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Title: Hypercapnic acidosis attenuates pulmonary epithelial wound repair by an NF-κB dependent mechanism.

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Short Title: HCA inhibits pulmonary epithelial wound repair.

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ABSTRACT

Rationale: Hypercapnic acidosis exerts protective effects in acute lung injury, but may also slow cellular repair. These effects may be mediated via inhibition of nuclear factor-κB (NF-κB), a pivotal transcriptional regulator in inflammation and repair.

Objectives: To determine the effects of hypercapnic acidosis in pulmonary epithelial wound repair, to elucidate the role of NF-κB, and to examine the mechanisms by which these effects are mediated.

Methods: Confluent small airway epithelial cell, human bronchial epithelial (HBE) cell and type II alveolar A549 cell monolayers were subjected to wound injury under conditions of hypercapnic acidosis (pH 7.0, PCO_2 11KPa) or normocapnia (pH 7.37, PCO_2 5.5KPa), and the rate of healing determined. Subsequent experiments investigated the role of hypercapnia versus acidosis, and elucidated the role of NF-κB and MAP kinases. The roles of cellular mitosis versus migration, and of matrix metalloproteinases, in mediating these effects were then determined.

Results: Hypercapnic acidosis reduced wound closure (33 ± 6.3% versus 64 ± 5.9%, $P < 0.01$), and reduced activation of NF-κB, compared to normocapnia. Buffering of the acidosis did not alter this inhibitory effect. Prior inhibition of NF-κB activation occluded the effect of hypercapnic acidosis. Inhibition of ERK, JNK and P38 did not modulate wound healing. Hypercapnic acidosis reduced epithelial cell migration but did not alter mitosis, and reduced matrix metalloproteinase-1 while increasing TIMP-2 concentrations.

Conclusions: Hypercapnic acidosis inhibits pulmonary epithelial wound healing by reducing cell migration, via an NF-κB dependent mechanism that may involve alterations in matrix metalloproteinase activity.

Keywords: hypercapnia; acidosis; buffering; pulmonary epithelial wound repair; ventilator-induced lung injury

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INTRODUCTION

Recent clinical trials have shown that in patients with ARDS, outcome is improved when a 'protective' ventilatory strategy is utilized (1). These ventilatory strategies minimize ventilator induced lung injury by reducing tidal volume and minute ventilation in order to limit the degree of lung stretch. The hypercapnic acidosis (HCA) that occurs in the setting of ALI/ARDS is a consequence both of these protective ventilatory strategies and of the disease itself which can limit carbon dioxide (CO_2) clearance. While this hypercarbia has traditionally been considered a passive phenomenon, it is increasingly clear that HCA is a potent biologic agent. HCA has been demonstrated to directly attenuate acute lung injury induced by free radicals (2), pulmonary (3) and systemic ischemia-reperfusion (4), and pulmonary endotoxin instillation (5), severe evolving (6) and established bacterial pneumonia (7) and systemic sepsis (8, 9) induced lung injury. Of importance in the context of protective ventilation, HCA directly attenuates ventilation induced lung injury (VILI) (10, 11). These protective actions of HCA appear to be due in part to its anti-inflammatory effects (12, 13).

The potential for HCA to modulate the repair process following injury is less well characterized. At a cellular level, HCA has been demonstrated to delay plasma membrane re-sealing, a key cellular reparative mechanism (14). Excessive lung stretch also produces gross alveolar wound lesions, due to the large shear forces produced, both in the clinical setting (15) and in experimental VILI models (16). Repair of wounds in the pulmonary epithelial surface is therefore critical to the repair process following mechanical injuries such as VILI.

The anti-inflammatory effects of HCA may be mediated – at least in part – via inhibition of the nuclear factor kappa B (NF-κB) pathway (17) , a key transcription factor during cell injury or stress. NF-κB is also an important cytoprotectant, and plays a key role in mediating cell survival and wound repair in other epithelia (18). HCA mediated inhibition of NF-κB activation could therefore retard pulmonary epithelial wound healing, slowing the recovery following VILI.

Matrix metalloproteinases (MMPs) are a family of enzymes that can degrade extracellular matrix proteins including basement membrane and tight junctions. Tissue inhibitors of metalloproteinases (TIMPs) exist, with TIMP-1 and TIMP-2 the major secreted TIMPs in the lung. MMPs have been implicated in the pathogenesis of ARDS, and may increase tissue breakdown and contribute to loss of the alveolo-capillary barrier and intercellular junctions (19). However MMPs are also central to lung repair, with a favourable balance of MMPs to TIMPs necessary to facilitate cell detachment from the basement membrane and migration during wound healing (20). The production of several MMPs, including MMP-1, and of TIMP-1 and-2 is NF-κB dependent (20).

In view of these considerations, we wished to explore the effects and mechanisms of action of HCA on pulmonary epithelial wound repair. We sought to determine whether this effect of HCA was a function of the acidosis or hypercapnia *per se*, and elucidate the role of NF-κB and MAP kinases in this process. Subsequent experiments determined the contribution of altered migration versus cell replication, and the role of MMPs and TIMPs in HCA induced inhibition of pulmonary epithelial wound healing. This work was presented in part at the American Thoracic Society annual conference,

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MATERIALS AND METHODS

Primary small airway epithelial cells (SAEC's), type II alveolar A549 cells, and human bronchial epithelial (HBE) cells and were used in all experiments. The small airway epithelial cells (SAEC) primary cells, which are harvested human lung cells from healthy donors, were purchased from Lonza Walkersville, Inc. (Walkersville, MD) as cryopreserved passage 1 cultures and used at passages 3 – 5. A549 cells were purchased from the European collection of cell cultures (Porton Down, UK) as cryopreserved 90-passage cultures and used at passages 91-95. The 16HBE14o- (HBE) transformed epithelial cell line was a gift from D. Gruender (University of Vermont, Burlington, VT) (21). All cells were then grown to confluence on plastic plates or tissue culture flasks of the required size (Corning Ltd, New York, US) at 37°C in a humidified incubator saturated with a gas mixture containing 5% CO_2 in air. Once the cells had grown to confluence, single or multiple wounds were made in each well, by scraping off cells with a 200μL pipette tip, as previously described (18). Pre-equilibrated and prewarmed media was then added to the wells, and the plates randomized to incubation in either normocapnia (F_ICO_2 0.05, balance air) or hypercapnic acidosis (F_ICO_2 0.15, balance air) as per group allocation. At 24 and/or 48 hours later, the monolayers were fixed with 4% paraformaldehyde in PBS (w/v), and stained with hemotoxylin and eosin. The extent of epithelial restitution was determined by imaging each plate on a flatbed scanner and assessing the area of each wound using edge-finding software (Photoshop v8.0, Adobe Systems Inc, San Jose, California). Additional detail in regard to these cell lines and the wound assessment technique is provided in an online data supplement.

