

α -Tocopherol transfer protein mediates protective hypercapnia in murine ventilator-induced lung injury

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ABSTRACT

Rationale Hypercapnia is common in mechanically ventilated patients. Experimentally, 'therapeutic hypercapnia' can protect, but it can also cause harm, depending on the mechanism of injury. Hypercapnia suppresses multiple signalling pathways. Previous investigations have examined mechanisms that were known a priori, but only a limited number of pathways, each suppressed by CO₂, have been reported.

Objective Because of the complexity and interdependence of processes in acute lung injury, this study sought to fill in knowledge gaps using an unbiased screen, aiming to identify a specifically upregulated pathway.

Methods and results Using genome-wide gene expression analysis in a mouse model of ventilator-induced lung injury, we discovered a previously unsuspected mechanism by which CO₂ can protect against injury: induction of the transporter protein for α -tocopherol, α -tocopherol transfer protein (α TTP). Pulmonary α TTP was induced by inspired CO₂ in two in vivo murine models of ventilator-induced lung injury; the level of α TTP expression correlated with degree of lung protection; and, absence of the α TTP gene significantly reduced the protective effects of CO₂. α -Tocopherol is a potent antioxidant and hypercapnia increased lung α -tocopherol in wild-type mice, but this did not alter superoxide generation or expression of NRF2-dependent antioxidant response genes in wild-type or in α TTP^{-/-} mice. In concordance with a regulatory role for α -tocopherol in lipid mediator synthesis, hypercapnia attenuated 5-lipoxygenase activity and this was dependent on the presence of α TTP.

Conclusions Inspired CO₂ upregulates α TTP which increases lung α -tocopherol levels and inhibits synthesis of a pathogenic chemoattractant.

INTRODUCTION

Hypercapnia is common in mechanically ventilated patients with injured lungs, where tidal volume is deliberately lowered to lessen the risk of ventilator-induced lung injury (VILI).¹ However, accumulating evidence indicates that CO₂ can have potent effects in injured tissue that are independent of changes in mechanical ventilation.² Depending on the context, these effects may be protective, for example, attenuating reperfusion injury,³ acute sepsis⁴ or ventilator-induced injury;^{5,6} or, injurious, for example, impairing alveolar fluid clearance.⁷

Several molecular mechanisms of hypercapnic action have been described. For example,

Key messages

What is the key question?

- Can molecular mechanisms induced by hypercapnia during mechanical ventilation reveal potential therapeutic pathways for lung injury?
- Does hypercapnia protect against lung injury by activating protective genes?

What is the bottom line?

- Inspired CO₂ upregulates a transport protein (α -tocopherol transfer protein); this mobilises vitamin E in the lung which inhibits synthesis of a pathogenic chemoattractant, leukotriene B₄.

Why read on?

- Experimental elevation of CO₂ can protect (or cause harm); this new induced mechanism of protection from CO₂ provides new potentially testable therapies for lung injury.

elevated CO₂ inhibits expression of inflammatory cytokines,⁵ activation of nuclear factor κ B (NF κ B)⁸ and sheddase (a disintegrin and metalloprotease 17; ADAM17)⁶ signalling, and functional protein expression (eg, endocytosis of ion channels).⁷ However, these observations are limited in scope because all were based on injury processes known a priori. Also, many of the mechanisms are pivotal biological processes and thus have limited translational potential (eg, inhibition of NF κ B).

We sought to address this knowledge gap using an unbiased screen. We chose a microarray approach because the technology is mature, and because regulation at the level of gene expression would be a proximal and fundamental step in most identified signalling or synthetic processes. Microarray analyses have led to identification of key molecular pathways in VILI⁹ and of genes regulated by hypercapnia in neonatal lung development,¹⁰ and provide the possibility of identifying a specifically upregulated mechanistic pathway. Hypercapnia-dependent transcriptional responses have been recently reviewed.¹¹ While not fully characterised, they include inhibition of NF κ B¹² and hypoxia-inducible factor 1 α (HIF-1 α)¹³ responses, and activation of CREB¹⁴ and FoxO3a.¹⁵



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We utilised a mouse model of VILI in which addition of CO₂ to the inspired gas protects against injury.⁵ We found that hypercapnia activated few genes; however, hypercapnia was protective in VILI, and here it increased the expression of α -tocopherol transfer protein (α TTP). We describe how increased expression of α TTP may contribute to the protection afforded by hypercapnia in this multifaceted *in vivo* model.

METHODS

Additional details can be found in the online supplementary data.

Animal model

All animal procedures were approved by the animal care committee of the Hospital for Sick Children (Toronto, Ontario, Canada) in accordance with the Guidelines of the Canadian Council on Animal Care. C57BL/6J male mice (20–25 g, Charles River, St Constant, Quebec, Canada) were anaesthetised and ventilated: tidal volume (V_T) 10 mL/kg, positive end-expiratory pressure (PEEP) 2.0 cm H₂O, frequency 135/min, FiO₂ 0.21. Two models were employed. Model-1: mice were randomised to Severe Injury as previously described⁵ (peak inspiratory pressure 27 cm H₂O, V_T 35–40 mL/kg, PEEP 0 cm H₂O, frequency 30–35/min) versus continuation of baseline (low V_T) ventilation with normocapnia (FiO₂ 0.75, FiCO₂ 0, balance N₂) or hypercapnia (FiO₂ 0.75, FiCO₂ 0.12, balance N₂) for 3 hours. Model-2: mice were subjected to Moderate Injury (V_T 20 mL/kg, PEEP 0 cm H₂O, frequency 45/min) for 4 hours, randomised to normocapnia (room air) or hypercapnia (FiCO₂ 0.12, balance room air).

Genetically modified mice

Male knockout (α TTP^{-/-}) (strain B6.129S4-Ttpa^{1Far}/J, Jackson Laboratories, Sacramento, California, USA) and wild-type sibling (α TTP^{+/+}) controls were subjected to severe injury (Model-1) and randomised to normocapnia or hypercapnia.

Microarray analysis

We performed gene expression analysis using lung samples from mice included in our previous study,⁶ ventilated with the Severe Injury protocol (Model-1). RNA from five groups: (1) non-ventilated, (2) low V_T normocapnia, (3) low V_T hypercapnia, (4) high V_T normocapnia, and (5) high V_T hypercapnia ($n=5$ /group non-ventilated and low V_T ; $n=10$ /group high V_T) was hybridised to Affymetrix (Santa Clara, California, USA) mouse gene 1.0 ST arrays. Primary datasets are accessible through NCBI GEO (series accession number GSE86229). Data were subjected to robust multichip analysis normalisation; preliminary principal components analysis (PCA) identified two outliers which were subsequently excluded from downstream analysis. Analysis of changes in gene expression was performed using Partek Genomics Suite (Partek Inc, St Louis, Michigan, USA), by two-way analysis of variance (ANOVA) using ventilation (non-ventilated, high V_T , low V_T) and CO₂ (normocapnia, hypercapnia) as factors, Benjamini and Hochberg false discovery rate 5%, and fold-change cutoff at 1.5. Overrepresented canonical pathways associated with V_T and hypercapnia were identified using Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Redwood City, California, USA).

RT-PCR

Changes in gene expression were measured using relative quantitative real-time PCR by the ddCt method. Primers are shown in online supplementary table S1.

Inflammatory mediators

Myeloperoxidase (MPO) was measured in lung homogenates using *o*-dianisidine dihydrochloride and H₂O₂ as substrates. Cytokines in bronchoalveolar (BAL) fluid were quantitated using a Milliplex mouse Cytokine Immunoassay Kit (Millipore, Billerica, Massachusetts, USA). Eicosanoids in BAL were quantitated by liquid chromatography (LC)–tandem mass spectrometry (MS), with cysteinyl leukotrienes (CysLTs) measured by enzyme immunoassay (Enzo Life Sciences, Farmingdale, New York, USA). Superoxide radical in hydroethidine-treated mouse lung was detected through quantification of 2-hydroxyethidium using LC-MS-MS analysis.

