Oxygen regulation of the epithelial Na channel in the collecting duct

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Husted RF, Lu H, Sigmund RD, Stokes JB. Oxygen regulation of the epithelial Na channel in the collecting duct. Am J Physiol Renal Physiol 300: F412–F424, 2011. First published December 1, 2010; doi:10.1152/ajprenal.00245.2010.—The PO2 within the kidney changes dramatically from cortex to medulla. The present experiments examined the effect of changing PO2 on epithelial Na channel (ENaC)-mediated Na transport in the collecting duct using the mpkCCD-c14 cell line. Decreasing ambient O2 concentration from 20 to 8% decreased ENaC activity by 40%; increasing O2 content to 40% increased ENaC activity by 50%. The O2 effect required several hours to develop and was not mimicked by the acid pH that developed in monolayers incubated in low-O2 medium. Corticosteroids increased ENaC activity at each O2 concentration; there was no interaction. The pathways for O2 and steroid regulation of ENaC are different since O2 did not substantially affect Sgk1, ENaC activity at each O2 concentration; there was no interaction. The results demonstrate that ENaC activity is more sensitive to changes in PO2 than previously thought.

MATERIALS AND METHODS

Materials. mpkCCD-c14 cells, a line of mouse collecting duct cells, were a generous gift from Alain Vandewalle. Human embryonic kidney (HEK)-293 cells were a kind gift from Jeffrey Pessin. Cell culture medium was from Gibco; 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) was from Calbiochem; COS7 cell extracts were from Santa Cruz. Antibodies were purchased from the following sources: anti-heme oxygenase-1 (Heme oxygenase-1) from Assay Designs (catalog no. SPA-896, dilution 1:1,000); anti-heat shock protein-70 (HSP-70) from Santa Cruz (catalog no. sc-32239, dilution 1:200); anti-actin from Sigma (catalog no. A2066, dilution: 1:1,000); anti-histone-H2B from Imgenex (catalog no. IMG-359, dilution: 1:250); anti-AMP kinase (AMPK) from Santa Cruz (catalog no. sc-35324-R, dilution 1:100). We used two hypoxia-inducible factor-2 antibodies from Novus Biologicals (catalog no. NB100-132, 1:500 dilution, and catalog no. NB100-122, 1:200 dilution). We also used four HIF-1α antibodies: Abcam (catalog no. ab1, dilution: 1:100); Chemicon (catalog no. MAB5382, dilution: 1:500); and two from Santa Cruz (catalog no. sc-8711 dilution: 1:100, and catalog no. sc-10790, dilution 1:100). Secondary antibodies were horseradish peroxidase (HRP) conjugated and used at 1:50,000 dilution: goat anti-rabbit (Chemicon. catalog no. AP132P); goat anti-mouse (Chemicon. catalog no. AP124P); and donkey anti-goat (Santa Cruz Cat no. sc-2020). All other materials were purchased from Sigma.

Cell culture and electrical measurements. mpkCCD-c14 cells were grown in Corning T-75 culture flasks in Dulbecco's modified Eagle's medium/Ham's F-12; 60 mM sodium selenite; 5 μg/ml transferrin; 2 mM glutamine; 50 mM dexamethasone; 110 ng/ml epidermal growth factor; 5 μg/ml insulin; 20 mM t-glucose; 2% fetal calf serum; and 20 mM HEPES, pH 7.4; 50 μg/ml gentamicin at 37°C in 5% CO2/95% air. Cells were passaged using 0.05% trypsin and 1 mM EDTA in phosphate-buffered saline. Cells were seeded at confluent density (20 μg of DNA/cm2) on 12- or 30-Millicell PCF filters or on 75-mm Transwell filters pretreated with human placental collagen using the previously described protocol (27). The cells were grown 4 days on filters in the medium indicated above without epidermal growth factor, at which time they were confluent monolayers, and serum and dexamethasone were removed. Medium was changed daily beginning on day 3. Electrical measurements were conducted on day 4, and, based on the value of the short-circuit current (Isc), monolayers were randomized to treatment groups. Monolayers were placed into chambers (model MIC-101
Billups-Rothenberg, Del Mar, CA) and purged with the indicated O₂ content; all incubation conditions had 5% CO₂. The chambers were then placed in an incubator at 37°C. Pilot testing of the chambers with 95% O₂ demonstrated no change in the PO₂ of the media for 72 h. HEK-293 cells were grown according to the recommendations of American Type Culture Collection.