Confluent SAEC, pulmonary alveolar A549, and HBE epithelial layers were each subject to single wound injury as described above, and subsequently incubated under conditions of normocapnia [5% CO_2, pH 7.37, PCO_2 5.5KPa] or hypercapnic acidosis [15% CO_2; pH 7.0, PCO_2 11KPa]. The dose response characteristics of hypercapnic acidosis were then determined. Confluent alveolar epithelial A549 cell cultures were incubated under conditions of normocapnia [5% CO_2, pH 7.37, PCO_2 5.5KPa], and moderate [10% CO_2; pH 7.15, PCO_2 8KPa] or more severe [15% CO_2; pH 7.0, PCO_2 11KPa] hypercapnic acidosis. The effect of hypercapnia versus acidosis on pulmonary epithelial healing was then determined. The RPMI-1640 medium, which includes 2.2g/L $NaHCO_3$ with 10% Fetal Bovine Serum, was used in these experiments. In preliminary studies, we determined that an additional 3.2g/L $NaHCO_3$ was required to buffer the pH of the medium back to 7.35 – 7.40 when incubated in 15% CO_2, and to produce normocapnic alkalosis when cells are incubated in 5% CO_2. An equiosmolar amount of NaCl was added to the cells exposed to normocapnia and HCA, to eliminate any potential effect of differences in medium osmolarity on wound healing. This produced the following groups: normocapnia (pH 7.37, PCO_2 5.5KPa); metabolic alkalosis (pH 7.8, PCO_2 5.5KPa); hypercapnic acidosis (pH 7.0, PCO_2 11KPa); and buffered hypercapnia (pH 7.37, PCO_2 11KPa). The effect of these conditions on the extent of wound healing was then determined. Additional experiments were carried out using the cell permeant pH sensitive fluorimetric dye carboxy SNARF®-1, acetoxymethyl ester, acetate (10μM, Invitrogen) to determine the intracellular pH in the epithelial layers exposed to normocapnia, normocapnic alkalosis, hypercapnic acidosis, and buffered hypercapnia.

Subsequent experiments determined the effect of wound injury and HCA on NF-κB activation. A549 cells transfected with κB-luciferase and TK-renillin control reporter

constructs were subjected to multiple wound injury and the extent of NF-κB activation determined under conditions of normocapnia or HCA. Western blotting was utilized to determine the degradation profile of IκBα in both uninjured and wounded A549 epithelial cultures under conditions of normocapnia and HCA. In addition, the extent of wound healing under HCA and normocapnia was determined in the presence of pharmacologic (PDTC (90μM, Sigma, Poole, UK), BAY 11-7085 (10μM, Calbiochem, San Diego, CA)) and gene overexpression (IκB-α-SR "super-repressor") mediated inhibition of NF-κB. The role of the MAP kinases ERK, JNK and P38 in mediating the effect of HCA was also determined using pharmacologic inhibitors.

The contribution of alterations in cell replication and cell migration to the inhibitory effect of HCA was determined. Confluent A549 monolayers were incubated with the mitosis inhibitors busulfan (10uM, Sigma) or indirubin-3′-monoximine (1uM, Calbiochem) in the presence of normocapnia and HCA. Subsequently, A549 cells were seeded into each upper chamber of an Invasion Assay plate (R&D Systems, Minneapolis, MN), and the migration of these cells in response to chemoattractant (IL8, MCP-1) and pro-inflammatory (TNF-α) cytokines was determined under conditions of normocapnia and HCA. The chemotactic potential of conditioned medium from wounded A549 cultures incubated under conditions of normocapnia or HCA, was then determined. Finally, the concentrations of matrix metalloprotineases (MMPs) and their tissue inhibitors (TIMPs) were measured in cell culture supernatants from epithelia subjected to multiple wounds under conditions of normocapnia and HCA, as previously reported (22).

Results are expressed as mean ± (SD) for normally distributed data, and as median (interquartile range, IQR) if non-normally distributed. Data were analyzed by t test, one way ANOVA or Kruskalis-Wallis followed by Mann-Whitney U test with the Bonferroni correction for multiple comparisons, as appropriate. A p value of <0.05 was considered statistically significant.

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RESULTS

HCA inhibits Pulmonary Epithelial Wound Healing

HCA reduced the rate of wound closure in primary small airway epithelial cell (SAEC) cultures 24 hours following wound injury, compared to normocapnia [***Figure 1 Panel A***]. This inhibition of pulmonary epithelial wound closure was also demonstrated in alveolar epithelial A549 cell monolayers, compared to control conditions, at both 24 and 48hrs (33 ± 6.3% versus 64 ± 5.9%, $P < 0.01$) following wound injury, compared to control conditions [***Figure 1 Panel B***]. In subsequent similar experiments, HCA also reduced the rate of wound closure in HBE cell monolayers [***Figure A, Online Repository***], demonstrating that this effect of HCA occurs in multiple pulmonary epithelial cell types. There was no effect of HCA on epithelial cell morphology in the 48 hours following wound injury [***Figure B, Online Repository***]. The effect of HCA on the rate of wound closure is dose dependent. Moderate degrees of hypercapnic acidosis [10% CO_2; pH 7.15; PCO_2 8KPa] reduced wound closure in A549 epithelial layers over 48 hours compared to normocapnia. This inhibition of wound closure was significantly less than that seen with exposure to more severe (15% CO_2; pH 6.9; PCO_2 11KPa) HCA [***Figure 1 Panel C***].

Buffered Hypercapnia inhibits Pulmonary Epithelial Wound Healing

Buffered hypercapnia retarded wound repair in A549 alveolar epithelial cell monolayers. The degree of inhibition of wound closure was not different from that seen with HCA [***Figure 1 Panel D***]. Furthermore, normocapnic alkalosis did not alter wound repair compared to normocapnia. Additional experiments which utilized the cell permeant pH sensitive fluorimetric dye SNARF-1® to determine intracellular pH, demonstrated that the intracellular pH in the epithelial layers exposed to buffered hypercapnia was not different from that seen with normocapnia [***Figure C, Online Repository***]. These findings demonstrate that the effect of HCA is a function of the hypercapnia rather than pH *per se.*

HCA inhibits wound healing via reduced NF-κB activation

HCA reduced the activation of NF-κB following wound injury to the pulmonary epithelium. The transcription of a κB-luciferase construct in A549 epithelial layers following wound injury was significantly reduced with HCA compared to normocapnia. [***Figure 2 Panel A***]. Direct inhibition of NF-κB activation with PDTC and BAY 11-7085 significantly inhibited wound healing under conditions of normocapnia [***Figure 2 Panel B***]. The degree of inhibition seen with PDTC and BAY 11-7085 was similar to that with HCA. Furthermore HCA did not further inhibit wound healing in the presence of PDTC or BAY 11-7085, demonstrating that the effect of HCA was completely occluded by direct inhibition of NF-κB.

Overexpression of the IκB-α transgene also inhibited wound healing under conditions of normocapnia [***Figure 2 Panel C***]. The extent of inhibition was similar to that seen with HCA. There was some further inhibition of wound healing by HCA in the presence of IκB-α overexpression, suggesting incomplete NF-κB blockade by IκB-α.

A Western blot of protein from HBE epithelial cell cultures demonstrated degradation of IκB-α, a key event in the propagation of the NF-κB response, at 60 minutes following wound injury. HCA inhibited this degradation when compared with normocapnic controls [***Figure 2 Panel D***].

Effect of HCA is MAP kinase and mitosis independent

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The effect of HCA on wound healing was MAP kinase independent. Inhibition of the MAP kinases ERK, JNK and P38 did not attenuate wound healing, in the presence or absence of HCA. Interestingly, inhibition of both ERK and JNK induced a minor, but statistically significant, increase in wound healing. This effect of JNK was seen in the presence and absence of HCA, and was therefore independent of HCA [***Figure D, Online Repository***].
Pharmacologic inhibition of mitosis with busulfan inhibited wound healing in the setting of both normocapnia and HCA. The mitosis inhibitor indirubin-3′-monoximine also inhibited wound healing in the presence of HCA [***Figure E, Online Repository***]. Taken together, these findings indicate that the effects of HCA on wound healing are not mediated via inhibition of mitosis.

