HIF and HOIL-1L–mediated PKCζ degradation stabilizes plasma membrane Na,K-ATPase to protect against hypoxia-induced lung injury


Organisms have evolved adaptive mechanisms in response to stress for cellular survival. During acute hypoxic stress, cells down-regulate energy-consuming enzymes such as Na,K-ATPase. Within minutes of alveolar epithelial cell (AEC) exposure to hypoxia, protein kinase C zeta (PKCζ) phosphorylates the α1,Na,K-ATPase subunit and triggers it for endocytosis, independently of the hypoxia-inducible factor (HIF). However, the Na,K-ATPase activity is essential for cell homeostasis. HIF induces the heme-oxidized IRP2 ubiquitin ligase 1L (HOIL-1L), which leads to PKCζ degradation. Here we report a mechanism of prosurvival adaptation of AECs to prolonged hypoxia where PKCζ degradation allows plasma membrane Na,K-ATPase stabilization at ~50% of normoxic levels, preventing its excessive down-regulation and cell death. Mice lacking HOIL-1L in lung epithelial cells (Creα1–HOIL-1Lfl/fl) were sensitized to hypoxia because they express higher levels of PKCζ and, consequently, lower plasma membrane Na,K-ATPase levels, which increased cell death and worsened lung injury. In AECs, expression of an α1,Na,K-ATPase construct bearing an S18A (α1–S18A) mutation, which precludes PKCζ phosphorylation, stabilized the Na,K-ATPase at the plasma membrane and prevented hypoxia-induced cell death even in the absence of HOIL-1L. Adenoviral overexpression of the α1–S18A mutant Na,K-ATPase in vivo rescued the enhanced sensitivity of Creα1–HOIL-1Lfl/fl mice to hypoxic lung injury. These data suggest that stabilization of Na,K-ATPase during severe hypoxia is a HIF-dependent process involving PKCζ degradation. Accordingly, we provide evidence of an important adaptive mechanism to severe hypoxia, whereby halting the exaggerated down-regulation of plasma membrane Na,K-ATPase prevents cell death and lung injury.

Significance

Exposure to hypoxia requires adaptive mechanisms for survival. During acute hypoxia, Na,K-ATPase endocytosis in alveolar epithelial cells occurs via protein kinase C zeta (PKCζ) phosphorylation of α1,Na,K-ATPase independently of the hypoxia-inducible factor (HIF). However, exaggerated Na,K-ATPase down-regulation leads to cell death. Here we report that during prolonged hypoxia plasma membrane Na,K-ATPase levels were maintained at ~50% of normoxic values due to HIF-mediated up-regulation of HOIL-1L, which targets PKCζ for degradation. Silencing HOIL-1L in the lung epithelium prevented PKCζ degradation, causing Na,K-ATPase down-regulation. Accordingly, HIF regulation of HOIL-1L targets the phosphorylated PKCζ for degradation and serves as an hypoxia-adaptive mechanism to stabilize the Na,K-ATPase, avoiding significant lung injury.


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the transcription of heme-oxidized IRP2 ubiquitin ligase 1L (HOIL-1L), which acts as the E3 ubiquitin ligase for PKCζ, targeting it for proteasomal degradation (20, 25). HOIL-1L together with HOIL-1–interacting protein (HOIP) and Shank-associated RH-domain–interacting protein (SHARPIN) form the linear ubiquitination assembly complex (LUBAC) (26–29). We and others have found that, when acting independently of LUBAC, HOIL-1L adds Lys-48–linked chains and serves as an ubiquitin E3 ligase (20, 30).

Here we report that, in lung epithelial cells exposed to prolonged hypoxia in vitro, the Na,K-ATPase is stabilized at a plateau lower than levels in normoxic conditions via a HIF-mediated up-regulation of HOIL-1L. Hypoxia promotes the translocation of phosphorylated PKCζ to the plasma membrane where it interacts with HOIL-1L, which targets it for degradation. This PKCζ degradation limits Na,K-ATPase down-regulation and safeguards alveolar epithelial function. To examine this pathway in vivo, we generated mice with lung epithelial-specific deletion of HOIL-1L (CreSPC/HOIL-1Lfl/fl). Exposure of these mice to prolonged hypoxia resulted in higher levels of PKCζ, excessive depletion of plasma membrane Na,K-ATPase, and more severe lung injury. This sensitivity was rescued by overexpression of a phosphorylation-resistant mutant, Na,K-ATPase. Collectively, these data suggest that HOIL-1L–mediated ubiquitination and degradation of PKCζ results in Na,K-ATPase stabilization at the plasma membrane as an important adaptive mechanism to preserve the alveolar epithelium from significant injury.

