oxidant generation in 5% CO_2-exposed neutrophils following activation. For unstimulated cells in the presence of both inhibitors, oxidant production in 0.04% CO_2-exposed cells was similar to 5% CO_2-exposed cells (**Fig. 5A**). In PMA-treated cells, the presence of both inhibitors prevented hypocarbia-induced enhancement of oxidant production (Fig. 5B). These data support the assertion that the alteration of pH_i in hypocarbia-exposed cells mediates the observed increase in oxidant production.

The effect of reduction in ambient pCO_2 on release of IL-8 from neutrophil and its modulation by acetazolamide

Consistent with previous literature, we observed significant IL-8 release from neutrophils over the 2-h study period. IL-8 production was sensitive to CO_2 tension in a manner similar to mononuclear cells. Exposure to 0.04% CO_2 versus 5% CO_2 led to a significant increase in IL-8 release from LPS-activated neutrophils at 2 h, and 10% CO_2 produced a contrasting effect. In the presence of acetazolamide, differences in IL-8 release were attenuated (**Fig. 6**).

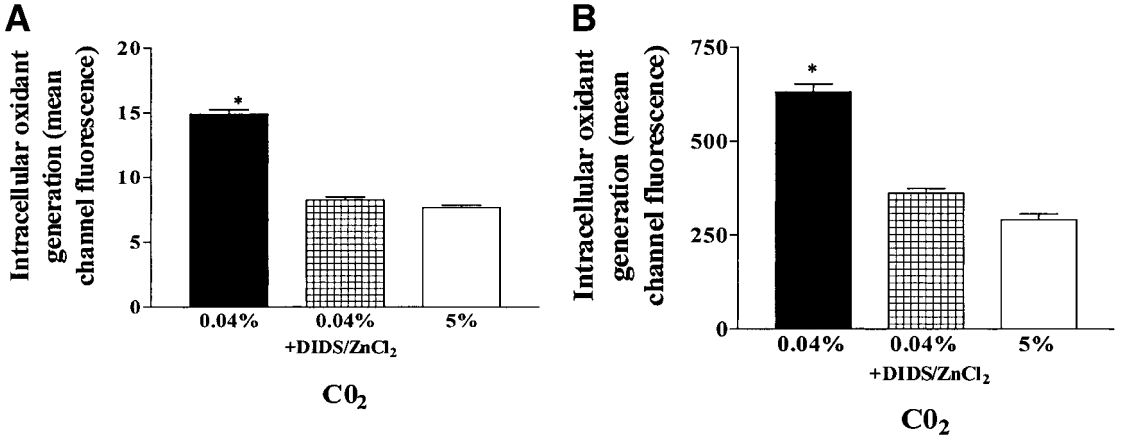

Fig. 5. Abrogation of hypocarbia-induced elevation in pH_i prevents alteration in intracellular oxidant generation. Isolated neutrophils (normal donor, $n=8$) were incubated under normocarbic, hypocarbic, or hypocarbic + DIDS (0.5 mM) + $ZnCl_2$ (50 μM) for 60 min, and intracellular oxidant generation was assessed with an oxidant-sensitive dye, as described in Materials and Methods. Intracellular oxidant generation was measured in (A) basal- and (B) PMA-activated states. The presence of DIDS + $ZnCl_2$ prevented the hypocarbia-induced increase in intracellular oxidant generation.