Measurements of transepithelial resistance (R₀) and Iₛc were made under sterile conditions by placing the 12-mm Millicell filters into modified Ussing chambers (Jim’s Instrument’s, Iowa City, IA) with a University of Iowa voltage clamp (53). Unless otherwise indicated, all measurements were made at 37°C in growth medium equilibrated to room air (20% O₂). In experiments in which we tested the effect of tin protoporphyrin (SnPP) on Iₛc, we kept the solutions and monolayers in the dark to prevent light activation of SnPP, which can produce potent biological effects (52). For all experiments involving nonelectrical measurements, we measured the Iₛc and R₀ of the 12-mm monolayers before making the biochemical determination. In the cases in which we used larger filters for the measurements, we set up identically treated 12-mm filters for electrical measurements.

**Chemical assays.** ATP was measured using a luciferase-based assay kit (Sigma FLAA Bioluminescent assay), according to the manufacturer’s directions using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). The standard curves were linear in a concentration range of 0.1–10 nM. The sensitivity of this assay is 2 pM; ATP concentration in the assay mixture was generally ~1 nM. ADP and AMP were measured by the increment in ATP measured following conversion of these products to ATP using phosphoenolpyruvate, pyruvate kinase, and adenylate kinase. Lactate, pH, and PO₂ were measured in the basolateral solutions following 24 h of incubation by pooling medium from three to four individual monolayers. The assay was conducted on a GEM Premier 3000 autoanalyzer (Instrumentation Laboratory, Bedford, MA).

**Generation of antibodies against ENaC.** We immunized rabbits using antigens that had been used by others for this purpose. The α-ENaC antigen was a gift from Olivier Staub and was a construct in pGEX encoding a glutathione-S-transferase (GST) fusion protein containing the rat sequence 2–77. The β- and γ-ENaC antigens were identical to the COOH-terminal peptides used by Maslamani et al. (38). The GST-α-ENaC fusion protein was expressed in E. coli and purified using a glutathione column (Pierce) to remove antibodies directed against GST; and then using SulfoLink Immobilization Kit (Pierce) using standard techniques. Serum from rabbits injected with this protein was purified using two sequential steps: first using immobilized GST beads (Pierce) to remove antibodies directed against GST; and then using the immunogen linked to beads via the Actigel-ALD reagent (Sterogene). The β- and γ-ENaC peptides were injected into rabbits with three to six boosts, and the resulting serum was purified using SulfoLink Immobilization Kit (Pierce). Characterization of the crude and immunopurified antisera is depicted in Supplementary Figs. S1–S3. The immunopurified α-ENaC antibody was used at a 1:500 dilution, the β-ENaC antibody at 1:100 dilution, and the γ-ENaC antibody at 1:1,000 dilution.

**Immunoblotting and immunocytochemistry.** Monolayers grown on filters were washed with 2 ml of PBS, and the filters were cut out and placed in a 1.5-ml Eppendorf tube. For each filter in the tube (usually 4), 100 μl of Laemmli buffer (2% SDS, 8 mm Tris buffer, 40 mm dithiothreitol, and 6% glycerol) were added. All samples were incubated for 15 min at 60°C and stored at 4°C until used. Protein analysis was conducted by fluorescence assay (2) with bovine albumin as the standard. In general, 100 μg total protein were loaded into each lane, separated by SDS-PAGE using 8% acrylamide, and transferred to Immobilon-NC (Millipore) using a FB-SDB-2020 semidry blotting unit (Fisher). The membrane was blocked in phosphate-buffered saline with 0.05% Tween 20 (PBST) plus 5% milk for 20 min, followed by 1 or 24 h incubation of the primary antibody at the appropriate dilution. The membrane was washed twice in PBST and incubated for 1 h in a 1:50,000 dilution of the secondary HRP-conjugated antibody. After three PBST washes and two PBS washes,
the HRP was detected by exposing the membrane to SuperSignal West Femto chemiluminescent substrate (Pierce). The light intensity was captured by an OPTI Chemi digital imaging system (UVP) and quantitated using the proprietary software. All immunoblots underwent reprobing with an antibody (against actin or histone H2B) to normalize for loading. Unless otherwise indicated, all values are normalized.

