Hypoxia-Mediated Degradation of Na,K-ATPase via Mitochondrial Reactive Oxygen Species and the Ubiquitin-Conjugating System

Alejandro P. Comellas, Laura A. Dada, Emilia Lecuona, Liuska M. Pesce, Navdeep S. Chandel, Nancy Quesada, G.R. Scott Budinger, Ger J. Strous, Aaron Ciechanover, Jacob I. Sznajder

Abstract—We set out to determine whether cellular hypoxia, via mitochondrial reactive oxygen species, promotes Na,K-ATPase degradation via the ubiquitin-conjugating system. Cells exposed to 1.5% O2 had a decrease in Na,K-ATPase activity and oxygen consumption. The total cell pool of α1 Na,K-ATPase protein decreased on exposure to 1.5% O2 for 30 hours, whereas the plasma membrane Na,K-ATPase was 50% degraded after 2 hours of hypoxia, which was prevented by lysosome and proteasome inhibitors. When Chinese hamster ovary cells that exhibit a temperature-sensitive defect in E1 ubiquitin conjugation enzyme were incubated at 40°C and 1.5% O2, the degradation of the α1 Na,K-ATPase was prevented. Exogenous reactive oxygen species increased the plasma membrane Na,K-ATPase degradation, whereas, in mitochondrial DNA deficient ρ0 cells and in cells transfected with small interfering RNA against Rieske iron sulfur protein, the hypoxia-mediated Na,K-ATPase degradation was prevented. The catalase/superoxide dismutase (SOD) mimetic (EUK-134) and glutathione peroxidase overexpression prevented the hypoxia-mediated Na,K-ATPase degradation and overexpression of SOD1, but not SOD2, partially inhibited the Na+,K+-ATPase degradation. Accordingly, we provide evidence that during hypoxia, mitochondrial reactive oxygen species are necessary to degrade the plasma membrane Na,K-ATPase via the ubiquitin-conjugating system. (Circ Res. 2006;98:1314-1322.)

Key Words: ATP • oxygen • proteasome • antioxidants • cell adaptation

A daptation to hypoxia represents a well-defined means to improve ischemic tolerance. Unfortunately, there is no definitive understanding of the mechanisms associated with these phenomena.1 As mammalian cells encounter lower oxygen levels, they develop mechanisms to prevent depletion of oxygen to anoxia that might result in cell death.2 Cells respond to hypoxia through the stabilization of the transcription factor hypoxia-inducible factor (HIF)-1α.3 In normoxic conditions, prolyl hydroxylases hydroxylate conserved proline residues in HIF-1α.3,4 This substrate modification is recognized by a ubiquitin ligase enzyme (Von–Hippel–Lindau protein [VHL]) that ubiquitinates and targets HIF-1α to the proteasome. During hypoxia, VHL-mediated degradation of HIF-1α is suppressed, allowing its transcriptional activation.4,5 The intracellular mechanisms by which cells sense hypoxia to stabilize HIF-1α are not fully understood. The generation of mitochondrial reactive oxygen species (mROS) during hypoxia has been proposed as part of an oxygen sensing pathway for the hypoxia stabilization of HIF-1α.6

Another mechanism to prevent the depletion of oxygen during hypoxia is to decrease the cellular demand for oxygen by upregulating anaerobic ATP-producing pathways and downregulating ATP-consuming processes.2 This regulation allows ATP levels to remain constant, even while ATP turnover rates greatly decline. The ATP requirements of ion pumping are downregulated by generalized “channel” arrest in hepatocytes and by the arrest of specific ion channels in neurons.7

The Na,K-ATPase, a membrane protein critically important for the maintenance of the ion gradients required for cell homeostasis, consists of a catalytic α subunit and a regulatory β subunit.8–10 Active Na+ and K+ transport by this protein is responsible for ∼20% to 80% of the resting metabolic rate of the cell11 and approximately 30% of cellular ATP consumption.2,12–16 Hypoxia, within minutes, induces a decrease of the Na,K-ATPase activity by promoting the endocytosis of the α subunit from the plasma membrane into intracellular pools.17

Here we report that hypoxia via mROS induces the catalytic Na,K-ATPase α subunit to undergo ubiquitin-mediated degradation, downregulating the activity of a major metabolic protein.