Results

PKCζ Degradation by HOIL-1L Stabilizes Plasma Membrane Na,K-ATPase During Prolonged Hypoxia. During prolonged exposure of primary rat alveolar type II (ATII) cells to 1.5% O₂ (hypoxia), Na,K-ATPase activity and protein abundance at the plasma membrane decreased by ∼50%, compared with cells exposed to 21% O₂ (normoxia), and then stabilized (Fig. 1A and B). As observed in SI Appendix, Fig. S1A–C, mitochondrial ATP production decreased after 6 h of hypoxia compared with the normoxic values. However, all hypoxia time points showed similar intracellular ATP content (SI Appendix, Fig. S1D). In pVHL-deficient renal clear carcinoma cells (RCC4), where HIF is stabilized, HOIL-1L levels increased while PKCζ levels decreased (20, 31) and hypoxia-induced α₁-Na,K-ATPase endocytosis was prevented (Fig. 1C). Overexpression of

![Fig. 1. PKCζ degradation by HOIL-1L leads to the stabilization of plasma membrane Na,K-ATPase during hypoxia. (A and B) Rat ATII (r-ATII) cells were exposed to normoxia (21% O₂) or hypoxia (1.5% O₂) for up to 24 h. (A) Na,K-ATPase activity was measured in isolated membranes by a colorimetric assay as described in Experimental Procedures (n = 6). (B) Plasma membrane α₁-Na,K-ATPase (NKAα₁) was determined after cell-surface labeling with biotin in cells exposed to hypoxia or normoxia by immunoblot with a NKAα₁-specific antibody (n = 3). (C) RCC4 cells were transfected with empty vector (Vector) or YFP-PKCζ (PKCζ). Forty-eight hours after transfection, cells were exposed to normoxia or hypoxia for 2 h, and then the expression of plasma membrane (PM) α₁-Na,K-ATPase was determined after cell-surface labeling with biotin and determined by Western blot. Transfection efficiency was determined by performing a Western blot on cell lysates (CL) proteins using YFP-specific antibody and β-actin antibody as a loading control (n = 6). (D) PKCζ levels were assessed in the cell lysates of r-ATII cells exposed to normoxia or hypoxia for up to 24 h by Western blot (n = 6). (E–G) A549 cells were transfected with the indicated siRNA and exposed to normoxia or hypoxia for 24 h. Expression of α₁-Na,K-ATPase at the plasma membrane was determined by cell-surface biotinylation and Western blot (n = 6). (H) Schematic representation of the hypoxia-induced pathway for PM Na,K-ATPase stabilization. Representative immunoblots and bar graphs showing the densitometry quantifications of immunoblots in relation to the corresponding loading controls are shown. Data are expressed as mean ± SD. Statistical significance was calculated using one-way ANOVA and the Tukey multiple comparisons test (*P < 0.05, **P < 0.01, ***P < 0.001).](https://www.pnas.org/cgi/doi/10.1073/pnas.1713563114)
YFP-PKCζ in these cells restored the Na,K-ATPase endocytosis (Fig. 1C). Hypoxia does not affect PKCζ mRNA levels or protein synthesis (20). However, hypoxia caused a time-dependent decrease of PKCζ levels in rat ATII cells (Fig. 1D). To assess the role of LUBAC during the hypoxia-induced Na,K-ATPase down-regulation, we performed loss-of-function experiments using A549 cells. Even though A549 cells are tumor cells, we and many others have reported that they have many characteristics of ATII cells (32, 33), including the regulation of Na,K-ATPase (1, 33, 34). HOIL-1L in A549 cells led to a more significant decrease of plasma membrane α1-Na,K-ATPase abundance during hypoxia compared with cells transfected with control siRNA (Fig. 1E). As with HOIL-1L silencing, HIF1α silencing resulted in fewer Na,K-ATPase molecules observed at the plasma membrane of AECs exposed to hypoxia compared with cells transfected with a control siRNA and exposed to hypoxia (Fig. 1F). Overexpression of HOIL-1L rescues the effects of HIF1α silencing, preventing the Na,K-ATPase endocytosis (Fig. 1G). However, silencing of HOIP did not further alter the α1-Na,K-ATPase abundance at the plasma membrane after 24 h of hypoxia (SI Appendix, Fig. S1E). Collectively, these data suggest that, in AECs exposed to hypoxia, HIF regulates HOIL-1L, which, by promoting PKCζ degradation, leads to plasma membrane α1-Na,K-ATPase stabilization (Fig. 1H).