Lung tissue α -tocopherol

Lung tissue α -tocopherol was quantitated using HPLC from homogenates spiked with internal standard δ -tocopherol (Sigma-Aldrich Canada Co., Oakville, Ontario, Canada).

Statistics

Data are presented as dot plots with mean (for normally distributed data) or median (non-normal distributions) indicated by a horizontal bar. Statistical analyses were calculated with Sigmaplot V12.3 (Systat software Inc) using ANOVA for multi-group comparisons. Two group comparisons used *t* tests on normally distributed data, and Mann–Whitney *U* for non-normally distributed data. *p* values <0.05 were considered significant.

RESULTS

Gene expression and hypercapnic protection against ventilator-induced lung injury

High V_T ventilation was used to generate VILI in C57BL/6J mice, as previously published;⁶ CO₂ (hypercapnia) was added to the inspired gas to protect against VILI in two of five experimental groups: (1) non-ventilated, (2) low V_T normocapnia, (3) low V_T hypercapnia, (4) high V_T normocapnia, and (5) high V_T hypercapnia.⁶ Lung injury was significantly greater in high V_T normocapnia compared with all other groups, and hypercapnia protected against VILI.⁶ PaO₂, PaCO₂ and pH values are given in online supplementary table S2.

Microarray analysis of lung samples was used to determine gene expression patterns associated with hypercapnic protection. PCA confirmed that whole gene expression profiles were similar among samples within each group (figure 1A). Two-way ANOVA analysis of overall gene expression with ventilation and CO₂ as factors both showed significant sources of variation, but no significant interaction (figure 1B). High V_T ventilation (normocapnia) changed the expression of 1658 gene probe sets (contrasted with no ventilation or low V_T ; fold change 1.5, *p*<0.05), including large increases in inflammatory gene expression (see online supplementary table S3).

In high V_T ventilation, there were modest changes in gene expression when contrasting normocapnia with hypercapnia. Hierarchical clustering of this gene set (figure 1C) confirmed distinct expression patterns, with some overlap in low V_T (uninjured) ventilation. A small number of genes were overexpressed in high V_T hypercapnia versus high V_T normocapnia, and most differentially expressed genes displayed downregulation under hypercapnia. Twenty-nine independent known genes responding to hypercapnia during high V_T ventilation were identified (table 1). We selected 12 candidate genes from this list based on plausibly protective effect and regulation at the transcriptional level, and real time RT-PCR confirmed that nine were regulated by hypercapnia (gene symbols: *Ttpa*, *Pmuk*, *Pdgfb*, *Ereg*, *Egr2*,

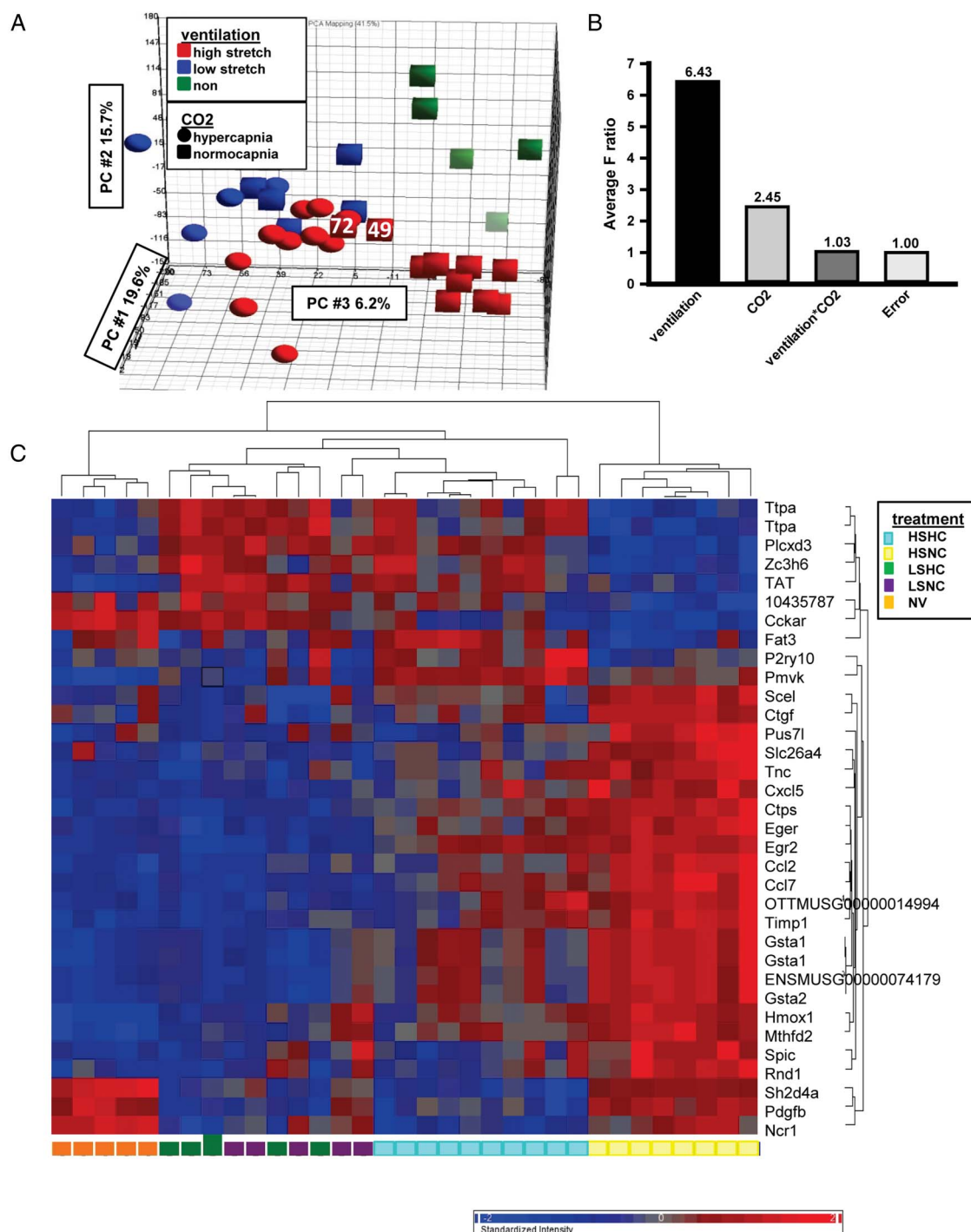


Figure 1 Microarray data in murine ventilator-induced lung injury and hypercapnia. (A) Principal component analysis plot for microarray data showing effects of ventilation and hypercapnia. Note: samples 72 and 49, from the high stretch normocapnia group, fell closer to the high stretch hypercapnia cluster than the high stretch normocapnia cluster and were excluded from further analysis. (B) Sources of variation in total gene expression estimated by two-way ANOVA. F ratio (y-axis) for each factor (x-axis) represents the F-statistics for that factor relative to F-statistic for error (noise) for all genes. (C) Cluster analysis showing changes in mRNA abundance during mouse lung ventilation as determined by microarray analysis. Each column represents RNA from a single mouse, and the heatmap shows genes detected by Robust Multichip Average analysis with expression increased (red) or decreased (blue) by 1.5 fold. HS, high stretch; LS, low stretch; HC, hypercapnia; NC, normocapnia; NV, non-ventilated.

Tnc, *Gsta1*, *Ncr1* and *Hmox1*); three trended as in microarrays, but were not significant (*P2ry10*, *Cckar*, *Ctgf*; table 1).

