Evolutionary Conserved Role of c-Jun-N-Terminal Kinase in CO2-Induced Epithelial Dysfunction

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Abstract

Elevated CO2 levels (hypercapnia) occur in patients with respiratory diseases and impair alveolar epithelial integrity, in part, by inhibiting Na,K-ATPase function. Here, we examined the role of c-Jun N-terminal kinase (JNK) in CO2 signaling in mammalian alveolar epithelial cells as well as in diptera, nematodes and rodent lungs. In alveolar epithelial cells, elevated CO2 levels rapidly induced activation of JNK leading to downregulation of Na,K-ATPase and alveolar epithelial dysfunction. Hypercapnia-induced activation of JNK required AMP-activated protein kinase (AMPK) and protein kinase C-specificity leading to subsequent phosphorylation of JNK at Ser-129. Importantly, elevated CO2 levels also caused a rapid and prominent activation of JNK in Drosophila S2 cells and in C. elegans. Paralleling the results with mammalian epithelial cells, RNAi against Drosophila JNK fully prevented CO2-induced downregulation of Na,K-ATPase in Drosophila S2 cells. The importance and specificity of JNK CO2 signaling was additionally demonstrated by the ability of mutations in the JNK homologs, jnk-1 and kgb-2 to partially rescue the hypercapnia-induced fertility defects but not the pharyngeal pumping defects. Together, these data provide evidence that deleterious effects of hypercapnia are mediated by JNK which plays an evolutionary conserved, specific role in CO2 signaling in mammals, diptera and nematodes.

Introduction

Hypercapnia is an emerging area of interest in the pathogenesis of pulmonary diseases including acute respiratory distress syndrome (ARDS) and chronic obstructive pulmonary disease (COPD). Under physiological conditions, the alveolar epithelium provides optimal gas exchange by minimizing fluid in the alveolar space through active vectorial Na+ transport driven in part by the Na,K-ATPase [1,2,3]. We have previously reported that hypercapnia, by downregulating the Na,K-ATPase, impairs alveolar fluid reabsorption (AFR), thereby leading to alveolar epithelial dysfunction [4,5]; however, the mechanisms regulating the effects of hypercapnia have not been fully elucidated.

While chemoreception of CO2 in mammalian neurons have been described decades ago [6], only recently did it become clear that non-excitable mammalian cells are also capable of sensing, and responding to, changes in CO2 concentrations [7,8,9,10,11]. The c-Jun-N-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) superfamily, plays a key role in cell adaptation to stress stimuli [12,13,14,15]. The ubiquitously expressed isoform, JNK1, is phylogenetically highly conserved with orthologs in Drosophila and C. elegans [12,13]. Activation of JNK requires its phosphorylation at the TPY motif (Thr-183 and Tyr-185) by MAPK kinases (MAPKK). Critically, phosphorylation of JNK1 at the Ser-129 residue by protein kinase C (PKC) has been shown to be required for maximal JNK induction [16,17,18].

Notably, not only mammalian cells sense and adapt to CO2 changes. For example, CO2 avoidance, which is mediated by specific neurons, has been demonstrated in both C. elegans and Drosophila [19,20,21]. Moreover, elevated CO2 levels also exhibit specific, non-neural effects in both C. elegans and Drosophila, which appear to be independent of any previously indentified stress adaptation pathways [22,23]. Strikingly, as in mammals, elevated CO2 levels lead to rapid endocytosis of the Na,K-ATPase in Drosophila S2 cells by a yet unidentified mechanism [23]. We therefore hypothesized that the cellular responses to elevated CO2 levels might be mediated by JNK in mammals, Drosophila and C. elegans, indicating that JNK may play a central, evolutionary conserved role in CO2 signaling and adaptation to hypercapnia.
**Results**

**JNK Activation is Required for Hypercapnia-induced Decrease in Na,K-ATPase Plasma Membrane Abundance and AFR**

To determine whether elevated CO2 activates JNK in the alveolar epithelium, we assessed JNK phosphorylation at residues Thr-183/Tyr-185, which reflects the activation status of JNK [13]. Exposure of rat alveolar epithelial type II (ATII) cells to elevated CO2 levels (60–120 mmHg at an extracellular pH (pHe) of 7.4) led to a concentration- and time-dependent phosphorylation of JNK (Figure 1A). Importantly, when ATII cells were exposed to extracellular acidosis, but normal CO2 levels (40 mmHg at a pHe 7.2), JNK phosphorylation was not observed (Figure S1).