HOIL-1L Silencing Leads to Exaggerated α1-Na,K-ATPase Down-Regulation in Lung Epithelium During Hypoxia. Analysis of peripheral lung tissue lyses from C57BL/6 (WT) mice exposed to 7% O2 (hypoxia) for up to 14 d showed a significant increase in HOIL-1L in parallel with a decrease in PKCζ protein abundance (Fig. 2A). We generated CreSPC/HOIL-1Lfl/fl mice, which bear a lung epithelial-specific deletion of the Rbck1 (HOIL-1L) gene (as described in Experimental Procedures). We analyzed, by RNA sequencing, the transcriptome of ATII cells isolated from WT and CreSPC/HOIL-1Lfl/fl mice in basal conditions. Only 20 of 13,617 detected genes were differentially expressed, suggesting that the deletion of HOIL-1L in the alveolar epithelium did not cause major changes in the transcriptome (Fig. S2). In contrast to the observed decrease in PKCζ levels in ATII cells from control mice exposed to hypoxia, PKCζ levels did not change in ATII cells isolated from CreSPC/HOIL-1Lfl/fl mice (Fig. 2B). The expression levels of neither HOIP nor SHARPIN, the other components of LUBAC, changed in lung tissue of mice exposed to hypoxia (Fig. S2B). We assessed, by flow cytometry, the effects of HOIL-1L deletion on α1-Na,K-ATPase abundance at the plasma membrane of ATII cells from mice exposed to 7% O2 for 7 d (SI Appendix, Fig. S2B, for gating strategy) and found no differences between the α1-Na,K-ATPase abundance in ATII cells from WT (black) or CreSPC/HOIL-1Lfl/fl mice exposed to 7% O2 (Fig. 2C and D, solid bars), whereas ATII cells from CreSPC/HOIL-1Lfl/fl mice exposed to 7% O2 had lower α1-Na,K-ATPase abundance compared with cells from WT mice exposed to hypoxia (Fig. 2C and D, striped bars). These data suggest that in AECs from mice exposed to hypoxia, the absence of HOIL-1L leads to increased PKCζ levels and decreased plasma membrane α1-Na,K-ATPase protein abundance.

Hypoxia Causes Lung Injury in Mice with HOIL-1L Deletion in the Lung Epithelium. To assess whether deletion of HOIL-1L in the alveolar epithelium affects lung function, we exposed mice to 7% O2 for 7 d and found that the epithelial permeability to small solutes increased (Fig. 3A), as well as the total number of cells and protein concentration in the bronchoalveolar lavage fluid (BALF) (Fig. 3B and C) in CreSPC/HOIL-1Lfl/fl mice compared with WT mice. H&E staining of lung tissue revealed that in lungs of CreSPC/HOIL-1Lfl/fl mice exposed to low O2 conditions there was increased thickening in the alveolar septa, more noticeable in the peribronchiolar area, compared with WT mice lungs exposed to hypoxia (Fig. 3D and E). Moreover, in lungs of CreSPC/HOIL-1Lfl/fl mice exposed to hypoxia there was increased apoptosis (as measured by TUNEL assay) compared with lungs of WT mice (Fig. 3F and G).

It has been reported that hypoxia induces an inflammatory response with recruitment of immune cells into the alveolar spaces (35). To assess whether the increased injury observed in CreSPC/HOIL-1Lfl/fl mice in response to hypoxia was due to an exaggerated immune response, we performed flow cytometric analysis of the immune cells in the lungs. While the numbers of neutrophils and interstitial macrophages in mice exposed to hypoxia were elevated, there was no difference in immune cell composition between WT and CreSPC/HOIL-1Lfl/fl mice either in basal state or after being exposed to hypoxia (SI Appendix, Fig. S3).