Identification of candidate gene α TTP in VILI

The most highly upregulated gene, encoding α TTP, was significantly more expressed in high V_T hypercapnia versus high V_T

normocapnia (confirmed by RT-PCR; table 1). In addition, the degree of protection (eg, decrement in static compliance) from hypercapnia was proportional to the degree of expression of the α TTP mRNA (figure 2). We used a second model of more moderate ventilation injury ($V_T=20$ mL/kg; $FiO_2=0.21$; see online supplementary figure S1), in which hypercapnia attenuated

Table 1 List of genes differentially expressed by hypercapnia in severe injury VILI

Symbol	Gene	Expression change by microarray		Expression by RTPCR (relative to NV; mean \pm SEM)	
		p Value* (hyper vs normo)	Fold change (hyper vs normo)	Normo	Hyper
<i>Ttpa</i>	α -tocopherol transfer protein	7.99E-07	1.74	0.94 \pm 0.12	2.11 \pm 0.42†
<i>Tat</i>	tyrosine aminotransferase	8.56E-04	1.71	not tested	
<i>Pmvk</i>	phosphomevalonate kinase	3.80E-05	1.66	4.70 \pm 0.70	8.50 \pm 1.20‡
<i>P2ry10</i>	purinergic receptor P2Y, G-protein coupled 10	2.33E-03	1.55	1.60 \pm 0.16	3.04 \pm 0.88
<i>Fat3</i>	FAT tumour suppressor homologue 3 (Drosophila)	5.31E-05	1.53	not tested	
<i>Zc3h6</i>	zinc finger CCCH type containing 6	1.56E-05	1.53	not tested	
<i>Cckar</i>	cholecystokinin A receptor	2.78E-04	1.52	0.32 \pm 0.03	0.48 \pm 0.06
<i>Plcxd3</i>	phosphatidylinositol-specific phospholipase C, X domain c	3.35E-08	1.50	not tested	
<i>Gsta2</i>	glutathione S-transferase, α 2 (Yc2)	1.30E-05	-1.50	not tested	
<i>Ctps</i>	cytidine 5'-triphosphate synthase	3.89E-07	-1.52	not tested	
<i>Pus7l</i>	pseudouridylate synthase 7 homologue (S. cerevisiae)-like	3.67E-06	-1.52	not tested	
<i>Pdgfb</i>	platelet-derived growth factor, B polypeptide	8.12E-06	-1.56	1.08 \pm 0.10	0.55 \pm 0.06†
<i>Scel</i>	scellin	1.16E-03	-1.58	not tested	
<i>Sh2d4a</i>	SH2 domain containing 4A	1.80E-09	-1.58	not tested	
<i>Ereg</i>	epiregulin	8.22E-07	-1.59	5.82 \pm 0.95	2.27 \pm 0.27‡
<i>Egr2</i>	early growth response 2	1.43E-04	-1.60	9.21 \pm 1.80	4.23 \pm 0.57‡
<i>Tnc</i>	tenascin C	2.94E-04	-1.61	4.21 \pm 1.01	1.60 \pm 0.28‡
<i>Gsta1</i>	glutathione S-transferase, α 1 (Ya)	1.71E-05	-1.62	6.91 \pm 1.33	2.58 \pm 0.45†
<i>Rnd1</i>	Rho family GTPase 1	1.74E-04	-1.63	not tested	
<i>Mthfd2</i>	methylenetetrahydrofolate dehydrogenase	2.44E-05	-1.64	not tested	
<i>Ccl7</i>	chemokine (C-C motif) ligand 7	1.26E-06	-1.65	not tested	
<i>Ctgf</i>	connective tissue growth factor	1.39E-04	-1.67	3.95 \pm 0.822	1.95 \pm 0.42
<i>Slc26a4</i>	solute carrier family 26, member 4	9.52E-06	-1.75	not tested	
<i>Timp1</i>	tissue inhibitor of metalloproteinase 1	5.00E-05	-1.82	not tested	
<i>Ncr1</i>	natural cytotoxicity triggering receptor 1	5.82E-07	-1.82	0.65 \pm 0.07	0.26 \pm 0.03†
<i>Cxcl5</i>	chemokine (C-X-C motif) ligand 5	7.43E-05	-1.88	not tested	
<i>Spic</i>	Spi-C transcription factor (Spi-1/PU.1 related)	1.07E-03	-1.96	not tested	
<i>Hmox1</i>	heme oxygenase (decycling) 1	6.72E-05	-1.98	12.9 \pm 1.99	4.51 \pm 0.59
<i>Ccl2</i>	chemokine (C-C motif) ligand 2	2.16E-06	-2.05	not tested	

*Adjusted p value, FDR <0.05, Benjamini Hochberg.

†p<0.01 vs high stretch normocapnia; N=10/group.

‡p<0.05 vs high stretch normocapnia.

injury (ie, BAL protein, lung MPO), and induced α TTP mRNA (figure 3).

The top canonical pathways associated with gene expression from high V_T (figure 4A) included inflammation-related signalling pathways known to be associated with VILI (NF κ B, IL-6, p38 MAPK, HMGB1). A single pathway, the NRF2-mediated oxidative stress response, was shared by high V_T and by hypercapnia (figure 4B); this is a protective response against oxidative and xenobiotic toxicity, and potentially against VILI.¹⁶ High V_T increased expression of NRF2 target genes, but these were decreased (not further increased) with hypercapnia (figure 5).

α TTP, the most upregulated gene, is a cytosolic protein that shuttles α -tocopherol (vitamin E), an important antioxidant, between cellular membranes. We hypothesised that increased α TTP expression in hypercapnia provides antioxidant protection upstream of the antioxidant NRF2 pathway, thereby limiting its induction.

α TTP gene deletion: impact on protection from CO₂

To examine the role of α TTP in hypercapnia-mediated protection from VILI, we used knockout (α TTP^{-/-}) and wild-type

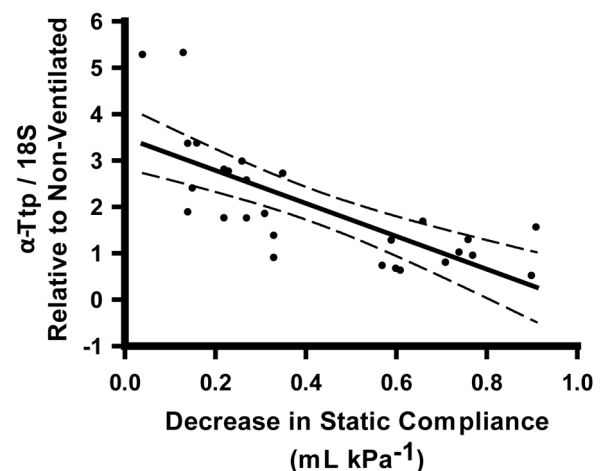


Figure 2 Hypercapnic protection and expression of α -tocopherol transferase protein (α TTP). Linear regression analysis indicating correlation between α TTP mRNA expression (RT-PCR) and decrease in static compliance in ventilated mice. Dotted lines indicate 95% CIs. R=-0.73, p<0.001.