Materials and Methods

Materials
Na,K-ATPase α-1 subunit monoclonal antibody (clone 464.6) was purchased from Upstate Biotechnology (Lake Placid, NY). GFP

Materials

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polyclonal antibody was purchased from Clontech (Palo Alto, Calif), and anti-ubiquitin monoclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). EUK-134 was provided to us by Eukariom Inc (Bedford, Mass). Superoxide dismutase (SOD) 2, SOD1, and glutathione peroxidase were gifts from Dr J. Engelhardt (purchased through University of Iowa, Viral Core). T-butyldihydroperoxide (t-H2O2) and chloroquine from Sigma-Aldrich (St Louis, Mo). EZ-link NHS-SS-biotin and pullified streptavidin beads purchased from Pierce Chemical Co (Rockford, Ill). Proteasome inhibitors MG-132 and lactacystin purchased from Calbiochem (San Diego, Calif). All other reagents were commercial products of the highest grade available.

Cell Culture
A549 cells (no. CCL 185; American Type Culture Collection), permanent cell lines of A549 cells expressing a GFP-tagged rat α1 subunit (GFP-A549) (a gift from Dr Bertorello, Department of Medicine, Karolinska Institutet and Atherosclerosis Research Unit, Karolinska University Hospital, Stockholm, Sweden), and V5-tagged rat α1 subunit (α1V5-A549) were generated in our laboratory (Lecuona and colleagues), as described18; pEx-A549 cells were generated as described previously.17,19 Cells were incubated under normoxic (16% O2, 5% CO2, 79% N2) and hypoxic conditions (1.5% O2, 93.5% N2, and 5% CO2) in a humidified atmosphere of 5% CO2 and were exposed to hypoxia (10% confluence).5,6,9 Chinese hamster ovary cell lines (CHO), CHO-ts20 (thermosensitive), and CHO-E36 (wild type)20,21 were incubated in a humidified workstation (Invivo O2; Ruskinn Technology). The murine hamster ovary cell lines (CHO), CHO-ts20 (thermosensitive), and CHO-E36 (wild type)20,21 were incubated in a humidified atmosphere of 5% CO2 and were exposed to hypoxia or normoxia in a humidified 1-L glass chamber at 30°C or 40°C. Experiments were performed with ~80% confluent cells. The use of animals for the present study was approved by the Northwestern University Institutional Animal Care and Use Committee. Rat alveolar epithelial type II cells (ATII cells) were isolated from pathogen-free male Sprague-Dawley rats (200 to 225 g; Harlan Inc, Indianapolis, Ind), as previously described.22,23

GFP-α1 Na,K-ATPase Subunit Immunoprecipitation
After cells were treated for the desired times, immunoprecipitation was done as previously reported.17

![Graph A](Image)

**Figure 1.** Hypoxia decreases the total pool of Na,K-ATPase α1 subunit. A, A549 cells were incubated at 16% O2 or 1.5% O2 for the indicated times. Equal amounts of cell lysates (100 μg) were analyzed by Western blot, and Na,K-ATPase abundance was determined using an antibody against Na,K-ATPase α1 subunit. Data were normalized to values obtained from normoxic controls (16% O2). Representative Western blot of n=3. B, ATII cells were incubated at 16% O2 or 1.5% O2 for the indicated times. Equal amounts of cell lysates (100 μg) were analyzed by Western blot, and Na,K-ATPase abundance was determined using an antibody against Na,K-ATPase α1 subunit. Data were normalized to values obtained from normoxic controls (16% O2). Representative Western blot of n=3. C, V5α1 Na,K-ATPase subunit in transfected A549 cells were incubated with methionine/cysteine-deficient DMEM for 60 minutes at 37°C. The cells were then pulse-labeled with 0.2 μCi/mL of [35S]methionine/cysteine (Amersham Biosciences, Arlington Heights, Ill) in methionine/cysteine-deficient DMEM for 120 minutes at 37°C and chased (DMEM containing 5 mmol/L unlabeled methionine and 5 mmol/L unlabeled cysteine) for 0 and 24 hours at 16% and 1.5% oxygen. After cells were treated for the desired times, media were aspirated and cells were washed twice with cold PBS, and A549-V5α1 cell lysates preparation and immunoprecipitation was done as previously reported by Lecuona (personal communication). Autoradiograph of [35S] labeled proteins was obtained, and membranes were analyzed by blotting against V5α1.