We have previously reported that hypercapnia leads to AFR impairment and promotes Na,K-ATPase endocytosis from the plasma membrane in ATII cells [4,5]. Importantly, Na,K-ATPase endocytosis was prevented when ATII cells were infected with an adenovirus expressing a dominant-negative variant of JNK (DN-JNK1-GFP), while hypercapnia-induced Na,K-ATPase endocytosis was preserved in ATII cells infected with a null (Ad-null) virus (Figure 1B). Similarly, in the presence of the specific JNK inhibitor, SP600125 (Figure 1C) or siRNA against JNK (Figure S2), Na,K-ATPase endocytosis was prevented upon elevated CO2. Consistent with our findings in ATII cells, CO2-induced impairment in AFR was prevented in rat lungs pretreated with SP600125 (Figure 2A) without effecting passive movement of small solutes (Figure 2B), suggesting that JNK activation was required for both hypercapnia-induced downregulation of the Na,K-ATPase in the alveolar epithelium and impairment of AFR.

**Activation of AMPK and PKC-ζ are Necessary to Stimulate JNK Upon Hypercapnia in Alveolar Epithelial Cells**

We have previously demonstrated that the AMP-activated protein kinase (AMPK) is an important element of CO2 sensing [5]. Since hypercapnia rapidly activates AMPK (within 1 min), we next examined whether JNK was a downstream target of AMPK. Indeed, JNK phosphorylation, induced by exposure of ATII cells to elevated CO2 levels for 10 min, was prevented when ATII cells were infected with an adenovirus expressing a dominant-negative variant of AMPK (DN-AMPK-α1; Figure 3A). Similarly, JNK phosphorylation was also inhibited by the AMPK inhibitor Compound C (Figure 3B), suggesting that AMPK acts upstream of JNK in the CO2-induced signaling cascade. Furthermore, treatment of ATII cells with AICAR, a chemical activator of AMPK, led to JNK phosphorylation (Figure S3); thus, AMPK activation was sufficient to stimulate JNK.

We and others have previously identified PKC-ζ as an important regulator of Na,K-ATPase [24,25], which is downstream of AMPK in the hypercapnia-induced signaling cascade [5]. Therefore, we next asked whether activation of JNK was regulated by PKC-ζ. Pretreatment of ATII cells with a myristoylated peptide inhibitor of PKC-ζ completely prevented the CO2-induced JNK activation (Figure 3C). Similar results were obtained in A549 cells overexpressing a dominant negative variant of PKC-ζ (DN PKC-ζ, Figure 3D), suggesting that PKC-ζ acts upstream of JNK. Moreover, preincubation of ATII cells with a high (but not with a low) dose of the PKC inhibitor bisindolylmaleimide I prevented the CO2-induced JNK activation (Figure S4A), further confirming that an atypical (as opposed to classical and novel) isoform of PKC was necessary for the CO2-induced JNK phosphorylation. In line with these findings, phorbol 12-myristate 13-acetate and Go6976, inhibitors of classical PKCs, did not affect activation of JNK upon hypercapnia (Figure S4B).
determined by biotin-streptavidin pull down and subsequent Western blot analysis. (C) ATII cells were exposed to 40 (open bars) or 120 (closed bars) mmHg CO₂ (pH 7.4) for 30 min in the presence or absence of SP600125 (5 μM, 30 min preincubation) and the amount of Na,K-ATPase protein at the plasma membrane was determined as in (B). Bars represent the mean ± SEM, n = 3. *, p<0.05, **, p<0.01.