HCA reduces cell migration

Incubation of alveolar A549 cells under conditions of HCA inhibited cell migration, in both the presence and absence of a chemotactic stimulus, compared to normocapnia. This inhibition of cell migration by HCA persisted at each concentration of the IL-8, MCP-1 and TNF-α cytokine combination tested [***Figure 3 Panel A***].
The conditioned medium from A549 monolayers subjected to wound injury and incubated under conditions of HCA reduced migration of A549 cells compared to normocapnia [***Figure 3 Panel B***]. This reduction in cellular migration by HCA was also demonstrated with conditioned media from A549 cells not subject to wound injury. These findings suggest that A549 cells exposed to HCA may produce lower concentrations of factors that promote migration and/or higher concentrations of factors that inhibit cellular migration.

HCA modulates MMP and TIMP Concentrations

Conditioned medium from A549 monolayers subjected to wound injury under both normocapnia and HCA conditions were assayed for MMP-1, -3 and -7 and for TIMP-1 and TIMP-2. These MMPs and TIMPs are central to cellular migration and wound healing. The concentration of MMP-1 in the medium was significantly reduced in wounded epithelial layers incubated in HCA compared to normocapnia [***Figure 4 Panel A***]. The concentration of MMP-7 was also reduced by HCA, but this difference was not statistically significant [***Figure 4 Panel B***]. MMP-3 was not detected in measurable quantities in the medium.
The concentration of TIMP-1 in the medium of scrape wounded cells was not altered by incubation in HCA [***Figure 4 Panel C***]. In contrast, the concentration of TIMP-2 in the medium was significantly increased in scrape wounded cells incubated in HCA compared to normocapnia [***Figure 4 Panel D***].

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DISCUSSION

We have demonstrated, for the first time, that hypercapnic acidosis impairs pulmonary epithelial wound healing. The mechanisms underlying this effect of HCA appear to be NF-κB dependent. The impairment of wound healing involves inhibition of cellular migration rather than mitosis, and is MAP kinase independent. The underlying mechanisms involve HCA induced alterations in the balance between matrix metalloproteinases and their tissue inhibitors. The relevance of these findings are clear given the prevalence of hypercapnia in patients managed with protective lung ventilation (12, 13), and the fact that pulmonary epithelial wounds, which result from mechanical disruption, are a key component of ventilator-injured lung injury (15, 16).

HCA – Risks and Benefits in Protective Lung Ventilation

We have proposed a new paradigm – 'Therapeutic Hypercapnia' – wherein the elevated CO_2 might contribute to lung protection (12). To explore this, we established several ALI models, and have demonstrated protective effects in ALI induced by free radicals (2, 23), endotoxin (5), both primary (3) and secondary (4) ischaemia-reperfusion, high lung stretch (10), severe evolving (6) and established bacterial pneumonia (7) and systemic sepsis (9) induced lung injury. Key mechanisms by which HCA exerts beneficial effects include attenuation of: free radical injury (2); apoptosis (3); NO-derived oxidant generation (5); and NF-κB activation (17). In the clinical setting, multivariate analysis of data from the ARDSnet trial (1) suggests that HCA increased survival in patients randomized to high tidal volume ventilation (24).

More recently, our group and others have questioned the safety of HCA (13, 25). HCA may potentiate protein nitration by peroxynitrite, a potent free radical, causing these proteins to malfunction (26). We have recently reported that HCA impairs bacterial killing in the setting of prolonged lung infection, ultimately worsening ALI (27). HCA may also delay healing following ALI/ARDS. HCA delays plasma membrane re-sealing following VILI, a key reparative mechanism following ALI/ARDS (14). Therefore, HCA may constitute a double-edged sword, attenuating injury pathways (2-5, 10, 11, 23), but retarding the response to pulmonary bacterial sepsis (27, 28) and slowing lung repair (14)

NF-κB – A key Transcriptional Regulator

The NF-κB protein complex consists of RelA, which is involved in dimerization, DNA binding and transcriptional regulation, and P50 or P65 homo- or heterodimers. NF-κB is normally sequestrated in the cytoplasm of non-stimulated cells, bound to IκB inhibitory proteins. NF-κB is activated by stimuli such as LPS, proinflammatory cytokines, and viral protein, via diverse signalling pathways that converge to phosphorylate IκB kinase complex proteins (IKK). IKK then phosphorylates IκB proteins on two N-terminal serine residues, which dissociates from NF-κB and is degraded. NF-κB then translocates to the nucleus and binds to specific cognate binding sequences in the promoter or enhancer regions of different inflammatory target genes where it initiates the transcriptional apparatus. NF-κB regulates genes central to lung injury, inflammation and repair, and is a key therapeutic target in ALI/ARDS (29, 30). Inhibition of NF-κB is protective in diverse models of inflammatory ALI (29, 31, 32). However, NF-κB is cytoprotective, promoting cell survival and wound repair (18), and resistance to infection (33).

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Effects and Mechanisms of Action of HCA in Epithelial Wound Repair

Our findings indicate that HCA potently reduces the rate of pulmonary epithelial wound closure. This effect of HCA appears to be a function of the hypercapnia rather than the acidosis *per se.* In fact, buffered hypercapnia retarded wound repair to an extent similar to that seen with HCA. The intracellular pH in buffered hypercapnia cultures was the same as that in epithelial cultures exposed to normocapnia. Normocapnic alkalosis did not modulate wound healing, which further illustrates that pH *per se* did not modulate wound healing in this model. In contrast, the effect of HCA to reduce pulmonary epithelial cell membrane resealing, a related but distinct component of pulmonary epithelial repair, is attenuated by buffering (34). In addition, the beneficial effects of HCA on lung permeability *in vitro* are abolished by buffering (23). Therefore, buffering appears to abolish the protective effects of HCA, but not its effect on restitution of the pulmonary epithelium.

The effects of HCA on pulmonary epithelial wound repair appear to be mediated via activation of NF-κB. Both PDTC and BAY 11-7085, which are pharmacologic NF-κB inhibitors, directly retarded wound healing, but did not have any effect in the presence of HCA. This suggests that these inhibitors shared a mechanism of action with HCA. This is supported by the finding that overexpression of the IκB-α transgene did not modulate wound healing in the presence of HCA. The effects of HCA on pulmonary epithelial wound healing appear to be MAP kinase independent. Pharmacologic inhibition of mitosis did not modulate wound healing in the setting of normocapnia or HCA, suggesting that the generation of new cells is not central to the mechanism of healing in this model. This finding is consistent with previous studies that report that cell replication does not play a role in restitution of pulmonary epithelial wounds (35). In contrast, migration of cells in response to inflammatory cytokines and media from wounded cell cultures was impaired by HCA. This finding suggests that HCA reduces wound healing by a process involving decreased cell migration. This finding is consistent with previous studies that report that cell migration is central to restitution of pulmonary epithelial wounds (36). HCA significantly reduced the concentrations of MMP-1 and increased TIMP-2 in the supernatant from wounded cell cultures. MMP-7 which is derived specifically from epithelial cells was also decreased, although this did not reach statistical significance. Overall this alteration in MMP/TIMP balance provides a mechanism for the reduced cellular migration potential induced by HCA. Furthermore, MMP-1, and TIMP-1 and-2 are all NF-κB dependent (20), which provides a potential mechanism by which HCA mediated NF-κB inhibition results in decreased cell migration and reduced wound healing.