Cell Surface Labeling
Cells were labeled for 1 hour using 1 mg/mL EZ-link Sulfo-NHS-SS-biotin and pulled down with streptavidin. Proteins were analyzed by 10% SDS-PAGE and Western blot.17

Pulse Chase Labeling
Cells permanently transfected with V5α1 Na,K-ATPase were incubated with methionine/cysteine-deficient DMEM for 60 minutes at 37°C. The cells were then pulse-labeled with 0.2 µCi/mL of [35S]methionine/cysteine (Amersham Biosciences, Arlington Heights, Ill) in methionine/cysteine-deficient DMEM for 120 minutes at 37°C and chased (DMEM containing 5 mM unlabeled methionine and 5 mM unlabeled cysteine) for 0 and 24 hours at 16% and 1.5% oxygen. After cells were treated for the desired times, media were aspirated and cells were washed twice with cold PBS, and A549-V5α1 cell lysates preparation and immunoprecipitation was done as previously reported by Lecuona (personal communication). Autoradiograph of [35S] labeled proteins was obtained, and membranes were analyzed by blotting against V5α1.

Oxygen Consumption
Oxygen consumption was measured by using the Oxymeter (Eurosep Instruments, Cergy Pontoise, France) that has been described previously.5,6,9

Determination of Na,K-ATPase Activity
Na,K-ATPase activity was determined by 2 methods. One using ATP-32P (Amersham Biosciences) labeled as described previously.25,26 The second method was ouabain-sensitive 86Rb assay (Amersham Biosciences), as described previously.27

Small Interference RNA
A549 cells were stably infected with small interference RNA (siRNA) for Rieske iron sulfur protein (RISP) generated with a siRNA targeted against the iron sulfur (FeS) protein as previously described.28

Adenoviral Infection
A549 cells (~70% confluent) were infected with 10 μL (10^6/μL pfu) of SOD1 (AdSCMVcuZnSOD), SOD2 (AdSCMVmSOD), and
glutathione peroxidase (Ad5CMVGpx). Na,K-ATPase degradation was studied 24 hours after adenoviral vector infection by plasma membrane biotin labeling as described above.

Statistical Analysis
Data are reported as mean±SE. Statistical analysis was performed by 1-way ANOVA and Tukey correction. When 2 groups were compared, analysis was performed using Student’s t test. Results were considered significant when P<0.05.

Results
Hypoxia Decreases the Total Cell Pool and Increases the Degradation of Plasma Membrane Na,K-ATPase, Resulting in Decreased Activity and Oxygen Consumption
As shown in Figure 1A and 1B, there was ∼30% decrease in Na,K-ATPase α1 subunit protein abundance in whole cell lysates of A549 and ATII cells after 30 hours of hypoxia. Next, we determined whether hypoxia would increase the degradation of the total pool of Na,K-ATPase protein by labeling cells with [135S]methionine/cysteine as described above. As shown in Figure 1C, there was a 50% decrease in Na,K-ATPase α1 subunit protein at 24 hours, which did not differ from cells incubated under hypoxia. Subsequently we determined whether hypoxia would increase the degradation of the Na,K-ATPase on the plasma membrane by labeling the cells previously with biotin. Figure 2A and 2B depicts that hypoxia (within 2 hours) caused a 50% degradation of the plasma membrane Na,K-ATPase α1 subunit in A549 and ATII cells. These effects were specific and not the result of bulk degradation, as HIF-1α was stabilized after 2 hours of hypoxia (Figure 2C), and total actin, Glut-1, and E-cadherin were not degraded; there was no decrease in ATP levels (data not shown). Paralleling the degradation of the Na,K-ATPase at the plasma membrane, we observed that in A549 and ATII cells exposed to hypoxia, there was a decrease in Na,K-ATPase activity of ∼50% at 2 hours (Figure 3A through 3C) and a similar decrease in oxygen consumption under hypoxia (Figure 3D).