Representative Western blots of Na,K-ATPase α-subunit at the plasma membrane and total protein abundance are shown. PM: plasma membrane, WCL: whole cell lysate.
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Interestingly, as opposed to PKC-ζ [4], JNK did not phosphorylate the Na,K-ATPase (Figure 4), suggesting that JNK may regulate the process of Na,K-ATPase trafficking.

Phosphorylation of JNK at Ser-129 by PKC-ζ Leads to JNK Activation and Na,K-ATPase Endocytosis during Exposure to Elevated CO₂

PKC-ζ, -β and -δ have been previously shown to phosphorylate JNK at its Ser-129 residue, thereby augmenting its activation by MAPKK [16,17,18]. Therefore, we next asked whether the PKC-ζ-mediated JNK activation upon hypercapnia was a consequence of JNK phosphorylation at Ser-129 by PKC-ζ. Immunokinase assays that used c-Jun as a substrate showed an increase in JNK activity after exposure to hypercapnia in A549 cells transfected with WT-JNK1-α. In contrast, overexpression of a mutant variant of JNK1 in which Ser-129 was mutated to alanine (S129A-JNK1-HA) prevented the hypercapnia-induced phosphorylation of the JNK downstream target c-Jun (Figure 5A), suggesting that the Ser-129 residue may serve as PKC phospho-acceptor site.

Furthermore, overexpression of S129A-JNK1-α-HA (as opposed to wild-type JNK) prevented endocytosis of the Na,K-ATPase during hypercapnia (Figure 5B), suggesting that phosphorylation of JNK at Ser-129 by PKC-ζ is required for JNK activation which in turn drives endocytosis of the Na,K-ATPase.

JNK is Required for CO₂-induced Na,K-ATPase Endocytosis in Drosophila S2 Cells and Mediates some, but not all, CO₂ Responses in C. elegans

Since CO₂ is a metabolite of all aerobic respiration, we reasoned that some of the mechanisms by which cells respond to CO₂ accumulation may be evolutionarily conserved. We therefore next investigated whether JNK might mediate CO₂ responses in Drosophila. Interestingly, exposure of Drosophila S2 cells to elevated CO₂ for 30 min resulted in a significant activation of Drosophila JNK (DJNK, also known as Basket; Figure 6A). Furthermore, RNA knockdown of the basket gene (bsk) completely prevented the CO₂-induced Na,K-ATPase endocytosis (Figure 6B). Thus, JNK is not only critically involved in CO₂ signaling in mammalian cells but also in diptera.

To further test for conservation of the role of JNK in CO₂ responses, we investigated if JNK was required for the CO₂-induced reductions in fertility and pharyngeal pumping in C. elegans that we had previously observed [22]. Deletion of the JNK homolog jnk-1 or kgb-2 significantly rescued the hypercapnia-induced impairment of fertility (Figure 7A). In contrast, pharyngeal pumping rate, which was markedly decreased upon exposure to elevated CO₂, was not affected by jnk-1(gk7) or kgb-2(gk161) null mutations (Figure 7B), highlighting the specificity of JNK action upon hypercapnia.

Discussion

CO₂ is a metabolite that has been produced by cells since aerobic respiration evolved over 2 billion years ago. One therefore would expect that some conservation of the mechanisms that cells use to respond to accumulation of CO₂ may exist. Here we provide evidence that JNK activation is an evolutionarily conserved mediator of CO₂ responses (Figure 8).