Clinical Significance

Doerr et al demonstrated that HCA reduced the rate of plasma membrane repair, an important component of the cellular repair process following VILI (14). In this study, we demonstrate that HCA also reduces repair of gross pulmonary epithelial wounds, similar to those seen following high stretch ventilation in patients with ARDS (16). Ultrastructural studies in models of VILI confirm that wounds of this type are commonly found in the lung epithelium following high stretch ventilation, (15) particularly at high capillary pressures (15). As our experiments were conducted in pulmonary epithelial cell cultures, caution is warranted prior to extrapolating to the clinical context. Our findings should be seen as hypothesis generating, and will need to be confirmed in further studies in pre-clinical models. Nevertheless, when taken together with the findings of Doerr *et al*, our studies raise clear concerns regarding the

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potential for HCA to delay repair of the pulmonary epithelium following mechanical injury, such as is seen in patients with ALI/ARDS undergoing mechanical ventilatory support. These findings may also be of relevance to repair in other organs in the setting of hypercapnia, and in other lung diseases where hypercapnia is prevalent, such as chronic obstructive airways disease.

Conclusions

We have demonstrated for the first time that HCA impairs pulmonary epithelial wound healing. The mechanism underlying this effect of HCA is NF-κB dependent, involves inhibition of cellular migration rather than mitosis, which may be mediated by modification of MMP/TIMP balance, and is MAP kinase independent. These findings suggest that HCA may constitute a double-edged sword in ALI/ARDS, attenuating injury pathways but slowing lung repair.

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Figure Legends

Figure 1

Panel A: Hypercapnic acidosis reduces wound closure in the first 24 hours following scrape injury in primary lung small airway epithelial (SAEC) cell layers, in comparison to normocapnic conditions.

Panel B: Hypercapnic acidosis reduces the rate of wound closure at both 24 and 48 hours following scrape injury in pulmonary alveolar (A549) cell epithelial layers, in comparison to normocapnic conditions.

Panel C: The effect of HCA on the rate of wound closure is dose dependent. Incubation of A549 epithelial layers under conditions of moderate hypercapnic acidosis [10% CO_2; pH 7.15; PCO_2 8KPa] reduces wound closure over 48 hours compared to normocapnia, but to a significantly lesser extent to that seen with exposure to more severe hypercapnic acidosis [15% CO_2; pH 7.0; PCO_2 11KPa]

Panel D: The effect of hypercapnic acidosis on wound healing appears to be a function of the hypercapnia rather than the acidosis *per se*. A549 epithelial cell layers were exposed to HCA, buffered hypercapnia, normocapnia, or metabolic alkalosis, subjected to scrape wounding, and the extent of restitution of the wound determined over 24 hours. There was no difference in the extent of wound healing in epithelial layers exposed to buffered hypercapnia compared to hypercapnic acidosis. In contrast, normocapnic alkalosis did not affect the rate of wound healing compared to normocapnia.

Abbreviations: HCA, hypercapnic acidosis.

* $P < 0.05$ versus normocapnia

Figure 2

Panel A: HCA reduced the activation of NF-κB following wound injury to the pulmonary epithelium. The transcription of a κB-luciferase construct in A549 epithelial layers following wound injury was significantly reduced with HCA compared to normocapnia.

Panel B: Direct inhibition of NF-κB by PDTC and BAY 11-7085 attenuated wound healing in A549 monolayers, and occluded the effect of HCA.

Panel C: Following transduction with an AAV construct encoding the IκB-α transgene or vehicle, HBE monolayers were grown to confluence, subjected to scrape wounding, and exposed to normocapnia or HA. Cells were then fixed at baseline (time zero), and 24 hours, and the extent of restitution of the wound determined.

Panel D: HCA reduced the degradation of IκB-α following wound injury, compared to normocapnia. HBE monolayers were subject to multiple wound scraping and exposed to normocapnia or HCA. Cells were then harvested at zero, 60 and 180 minutes and examined for IκB-α degradation by Western blotting.

* $P < 0.05$ versus all other groups (student-newman-keuls test post 1 way ANOVA)

Figure 3

Panel A: HCA inhibits pulmonary epithelial cell migration in response to a chemotactic stimulus. A549 cells were seeded into the upper chamber of a collagen type I coated invasion assay plate. HCA inhibited cell migration at all concentrations of a combination of TNFα, MCP-1 and IL-8 added to the lower chamber.

Panel B: HCA reduces the concentration of chemotaxins in media from wounded and uninjured epithelia. The migration of A549 cells towards conditioned medium from wounded and uninjured epithelial layers exposed to HCA is reduced compared to

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migration towards medium from wounded and uninjured epithelial cultures exposed to normocapnia. Values for migration in both experiments are normalized to migration towards serum free fresh medium.
* $P < 0.05$ versus normocapnia
Abbreviations: No wound, cell culture media from uninjured A549 cultures; Wound, media from wound injured A549 cultures.

Figure 4
Panel A: HCA significantly reduced the concentration of matrix-metalloproteinase-1 (MMP-1) in the medium from A549 epithelial layers subjected to wound injury in comparison to wounded epithelia exposed to normocapnia.
Panel B: The concentration of MMP-7 in the medium from A549 epithelial layers subjected to wound injury under HCA conditions was not different from that in wounded epithelia exposed to normocapnia.
Panel C: The concentration of TIMP-1 in the medium from A549 epithelial layers subjected to wound injury under HCA conditions was not different from that in wounded epithelia exposed to normocapnia.
Panel D: HCA significantly reduced the concentration of TIMP-2 in the medium from A549 epithelial layers subjected to wound injury in comparison to wounded epithelia exp
osed to normocapnia.
* $P < 0.05$ versus normocapnia (t test)