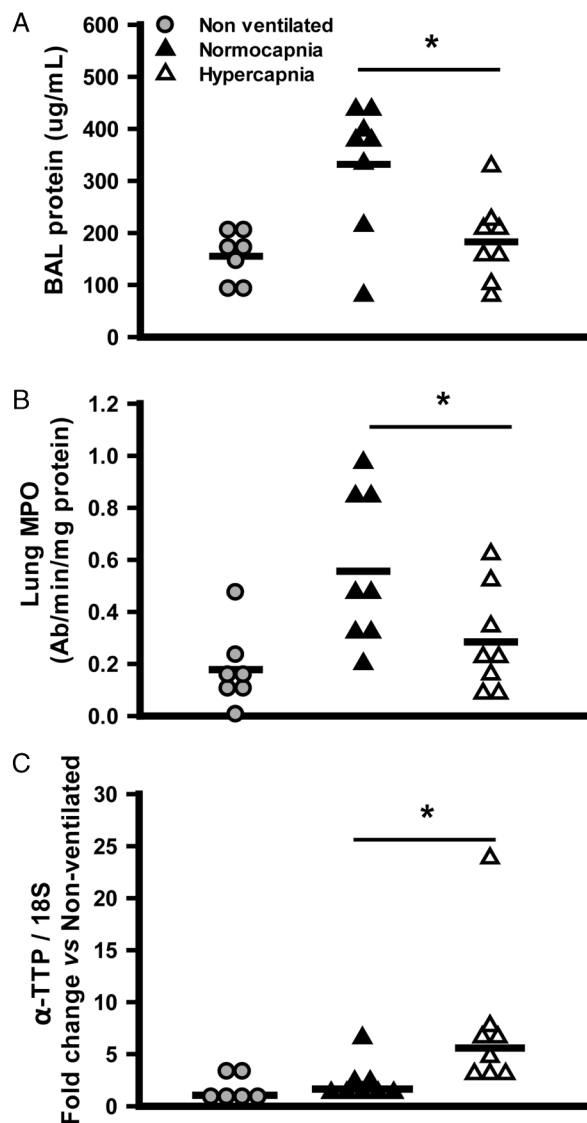


Figure 3 Hypercapnic protection and expression of α -tocopherol transferase protein (α TTP) in milder injury. Hypercapnia reduced protein concentration in the bronchoalveolar lavage (BAL) fluid (A), lung tissue myeloperoxidase (MPO) activity (B), and increased the tissue expression of α TTP mRNA (C), in a model of milder ventilator-induced lung injury (4 hours, V_T =20 mL/kg) (* p <0.05 vs normocapnia, t test). Data from small numbers of non-ventilated mice are presented for illustrative purposes but excluded from statistical analysis.

sibling (α TTP^{+/+}) mice subjected to high V_T ventilation. All mice had similar lung compliance at baseline, and similar decreases in compliance over 3 hours during high V_T ventilation with normocapnia (see online supplementary figure S2A). Hypercapnia attenuated several markers of injury in α TTP^{+/+} but not α TTP^{-/-} mice: compliance (figure 6A, B), lung tissue MPO and TNF α mRNA (figure 7A, B), and BAL levels of MCP-1 and KC (figure 7C, D). Also, hypercapnia tended to decrease BAL IL-6 in α TTP^{+/+} mice only (figure 7E).

Role of α TTP and α -tocopherol in protection with hypercapnia

Hypercapnia induced lung tissue expression of α TTP mRNA in α TTP^{+/+} sibs (figure 8A) following high V_T ventilation, and greater expression of α TTP mRNA was associated with greater

protection (figure 8B) (as with C57BL/6J mice: table 1, figure 2). In addition, lung tissue α -tocopherol levels were increased by hypercapnia during high V_T ventilation in α TTP^{+/+} sibs (figure 8C), while in α TTP^{-/-} sibs, levels were 20–50 fold less and were not altered by ventilation (figure 8D).

α -Tocopherol and oxidative stress in ventilator-induced lung injury

The decrease in NRF2 target gene expression due to hypercapnia in the C57BL/6J mice (figure 4B) suggested that hypercapnic induction of α TTP may exert antioxidant effects by shuttling α -tocopherol; reduced oxidative stress would then explain the lessened induction of NRF2 target gene expression. If this were true, then in the setting of lung injury, hypercapnia should lower the expression of NRF2 target genes in α TTP^{+/+} sibs, but not in α TTP^{-/-} mice. Constitutive expression of HMOX1 and GSTA1 was highest in lungs of α TTP^{-/-} (vs α TTP^{+/+}) and lowest in C57BL/6J mice (figure 9A, B), perhaps an adaption to low α -tocopherol levels. However, α TTP^{-/-} and α TTP^{+/+} mice showed similar changes in HMOX1 and GSTA1 in response to ventilation and hypercapnia (figure 9C, D), indicating that these effects are independent of α TTP expression.

To determine whether α TTP protected via an antioxidant mechanism, we measured 2-hydroxyethidium as a unique marker of superoxide generation in mice injected with hydroethidine; hypercapnia had no impact on α TTP^{-/-} or α TTP^{+/+} mice (figure 10A). Similar results were found using multiple markers of oxidative stress (eg, 8-isoprostane, protein carbonyls, and antioxidant capacity assay: data not shown).

α -Tocopherol and eicosanoids in lung injury

α -Tocopherol inhibits 5-lipoxygenase (5-LOX),¹⁷ and its downstream products, the leukotriene B4 (LTB4) and cysteinyl leukotrienes (CysLTs: LTC4, LTD4 and LTE4), are pathogenic in lung injury and ARDS.¹⁸ Hypercapnia suppressed the BAL level of LTB4 and CysLTs in α TTP^{+/+}, but not in α TTP^{-/-} mice (figure 10B, C). A similar trend was observed for the additional 5-LOX product 5-hydroxyeicosatetraenoic acid (5-HETE) (see online supplementary table S4). There were no differences in levels of the eicosanoid precursor arachidonic acid (figure 10D), or in other downstream products (eg, HETEs, prostaglandins or epoxyeicosatrienoic acids) (see online supplementary table S4).

DISCUSSION

The current study provides evidence of a previously unsuspected mechanism by which CO₂ can protect against lung injury: induction of a transporter protein for α -tocopherol, α TTP. Addition of CO₂ to the inspired gas increased pulmonary expression of α TTP mRNA in different in vivo murine models of VILI; the level α TTP expression correlated with degree of lung protection; and, absence of the α TTP gene abolished the protective effects of CO₂. These data provide new insights into the mechanisms of action of CO₂ on molecular pathways in lung injury beyond previously described multifactorial inhibitory effects such as NF κ B,⁸ ADAM17⁶ and Na,K-ATPase.⁷ In contrast to previously described mechanisms, the current data demonstrate that CO₂ upregulates expression of α TTP, which increases lung α -tocopherol levels; α -tocopherol can inhibit synthesis of the inflammatory mediator leukotrienes, products of 5-LOX which are pathogenic in acute lung injury.¹⁸

Although α TTP was required for hypercapnia's protective effects, we did not find that α TTP knockout (KO) mice injured more readily than wild-type mice in normocapnic conditions; in fact, the opposite may be true, since the decrease in static

Figure 4 Top canonical pathways in response to mechanical ventilation. Differences in mRNAs in lungs of non-ventilated and ventilated mice were identified through microarray analysis and submitted to functional enrichment analysis using Ingenuity Pathway Analysis. Highly significant genes were selected by Fisher's exact test (at $p < 0.0001$), and the statistical significance of each pathway is presented as negative log (p value). Pathways associated with genes affected by stretch (A) or associated with genes affected by hypercapnia (B) are illustrated.