The role of hypercapnia in the pathogenesis of pulmonary diseases associated with alveolar hypoventilation, such as ARDS and COPD, remains incompletely understood. Particularly, the mechanisms by which the alveolar epithelium, the primary site of gas exchange and thus CO₂ elimination, senses and adapts to changes in CO₂ levels are largely unknown. We have previously demonstrated that functional integrity of the alveolar epithelium is rapidly impaired by changes in CO₂ concentrations [4,26,27]. In this study, we report that clinically relevant elevated CO₂ levels led to rapid activation of JNK in alveolar epithelial cells. Importantly, and in line with our previous findings showing that the deleterious effects of hypercapnia on the alveolar epithelium were independent of mechanisms in changes in extracellular pH [4], the CO₂-induced activation of JNK was also independent of pH. Critically, our data

Figure 2. Activation of JNK by hypercapnia is required for inhibition of AFR in rat lungs. Isolated rat lungs were perfused for 1 h with 40 mmHg CO₂ (pH 7.4; open bars) or with 60 mmHg CO₂ (pH 7.2; solid bars) in the presence or absence of SP600125 (5 μM, 30 min preincubation) and (A) AFR and (B) and passive fluxes of 22Na⁺ (dark grey bars) and 3H-mannitol (light grey bars) were measured as described in the online supplementary material. Bars represent the mean ± SEM, n = 5, **, p<0.01. AFR: alveolar fluid reabsorption.
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reveals that JNK activation was required for the hypercapnia-induced downregulation of the Na,K-ATPase in the alveolar epithelium and impairment of AFR. Previously, JNK has been shown to have a key role in promoting cellular adaptation to various stress stimuli and has recently emerged as a novel regulator of epithelial transport function [28,29] and Na,K-ATPase activity [30,31], but JNK has not been implicated in CO2 responses.

Why elevated CO2 concentrations lead to downregulation of the Na,K-ATPase has not been fully elucidated. It is well known that the Na,K-ATPase accounts for approximately 40% of cellular energy expenditure [2] and hypercapnia suppresses select physiological functions that are known to be metabolically demanding [4,22,23]. Thus, it is possible that elevated levels of CO2 signal excessive metabolic load. Consistent with this hypothesis, we have previously reported that AMPK, a metabolic sensor that regulates energy-consuming events, is an important element of CO2 sensing [5,8]. Moreover, we and others have previously identified PKC-ζ as an important regulator of Na,K-ATPase [24,25,32], which is downstream of AMPK in the hypercapnia-induced signaling cascade [5]. Therefore, we next asked whether activation of JNK was regulated by AMPK and PKC-ζ.

By applying genetic and pharmacological approaches, we found that both AMPK and PKC-ζ act upstream of JNK in the CO2-induced signaling cascade in alveolar epithelial cells.

Figure 3. CO2-induced activation of JNK is dependent on AMPK and PKC-ζ. (A) ATII cells were infected with Ad-Null or HA-tagged Ad-DN AMPK-α1 and were exposed 24 h later to (open bars) or 120 (closed bars) mmHg CO2 (pH 7.4) for 10 min and JNK activation was assessed. (B) ATII cells were exposed to 40 (open bars) or 120 (closed bars) mmHg CO2 (pH 7.4) for 10 min in the presence or absence of DMSO (20 μM, 30 min preincubation). (C) ATII cells were exposed to 40 (open bars) or 120 (closed bars) mmHg CO2 (pH 7.4) for 10 min in the presence of a myristoylated peptide inhibitor of PKC-ζ (15 μM, 30 min preincubation) or a scrambled peptide. Activation of JNK was determined by Western blot as described above. (D) AS49 cells expressing an empty vector or DN PKC-ζ were grown confluent in the presence of G418 after which cells were exposed to 40 (open bars) or 120 (closed bars) mmHg CO2 (pH 7.4) for 10 min. JNK was immunoprecipitated and incubated with c-Jun and p-c-Jun was measured by Western blot. Bars represent the mean ± SEM, n ≥ 3. *, p < 0.05; **, p < 0.01. Representative Western blots of p-JNK and total JNK (A-C) or p-c-Jun (D) are shown.