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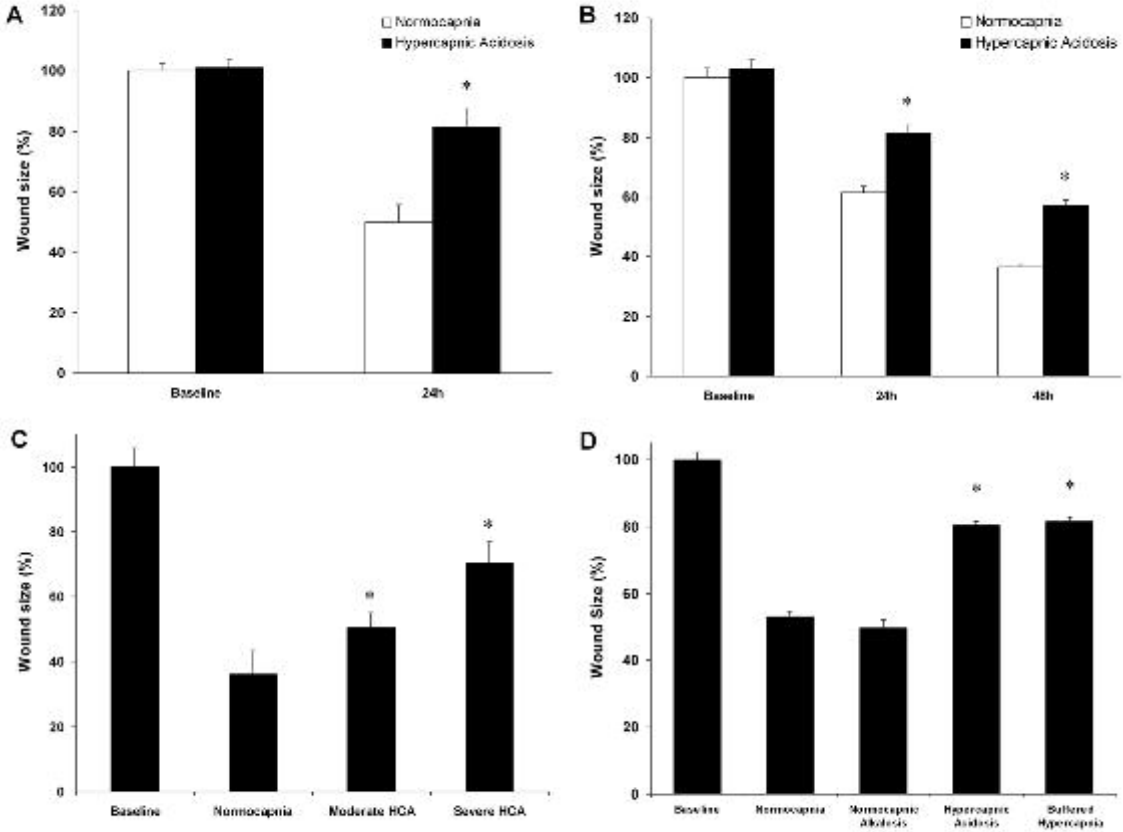
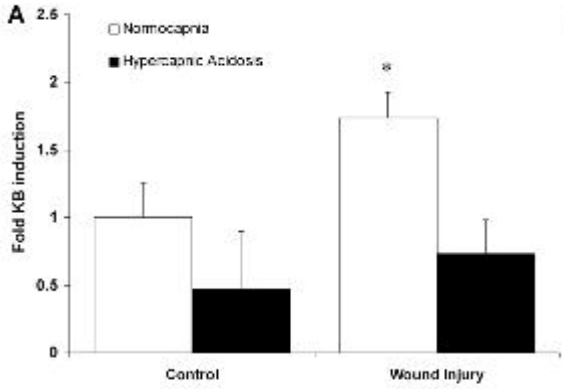
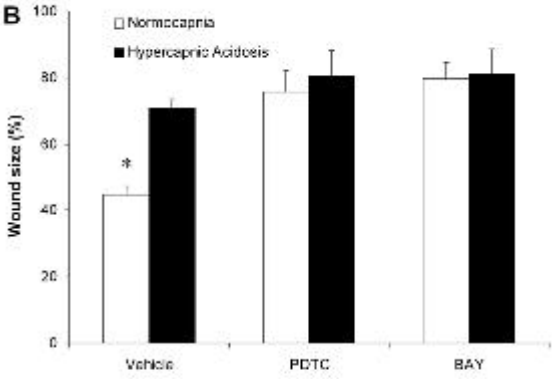
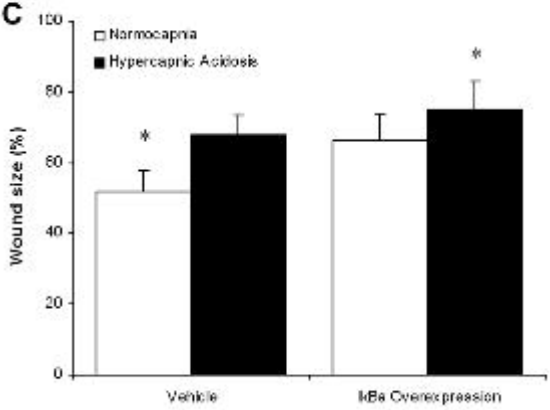
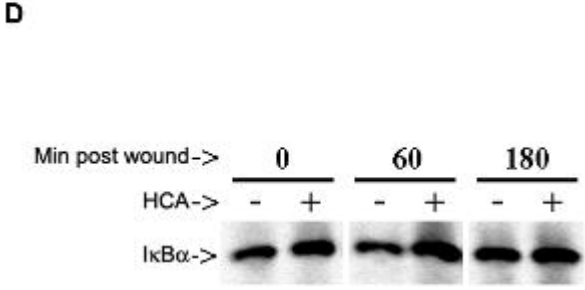
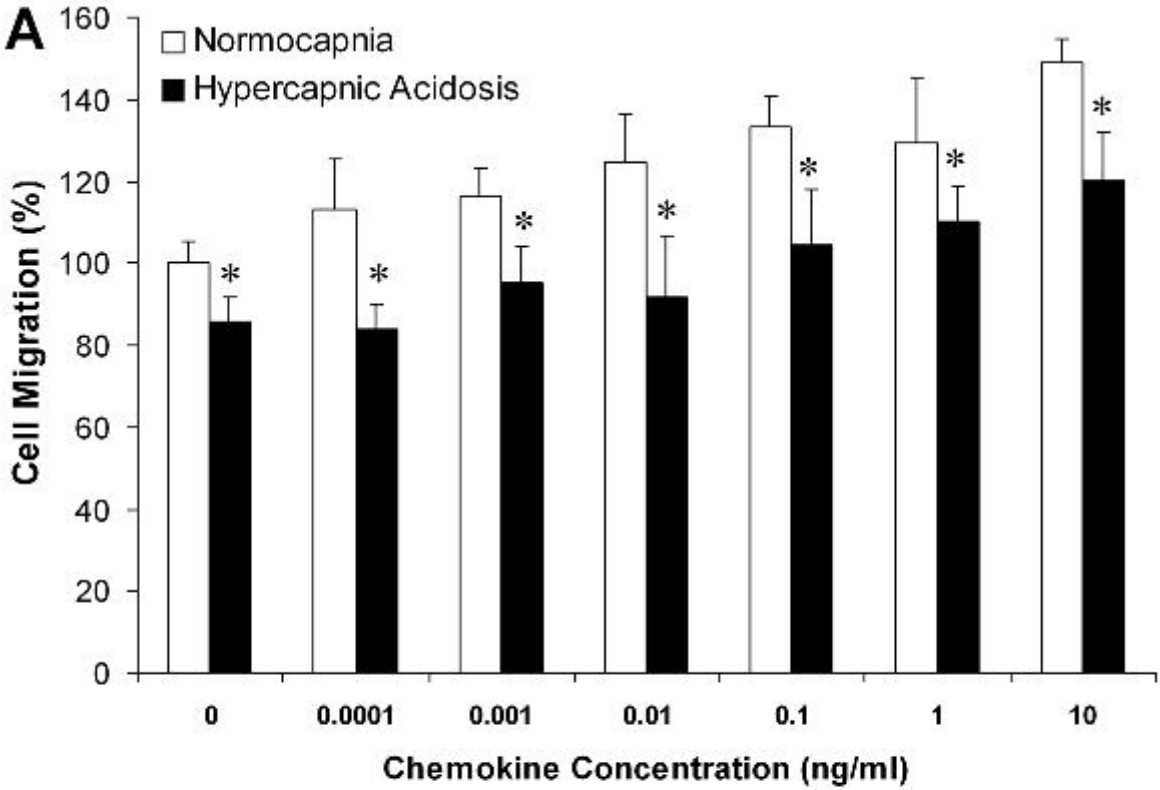