Lysosome and Proteosome Participate in the Hypoxia-Mediated Degradation of the Plasma Membrane Na,K-ATPase
Pretreatment with the lysosome inhibitor chloroquine and the proteosome inhibitor lactacystin prevented the degradation of the plasma membrane Na,K-ATPase (Figure 4A). This correlated with Na,K-ATPase activity, where pretreatment with proteosome inhibitor MG-132 and lysosome inhibitors chloroquine and E-64 prevented the hypoxia-mediated inhibition of Na,K-ATPase activity (Figure 4B).

ROS Participate in the Hypoxia-Mediated Degradation of the Na,K-ATPase
As shown in Figure 5A, incubation of A549 cells with exogenous ROS (t-H2O2) revealed increased Na+ pump degradation at the plasma membrane. Furthermore, pretreatment of A549 cells with the combined SOD/catalase mimetic EUK-134 prevented the hypoxia-mediated plasma membrane Na,K-ATPase degradation (Figure 5B).

Ubiquitin-Conjugating System Participates in the Hypoxic-Mediated Degradation of the Na,K-ATPase
We used a thermosensitive mutant of the ubiquitin-activating enzyme (E1) (CHO-ts20) that becomes inactivated when
incubated at 40°C. The progenitor cell line (CHO-E36), with the wild-type E1, served as control. As shown in Figure 6A, the Na,K-ATPase α1 subunit protein abundance in whole cell lysate was not degraded when CHO-ts20 cells were incubated in hypoxic conditions at 40°C. Next, we determined whether the Na,K-ATPase is directly ubiquitinated during hypoxia. A549 cells expressing GFP-α1–Na,K-ATPase were pretreated with the proteasome inhibitor MG132 for 2 hours and then incubated under hypoxia; cells were lysed and protein–ubiquitin conjugates were immunoprecipitated with an anti-GFP–specific antibody. As shown in Figure 6B, the extent of ubiquitin-conjugated Na,K-ATPase was noticeably increased after hypoxia exposure. Also, when cells were incubated with exogenous ROS (t-H2O2), ubiquitin-conjugated Na,K-ATPase was markedly increased (Figure 6C). Finally, to determine whether hypoxia-generated mROS participated in the increase Na,K-ATPase ubiquitination, we generated ρ0-A549 cells (mitochondrial DNA deficient) that are unable to generate ROS during hypoxia (data not shown). As shown in Figure 6D, ρ0-A549 cells, when incubated under hypoxia for 2 hours, demonstrated a lack of increase in ubiquitin conjugates associated with Na,K-ATPase α1 subunit. However, when these cells were incubated with exogenous ROS, ubiquitin-conjugated Na,K-ATPase was clearly increased.

Mitochondrial ROS Are Involved in the Hypoxia-Mediated Degradation of the Na,K-ATPase
To determine whether mROS participated in the hypoxia-mediated degradation of the Na,K-ATPase, besides generating ρ0-A549 cells as described above, we generated siRNA constructs against RISP, which is a ubiquitous component of cytochrome bc1 complexes of the mitochondria. When ρ0-A549 cells were exposed to hypoxia, the plasma membrane Na,K-ATPase was not degraded (Figure 7B); however, exposure of these cells to exogenous ROS increased the degradation of the Na,K-ATPase. In addition, A549 cells permanently transfected with siRNA against RISP blocked the hypoxia-mediated Na,K-ATPase degradation, with no effect when Drosophila HIF (dHIF) siRNA was transfected, as an internal control of the siRNA transfection, and exposed to hypoxia. Iron sulfur protein expression was confirmed in both cells transfected with siRNA, showing that there was an almost complete knockdown of RISP as opposed to the transfected with dHIF siRNA (Figure 7C), with no difference in tubulin expression as loading control (data not shown), all of which suggesting that during hypoxia, the mitochondria (complex III) is the source of ROS. We then sought to determine whether superoxide anion, H2O2, or both generated within the mitochondrial matrix or within the mitochondrial intermembrane space were required for hypoxia induced degradation of the Na,K-ATPase α1 subunit. Thus, we
infected A549 cells with adenoviral vectors encoding SOD1, SOD2, and glutathione peroxidase. As shown in Figure 8A, the overexpression of SOD1 attenuated Na,K-ATPase degradation, whereas the overexpression of SOD2 had no effect. Glutathione peroxidase overexpression prevented completely the degradation of the Na,K-ATPase. All cells were confirmed for infection of the respective adenoviruses, as shown in Figure 8B. Because SOD1 metabolizes superoxide anion generated in the cytosol and mitochondrial intermembrane space, whereas SOD2 metabolizes superoxide anion in the mitochondrial matrix, these results suggest that H₂O₂ generated from superoxide anion released into the intermembrane space is required for the hypoxia mediated degradation of the Na,K-ATPase α-1 subunit.