To determine whether the HOIL-1L-mediated cell adaptation to hypoxia was due to the stabilization of Na,K-ATPase at the plasma membrane, we used A549 cells expressing GFP-α1-Na,K-ATPase-WT (α1-WT) or GFP-α1-Na,K-ATPase-S18A (α1-S18A). The Na,K-ATPase bearing an S18A mutation is resistant to PKCζ phosphorylation and thus fails to internalize during hypoxia (1, 33, 36). In α1-WT cells transfected with HOIL-1L-specific siRNA, exposure to hypoxia significantly increased cell death compared with α1-WT cells transfected with a scrambled siRNA (Fig. 4A). In contrast, in α1-S18A A549 cells neither the silencing of HOIL-1L nor the exposure to hypoxia increased cell death (Fig. 4A), supporting the hypothesis that during prolonged hypoxia HOIL-1L exerts a protective effect by promoting PKCζ degradation, which stabilizes the α1-Na,K-ATPase at the AEC plasma membrane.
We also infected WT and Cre^{SPC}/HOIL-1L^{fl/fl} mice before exposure to 7% O_2 with an adenoviral construct coding for GFP-\(\alpha_1\)-Na,K-ATPase-WT (Ad \(\alpha_1\)-WT) or GFP-\(\alpha_1\)-Na,K-ATPase-S18A (Ad \(\alpha_1\)-S18A). The infection efficiency was assessed by measuring the expression of GFP-\(\alpha_1\)-Na,K-ATPase in membranes from lung peripheral tissue by immunoblotting with an anti-GFP antibody (Fig. 4B). As observed in noninfected mice (Fig. 3A), lungs of Cre^{SPC}/HOIL-1L^{fl/fl} mice expressing Ad \(\alpha_1\)-WT exposed to hypoxia had increased permeability and total cell counts in the BALF compared with WT mice (Fig. 4C and SI Appendix, Fig. S4).

However, Cre^{SPC}/HOIL-1L^{fl/fl} mice expressing \(\alpha_1\)-S18A exposed to hypoxia did not have significant changes in lung permeability or total cell counts compared with mice breathing room air (Fig. 4C and SI Appendix, Fig. S4). Moreover, the decrease in Na,K-ATPase activity observed in Cre^{SPC}/HOIL-1L^{fl/fl} mice exposed to hypoxia was rescued in mice infected with Ad-\(\alpha_1\)-S18A (Fig. 4D).

**Phosphorylation at Thr-410 Is Required for PKC\(\zeta\) Ubiquitination and Degradation.** Phosphorylation in the activation loop of the PKC\(\zeta\) kinase domain at Thr-410 exposes it to further phosphorylation and ubiquitination, leading to its degradation. This process is critical for the regulation of PKC\(\zeta\) activity and its role in various cellular processes including stress response and immune cell function. The effect of Thr-410 phosphorylation on PKC\(\zeta\) activity and stability suggests potential therapeutic targets for modulating PKC\(\zeta\) function in disease contexts.
and is considered the key event in stabilizing PKCζ in the active conformation at the plasma membrane (37). In COS-7 cells transfected with FLAG-PKCζ-WT, hypoxia-induced PKCζ phosphorylation increased after 4 h and returned to basal levels after 24 h of exposure (Fig. 5A). To assess whether AMPK-induced phosphorylation is necessary for PKCζ down-regulation during hypoxia, we infected rat ATII cells with a dominant-negative AMPK α1 subunit adenovirus (DN-AMPK) or an empty adenovirus (Null). The lack of AMPK activity was sufficient to prevent the hypoxia-induced PKCζ down-regulation (Fig. 5B). To further confirm the role of phosphorylation in PKCζ degradation, we used loss- and gain-of-function analysis. Expression of PKCζ bearing a T410A mutation, FLAG-PKCζ-T410A (T410A), prevented the hypoxia-induced degradation of PKCζ, whereas a PKCζ phosphorylation mimic, FLAG-PKCζ-T410E (T410E), was degraded during hypoxia similarly to PKCζ-WT (Fig. 5C). The degradation of FLAG-PKCζ-T410E during hypoxia was prevented in HOIL-1L-silenced cells (Fig. 5D).