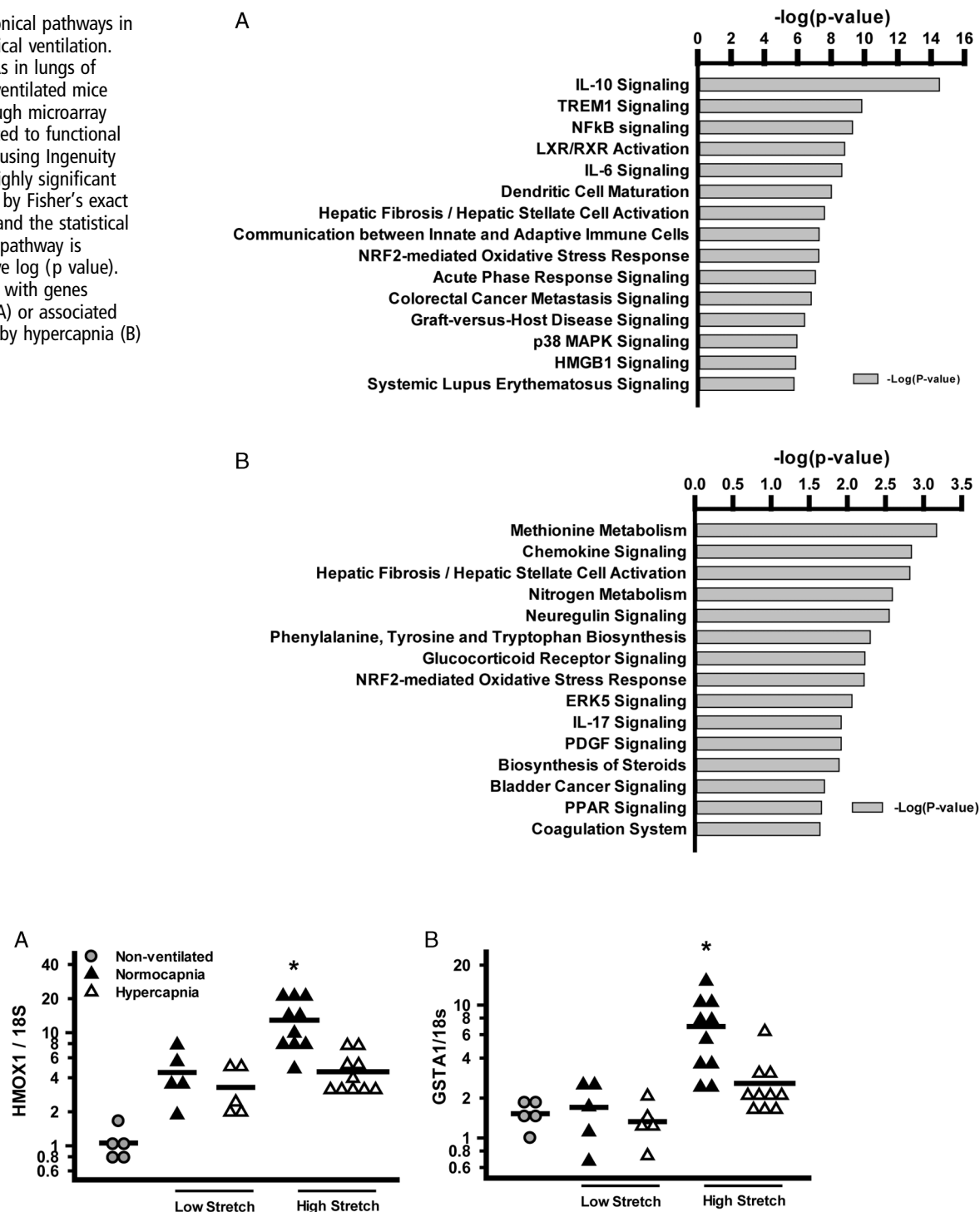


Figure 5 Relative expression of mRNAs of the antioxidant response pathway in lungs of mechanically ventilated mice. NRF2 target genes heme oxygenase (HMOX1; A) and glutathione-S-transferase (GSTA1; B) were more highly expressed in high stretch normocapnia than all other ventilated groups; that is, addition of hypercapnia reduced the expression of each during high stretch ventilation (* $p < 0.01$ vs all other groups, one-way ANOVA).

compliance in normocapnic KO mice appears slightly less than in wild-type mice (figure 6A,B). This may be due to higher constitutive levels of other protective mechanisms (eg, NRF2 responsive antioxidant genes; figure 9) as a compensatory mechanism in KO animals which have very low levels of α -tocopherol.

α TTP is a cytosolic protein that is most highly expressed in hepatocytes, where it traffics α -tocopherol and, with lower

affinity, other forms of vitamin E, from endosomes to the plasma membrane in exchange for PIP₂; this promotes α -tocopherol secretion into the bloodstream, bound to lipoprotein particles.¹⁹ Expression of α TTP has been described outside the liver (eg, brain, lung, prostate, placenta, retina and kidney), and while information about its non-hepatic roles is limited, some of the roles seem heterogeneous. In contrast to α -tocopherol efflux in hepatocytes,²⁰ overexpression in prostate

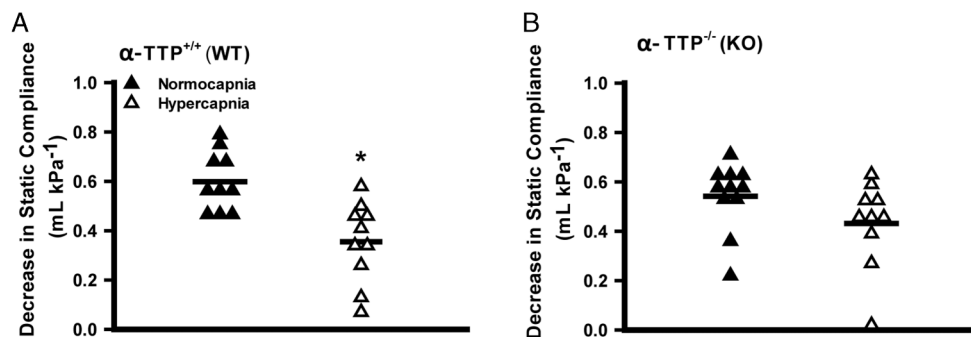


Figure 6 The α -tocopherol transferase protein (α TTP) gene is required for CO₂-mediated protection of compliance in murine ventilator induced lung injury. High-stretch (injurious) mechanical ventilation results in decreased static compliance in α TTP^{+/+} and α TTP^{-/-} mice. Hypercapnia is protective (ie, reduces the injury) in α TTP^{+/+} (A), but not in α TTP^{-/-} (B) mice (*p=0.001, t test). KO, knockout; WT, wild type.

cells promotes accumulation of α -tocopherol,²¹ indicating tissue-specific functions. In addition, human mutations in the α TTP gene cause progressive ataxia and vitamin E deficiency ('AVED').

Surprisingly, we did not demonstrate a role for α TTP against oxidative stress in VILI, in terms of superoxide (figure 10A) or lipid peroxidation (ie, 8-Iso PGF₂ α , see online supplementary table S4). This may be because of higher constitutive NRF2 antioxidant expression in α TTP colony mice versus C57BL/6J mice (figure 9C, D). Indeed, an increase in constitutive expression of NRF-2-responsive genes in α TTP KO mice has been previously noted²² and may reflect an adaptation to lower levels of α -tocopherol. Our observation that WT sibs also display elevation may be due to copy insufficiency during development in a heterozygote mother. It is possible that induction of α TTP by hypercapnia in other strains (eg, C57Bl/6J) or species reduces oxidative stress.

There is nevertheless considerable evidence that vitamin E (including α , β , γ and δ tocopherols and tocotrienols) modulates the innate immune response in lung. α -Tocopherol suppresses inflammatory responses and lipopolysaccharide (LPS)-induced oxidative injury in lung epithelial cells,²³ and multiple formulations of vitamin E protect in lung injury models.²⁴ For example, in rodent models of LPS-induced lung injury, intraperitoneal α -tocopherol decreased lung oedema and neutrophil transmigration without affecting cytokine levels or NF κ B activation,²⁵ while enteral γ -tocopherol (a more powerful antioxidant than α -tocopherol) decreased neutrophils and the key inflammatory mediators MIP-2, CINC-1 and PGE₂.²⁶ A small human study demonstrated that oral γ -tocopherol reduced sputum neutrophil numbers following intranasal LPS.²⁷

However, important gaps exist in our understanding of the protective effects of α -tocopherol: supplementation protects against lung injury in murine bacterial pneumonia (*Streptococcus pneumoniae*), reducing mortality, bacterial load, and neutrophil transepithelial migration.²⁸ In addition, while α -tocopherol protects against hyperoxic lung injury through antioxidant mechanisms,²⁹ the mechanisms of protection in other lung injury (eg, sepsis) is unclear.