Immunocytochemical analysis was conducted on monolayers grown on filters. The monolayers were fixed for 15 min in 4% paraformaldehyde, then blocked and permeabilized overnight in PBS with 2% non-fat dry milk and 0.1% Triton X-100 at 4°C. They were exposed to the antibody directed against HIF-1α or HIF-2α for 1 h at 37°C in PBS with 2% BSA and 0.01% Triton X-100. They were incubated with a 1:500 dilution of Cy3-conjugated anti-mouse antibody for 1 h at 37°C in PBS with 2% BSA and 0.1% Triton X-100. The filters were then stained with 4,6-diamidino-2-phenylindole for 5 min. The filters were cut out, mounted on a slide, and visualized on an Olympus Provis inverted fluorescent microscope. Images were captured with a SPOT digital imaging system (Diagnostic Instruments).

Quantitative PCR: RNA was isolated from the filters using the methods previously described (15, 29). After processing and exposure to DNase, the RNA was reverse transcribed, as described previously (29). Primers were designed using the Primer Express software (Applied Biosystems) and usually spanned an intron. The primers were manufactured by IDT (Coralville, IA) and had the sequences listed in Table 1. The quantitative PCR reaction was run on a PRISM 7700 Sequence Detection System (Applied Biosystems) using the SyBr Green Master mix. Quantitation was done by the ΔΔ method, as described in the manufacturer’s instructions using actin as the normalizing factor.

Statistical analysis: Comparisons of two groups were made using unpaired t-test. Comparisons using more than two groups were made using ANOVA, and, if further analyses were made, we used unpaired t analysis with correction for multiple comparisons.

RESULTS

Effects of O2 and corticosteroids on Na transport. Figure 1 shows the effects of four different levels of O2 on Isc and Rr of mpkCCD-14 monolayers. Compared with ambient O2 (20%), 40% O2 produced a 50% increase in Isc; 95% O2 produced no further increase. The effects of 95% O2 were variable; sometimes there was a higher Isc than 40% O2, and sometimes it was lower. In contrast, Isc of monolayers incubated in 8% O2 were consistently 40% lower than those in ambient O2. Monolayers with a greater Isc had a lower Rr. When 10 μM benzamil were added to the apical surface of the monolayers, Isc fell to ~1 μA/cm2, and Rr increased substantially (data not shown). These results demonstrate that ambient PO2 has a substantial effect on the magnitude of Na transport mediated by ENaC.

We asked if the effects of O2 on Isc would interact with the well-known effects of corticosteroids. As shown in Fig. 1B, addition of a saturating concentration of steroids produced an approximate doubling of the Isc at each O2 concentration. Benzamil reduced Isc to ~1 μA/cm2 and greatly increased Rr. There was no interaction between the O2 and steroid treatments by two-way ANOVA. These results suggest that steroids and O2 act by independent pathways to alter Na transport.

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**Fig. 1.** A: effect of 24-h O2 exposure on short-circuit current (Isc) and transepithelial resistance (Rr) of mpkCCD monolayers. n = 42 monolayers; 11 experiments. P < 0.05; *less than 20, 40, and 95% O2 values; #less than 40 and 95% O2 values. B: additive effects of corticosteroids on Isc. Aldosterone and dexamethasone (100 nM each, hatched bars) were added to medium for the 24-h period where O2 was changed. n = 12 monolayers, 3 experiments. Two-way ANOVA showed no significant interaction between O2 and steroids. Values are means ± SE.
Time-dependent and acute \( O_2 \) effects. Changes in \( O_2 \) can have rapid (minutes) effects on some ion channels (55). The time course of the present \( O_2 \) effect (Fig. 2) indicates that these effects take hours to develop. At 6 h, the differences in \( I_{sc} \) between the groups treated with \( O_2 \) was not significant by ANOVA. At 24 h, the effects of 8, 20, and 40% \( O_2 \) were different from each other (\( P < 0.001 \)).