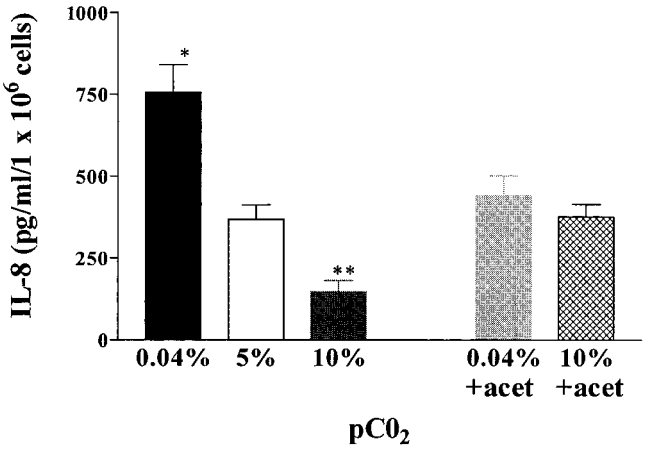

Fig. 6. The effect of pCO_2 on IL-8 release from LPS-stimulated, normal neutrophils and its modulation by acetazolamide (acet). Neutrophils isolated from normal donors ($n=10$) were cultured in the presence of 10% autologous serum and stimulated with LPS (10 μg/ml). They were incubated in 0.04%, 5% or 10% CO_2 for 2 h in the presence or absence of acetazolamide (1 mM). IL-8 levels in supernatants were determined by enzyme-linked immunosorbent assay. CO_2 suppressed IL-8 release, but acetazolamide (which abrogates the pCO_2-induced change in pH_i) eliminated pCO_2-induced changes in IL-8 release. *, $P < 0.05$, 0.04% versus 5% CO_2; **, $P < 0.05$, 5% versus 10% CO_2, 0.04% CO_2 + acetazolamide and 10% CO_2 + acetazolamide = not significant versus 5% CO_2.

DISCUSSION

Our results suggest that changes in pCO_2 are associated with significant alterations of diverse, critical effector responses in human neutrophils, including the production of IL-8 and the generation of intracellular oxidants. These effects appear to be mediated by changes in pH_i and are independent of alterations in extracellular pH. Such observations support the role of pH_i in the modulation of neutrophil function [28, 29]. The elevation in pH_i we observed in hypocarbia-exposed neutrophils is of similar magnitude to that associated with activation of neutrophils by cytokines or bacterial products. These results are likely to be relevant in vivo, because experiments were performed over pCO_2 ranges encountered physiologically and pathologically.

Our observation of altered neutrophil function in response to ΔpCO_2 is consistent with results that alterations in pCO_2 can also affect cellular function in other mammalian cells. Activity of neutrophils requires tight regulation. Excessive neutrophil-mediated inflammation has been implicated in tissue damage and disease [30]. Activation of secretory function and oxidant generation in neutrophils in the circulation would be deleterious, and enhancement of these functions in the appropriate inflammatory milieu is beneficial. Direct exposure of surface liquid in airways and wounds to the atmosphere is likely to result in a $pCO_2 < 5\%$. Our results suggest that neutrophils present at sites of low pCO_2 would be sufficient to initiate or exaggerate inflammatory responses in the cells, whereas intracellular acidification as a result of high pCO_2 might impair inflammatory processes. Laffey and Kavanagh [31] have outlined the disease states in which raised concentrations of CO_2 have been found to be protective and low concentrations, injurious, and have suggested that supplemental CO_2 could reduce the harmful affects of hypocapnia by promoting the beneficial affects of hypercapnia.

Our data suggest that hypocarbic alkalinization modulates increased release of the chemokine IL-8 from neutrophils, and hypercarbia produced the opposite effect. This is consistent with previous observations showing that CO_2 regulates TNF-α and IL-1 secretion from peritoneal macrophages [22] and that laparotomy versus CO_2 laparoscopy is associated with a systemic, inflammatory response, including elevated cytokine levels [20]. The effects of CO_2 are likely to be similar in these cells. In any case, the response to a fall in CO_2 is the secretion of factors that are capable of perpetuating the inflammatory responses. Although the amounts of IL-8 released are lower than those produced by monocytes and macrophages, they still may achieve sufficient local concentration to have significant autocrine effects. It is interesting that the observed pCO_2 sensitivity of IL-8 release and the increase in intracellular oxidant generation were sensitive to pharmacological manipulation.