A
Normocapnia
Hypercapnic Acidosis
Wound size (%)
120
100
80
60
40
20
0
*
Baseline
24h
B
Normocapnia
Hypercapnic Acidosis
Wound size (%)
120
100
80
60
40
20
0
*
*
Baseline
24h
48h
C
Wound size (%)
100
80
60
40
20
0
*
*
Baseline
Normocapnia
Moderate HCA
Severe HCA
D
Wound Size (%)
100
80
60
40
20
0
*
*
Baseline
Normocapnia
Normocapnic Alkalosis
Hypercapnic Acidosis
Buffered Hypercapnia

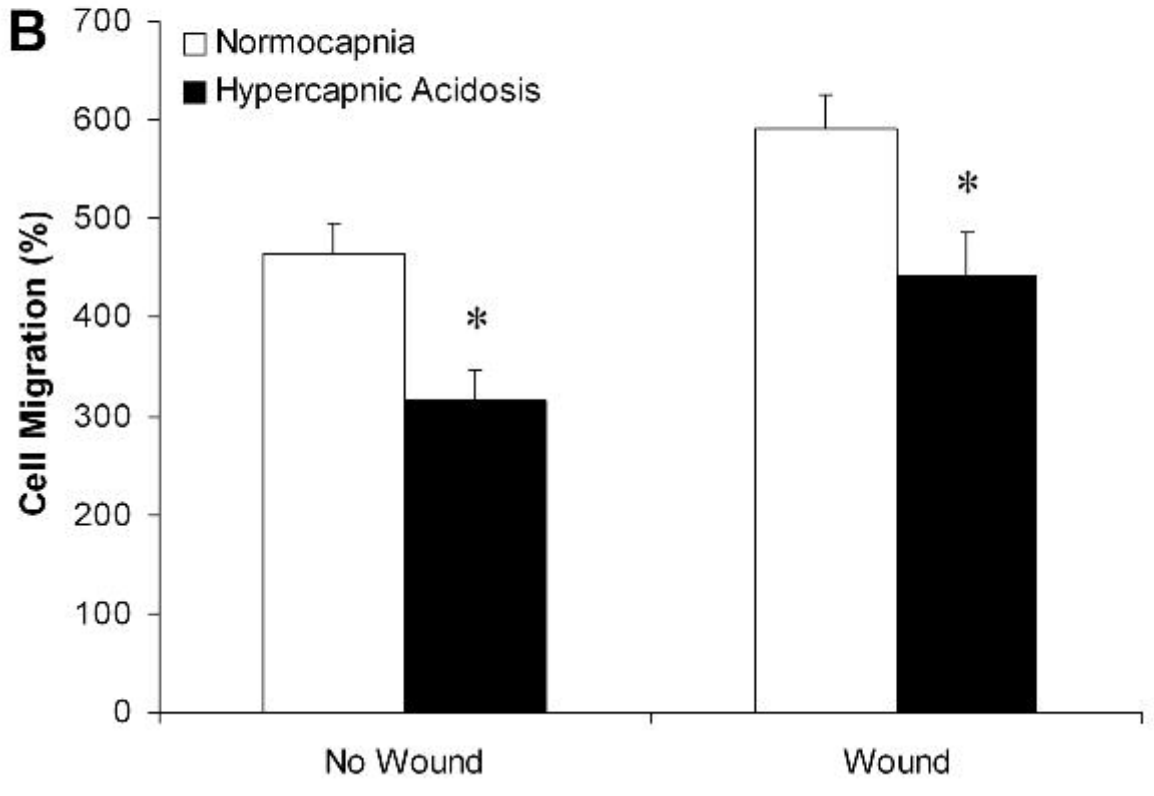

A
Normocapnia
Hypercapnic Acidosis
Fold KB induction
2.5
2
1.5
1
0.5
0
*
Control
Wound Injury

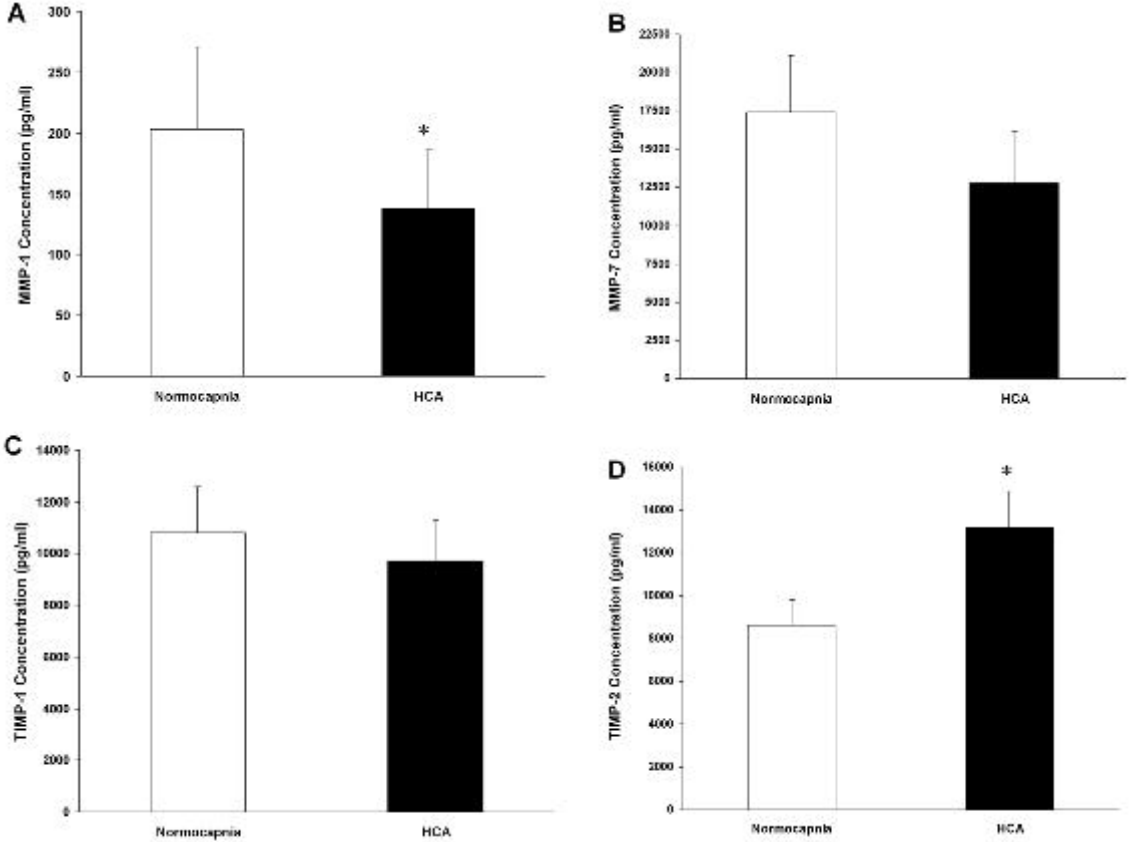

B
Normocapnia
Hypercapnic Acidosis
Wound size (%)
100
80
60
40
20
0
*
Vehicle
PDTC
BAY


C
Normocapnia
Hypercapnic Acidosis
Wound size (%)
100
80
60
40
20
0
*
*
Vehicle
IκBα Overexpression