**Discussion**

The mechanisms of hypoxia-induced pulmonary edema are not well understood. Recent evidence suggests that hypoxia significantly reduces the capacity for active Na⁺ transport across the alveolar epithelium.²⁹⁻³¹ It has been described that β-2 agonists can overcome the depressant effects of hypoxia on alveolar fluid clearance,³¹,³² which in part explains the preventive effect of β agonists in the development of high altitude pulmonary edema (HAPE) and the observed reduction in extraalveolar water in patients with acute respiratory distress syndrome (ARDS).³³,³⁴

Adaptation to hypoxia is key for cell survival. To accomplish this, there is a need for cellular energy reallocation between essential and nonessential ATP demand processes.¹¹

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**Figure 4.** Lysosome and proteasome are involved in the hypoxia-mediated degradation of the Na,K-ATPase α-1 subunit. A, A549 cells were surfaced labeled with biotin, pretreated for 1 hour in the presence or absence of 100 μmol/L of chloroquine (C) or 10 μmol/L of lactacystin (L), and then exposed to 1.5% O₂ for 2 hours. Equal amounts of cell lysate (150 μg of protein) were pulled down with streptavidin beads and Na,K-ATPase protein abundance was determined by performing a Western blot against Na,K-ATPase α-1 subunit. Representative Western blot of n=5. B, Na,K-ATPase activity was determined in A549 cells pretreated for 1 hour in the presence or absence of proteasome inhibitor MG-132 (20 μmol/L) or lysosome inhibitors chloroquine (100 μmol/L) and E64 (10 μmol/L), and then exposed to 1.5% O₂ for 2 hours by ouabain-sensitive ⁸⁶Rb⁺ uptake and expressed as a percentage of normoxic controls (16% O₂). n=3; *P<0.05, **P<0.01.

**Figure 5.** ROS are involved in the degradation of the Na,K-ATPase under hypoxia. Cells were surfaced labeled with biotin before exposing to different experimental conditions. Equal amounts of proteins (150 μg) were pulled down with streptavidin beads, and Na,K-ATPase protein abundance was determined by performing Western blot against Na,K-ATPase α-1 subunit. A, A549 cells were exposed to either 16% O₂ or 100 μmol/L t-H₂O₂ every 40 minutes for 2 hours. Representative Western blot of n=3. B, A549 cells were preincubated overnight with EUK-134 (20 μmol/L) and exposed to either 16% O₂ or 1.5% O₂. Representative Western blot of n=2. *P<0.05.
Consistent with previous reports,30,35,36 the total Na,K-
ATPase α1 cell pool decreased on exposure to prolonged
hypoxia (Figure 1A and 1B). Its half-life is approximately 24
hours and is not shortened in hypoxic conditions (Figure 1C),
suggesting a decrease of the total pool protein synthesis and
not of the total pool degradation, as it has been suggested
previously.30 However, 15% to 20% of the Na+
pumps are
present at the plasma membrane of A549 cells (E. Lecuona
and J.I. Sznajder, personal communication) and are enzymat-
ically active as opposed to the 80% of Na,K-ATPase in the
intracellular pools.37 Therefore, we decided to determine
whether the plasma membrane Na+ pumps, which are con-
suming ATP, would be affected first during hypoxia. We
observed that within a short period of time, hypoxia increased
the plasma membrane Na,K-ATPase degradation (Figure 2A
and 2B). As shown in Figure 3A through 3D, A549 and ATII
cells had a similar decrease in Na,K-ATPase activity when
incubated in hypoxic conditions, being prevented by either a
lysosome or proteasome inhibitor (Figure 4A and 4B). During
hypoxia, the cells appear to adapt, as described in hepatocy-

cies, by downregulating consumption of ATP in response to
a decreasing oxygen availability.24,38