Hypocarbia-induced changes in pH_i occurred in culture media that were not only physiologically buffered with bicarbonate but were also with cell-impermeant zwitterionic buffers, demonstrating the capacity of pCO_2 to alter pH_i and effector responses in the absence of significant changes of extracellular pH. The lack of a complete cellular compensation for these changes in pH_i is surprising and suggests that the functional changes we observed are likely to be duplicated during alterations in pCO_2 in vivo. The CO_2-induced changes in pH_i did not occur rapidly; i.e., pH_i changed by only 0.13 units in 2 min in the presence of 10% CO_2. This is in direct comparison to pH_i changes induced by stimuli, such as propionic acid, where pH_i dropped by almost 0.3 of a pH unit [23]. The reasons for the differences in pH_i changes induced by 10% CO_2 compared with propionic acid are not clear but are obviously worthy of further consideration in future experiments. The results of our assays on intracellular oxidant generation in whole-blood samples also suggest that functional compensation for acute changes in pCO_2 is not achieved through the buffering capacity of whole blood. However, the paracrine effects of other blood cells, such as monocytes that may be affected similarly, may affect proton translocation in neutrophils. Intracellular oxidant generation experiments carried out at earlier time points also demonstrated significant differences; that is, oxidant generation was higher in neutrophils exposed to 0.04% CO_2 compared with cells exposed to 5% CO_2, which in turn was higher than oxidant generation in neutrophils exposed to 10% CO_2. The partial recovery of pH_i under hypercarbic conditions depended on Na^+/H^+ exchange, as demonstrated previously [32]. However, we also detected a partial sensitivity to bafilomycin A_1. The implied involvement of proton-translocating ATPases was unexpected because these pumps are expressed in the membranes of intracellular organelles and are conveyed to the outer membrane during degranulation. Indeed, as a marker of degranulation, we have observed that significantly greater amounts of neutrophil elastase are released from neutrophils exposed to 10% CO_2 compared with neutrophils incubated in 5% CO_2 (unpublished results). Our observations in relation to hypocarbic alkalinization were unexpected. We did not detect

a DIDS-sensitive compensation for this process but rather the opposite, although previous studies have cited the involvement of HCO_3^-/Cl^- exchange in recovery of pH_i from alkalinization. This apparent discrepancy may be explained by differences in experimental protocols. Simchowitz and Roos [32] induced alkalinization over seconds by the immediate removal of 18% CO_2 and kept extracellular pH constant by varying HCO_3^- in solutions that were relatively weakly buffered compared with ours. The result was a change in extracellular (HCO_3^-) from ~100 mM (18% CO_2) to 0 mM (0 CO_2) conditions during alkalinization. This would impose an outwardly directed HCO_3^- chemical gradient under their alkalinized conditions, which would be absent under ours, which mimic acute respiratory alkalosis in vivo where extracellular HCO_3^- is preserved, but pCO_2 falls. In fact our data suggest that a DIDS-sensitive process facilitates HCO_3^- entry into the cell under these conditions. This could occur because of an inwardly directed HCO_3^- gradient facilitating HCO_3^- entry and Cl^- efflux via DIDS-sensitive anion exchange or alternatively, because of the presence of a hitherto-undescribed Na^+/HCO_3^- cotransport (NBC) in neutrophils. The latter hypothesis is supported by the observations that NBC is DIDS-sensitive, bi-directional—depending on concentration gradients—and sensitive to voltage with Km in the ~−40 mV, close to the values demonstrated for neutrophils (−53 mV) [33]. Together, all of these data support the contention that neutrophils compensate poorly for hypocarbia without a marked reduction in extracellular HCO_3^-, as would occur eventually with renal compensation for respiratory alkalosis in vivo but not during acute respiratory alkalosis. Limitations of hypocarbic alkalosis in neutrophils in the presence of $ZnCl_2$ are explained on the basis of the observed increase in oxidant production associated with reduced pCO_2 and the association of NADPH oxidase with passive proton conductance channels. The fact that pH_i was unaltered in the presence of both inhibitors under hypocarbic conditions was fortuitous in that it allowed us to test the hypothesis that the observed changes in effector function were manifestations of alterations in pH_i and could be ameliorated by eliminating the pH_i change.

In conclusion, we show functional sensitivity of several critical effector responses in neutrophils to pCO_2/pH_i, suggesting that inflammation may be modulated by pharmacological strategies targeting mechanisms of pH_i regulation. This may be of benefit in diseases associated with neutrophil-mediated tissue damage, such as adult respiratory distress syndrome, cystic fibrosis, Wegener's granulomatosis, and bronchopulmonary dysplasia.

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