To determine whether the interaction between PKCζ and HOIL-1L during hypoxia requires PKCζ phosphorylation, cells were cotransfected with HA-HOIL-1L and either FLAG-PKCζ-WT or FLAG-PKCζ-T410 and exposed to hypoxia, and then proteins in the cell lysate were coimmunoprecipitated by use of an anti-FLAG antibody. Exposure to hypoxia increased PKCζ-WT phosphorylation and the interaction between HOIL-1L and PKCζ-WT (Fig. 5E, lanes 1 and 2). Overexpression of PKCζ-T410A prevented not only the hypoxia-induced PKCζ phosphorylation but also the interaction between HOIL-1L and PKCζ (Fig. 5E, lanes 3 and 4). Similarly, transfection with FLAG-PKCζ-T410A prevented the hypoxia-induced PKCζ ubiquitylation (Fig. 5F).

**PKCζ Degradation Is Initiated in the Plasma Membrane.** Hypoxia-induced PKCζ phosphorylation is required for its translocation to the plasma membrane, as translocation was prevented in cells overexpressing FLAG-PKCζ-T410A (Fig. 6A). PKCζ remained phosphorylated for at least 4 h (Fig. 5A), and its removal from the plasma membrane had a significant effect after 6 h of hypoxia exposure (Fig. 6B). These data suggest that the interaction between HOIL-1L and the phosphorylated PKCζ occurs at the plasma membrane, which was confirmed by transfecting COS-7 cells with FLAG-PKCζ-WT and HA-HOIL-1L, exposing the cells to hypoxia for 4 h, isolating the membranes, and coimmunoprecipitating the solubilized proteins with an anti-FLAG antibody (Fig. 6C). Moreover, the hypoxia-induced PKCζ ubiquitylation occurs at the plasma membrane (Fig. 6D).

**Discussion**

Adaptation to hypoxia requires coordination between energy demand and supply to promote cell homeostasis. The Na,K-ATPase is a major energy consumer, accounting for 20–70% of metabolic ATP depending of the tissue (38, 39). To conserve energy during acute hypoxia, the Na,K-ATPase α1 subunit is phosphorylated by PKCζ, which triggers its endocytosis, independently of HIF (1, 36). Here we describe that AEC exposure to prolonged hypoxia results in decreased Na,K-ATPase levels; however, the cells survive with Na,K-ATPase activity calculated for

![Fig. 4. Alveolar epithelial function in Cre<sup>SPC</sup>/HOIL-1L<sup>fl/fl</sup> mice exposed to hypoxia is rescued by Na,K-ATPase α1-S18A overexpression.](image-url)

(A) Cell death was measured using a lactate dehydrogenase (LDH) assay in incubation medium from A549 cells stably expressing rat Na,K-ATPase α1-WT (A549 α1-WT) or α1-S18A (A549 α1-S18A) that were transfected with scrambled (control, white bars) or HOIL-1L (HOIL-1L, black bars) siRNA and exposed to normoxia or 1.5% O2 for 24 h (n = 3). (B) WT (white bars in C and D) and Cre<sup>SPC</sup>/HOIL-1L<sup>fl/fl</sup> (black bars in C and D) mice were infected with either adenovirus expressing GFP-NaK-ATPase α1-WT (Ad-α1-WT) or GFP-NaK-ATPase α1-S18A (Ad-α1-S18A) and kept in room air or exposed to 7% O2 for 7 d. (B) Adenovirus infection efficiency was determined by measuring GFP expression in lung peripheral tissue by Western blot. CD29 was used as a loading control. (C) Alveolar epithelial permeability is expressed as the ratio of the fluorescence present in BALF to the plasma (n = 3). (D) Na,K-ATPase activity measured in isolated plasma membranes from lung peripheral tissue by a colorimetric assay as described in Experimental Procedures. Graph bars represent mean ± SD. Statistical significance was calculated for E and G using one-way ANOVA and the Tukey multiple comparisons test (*P < 0.05, ***P < 0.001).

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normoxia levels) (Fig. 1 A and B) to conserve energy. This is achieved via HIF-induced up-regulation of the E3 ubiquitin ligase HOIL-1L and phosphorylation of PKCζ by AMPK. HOIL-1L then interacts with and ubiquitylates the phosphorylated PKCζ at the plasma membrane, triggering its degradation.