Monolayers incubated in 95% \( O_2 \) were not different from monolayers incubated in 20% \( O_2 \). At 24 h, the differences in \( I_{sc} \) between groups was eliminated. \( n = 12 \) monolayers, 3 experiments/group. *?, #?. B: by 24 h after return to 20% \( O_2 \) (recovery), the difference in \( I_{sc} \) between groups was eliminated. \( n = 9 \) monolayers, 3 experiments per group. Darker shaded bars indicate higher \%\( O_2 \). *\( P < 0.05 \) compared with all other conditions. #\( P < 0.05 \) compared with 40% oxygen. C: after 24-h exposure to different \( P_O_2 \), \( I_{sc} \) was measured either in medium containing 20% \( O_2 \) (open bars) or the same \( O_2 \) concentration in which they had been incubated for 24 h (hatched bars). There was no difference in the values. \( n = 9 \) monolayers, 3 experiments per group. Values are means ± SE.

**Fig. 2.** Time-dependent effects of \( O_2 \) on \( I_{sc} \) in mpkCCD cells. A: monolayers measured at 0, 6, and 24 h after changing \( O_2 \). At 6 h, the difference in \( I_{sc} \) between the groups treated with \( O_2 \) was not significant by ANOVA. At 24 h, the effects of 8, 20, and 40% \( O_2 \) were different from each other (\( P < 0.001 \)). Monolayers incubated in 95% \( O_2 \) were not different from monolayers incubated in 20% \( O_2 \). At 24 h after return to 20% \( O_2 \) (recovery), the difference in \( I_{sc} \) between groups was eliminated. \( n = 12 \) monolayers, 3 experiments/group. *?, #?. B: by 24 h after return to 20% \( O_2 \) (recovery), the difference in \( I_{sc} \) between groups was eliminated. \( n = 9 \) monolayers, 3 experiments per group. Darker shaded bars indicate higher \%\( O_2 \). *\( P < 0.05 \) compared with all other conditions. #\( P < 0.05 \) compared with 40% oxygen. C: after 24-h exposure to different \( P_O_2 \), \( I_{sc} \) was measured either in medium containing 20% \( O_2 \) (open bars) or the same \( O_2 \) concentration in which they had been incubated for 24 h (hatched bars). There was no difference in the values. \( n = 9 \) monolayers, 3 experiments per group. Values are means ± SE.

**Fig. 3.** Chemistry of basolateral medium 24 h after changing the ambient \( O_2 \) to the indicated values. Samples from 3–4 monolayers per experiment were pooled; the results represent 9 experiments. ANOVA with subsequent comparisons between groups showed that pH of medium incubated in 95% \( O_2 \) was higher than 8% or 20% \( O_2 \); lactate concentration and \( P_O_2 \) were different in all groups. Values are means ± SE. *\( P < 0.05 \) compared with 20% \( O_2 \).
highest PO₂ produced greater ATP levels compared with 8 or 20% O₂. The sum of ADP and AMP was not affected by O₂. We examined this question further by conducting a functional study using AICAR, an activator of AMPK (58). AICAR (1 mM for 24 h) did reduce $I_{sc}$ in mpkCCD-c14 cells (Fig. 4B) as expected, but, when PO₂ was altered in its presence, the $I_{sc}$ response was qualitatively similar to the response in the absence of AICAR. We also measured the abundance of phosphorylated AMPK, an index of its activation. The phosphorylated form was not altered by O₂ concentrations of 8–40%, but phosphorylation was increased by incubation in 1% O₂ (Fig. 4C). Total AMPK was not altered by O₂ from 8 to 40% (data not shown). We conclude that, while very low levels of O₂ (1%) can activate AMPK in these cells, changes in O₂ concentrations 8% and above do not cause activation of AMPK. The regulation of ENaC function at O₂ concentrations between 8 and 40% must be mediated by another signaling system.