D
Min post wound->
0
60
180
HCA->
- + - + - +
IκBα->


A
Normocapnia
Hypercapnic Acidosis
Cell Migration (%)
160
140
120
100
80
60
40
20
0
*
0
0.0001
0.001
0.01
0.1
1
10
Chemokine Concentration (ng/ml)


B
Normocapnia
Hypercapnic Acidosis
Cell Migration (%)
700
600
500
400
300
200
100
0
*
No Wound
Wound


A
MMP-1 Concentration (pg/ml)
300
250
200
150
100
50
0
*
Normocapnia
HCA
B
MMP-7 Concentration (pg/ml)
22500
20000
17500
15000
12500
10000
7500
5000
2500
0
Normocapnia
HCA
C
TIMP-1 Concentration (pg/ml)
14000
12000
10000
8000
6000
4000
2000
0
Normocapnia
HCA
D
TIMP-2 Concentration (pg/ml)
16000
14000
12000
10000
8000
6000
4000
2000
0
*
Normocapnia
HCA

Online Data Supplement

Title: Hypercapnic acidosis attenuates pulmonary epithelial wound repair by an NF-κB dependent mechanism.

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MATERIALS AND METHODS

Pulmonary Epithelial Cell Cultures

Primary small airway epithelial cells (SAEC's), type II alveolar A549 cells, and human bronchial epithelial (HBE) cells and were used in all experiments. The small airway epithelial cells (SAEC) primary cells, which are harvested human lung cells from healthy donors, were purchased from Lonza Walkersville, Inc. (Walkersville, MD) as cryopreserved passage 1 culture and used at passages 3-10. These cells were subcultured into growth medium (Bronchial Epithelial Basal Medium), supplemented with Bronchial Epithelial Growth Media BulletKit (both Lonza Walkersville, Inc.), penicillin G (100 U/ml) and streptomycin (100 μg/ml). All cells were grown at 37°C in a humidified incubator saturated with a gas mixture containing 5% CO_2 in air, on plastic plates or tissue culture flasks of the required size (Corning Ltd, New York, US).

A549 cells were purchased from The European Collection of Cell Cultures (Porton Down, UK) as cryopreserved 90-passage culture and used at passages 91-95. The epithelial cells were grown on 75-cm^2 tissue culture flasks (Corning Ltd, New York, US), and then subcultured with 0.025% trypsin-0.05 mM ethylenediamine tetraacetic acid (EDTA; GIBCO BRL). The cells were then grown to confluence in growth medium (RPMI-1640, Sigma, Poole, UK), supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml; GIBCO BRL, Grand Island, NY).

The 16HBE14o- (HBE) transformed epithelial cell line was a gift from D. Gruender (University of Vermont, Burlington, VT) as cryopreserved passage 1 culture and used at passages 4-14. These are virally immortalized primary human cells which form correctly polarised cell layers *in vitro* (1). These cells were subcultured into growth medium (Minimum Essential Medium, Alpha Modification, Sigma, Poole, UK), supplemented with 10% fetal calf serum, penicillin G (100 U/ml) and streptomycin (100 μg/ml).

Scrape Wounding and Restitution of Epithelial Monolayer

Single Wound Technique: Once the cells had grown to confluence, a single wound was made in each well, by scraping off cells with a 200μL pipette tip, as previously described (2). The medium was then aspirated, and the cells washed once with phosphate buffered saline (PBS). Washing with PBS removed those cells scraped from the plate surface and prevented their re-adherence to the plate. In each 24 well plate, three scrapes were immediately fixed for 10 minutes with 4% paraformaldehyde in PBS (w/v). Pre-equilibrated and pre-warmed media was then added to the remaining wells, and the plates randomized to incubation in either normocapnia (F_ICO_2 0.05, balance air) or hypercapnia (F_ICO_2 0.15, balance air) as per group allocation (see below). At 24 or 48 hours later, the medium was aspirated and the cells washed with PBS and fixed for 10 minutes with 4% paraformaldehyde in PBS (w/v). The epithelial monolayers were then stained with hemotoxylin and eosin to delineate the borders of the scrape wounds.

The extent of epithelial restitution was determined by imaging each plate, following fixation of the monolayer, on a flatbed scanner (Canoscan 3200F, Canon Ireland Ltd), and assessing the area of each wound using edge-finding software (Photoshop v8.0, Adobe Systems Inc, San Jose, California). Briefly, randomly chosen images from each scrape were cropped to 5mm in length, and the Magic Wand tool, set to a constant tolerance, was used to determine the number of pixels still in the wounded area. The

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remaining wound area was then compared to the area of a wound in an adjacent well fixed at baseline to determine the extent of wound restitution.

Multiple Wounds Technique: For these experiments, cells were grown to confluence in 3.5cm tissue culture dishes. The monolayer was cross-scraped 8 times horizontally and vertically (16 scrapes total) with a 200μL pipette tip to maximize the length of the wound edge, after which the monolayer was washed as described above. Plates were returned to the relevant incubators for 24 hours and the medium and/or cells harvested.

Effect of HCA on wound healing

Confluent pulmonary alveolar A549, HBE and SAEC monolayers were each subject to single wound injury as described above, and subsequently incubated under conditions of normocapnia [5% CO_2, pH 7.3, PCO_2 5.5KPa] or hypercapnic acidosis [15% CO_2; pH 6.9, PCO_2 11KPa].

Effect of pH versus CO_2 on wound healing

The effect of hypercapnia versus acidosis on pulmonary epithelial healing was then determined. The RPMI-1640 medium, which includes 2.2g/L $NaHCO_3$ with 10% Fetal Bovine Serum, was used in these experiments. In preliminary studies, we determined that an additional 3.2g/L $NaHCO_3$ was required to buffer the pH of the medium back to 7.35 – 7.40 when incubated in 15% CO_2, and to produce normocapnic alkalosis when cells are incubated in 5% CO_2. An equiosmolar amount of NaCl was added to the cells exposed to normocapnia and HCA, to eliminate any potential effect of differences in medium osmolarity on wound healing. Addition of these pre-equilibrated media to confluent pulmonary alveolar A549 monolayers, and incubation in normocapnia or hypercapnia, produced 4 distinct experimental conditions: normocapnia (pH 7.37, PCO_2 5.5KPa); and metabolic alkalosis (pH 7.8, PCO_2 11KPa); hypercapnic acidosis (pH 7.0, PCO_2 11KPa); and buffered hypercapnia (pH 7.37, PCO_2 11KPa). The effect of these conditions on the extent of wound healing was determined as described above.

The intracellular pH of epithelial layers exposed to normocapnia, normocapnic alkalosis, hypercapnic acidosis, and buffered hypercapnia, was determined fluorimetrically as previously described (3, 4). In brief, A549 cells were seeded to 96 well plates at 50,000 cells per well and returned to the incubator overnight. Cells were then loaded with the cell permeant pH sensitive fluorimetric dye carboxy SNARF®-1, acetoxymethyl ester, acetate (10μM, Invitrogen) for 1 hour. Cells were washed three times with PBS and re-fed with Hepes bicarbonate buffer (15mM Hepes, pH 7.4, 110mM NaCl, 20mM $NaHCO_3$, 5mM KCl, 0.8mM $MgCl_2$, 1.8mM $CaCl_2$, and 5.5mM glucose), or Hepes high bicarbonate buffer (identical except with 40mM $NaHCO_3$). Plates were then incubated in either normocapnic (5% CO_2) or hypercapnic (15% CO_2) conditions for one hour. Intracellular pH was estimated in a Victor II plate reader (Perkin Elmer) using 485nm excitation and 572nm emission wavelengths. The signal was calibrated using A549 cells fed with high potassium Hepes buffer (25mM Hepes, 145mM KCl, 0.8mM $MgCl_2$, 1.8mM $CaCl_2$, 5.5mM glucose) containing the H+/K+ ionophore nigericin (10mg/mL) (Sigma) at defined pH (6.64, 7.28, and 7.50).