HOIL-1L is a member of LUBAC, which catalyzes the non-degradative linear ubiquitination of proteins. However, not only is the activity of HOIL-1L on PKCζ independent of the function of HOIP (the LUBAC catalytic component), but also HOIP competes with PKCζ for binding to HOIL-1L (20). We found that silencing of HOIP does not affect Na,K-ATPase levels at the plasma membrane during hypoxia (SI Appendix, Fig. S1E).

In RCC4 cells, which express constitutive levels of HIF1α, high levels of HOIL-1L, and low levels of PKCζ, hypoxia fails to induce Na,K-ATPase endocytosis, which is rescued by overexpression of PKCζ (Fig. 1D). In agreement with these data, silencing of HOIL-1L in hypoxia-exposed cells increased Na,K-ATPase down-regulation and cell death. The increase in cell death in the absence of HOIL-1L is rescued by expressing a phosphorylation-deficient Na,K-ATPase α1 subunit (Fig. 4A), indicating the specificity of the effect. As we have previously reported, the phosphorylation by PKCζ of the plasma membrane Na,K-ATPase-α1 subunits triggers the internalization of Na,K-ATPase molecules during hypoxia. It has been described that myristoylated alanine-rich C kinase substrate proteins (MARCKS) in the nonphosphorylated state sequester PIP2, preventing the formation of signaling complexes and thereby promoting the stability of plasma membrane proteins (40, 41). However, MARCKS is not a substrate for the atypical PKCs.

Concordant with the in vitro results, ATII cells isolated from WT mice exposed to 7% O2 for 7 d had increased HOIL-1L and...
decreased PKCζ levels (Fig. 2A). In contrast, PKCζ levels did not decrease in ATII cells from Cre<sup>SPC/HOIL-1L<sup>fl/fl</sup></sup> mice exposed to hypoxia as they lacked HOIL-1L. We found significant α1-Na,K-ATPase down-regulation (Fig. 2E), more injury (Fig. 3), and increased apoptosis (Fig. 3) in lungs from Cre<sup>SPC/HOIL-1L<sup>fl/fl</sup></sup> mice than in lungs from WT mice after low O<sub>2</sub> treatment. We have previously reported that hypoxia moderately impairs the alveolar fluid clearance (42). However, this impairment did not result in evident alveolar edema, allowing the mice to adapt and survive hypoxic conditions. Interestingly, alveolar type I cells express lower levels of LUBAC compared with ATII cells (SI Appendix, Fig. S5). While ATII cells express only the Na,K-ATPase α<sub>1</sub> isoform, ATI cells express both α<sub>1</sub> and α<sub>2</sub> Na,K-ATPase isoforms. The α<sub>2</sub> isoform does not have a PKC consensus phosphorylation domain (Scansite 3 beta). Therefore, the alveolar fluid clearance could be conserved in ATII cells and protect against exaggerated lung injury.

The importance of HOIL-1L in the stabilization of α1-Na,K-ATPase during prolonged hypoxia was confirmed by the rescue of the epithelial function in Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup> mice overexpressing a phosphorylation-resistant Na,K-ATPase-α<sub>1</sub> subunit (Fig. 4). Low O<sub>2</sub> exposure can be associated with increased immune cell recruitment and inflammatory response that has been linked with endothelial injury (43, 44). Interestingly, the increased lung injury observed in Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup> mice during prolonged hypoxia does not appear to increase the hypoxia-induced inflammatory response in the lung but rather results from Na,K-ATPase down-regulation.

The activation of atypical PKCs is analogous to the activation of Akt, in which the loop phosphorylation is agonist-dependent and required for its translocation to the plasma membrane where the autoinhibition is removed, enabling the phosphorylation of substrates (45). In the current study, we provide evidence that the AMPK-induced phosphorylation at Thr-410 and activation are necessary for the ubiquitination and degradation of PKCζ. Translocation of PKCζ to the plasma membrane occurs within 10 min of hypoxia exposure, and it remains membrane-bound for at least 4 h (Fig. 5A). Similarly to PKCζ-WT, the degradation of PKCζ-T410E increased during hypoxia (Fig. 5C). These data suggest that both the hypoxia-induced phosphorylation of PKCζ and the HIF-dependent HOIL-1L up-regulation are required for PKCζ degradation to occur. Activation-dependent ubiquitylation and proteasomal degradation have been described for PKC isoforms (22, 46), suggesting that the ubiquitin proteasome system is critical for PKC regulation. Our data suggest that HOIL-1L interacts with and ubiquitylates the phosphorylated PKCζ at the plasma membrane to trigger PKCζ degradation (Fig. 6).