Most clinical studies of α -tocopherol supplementation show minimal or no benefit in patients who have adequate dietary intake,²⁴ including the critically ill.³⁰ An important limitation not addressed in other studies is intracellular accumulation and trafficking of α -tocopherol in non-hepatic tissue. Increased α TTP expression could overcome such a limitation, although achieving increased expression would be a challenge. Alternatively, bypassing limited endogenous transport

mechanisms by direct application of tocopherols, such as aerosolisation or incorporation into surfactant, might enhance their effects. In addition, recent data indicate that metabolites, for example, γ -tocopherol or tocotrienols, may prove more efficacious.²⁴

Beyond neutralisation of oxygen-derived or nitrogen-derived radicals, vitamin E can regulate eicosanoid metabolism.¹⁷ Eicosanoids, including prostaglandins, thromboxanes, leukotrienes and HETEs, are an important network of signalling molecules that regulate inflammatory responses in critical illness. For example, cyclic stretch of lung epithelial cells (mimicking dyspnoea or mechanical ventilation) activates cPLA₂,³¹ thereby providing free arachidonic acid for eicosanoid biosynthesis. α -Tocopherol modulates eicosanoid synthesis through multiple means, such as preventing the oxidation of lipid precursors, modulating mediators derived from 12-LOX and 12/15-LOX,³² and specific inhibition of 5-LOX.¹⁷ Among a panel of arachidonate products, we identified significant reduction in LTB₄ and CysLTs (and a trend to decreased 5-HETE) resulting from hypercapnic induction of α TTP. Hypercapnia did not influence levels of other eicosanoids, including COX-derived prostaglandins, products of other LOX enzymes (12-HETE or 15-HETE) or non-enzymatic oxidation products of arachidonic acid (9-HETE, 11-HETE) (see online supplementary table S4). Together these data indicate that in our model, hypercapnia increased expression of α TTP; this increased accumulation and/or trafficking of α -tocopherol, leading in turn to specific inhibition of 5-LOX, and lower levels of leukotrienes.

Such lowering of leukotriene levels is a plausible downstream mechanism for protection against lung injury in the current experiments. LTB₄ is a potent PMN chemoattractant, providing a key signal for transmigration into the airway mediated by specific G-protein coupled receptors (BLT1, BLT2), and CysLTs increase vascular permeability and modulate vascular smooth muscle tone via CysLT1 and CysLT2 receptors. A small clinical study has demonstrated that plasma levels of leukotrienes were elevated in patients with ARDS early in their illness, and early LTB₄ levels predicted mortality from ARDS.³³

In other models of lung injury, antagonism of 5-LOX is protective; for example, pharmacological inhibition and gene knockout of 5-LOX protect against murine VILI,¹⁸ and in murine sepsis KO of the 5-LOX gene (or inhibition of the enzyme) provided more protection than use of leukotriene receptor antagonists (eg, Montelukast).³⁴ Antagonism of LTB₄ is also effective in lung injury caused by haemorrhagic shock³⁵ or in carrageenan-induced pleurisy.³⁶

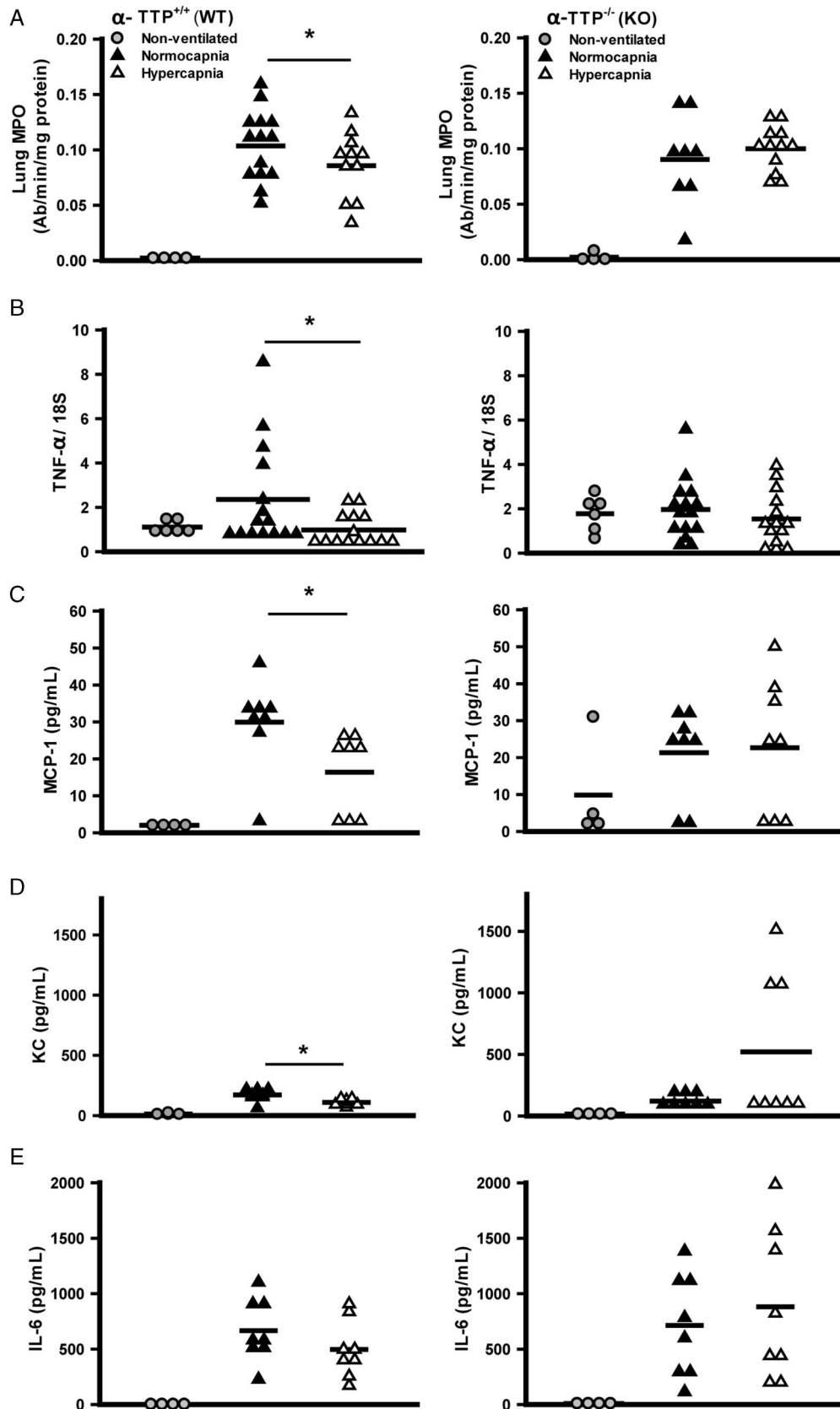


Figure 7 CO₂-mediated protection and impact of α -tocopherol transferase protein (α TTP) on biochemical injury markers. Tissue levels of myeloperoxidase (MPO) activity (A) and TNF α mRNA (B) are significantly less after 3 hours of injurious ventilation with hypercapnia versus normocapnia (ie, hypercapnia protective) in α TTP^{+/+} (left panels) mice, but not in α TTP^{-/-} (right panels) mice. Trends for these patterns (hypercapnic protection in α TTP^{+/+}, not in α TTP^{-/-}) were preserved for bronchoalveolar lavage levels of monocyte chemoattractant protein 1 (MCP-1; C), keratinocyte chemoattractant (KC; D), and interleukin 6 (IL-6; E) (* $p < 0.05$ vs normocapnia, t test). Data from non-ventilated mice are presented for illustrative purposes but excluded from statistical analysis. KO, knockout; WT, wild type.