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Recently, the extracellular signal-regulated kinase (ERK), another member of the MAPK superfamily, has been also shown to play an important role in CO₂ signaling [33]. However, activation of ERK is extremely fast, peaking within seconds after CO₂ exposure and returning to baseline levels within 5 min [33] as opposed to JNK activation which occurs after 5–10 min. Interestingly, and in contrast to the role of JNK during CO₂ signaling, ERK was found to be upstream of AMPK playing a modulatory role in the AMPK-mediated sensing of CO₂. Thus these two members of the MAPK superfamily are both involved but play distinct roles in CO₂ sensing and signaling.

Once activated, PKC-ζ phosphorylates the Na,K-ATPase α₁-subunit at the Ser-11 or Ser-18 (depending on the species) thereby promoting its endocytosis [4,25,34]. Since mutation of Ser-18 of the Na,K-ATPase α₁-subunit prevents the CO₂-induced endocytosis of the Na,K-ATPase upon hypercapnia [4] one might ask how JNK (which is apparently downstream of PKC-ζ) can be involved in this process. Since JNK did not directly phosphorylate the Na,K-ATPase, it is possible that JNK regulates the process of Na,K-ATPase trafficking. Indeed, various reports described that JNK may modulate cytoskeletal rearrangement and function of molecular motors involved in trafficking of membrane proteins [35,36,37]. Also, we cannot fully exclude the involvement of other intermediates in the CO₂-induced signalling pattern. These possibilities are currently under investigation in our laboratory.

It is well established that JNK activation requires its phosphorylation at the TPY motif by MAPKK [12]. Interestingly, classical PKCs and PKC-δ, a novel PKC isoform, have been shown to be required for JNK induction by diverse stimuli including cytokines and UV-irradiation by phosphorylating JNK at Ser-129, thereby further augmenting its activation [16,17,18]. In the current study we found that phosphorylation of JNK at the Ser-129 residue is required for JNK activation and Na,K-ATPase endocytosis upon hypercapnia and that this residue may serve as PKC-ζ phosphorylation acceptor site.

Recently, it has become increasingly clear that cells and organisms respond to CO₂ and that some of those responses are highly similar [7]. For example, both mammalian alveolar epithelial cells and Drosophila S2 cells reduce their surface Na,K-ATPase levels in elevated CO₂ conditions [23]. However, it has not been clear whether the similar cellular responses were controlled by conserved intracellular processes. Remarkably, blocking JNK signaling prevented Na,K-ATPase endocytosis in both mammalian and Drosophila cells, strongly supporting the hypothesis that at least some responses to CO₂ are evolutionary conserved. Furthermore, downregulation of the C. elegans JNK homologs jnk-1 or kgb-2 significantly rescued the reduction in fertility but not the pharyngeal pumping rate defects caused by elevated CO₂ levels, suggesting the existence of multiple pathways that can mediate CO₂ responses and highlighting the specificity of JNK action.

In summary, we provide evidence that JNK activation is an evolutionary conserved mediator of CO₂ responses in mammals, Drosophila melanogaster and C. elegans. Further, we identify mammalian PKC-ζ as a novel upstream kinase responsible for JNK phosphorylation at Ser-129, leading to downregulation of the Na,K-ATPase and thus alveolar epithelial dysfunction.
Figure 5. CO₂-induced activation of JNK is dependent on Ser-129 phosphorylation downstream of PKC-ζ. (A) A549 cells transfected with a wild-type JNK (WT-JNK₁-HA) or with a mutant variant in which the Ser-129 residue was mutated to alanine (S129A-JNK₁-HA) were exposed to 40 (open bars) or 120 (closed bars) mmHg CO₂ (pH₇.4) for 10 min. JNK was immunoprecipitated and incubated with c-Jun and p-c-Jun was measured by Western blot. n.s.: non-specific bands. (B) A549 cells transfected with WT-JNK₁-HA or S129A-JNK₁-HA were exposed to 40 (open bars) or 120 (closed bars) mmHg CO₂ (pH₇.4) for 30 min. The amount of Na,K-ATPase protein at the plasma membrane was determined by biotinylation as described above. PM: plasma membrane, WCL: whole cell lysate. Values are expressed as mean ± SEM, n = 3. **, p<0.01.