Proteins that are altered by oxidative stress. We hypothesized that varying the O₂ concentration might alter the expression of genes that reflect a degree of oxidative stress. We tested HSP-70, since it has been shown to inhibit ENaC activity when expressed in oocytes (23). As shown in Fig. 5A, the protein levels of HSP-70 were unaffected by varying concentrations of O₂. We also tested HO-1, since heme and its metabolic products have been shown to alter ENaC function in excised membrane patches from M1 cells (54). As shown in Fig. 5B, protein abundance of HO-1 was significantly lower at higher concentrations of O₂, but there was no difference between 8 and 20%. We also tested the ability of SnPP, an inhibitor of the enzyme (52), to alter the effects of varying O₂; there was no effect (Fig. 5C). Thus, while HO-1 appears to be responsive to changes in O₂ concentration, three lines of evidence suggest that it is an unlikely candidate to mediate these O₂-mediated changes in Na transport: 1) the lack of a difference in the two lower concentrations of O₂; 2) the lack of effect of an inhibitor of the enzyme; and 3) the regulation of HO-1 by O₂ in the direction opposite to that which would be predicted to produce the changes in ENaC function. Some of the most widely studied proteins mediating the effects of low O₂ are HIF proteins (44). We were unable to detect HIF-1α by immunoblot in mpkCCD-c14 cells, even under 1% O₂ with cobalt and N-acetyl-l-leucyl-l-leucyl-l-norleucinal (ALLN) (a proteasome inhibitor, Fig. 6A), despite detection in two different positive controls. We tried four different antibodies for immunoblot and two different antibodies for immunohistochemistry (data not shown), all with similar results. However, we were able to detect a signal for HIF-1α using immunohistochemistry and extreme conditions in HEK-293 cells and COS7 cells (Fig. 6A). In contrast, HIF-2α was detectable in mpkCCD-c14 cells, but the amount

Fig. 4. ATP and AMP kinase (AMPK) relationships to O₂ concentration. A: ATP (open bars) and ADP + AMP (hatched bars) content of monolayers incubated in the indicated O₂ concentrations. ATP content of monolayers incubated in 95% O₂ had higher ATP content than those incubated with 8 or 20% (*P < 0.05). Content of ADP + AMP was not different in the 4 groups. Analysis by ANOVA, $n = 3$ monolayers from 3 experiments in each group. B: 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR; 1 mM for 24 h, hatched bars), an activator of AMPK, reduced $I_{sc}$ but did not alter the qualitative effect of O₂ (by ANOVA, *P < 0.05 compared with controls, $n = 4$ monolayers). C: top: immunoblot of phosphorylated AMPK (p-AMPK) in response to O₂. Middle: there was no difference in the abundance of p-AMPK normalized to actin in monolayers exposed to 8, 20, or 40% O₂, but monolayers exposed to 1% O₂ had greater p-AMPK (*P < 0.05). Bottom: relative $I_{sc}$ values for monolayers used for these assays. *P < 0.05 compared with all other monolayers by ANOVA and subsequent unpaired analysis. Nos. in the bars represent no. of experiments; no. of monolayers. Values are means ± SE.
did not change with different O2 concentrations (Fig. 6A). We further tested the possible role of HIF proteins in ENaC regulation by using dimethylxallyl glycine (DMOG), a prolyl-4-hydroxylase inhibitor. Such inhibitors increase the abundance of HIF proteins by inhibiting the major degrading enzyme. While DMOG inhibited \( I_{sc} \) modestly, it had no effect on the changes produced by O2 (Fig. 6B).

We tested the idea that reactive oxygen species (ROS) might be involved in the regulation of ENaC by O2. Incubating monolayers in TEMPOL (a ROS scavenger) did reduce \( I_{sc} \), a result suggesting that endogenous ROS may stimulate ENaC activity (49, 59). However, even in the presence of TEMPOL, O2 produced its concentration-dependent effects, albeit at a lower absolute level (Fig. 6C). Taken together, it appears that the O2 effects on ENaC are not mediated by ROS, nor do they appear to be mediated by HIF-1\( \alpha \), HIF-2\( \alpha \), HSP-70, or HO-1.