Effect of wound injury and HCA on NF-κB Activation

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A549 cells were seeded at $2x10^4$ cells per well to 96 well plates, transfected with κB-luciferase and TK-renillin control reporter constructs (Promega Corp, Madison, Wisconsin) using Lipofectamine 2000 (Invitrogen Corp, Carlsbad, California), and allowed to recover overnight. The A549 monolayers were then subjected to multiple wound injury as described above, and incubated under conditions of normocapnia or HCA for 24hrs. Cells were then harvested into reporter lysis buffer (Promega), freeze-thawed, and assayed for luciferase activity using luciferase substrate (Promega). Values were normalized for a TK-renillin internal control.

A Western blot for IκB-α was performed on homogenates from HBE epithelial cultures subjected to multiple wound injury, as described above, and allowed to heal under conditions of normocapnia and HCA for 0hr, 1hr or 3h. Cells were pelleted and boiled in Laemmli sample buffer (2% SDS w/v, 10% glycerol v/v, 5% 2-mercaptoethanol v/v, 0.002% bromphenol blue w/v, 0.0625M Tris-HCl) and proteins separated on 4-20% polyacrylamide gels (Pierce Biotechnology, Rockford, Illinois). Proteins were transferred to nitrocellulose and probed using mouse-anti-IκB-α antibody followed by goat-anti-mouse-HRP secondary antibody (both Cell Signaling Technology, Danvers, Massachusetts). Membranes were then soaked in chemiluminescent substrate (Pierce) and imaged in a FluorChem imager (Alpha Innotech Corporation, San Leandro, California)

Role of NF-κB and MAP Kinases

Confluent A549 monolayers were incubated with the NF-κB inhibitors PDTC (90μM, Sigma), BAY 11-7085 (10μM, Calbiochem, San Diego, CA), or DMSO vehicle, in the presence of normocapnia and HCA, subjected to wound injury, and the extent of epithelial restitution over 24hrs determined.

HBE cells were then seeded at $5x10^5$ cells per well to 24 well plates, and transduced with AAV-5 vectors encoding the IκB-α-SR "super-repressor" transgene. Following growth to confluence the monolayers were subjected to wound injury in the presence of normocapnia and HCA, and the extent of epithelial restitution determined.

In subsequent experiments, confluent A549 monolayers were incubated with inhibitors of the MAP kinases ERK (PD98059, 10μM), JNK (SP600125, 5 μM), and P38 (SB203580, 1μM, all Calbiochem), subjected to wound injury in the presence of normocapnia and HCA, and the extent of epithelial restitution determined.

Role of Mitosis and Migration

Confluent A549 monolayers were incubated with the mitosis inhibitors busulfan (10uM, Sigma) and indirubin-3′-monoximine (1uM, Calbiochem) in the presence of normocapnia and HCA, subjected to wound injury, and the extent of epithelial restitution determined over 24hrs as described above.

To determine the effect of HCA on pulmonary epithelial cell migration, $5x10^4$ A549 cells were seeded into each upper chamber of an Invasion Assay plate (R&D Systems, Minneapolis, Minnesota) that had been pre-coated with Type I collagen. A cocktail of chemoattractant (IL8, MCP-1) and pro-inflammatory (TNF-α) cytokines were added to the bottom chamber, in progressively increasing concentrations from 0.001 – 10ng/ml of

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each cytokine. These plates were then incubated under conditions of normocapnia and HCA.

In further experiments, $5x10^4$ A549 cells were seeded into each of the pre-coated type I collagen chambers of an invasion assay plate, and either fresh medium (containing serum or serum free), conditioned medium from A549 wounds incubated under conditions of normocapnia or HCA, and conditioned medium from intact A549 monolayers incubated under conditions of normocapnia or HCA. In both experiments, 24 hours later the chambers were washed and the cells which had crossed the membrane were assessed using a Calcein-AM substrate (R&D Systems) which has a fluorescent product when metabolized by viable cells.

MMP and TIMP Analysis

In pilot studies, the concentrations of the matrix metalloprotineases MMP-1, -2, -3 -7, -8, -9,-12 and -13 were measured in cell culture supernatants from epithelia subjected to multiple wounds using a multi-analyte profiling bead array (R&D Systems) according to manufacturer's instructions. Only MMP-1, -3, and -7 were detectable and for the experiments described, this limited array was performed. In subsequent experiments, the concentrations of the MMP inhibitors TIMP-1 and TIMP-2 were measured in cell culture supernatants from epithelial monolayers subjected to multiple wounds by ELISA (R&D Systems) according to manufacturer's instructions, and as previously described (3).

Statistical Analysis

Results are expressed as mean ± (SD) for normally distributed data, and as median (interquartile range, IQR) if non-normally distributed. Data were analyzed by t test, one way ANOVA or Kruskalis-Wallis followed by Mann-Whitney U test with the Bonferroni correction for multiple comparisons, as appropriate. A p value of <0.05 was considered statistically significant. All experiments were repeated at least four times and representative results are shown.

FIGURE LEGENDS (ONLINE REPOSITORY)

Figure A: Hypercapnic acidosis reduces the rate of wound closure in the first 24 hours following scrape injury in human bronchial epithelial (HBE) cell cultures, in comparison to normocapnic conditions.
* $P < 0.05$ versus normocapnia

Figure B: High resolution picture showing initial wound at time zero and subsequent closure at 48 hours under following incubation under conditions of normocapnia and hypercapnic acidosis (HCA). HCA clearly reduces wound healing compared to normocapnia. There are not changes in epithelial cell morphology compared to baseline following exposure to hypercapnic acidosis.

Figure C: The intracellular pH of epithelial layers at one hour following incubation under conditions of normocapnia, normocapnic alkalosis, hypercapnic acidosis, and

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buffered hypercapnia. The intracellular pH of the epithelial layers exposed to buffered hypercapnia is the same as that in cells exposed to normocapnia.

Figure D: The inhibitory effect of HCA on pulmonary epithelial wound healing is not mediated via inhibition of the MAP kinases ERK, P38 and JNK. A549 cultures were subjected to scrape wounding in the presence of a MAP kinase inhibitor (ERK, P38 or JNK) or vehicle, exposed to normocapnia or hypercapnic acidosis, and the extent of wound restitution over 24 hours determined.

Figure E: The inhibitory effect of HCA on pulmonary epithelial wound healing is not mediated via inhibition of cell replication. A549 cultures were subjected to scrape wounding in the presence of the busulfan (10μM), indirubin-3-monoxime (1μM) or vehicle, and exposed to normocapnia or HCA, and the extent of wound restitution over 24 hours determined.
* $P < 0.05$ versus normocapnia + vehicle
† $P < 0.05$ versus hypercapnic acidosis + vehicle

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