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Materials and Methods

The CO₂ media, cell surface biotinylation and isolated-perfused lung preparation have been described in detail previously [4,5,25,38]. A brief description of methodologies and reagents is provided in the Supplemental Material.

Ethics Statement

Animals were handled according to National Institutes of Health guidelines and the experimental protocol for the use of rats (2010–2177) was approved by the Animal Care and Use Committee at Northwestern University.

Adenoviral Infection of ATII Cells

Day 2 ATII cells, plated on 60-mm cell culture dishes were incubated with null adenovirus (Ad-Null, 20 pfu/cell) or with adenovirus expressing a dominant-negative, JNK1 tagged with GFP (Ad-DN JNK1-GFP) as previously described [39], or carrying a dominant-negative, kinase dead (K45R) variant of the AMPK-α1-subunit (Ad-DN AMPK-α1, a generous gift from

Figure 6. CO₂-induced downregulation of Na,K-ATPase in Drosophila is mediated by JNK. (A) Drosophila S2 cells were allowed to attach to 6-well plates, were exposed to air (open bars) or 120 mmHg CO₂ (pHe 7.4, closed bars) for 30 min and phosphorylation of DJNK at Thr-183/Tyr-185 (p-DJNK) and total DJNK were measured by Western blot. E. coli peptidoglycan (PGN; 25 mg/ml, 15 min) was used as positive control. (B) Drosophila S2 cells were grown for 5 days after incubation with RNAi against DJNK/basket (RNAi-bsk) or a non-relevant RNAi (RNAi-lux). Thereafter cells were exposed to air (open bars) or 120 mmHg CO₂ (pH₇.4, closed bars) for 1 h and membrane abundance of the Drosophila Na,K-ATPase was assessed by cell surface biotinylation, Toll served as loading control. Values are expressed as mean ± SEM, n = 3, *, p<0.05; **, p<0.01. PM: plasma membrane, WCL: whole cell lysate.

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Figure 7. JNK mediates CO₂-induced inhibition of fertility but not impaired pharyngeal pumping rate in C. elegans. (A) Gravid C. elegans worms of genotype wild type (N2) or the null mutants jnk-1 (gk7) or kgb-2(gk361) were allowed to lay eggs for 6 hours at 20°C in either 19% CO₂ or in control air condition and the number of eggs laid in 19% CO₂ was normalized to the number of eggs laid in air condition. (B) Wild type and mutant worms were grown in normal air conditions until their first day of adulthood, exposed to air (open bars) or 19% CO₂ (closed bars) for 10 min and pharyngeal pumping rate was scored. Values are expressed as mean ± SEM, n = 30, **, p<0.01; ***, p<0.001.

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RNA Knockdown and Na,K-ATPase Membrane Abundance in Drosophila S2 Cells
For RNA knockdown, 5×10^5 Drosophila S2 cells (Invitrogen, Carlsbad, CA) were incubated with 7.5 µg double-stranded RNA for 30 min in serum-free medium in 6-well plates, and thereafter grown in 10% FBS-containing medium for 5 days. CO2 treatments were performed at 15% CO2 using pre-equilibrated media buffered to pH equal to air condition, with S2 cells attached to 6-well plates. Cell surface biotinylation was performed as previously described after 1 h in 15% CO2 [23].

Progeny Number and Pharyngeal Pumping Measurements in C. elegans
Gravid worms of wild type (N2), jnk-1(gk7) and kgb-2(gk361) null mutations were allowed to lay eggs for 6 hours at 20°C in either 19% CO2 or in control air condition. After 6 hours adult worms were removed, progeny was scored as described previously [22]. In experiments assessing pharyngeal pumping, animals were grown in normal air conditions until their first day of adulthood. Pumping rate was scored after 10 minutes exposure to air or 19% CO2.