Comparative expression of genes: O2 and corticosteroids. Because it appeared that the regulation of ENaC activity by O2 was independent of the action of corticosteroids (Fig. 1), we compared the expression of several genes that are known to be regulated by either corticosteroids or O2. Figure 7 shows that 24-h corticosteroid exposure increased the mRNA of Sgk1 (serum/glucocorticoid regulated kinase 1), Gilz (TSC22 domain family, member 3), and Usp2-45 (ubiquitin-specific peptidase 2), but O2 had little or no effect on these transcript levels. Corticosteroids also increased \( \alpha \)-ENaC mRNA, as has been demonstrated on many occasions (data not shown). In contrast, O2 had a marked effect on the facilitated glucose transporter-1 (Slc2a1, Glut1) and HO-1 expression, whereas steroids did not. These results support the idea that the effects of O2 and steroids regulate Na transport by independent pathways.

Effects of O2 on ENaC mRNA and protein. As shown in Fig. 8, varying the PO2 had no effect on \( \alpha \)-ENaC mRNA abundance, but low PO2 did produce a modest effect on \( \beta \)- and \( \gamma \)-ENaC mRNA. There was a similar pattern on ENaC protein abundance; there was no effect of O2 on \( \alpha \)- or \( \gamma \)-ENaC, but there was a moderate effect on \( \beta \)-ENaC protein (Fig. 9). We note that there was probably less total amount of \( \beta \)-ENaC protein in mpkCCD-c14 cells than in native collecting ducts based on the different relative magnitudes of \( \beta \)- and \( \gamma \)-ENaC (data not shown). M1 cells (another collecting duct line) show relatively
lower γ-ENaC protein abundance (29). The reason for these apparent differences in ENaC subunit abundance is not clear.

Surface expression of ENaC. We used surface protein biotinylation to estimate the abundance of ENaC subunits on the apical membrane. We found that the ~90-kDa α-ENaC subunit was readily detectable in cell lysates, but that surface expression was difficult to detect. On the few occasions when we were able to detect α-ENaC on the surface, there was minimal difference between 8 and 40% O2 (Fig. 10A). We were unable to detect an ~30-kDa α-ENaC fragment that has been reported in kidney tissue using antibodies directed at the NH2-terminus (16).

In contrast, we were able to detect β-ENaC on the apical membrane of monolayers incubated in 40% O2, but detecting this subunit on the surface of monolayers incubated in 8% O2 was much more difficult. As shown in Fig. 10B, monolayers exposed to 40% O2 demonstrated a greater abundance of the ~80-kDa β-ENaC band, as well as the abundance of an ~90-kDa band in the cell lysate. The surface expression of the ~90-kDa band in monolayers incubated at 40% O2 was considerably greater than that of the ~80-kDa band, a reversal of their relative abundance in cell lysates.

Using our antibody that recognizes the COOH-terminus of γ-ENaC in surface biotinylation experiments, we found that monolayers exposed to 40% O2 had three to four times more total surface γ-ENaC than monolayers incubated in 8% O2 (Fig. 10C). Actin was not detected in the streptavidin precipitate (data not shown). The abundance of γ-ENaC from the total cell lysate was similar in the two groups (as in Fig. 9). The 40% O2 concentration produced greater full-length γ-ENaC (upper band, ~83 kDa), as well as the cleaved (shorter) γ-ENaC (~72 kDa). We seldom were able to detect the cleaved form of γ-ENaC in whole cell lysates, but the surface expression of this form was much more readily observed at 40% O2. These results are consistent with the idea that the higher ENaC activities produced by high O2 concentration are the result, at least in part, of a greater amount of active ENaC on the apical surface.

We tested one mechanism whereby surface ENaC activity might be regulated. We asked if O2 might alter the activity of a secreted protease, which then could influence the cleavage of α- and/or γ-ENaC. This mechanism of ENaC activation has received considerable attention recently (33), but the role of regulated protease secretion is unclear. We treated monolayers incubated with different O2 concentrations for 24 h with the protease inhibitor aprotinin. Six hours later, we repeated the electrical measurements, reasoning that 6 h would be adequate to allow enough endocytosis and exocytosis to detect significant functional differences. Figure 11 shows that aprotinin reduced the Isc of monolayers in each of the O2 groups proportionally, but there was no differential effect of O2 treatment. These results do not support the idea that O2 regulates ENaC activity by altering the protease activity of the monolayer.