Accordingly, we provide evidence for an adaptive mechanism of cells to hypoxia, linking a HIF-regulated pathway to Na,K-ATPase stabilization at the plasma membrane (Fig. 7). During prolonged hypoxia, HIF up-regulates HOIL-1L, which targets PKCζ for degradation, thereby preventing it from further downregulating the Na,K-ATPase to conserve ATP and promote AEC survival. This mechanism represents a noncanonical pathway in which HOIL-1L acts as an E3 ligase for PKCζ to protect the alveolar epithelium against lung injury during severe hypoxia.

**Experimental Procedures**

**Generation of Cre<sup>SPC/HOIL-1L<sup>fl/fl</sup></sup> Mice.** We generated a targeting vector containing LoxP sites flanking exon 8 in the HOIL-1L genomic allele, followed by a neomycin resistance gene (SI Appendix, SI Experimental Procedures). Targeted ES cells were subjected to homologous recombination. Southern blot analysis was performed to screen for homologous integration of the targeting vector. Selected ES cells were microinjected into the blastocysts of male C57BL/6J <s>+</s> C57BL/6J females. Chimeric mice were bred with C57BL/6J males to generate offspring mice for the experiments. Oligonucleotides for the Cre<sup>SPC/HOIL-1L<sup>fl/fl</sup></sup> mice were designed to target the Cre<sup>SPC/HOIL-1L<sup>fl/fl</sup></sup> allele to generate the Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup> mice.

**Generation of Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup>/Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup> Mice.** We generated a targeting vector containing LoxP sites flanking exon 8 in the HOIL-1L genomic allele, followed by a neomycin resistance gene (SI Appendix, SI Experimental Procedures). Targeted ES cells were subjected to homologous recombination. Southern blot analysis was performed to screen for homologous integration of the targeting vector. Selected ES cells were microinjected into the blastocysts of male C57BL/6J <s>+</s> C57BL/6J females. Chimeric mice were bred with C57BL/6J males to generate offspring mice for the experiments. Oligonucleotides for the Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup>/Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup> mice were designed to target the Cre<sup>SPC/HOIL-1L<sup>fl/fl</sup></sup> allele to generate the Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup>/Cre<sup>SPC/HOIL-1L<sup>β/β</sup></sup> mice.

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Alveolar Epithelial Cell Isolation and Culture. Mice and rats were provided with food and water ad libitum, maintained on a 14-h/10-h light-dark cycle, and handled according to National Institutes of Health guidelines and the experimental protocol approved by the Northwestern University Institutional Animal Care and Use Committee. Rat ATII cells from the lungs of male Sprague-Dawley rats (weight: 200–225 g) (29) or mouse ATII cells from WT and Cre\(^{ΔE694}\)/HOIL-1\(^{−/−}\) mice (age: 10–12 wk) (50) were isolated by the Pulmonary Division Cell Culture and Physiology Core from Northwestern University. The day of isolation and plating of rat ATII cells was designated day 0. All experiments were conducted on day 3.

The following cell lines were used: human adenocarcinoma A549 (ATCC CCL 185), African green monkey kidney COS-7 (ATCC CRL 1651), and pVHL-deficient human RCC4 (51). A549 cells stably expressing the rat Na,K-ATPase-\(α_1\) subunit WT or with a Ser-18 mutation have been described elsewhere (1). ATII cells from the lungs of male Sprague-Dawley rats (weight: 200–225 g) (29) or mouse ATII cells from WT and Cre\(^{ΔE694}\)/HOIL-1\(^{−/−}\) mice (age: 10–12 wk) (50) were isolated by the Pulmonary Division Cell Culture and Physiology Core from Northwestern University. The day of isolation and plating of rat ATII cells was designated day 0. All experiments were conducted on day 3.

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Additional materials and methods can be found in SI Appendix, SI Experimental Procedures.

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Fig. 7. Cartoon depicting the proposed mechanism for Na,K-ATPase stabilization during chronic hypoxia. Hypoxia leads to stabilization of HIF, which regulates HOIL-1L, leading to PKCζ ubiquitination to avoid an excessive depletion of Na,K-ATPase, and safeguarding the alveolar epithelial function as a mechanism of adaptation to hypoxic conditions.