Data Analysis
Data are expressed as mean ± SEM. Data were compared using analysis of variance adjusted for multiple comparisons with the Dunnet test. When comparisons were performed between two groups of values, significance was evaluated by Student’s t test. A p value < 0.05 was considered significant.

Supporting Information
Figure S1 ATII cells were exposed to 40 mmHg CO2 with a pHe of 7.4 or to 40 mmHg CO2 with a pHe of 7.2 for 10 min and the phosphorylation of JNK at Thr-183/Tyr-185 (p-JNK) and the total amount of JNK (JNK) was measured by Western blot analysis. Top: Graph represents the p-JNK/JNK ratio. Values are expressed as mean ± SEM, n = 3. Bottom: Representative Western blots of p-JNK and total JNK. pHe: extracellular pH.

Figure S2 (A) A549 cells were transfected with siRNA against JNK1 (siRNA - JNK1) or scrambled siRNA (scr siRNA) as described in the Supplemental methods. Twenty four hours after transfection cells were exposed to 40 or 120 mmHg CO2 (pH 7.4) for 10 min. Representative Western blots of p-c-Jun, JNK1 and JNK2 as well as actin (loading control) from A549 whole cell lysates (WCL) are shown. (B) Twenty four hours after transfection A549 were exposed to 40 (open bars) or 120 (closed bars) mmHg CO2 (pH 7.4) for 30 min. Na,K-ATPase at the plasma membrane was determined by biotin-streptavidin pull down and subsequent Western blot analysis. Bars represent the mean ± SEM, n = 3, **, p<0.01. Representative Western blots of Na,K-ATPase α1-subunit and E-cadherin (E-cad) at the plasma membrane (PM) are shown. n.s.: non-specific band.

Figure S3 ATII cells were treated with 2 mM AICAR or its vehicle for 30 or 60 min and phosphorylation of AMPK, acetyl-CoA carboxylase (ACC) and JNK (p-AMPK, p-ACC and p-JNK, respectively) and the amount of total AMPK and JNK were determined by Western blot. Representative Western blots are shown.

Figure 8. Schematic representation of the evolutionary conserved CO2-induced effects. Elevated CO2 levels rapidly activate JNK in C. elegans, Drosophila, rat lungs and human alveolar epithelial cells. In C. elegans (light grey arrows) the CO2-induced JNK activation leads to impaired fertility. In Drosophila (dark gray arrows) and in the rat and human alveolar epithelium (black arrows) the hypercapnia-induced JNK activation decreases Na,K-ATPase membrane stability leading to impaired alveolar epithelial barrier function in mammals. doi:10.1371/journal.pone.0046696.g008

Figure S1, S2 and S3 (TIF)
were exposed to 40 (open bars) or 120 mmHg CO2 (closed bars) mmHg CO2 (pHe 7.4) for 10 min in the presence or absence of GO6976 (1 μM, 30 min preincubation). p-JNK and total JNK were determined by Western blot. Graph represents the p-JNK/JNK ratio, values are expressed as mean ± SEM, n = 3. ** p < 0.01. Representative Western blots of p-JNK and total JNK are shown. (B) ATII cells were exposed to 40 (open bars) or 120 (closed bars) mmHg CO2 (pH 7.4) for 10 min in the presence or absence of GO6976 (1 μM, 30 min preincubation) or PMA (25 μM, 24 h preincubation). p-JNK and total JNK were determined by Western blot. Graph represents the p-JNK/JNK ratio, values are expressed as mean ± SEM, n = 3. ** p < 0.01. Representative Western blots of p-JNK and total JNK are shown.

Methods S1 Supporting Methods. (PDF)

Author Contributions
Conceived and designed the experiments: IV LAD YG JIS. Performed the experiments: IV LAD GTH KS LCW AMK BG. Analyzed the data: IV LAD YG JIS. Contributed reagents/materials/analysis tools: GRSB JL WS GJB. Wrote the paper: IV LAD JIS GJB YG.

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