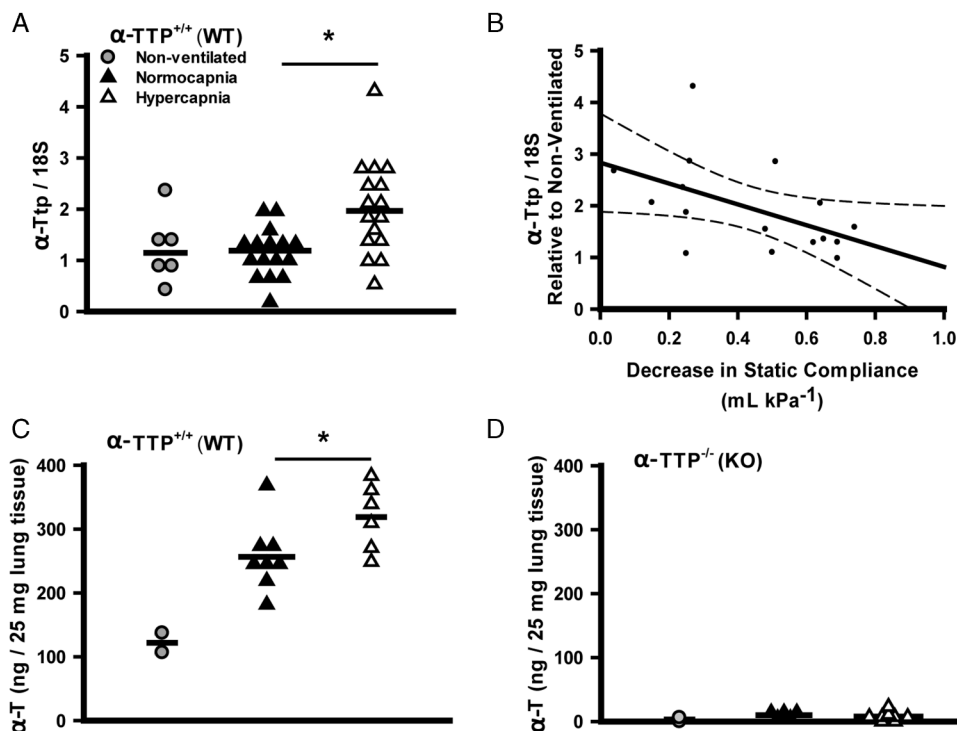
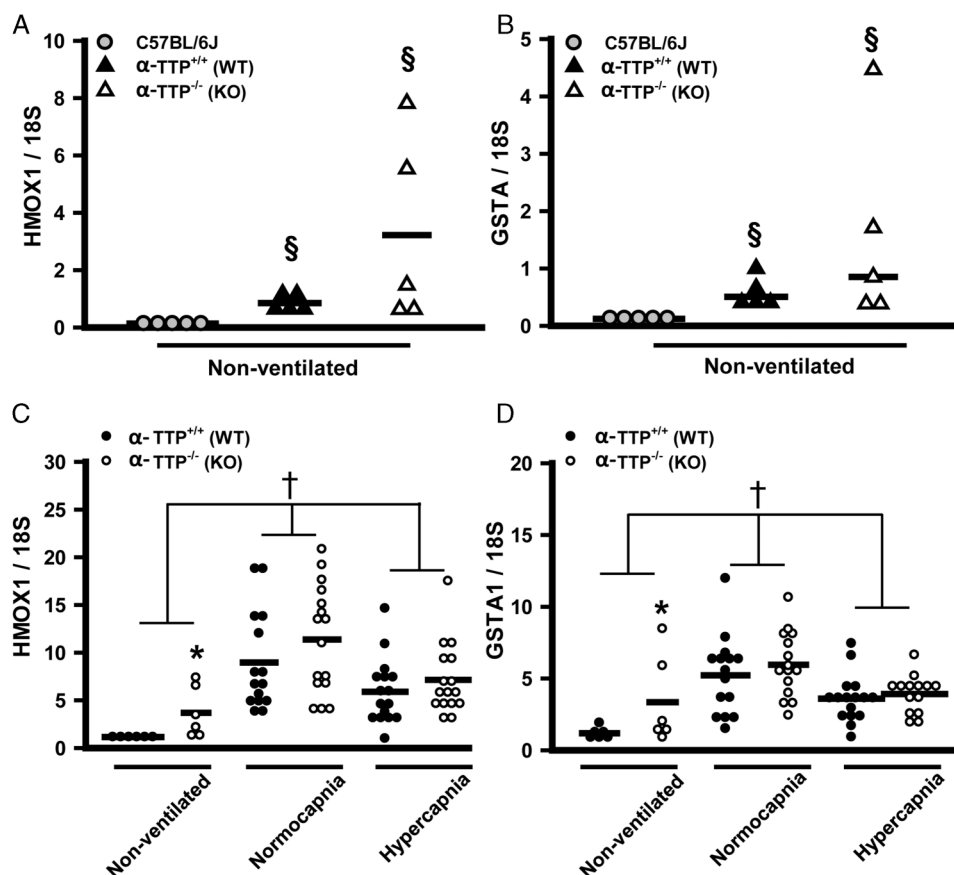


Figure 8 Effects of mechanical ventilation and hypercapnia on lung α -tocopherol transferase protein (α TTP) mRNA expression and α -tocopherol levels. Hypercapnia induced expression of α TTP mRNA in lung tissue of α TTP^{+/+} mice following high-stretch ventilation (Mann–Whitney) (A). Linear regression demonstrates the correlation between expression of α TTP mRNA and CO₂ protective effect (regression expressed \pm 95% CI; $R = -0.50$, $p < 0.05$) (B). Lung tissue α -tocopherol (α -T) levels in α TTP^{+/+} mice are increased by hypercapnia during ventilation (t test) (C), and are 20–50 fold less in α TTP^{-/-} mice (D) (* $p < 0.05$ vs normocapnia). Data from ($n = 2$ –4) non-ventilated mice are presented for illustrative purposes but excluded from statistical analysis. KO, knockout; WT, wild type.

Figure 9 Antioxidant response pathway in α -tocopherol transferase protein (α TTP^{+/+}) and α TTP^{-/-} murine model of ventilator-induced lung injury, effect of hypercapnia. α TTP^{+/+} and α TTP^{-/-} mice expressed higher constitutive levels of mRNA for NRF2 target genes heme oxygenase (HMOX1) (A) and glutathione-S-transferase (GSTA1) (B) than C57BL/6J mice (§ $p < 0.05$ vs C57BL/6J, ANOVA on RANKS). Expression of HMOX1 (C) and GSTA1 (D) during injurious mechanical ventilation is similar in α TTP^{+/+} and α TTP^{-/-} mouse lungs. In non-ventilated mice, constitutive levels of HMOX1 and GSTA1 were higher in α TTP^{-/-} versus α TTP^{+/+} mice (* $p < 0.01$ vs α TTP^{+/+}, two-way ANOVA). Both genes were similarly induced by VILI and this effect was abrogated by hypercapnia († $p < 0.05$ vs all other ventilation groups). KO, knockout; WT, wild type.