DISCUSSION

The present results demonstrate a potent role for O2 in regulating ENaC-mediated Na transport by collecting duct cells. The effect takes hours to develop and appears insensitive to acute changes in O2 concentration, and the molecular machinery responsible for this regulation appears parallel to, and independent of, corticosteroid-mediated ENaC regulation.
Most of the existing studies of the effects of O2 on ENaC function have used lung epithelial cells. In general, these studies have found that lowering O2 from 20 to 3% or lower causes a decrease in ENaC function. For example, Planes et al. (42) showed that 0 or 3% O2 (but not 5% O2) reduced Na influx in cultured alveolar cells with a time course requiring hours. Their results, like ours, showed a modest effect of low O2 on ENaC mRNA and protein. Similar results have been reported using lung epithelial cells (13, 41), fetal lung cells (3, 50), and cultured epithelial cells from the middle ear, where O2 content averages 7% (37).

Two general features of the present results appear different from those in the existing literature. First, Na transport in the present experiments appears substantially more sensitive to changes in O2 than previous reports. We saw a consistent difference in Na transport in monolayers incubated in 8 and 20% O2 (Fig. 1), whereas others have not demonstrated effects until O2 content falls well below 5%. We do not have a clear explanation for this difference. While there may be important methodological differences between the present experiments and those using lung cells, the sensitivity to low O2 that we report here is probably physiologically relevant. One group of investigators (13, 51) has shown that rats breathing 10% O2 for 24 h have a reduced amiloride-sensitive voltage in native airways. The lungs of these animals did not demonstrate significant reduction in ENaC protein or mRNA. Thus it seems reasonable to suspect that even modest reductions in O2 can reduce ENaC function.

The second feature of the present experiments is that higher than “normal” levels of O2 can increase ENaC function. This effect is evident for values up to 40% O2; 95% O2 did not consistently produce any further increase in Na transport (Fig. 1). Superficially, it may appear that this effect is pharmacological rather than physiological. However, reducing the volume of the apical solution by 50% increased the Ifc by ~40% in monolayers exposed to 20% O2. Eliminating the apical solution in cultured lung epithelia is now a common practice as it appears to increase ENaC function (31). It seems likely that reducing or eliminating the volume of the apical solution reduces the diffusion distance for O2 from the ambient air to the mitochondria and thus increases O2 availability. From this perspective, cells in culture may be relatively hypoxic compared with cells in their native environment (24). The measurements demonstrating an inverse relationship between O2 content and lactate production (Fig. 3) are consistent with this idea. That increasing O2 from 40 to 95% further decreased lactate production suggests that even incubated in 40% O2 the monolayers may be relatively hypoxic. We speculate that the reason we did not consistently measure an increase in Ifc at 95% O2 is that some monolayers developed O2 toxicity. It is interesting that these effects occur, despite apparently adequate O2 content of the bulk solutions (Fig. 3).

Mechanism of the effect of O2 on ENaC. Despite efforts by several investigators, there is relatively little information on the molecular mechanisms responsible for regulating ENaC func-
tion by O2. The time course of the present O2 effects clearly separates this mechanism from those responsible for changing activity of the carotid body or the pulmonary artery pressure, processes that are influenced by O2 within seconds (55). Thus regulation of ENaC in collecting duct cells probably does not depend on the generation of ROS (59), since TEMPOL, while it decreased baseline ENaC activity, did not alter the response to varying O2 concentrations (Fig. 6C).

In the context of the existing literature, the present experiments permit some general conclusions. First, it is unlikely that there is much overlap in the O2-mediated pathways of ENaC regulation and those mediated by corticosteroids. Whereas steroids increase expression of α-ENaC, Sgk1, Gilz, and Usp2–45 (Fig. 7), O2 has relatively little or no effect on these gene products. Instead, O2 alters expression of HO-1 and Glut1, genes that are minimally altered by steroids. Given these
mediators of O2-related effects: HIFs and AMPK. We were unable to detect HIF-1α by immunoblot in mpkCCD-c14 cells (despite trying 4 different antibodies), even under conditions of CO and ALLN treatment (Fig. 6A), although we were able to detect it in other cells. In addition, we were not able to detect HIF-2α changes in mpkCCD-c14 cells, even though we were able to detect changes in COS7 and HEK-293 cells (Fig. 6A). Finally, an inhibitor (DMOG) of the degrading enzyme prolyl hydroxylase did not alter the qualitative response to O2 (Fig. 6B). Thus these mpkCCD-c14 cells appear to be different from models of airway epithelial cells, where HIF proteins are induced by hypoxia (1, 24). While these mpkCCD-c14 cells probably contain modest amounts of HIF proteins, it appears that they play at most a minor role in regulating ENaC in mpkCCD-c14 cells under these conditions.