The involvement of both the proteasome and the lysosome
in degrading the Na,K-ATPase α1 subunit is still puzzling.
Following ubiquitination, soluble and endoplasmic reticulum
proteins are usually targeted to the proteasome, whereas cell
surface membrane proteins are usually targeted to the lyso-
some.39 Exceptions to these rules have been observed in other
systems; for example, proteolytic cleavage of the growth
hormone receptor is necessary before the protein can be
delivered to the lysosome40 and the glutamate receptor
requires the activity of the proteasome for its endocytosis.41
Also, it has been reported that specific inhibitors of lysosomal
proteases and inhibitors of the proteasome are effective in
reducing the ligand-induced platelet-activating factor recep-
tor downregulation, indicating the importance of receptor
targeting to both lysosomes and proteasomes.42

The ubiquitin-conjugating system plays a crucial role in
cell homeostasis, including the regulation of membrane
proteins such as the Na+ channel and the growth hormone

Figure 6. Ubiquitin-conjugating system is involved in the hypoxia-mediated degradation of the Na,K-ATPase α1 subunit. A, CHO-ts20
and E36 cells were incubated under 16% O2 or 1.5% O2 for 24 hours at either 30°C or 40°C. Equal amount of cell lysates (150 µg)
were analyzed by Western blot against the Na,K-ATPase α1 subunit. Representative Western blot of n=3. B, GFP-α1 A549 cells were
pretreated with 20 µmol/L of MG-132 and then incubated at 16% O2 or 1.5% O2 for 2 hours. The Na,K-ATPase α1 subunit was immu-
noprecipitated using a GFP antibody. Immunoprecipitates were immunoblotted against ubiquitin. The membranes were then stripped
and rebotted against GFP as a loading control. Representative Western blot of n=3. C, GFP-α1 A549 cells were pretreated with
20 µmol/L MG-132 and then incubated at 16% O2 or t-H2O2 (100 µmol/L) for 2 hours. The Na,K-ATPase α1 subunit was immuno-
precipitated using a GFP antibody. Immunoprecipitates were immunoblotted against ubiquitin. The membranes were then stripped
and rebotted against GFP as a loading control. Representative Western blot of n=3. **P<0.01, ***P<0.001.
receptor.20,21,43–49 Multiubiquitination of proteins leads to their recognition and subsequent degradation by the proteasome,45,50,51 and for most cell surface membrane proteins, ubiquitin-tagging triggers the endocytic machinery that direct proteins to the lysosome wherein they are degraded.44,48,52 Our data provide evidence that hypoxia ubiquitinates the Na,K-ATPase, targeting it for degradation (Figure 6A and 6B). This phenomenon contrasts with the regulation of HIF-1 \( {\text{H}} \) which is stabilized as a result of the inhibition of its constitutive ubiquitin mediated degradation during hypoxia by the E3 ligase VHL.4,5,53 These data suggest that signaling events initiated by exposure to hypoxia activates distinct mechanisms that regulate the stabilization or degradation of proteins through the ubiquitin/proteasome system.

During hypoxia, the mitochondrial electron transport is partially inhibited causing redox changes in the electron carriers that result in the generation of superoxide anions (O) and hydrogen peroxide (H\( {\text{2}} \)), which on entering the cytosol, can act as second messengers.54–56 The mitochondria generate ROS in response to a change in redox of the electron transport proteins, specifically from complex III.54,57 Recently, Dada et al, reported that exposing cells to hypoxia for 60 minutes decreased Na\( {\text{+}} \) pump activity by promoting endocytosis of plasma membrane Na,K-ATPase but without degradation.17 We provide new evidence that exposing cells to severe hypoxia or exogenous ROS (H\( {\text{2}} \))O\( {\text{2}} \) for 2 hours results in an increased Na,K-ATPase ubiquitination and degradation (Figures 5A and 6C). When A549 cells were preincubated with the combined superoxide dismutase/catalase mimetic EUK-134,58 which blocks completely the generation of ROS,59 the hypoxia-mediated degradation of the plasma membrane Na,K-ATPase was prevented (Figure 5B).