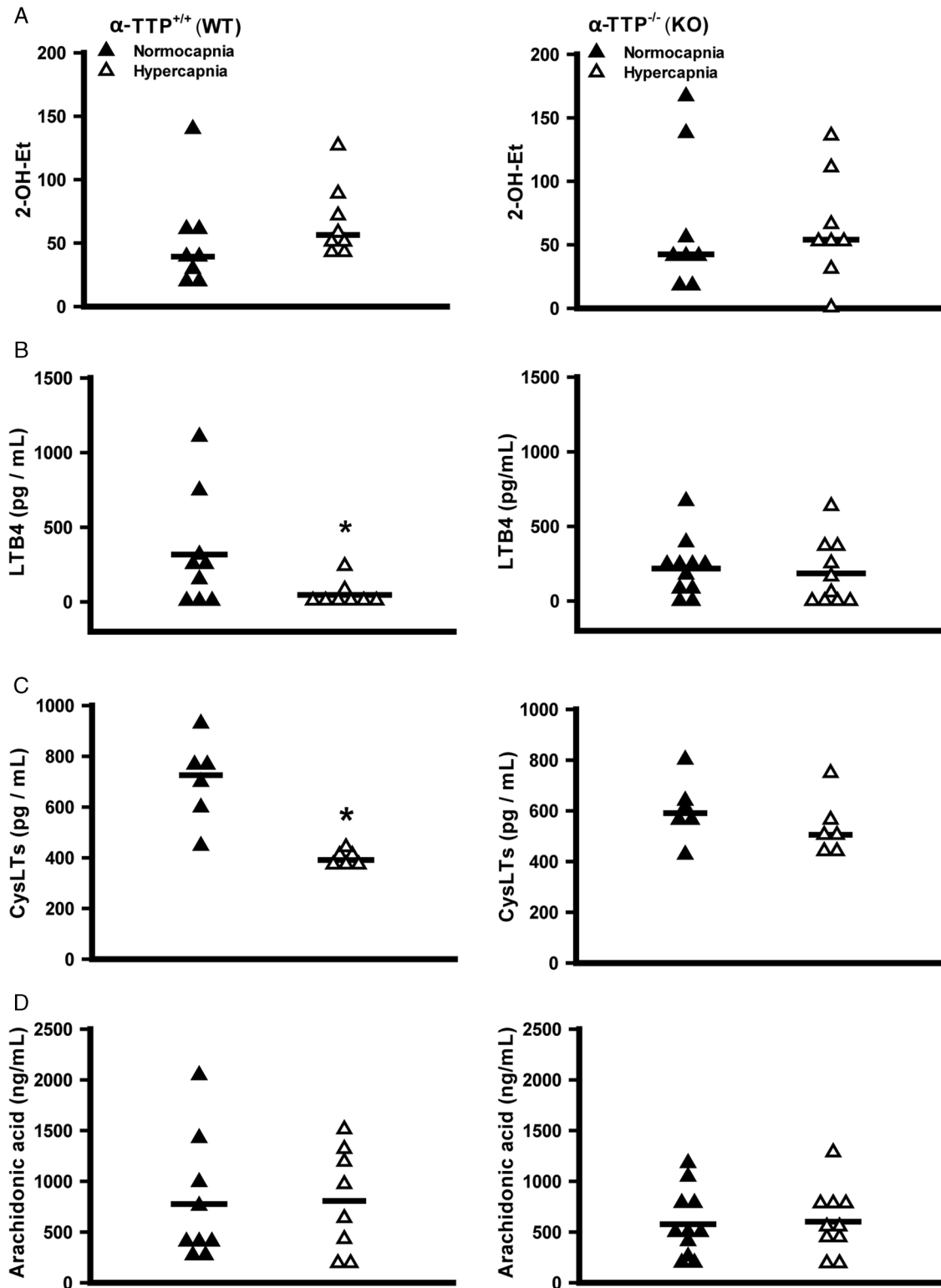


Figure 10 Hypercapnia-induced α tocopherol transfer protein (α TTP) attenuates leukotriene synthesis, not reactive oxygen species production, in murine ventilator-induced lung injury. Relative levels of superoxide in α TTP^{+/+} and α TTP^{-/-} mouse lungs were measured as 2-hydroxy-ethidium (2-OH-Et, arbitrary units) in mice injected with hydroethidine and subjected to 3 hours of injurious ventilation (A). There was no significant difference in superoxide levels between normocapnia and hypercapnia in α TTP^{-/-} or α TTP^{+/+} animals (Mann–Whitney). Hypercapnia suppressed levels of leukotriene B4 (LTB4, t test) and cysteinyl leukotrienes (CysLTs, Mann–Whitney) in the bronchoalveolar fluid from α TTP^{+/+} mice subjected to injurious ventilation, but had no effect on leukotriene levels in α TTP^{-/-} mice (B and C). Hypercapnia did not alter arachidonic acid levels in bronchoalveolar fluid from mice of either genotype (D) (* $p < 0.05$ vs normocapnia). KO, knockout; WT, wild type.

Despite preclinical success in experimental lung injury, caution is required in therapeutic targeting of leukotrienes. Many patients with lung injury have concomitant sepsis, and reduced leukotriene function could impair pathogen clearance. In addition, 5-LOX inhibition could divert arachidonic acid via alternative pathways (eg, COX), thereby altering the balance of eicosanoid signalling; in later stages this could impair production of the pro-resolving 5-LOX product, lipoxin A4. Targeting leukotriene receptors is effective in lung injury models.^{18–37} However, the high affinity LTB₄ receptor, known as BLT₁, is also a receptor for Resolvin E1, a pro-resolving mediator which enhances resolution of lung injury. Leukotriene A₄ hydrolase (LTA₄H), the enzyme that converts LTA₄ (the unstable direct product of 5-LOX) into LTB₄, can be targeted by several antagonists (eg, SC57461A, JNJ-26993235, or ARM1)³⁸ to reduce LTB₄ synthesis, while protecting pro-resolving mediators, but optimal blockade may also require CysLT inhibition.

There are important limitations to this study. Only male mice were used and impact of gender is unknown. We have been unable to identify the specific cell types in the lung that express α TTP due to a lack of suitable antibodies for immunohistochemistry. It is unclear whether the protective effects of α TTP (\approx 20% increased expression) result from the modest increase across all lung tissue, large changes in a subset of cells, or from α TTP's trafficking effect to particular subcellular locations. We have not tested whether α TTP has a protective role in other models of lung injury,^{3–4} and did not explore the mechanism by which hypercapnia upregulates α TTP expression. Finally, the α TTP gene promoter is not yet well characterised. Animal experiments investigating effects of dietary α -tocopherol and oxidative stress on hepatic α TTP expression in vivo provided conflicting data.³⁹ However, immortalised hepatocytes upregulate α TTP in response to peroxide, hypoxia and agonists of nuclear receptors peroxisome proliferator-activated receptor α and retinoid X receptor;⁴⁰ and the α TTP gene is a direct target for transactivation by the liver X receptor, a transcription factor that modulates cholesterol metabolism and lipid biosynthesis.³⁹

While deliberate induction of hypercapnia remains an experimental intervention,² so-called 'permissive hypercapnia' is widely accepted in mechanically ventilated patients; however, the case for net harm versus benefit in permissive hypercapnia is uncertain. Nevertheless, hypercapnia is an important tool in laboratory studies such as this to elucidate relevant, previously unsuspected pathways, such as upregulation of α TTP as shown here, or discovering the role of ADAM-17.⁶ Although the role of α TTP in lung tissue has received little attention, our data showing induction in multiple mouse models suggest broad applicability, and the correlation with lung protection is evidence of an induced, protective pathway. Targeted interventions such as aerosolisation of vitamin E formulations into the lung, or specific inhibition of LTA₄H, are related to new approaches to protecting the injured lung.

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Contributors GO conceived the study, conducted the study, and analysed the data. DE, HA and HH participated in study design, conducted experiments, analysed data. HB, MP and BPK participated in study design, data analysis and interpretation. All authors participated in drafting or critically revising the manuscript and approved the final version.

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