AMPK is activated by low O2 levels (40), and the activated form inhibits ENaC function in heterologous expression systems (6, 11). However, changing O2 between 8 and 40% did not produce changes in the active (phosphorylated) form (Fig. 4C), although lowering O2 to 1% did increase phosphorylation. Furthermore, AICAR, an activator of AMPK, did not mitigate the O2 effects on Na transport, although it did reduce the baseline (Fig. 4B). Thus AMPK is present in mpkCCD-c14 cells, but does not appear to mediate the entire range of O2 effects on Na transport. However, it is possible that AMPK may play a role in inhibiting ENaC at much lower O2 concentrations, such as 1% (Fig. 4C).

Surface expression of ENaC. The most dramatic effects of changes in O2 exposure to ENaC biochemistry were not in ENaC protein or mRNA expression (Figs. 8 and 9), but in ENaC surface expression (Fig. 10). Others have reported that more severe reductions in O2 levels reduce ENaC surface expression in lung epithelial cells (7, 43).

The nature of the β- and γ-ENaC expressed on the surface was different from that detected in cell lysates. The β-ENaC subunit displayed two bands, an ~80-kDa band representing the full length form and a ~90-kDa band probably representing a glycosylated form previously reported by Ergonul et al. (16) in kidney. The abundance of this glycosylated form appears to
be increased by feeding animals a low-NaCl diet (16). The ratio of the ~90-kDa form and the ~80-kDa form was different on the surface than in the cell lysate; there was relatively greater amounts of the glycosylated form on the cell surface, at least in monolayers exposed to 40% O2 (Fig. 10B). The extent to which this glycosylated form is required for surface expression is not known, but Butterworth et al. (9) reported that to which this glycosylated form is required for surface expres-

The observation that changes in PO2 can, in turn, influence Na transport (and hence O2 consumption) represents another variable that must be considered in the factors that regulate PO2 and tubular transport.

Recent studies are reviving interest in trying to understand the factors that contribute to intrarenal PO2. Using blood oxygen level-dependent (BOLD) MRI, Prujim et al. (45) showed that a low-NaCl diet increased the PO2 of the renal medulla (17, 36, 47). Markedly lower PO2 within the medulla also involves O2 shunting, but in the setting of a substantially lower blood flow. The factors responsible for determining the intrarenal PO2 include local perfusion, local O2 consumption, and O2 shunting (17). The observation that changes in PO2 can, in turn, influence Na transport (and hence O2 consumption) represents another variable that must be considered in the factors that regulate PO2 and tubular transport.

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Local tissue PO2 is illustrated by an analysis of differences within small regions of the kidney. Baumgartl et al. (4, 5) found that PO2 varied by up to 40 Torr in regions of the kidney within a distance of only 50 μm.

Can we infer that regional differences of PO2 might influence ENaC activity in vivo? The strikingly lower PO2 in the medulla within the cortex might influence the transport capacity of segments within that region. For example, it seems possible that regional differences in PO2 might play a role in the higher ENaC-mediated Na transport rates in the connecting tubule compared with the collecting duct (21). The connecting tubule might reside in a region in which PO2 is greater than in the medullary rays. We are not aware of specific information on PO2 gradients within the cortex related to structure, but note that the connecting tubules, as well as the adjacent distal convoluted tubules, have abundant mitochondria (32), consistent with a generous supply of O2.

The PO2 of renal tissue may play a role in kidney disease. There has been considerable discussion about how renal hypoxia may contribute to progression of renal disease (18, 39). However, it is well recognized that obesity can predispose to kidney disease. Furthermore, this state is often accompanied by local tissue hypoxia; given the time course of the response, gene regulation may play an important role. This relationship between PO2 and Na transport prompts a reevaluation of the intrarenal mechanisms responsible for Na transport and further consideration of how differences in renal PO2 within the kidney might alter tubular function.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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