![Figure 7. Mitochondrial derived ROS are involved in hypoxia-mediated Na,K-ATPase degradation. A, Southern blot analysis of total cellular DNA from wild type (A549) and A549 \( {\text{H}} \) cells. Hybridization was performed with a cytochrome oxidase subunit II probe, spanning bp 7757 to 8195, generated by RT-PCR. B, A549 \( {\text{H}} \) cells were exposed to 16% O, 1.5% O, or 100 \( {\text{m}} \)mol/L H\( {\text{2}} \)O\( {\text{2}} \) every 40 minutes for 2 hours. Representative Western blot of n=3. C, A549cells transfected with siRNA against dHIF and RISP exposed to 16% O and 1.5% O for 2 hours and Western blot showing expression of iron sulfur protein in dHIF and RISP siRNA-transfected cells. Representative Western blot of n=4. *P<0.05, ***P<0.001.](http://circres.ahajournals.org/)

![Figure 8. SOD1 and GPX overexpression protect against hypoxia-mediated Na,K-ATPase degradation. A, A549 cells were infected with adenovirus vectors overexpressing SOD2, SOD1, and glutathione peroxidase (Gpx) 24 hours before treatment; cells were then exposed to 16% O, or 1.5% O for 2 hours. The membranes were then stripped and rebotted against tubulin. Data were normalized to values obtained from normoxic controls (16% O). Representative Western blot of n=3. B, Western blot stripped and rebotted against SOD2 (MnSOD), SOD1 (CuZnSOD), and Gpx (anti-myc) antibodies. *P<0.05, **P<0.01.](http://circres.ahajournals.org/)
When A549 ρ0 cells and siRNA RISP cells, which cannot generate mitochondrial ROS,17,40 were exposed to hypoxia, ubiquitination and degradation of α1 Na,K-ATPase was prevented, suggesting that ROS are generated at the mitochondria, specifically at complex III, playing a key role in the degradation of the Na+,K+ pump during hypoxia (Figures 6D and 7). Collectively, these results suggest that during hypoxia the mitochondrial generation of ROS is required for the degradation of the Na+,K-ATPase and H2O2 acts downstream of the mitochondria.

The mitochondria possess antioxidant defenses against O2·− and H2O2. These include SOD2 (MnSOD), which is transported to the mitochondrial matrix where it forms the active homotetramer.41 Although the O2·− generated in the matrix is eliminated in that compartment, part of the O2·− produced in the intermembrane space may be either dismutated by a different SOD isoform (SOD1) that contains copper and zinc instead of manganese (CuZnSOD) or carried to the cytoplasm via voltage-dependent anion channels (VDAC), where it can be eliminated via CuZnSOD, which is also found in the cytoplasm of eukaryotic cells. Moreover, H2O2, the byproduct of O2·− dismutation, is eliminated by glutathione peroxidase localized in the cytosol.61 We found that the overexpression of glutathione peroxidase in A549 cells prevented the hypoxia-mediated plasma membrane Na+,K-ATPase degradation, whereas SOD1 overexpression partially blocked the Na+,K-ATPase degradation. However, SOD2 overexpression was not protective (Figure 8A and 8B), suggesting that during hypoxia, mitochondria-intermembrane-space, but not mitochondrial-matrix, generated ROS participate in the degradation of the Na+,K-ATPase.

In summary, we provide evidence that hypoxia-generated mROS increases Na+,K-ATPase degradation via the ubiquitin-conjugating system. Whether ROS posttranslationally modify specific residues of the Na+,K-ATPase targeting it for recognition by the ubiquitin pathway and degradation warrants further studies. This report supports the hypothesis that during hypoxia, mROS play a physiological role in the degradation of energy-consuming proteins, such as the Na+,K-ATPase, although it stabilizes others that are energy-producing reactions, such as HIF-1α.61

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Comellas et al Hypoxia-Mediated Na,K-ATPase Degradation


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