Oxygen-evoked changes in transcriptional activity of the 5'-flanking region of the human amiloride-sensitive sodium channel (α ENaC) gene: role of nuclear factor κ B

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Expression of the α -subunit of the amiloride-sensitive sodium channel (α ENaC) is regulated by a number of factors in the lung, including oxygen partial pressure (P_{O_2}). As transcriptional activation is a mechanism for raising cellular mRNA levels, we investigated the effect of physiological changes in P_{O_2} on the activity of the redox-sensitive transcription factor nuclear factor κ B (NF- κ B) and transcriptional activity of 5'-flanking regions of the human α ENaC gene using luciferase reporter-gene vectors transiently transfected into human adult alveolar carcinoma A549 cells. By Western blotting we confirmed the presence of NF- κ B p65 but not p50 in these cells. Transiently increasing P_{O_2} from 23 to 42 mmHg for 24 h evoked a significant increase in NF- κ B DNA-binding activity and transactivation of a NF- κ B-driven luciferase construct (pGLNF- κ Bpro), which was blocked by the NF- κ B activation inhibitor sulphasalazine (5 mM). Transcriptional activity of α ENaC-luciferase constructs containing

5'-flanking sequences (including the NF- κ B consensus) were increased by raising P_{O_2} from 23 to 142 mmHg if they contained transcriptional initiation sites (TIS) for exons 1A and 1B (pGL3E2.2) or the 3' TIS of exon 1B alone (pGL3E0.8). Sulphasalazine had no significant effect on the activity of these constructs, suggesting that the P_{O_2} -evoked rise in activity was not a direct consequence of NF- κ B activation. Conversely, the relative luciferase activity of a construct that lacked the 3' TIS, a 3' intron and splice site but still retained the 5' TIS and NF- κ B consensus sequence was suppressed significantly by raising P_{O_2} . This effect was reversed by sulphasalazine, suggesting that activation of NF- κ B mediated P_{O_2} -evoked suppression of transcription from the exon 1A TIS of α ENaC.

Key words: lung, oxygen, transcription factor.

INTRODUCTION

Expression of the amiloride-sensitive sodium channel (ENaC) is critical for fluid homeostasis in a number of epithelial tissues, including the lung [1]. The channel comprises three subunits, α , β and γ . The presence of the α -subunit is essential for functional transport of Na^+ from the lung lumen and the concomitant osmotic movement of water [2], whereas the β - and γ -subunits modulate the activity and functional characteristics of the channel [3]. The α -subunit abundance and channel activity are known to be up-regulated by a number of factors in the lung. These include glucocorticoids, thyroid hormones (T3), catecholamines and, most recently, changes in the atmospheric partial pressure of oxygen (P_{O_2}) [4–8]. Raised fetal corticosteroids dramatically increase mRNA levels of the α -subunit of ENaC (α ENaC) around the time of birth, increasing fluid absorption, but levels rapidly decline postnatally. The increase in alveolar P_{O_2} that occurs when the infant takes its first breath has also been shown to increase Na^+ transport and α ENaC mRNA levels in fetal distal lung epithelial cells (FDLE cells) [4,7,8]. As the increase in P_{O_2} is maintained after birth, it has been speculated that this change could stabilize and maintain the transition of the lung from a secretory organ in the fetus to an absorptive phenotype postnatally. Furthermore, in the adult, reducing alveolar P_{O_2} could potentially decrease α ENaC levels, depressing fluid ab-

sorption and leading to pulmonary oedema, e.g. high-altitude pulmonary oedema.

Although not the only mechanism, activation of transcription of the ENaC subunit genes is an important regulatory strategy for increasing mRNA levels and functional protein within the epithelial cell. Recently, cloning of the promoter region of the α ENaC gene revealed that transcription of the human and rat genes can be increased in the adult lung epithelial cell lines A549 and H441, by glucocorticoids acting via a glucocorticoid response element (GRE) in the 5'-flanking region of the gene [9,10]. Furthermore, the response to glucocorticoids can also be augmented by thyroid hormone (T3) [10]. Increasing atmospheric P_{O_2} from fetal (23 mmHg) to neonatal (100 mmHg, alveolar P_{O_2}) levels up-regulates transcriptional activity of the human α ENaC gene promoter, mRNA levels, protein levels and function of the channel in rat primary cultured FDLE cells [8]. Conversely, reducing P_{O_2} from 142 mmHg (water-saturated atmospheric P_{O_2} + 5% CO_2) to 23 mmHg or below (anoxia) has been demonstrated to decrease α ENaC mRNA levels (and function) in adult rat alveolar type II (ATII) cells and the human alveolar epithelial A549 cell line [11,12]. The pathway by which increased P_{O_2} potentially mediates changes in transcriptional activity of α ENaC has been postulated to work through the redox-sensitive transcription factor, nuclear factor κ B (NF- κ B) [5] (Figure 1). NF- κ B DNA-binding activity is increased in FDLE cells subjected to

Abbreviations used: ENaC, amiloride-sensitive sodium channel; α ENaC, α -subunit of ENaC; EMSA, electrophoretic mobility-shift assay; FDLE cell, fetal lung distal epithelial cell; GRE, glucocorticoid response element; NF- κ B, nuclear factor κ B; I κ B, NF- κ B inhibitor protein; P_{O_2} , partial pressure of oxygen; TIS, transcriptional initiation site; RT, reverse transcriptase; DMEM, Dulbecco's modified Eagle's medium.

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Figure 1 Schematic diagram of the 5'-flanking region of the human α ENaC gene showing known regulatory pathways affecting transcriptional activity and mRNA generation

5'-Flanking region of α ENaC (834–2933 bp of the sequence of GenBank accession number HSU81961). Known regulatory pathways are marked with solid lines and possible regulatory pathways involving NF- κ B with broken lines. 5' and 3' Transcriptional initiation sites (TIS) give rise to exon 1A and exon 1B, respectively, which can be alternatively spliced to exon 2 to produce different α ENaC mRNA products. Glucocorticoid hormones enhance transcriptional activity from both TIS via the GRE (+) [15] and H_2O_2 suppresses GRE-mediated transcriptional activity (–) [9,14]. How changes in P_{O_2} and the activation of the transcription factor NF- κ B potentially interact with these known transcriptional regulatory pathways are illustrated (1; see the Introduction and Discussion for more details). The position of translation-initiation sites (ATG) are indicated and the GRE and NF- κ B consensus binding sequence are depicted as boxes.

shifts in P_{O_2} from 23 to 100 or 142 mmHg and is associated with the increase in α ENaC mRNA levels [5,8]. More recently, inhibition of NF- κ B activity has been demonstrated to reduce functional activity of amiloride-sensitive sodium channels in these cells [13].

Although oxidative stress created by the exogenous addition of H_2O_2 has been shown to modulate the α ENaC transcriptional response to glucocorticoids in A549 cells [14], there is little direct evidence linking physiological changes in P_{O_2} and NF- κ B activity to transcriptional modulation of the α ENaC gene (Figure 1). Nevertheless, NF- κ B consensus binding sites have been demonstrated in the rat, guinea-pig (D.L. Baines, unpublished work) and human α ENaC promoter regions [10,15]. In this paper we have determined whether physiological changes in P_{O_2} , i.e. exposure to fetal (23 mmHg) or incubator atmospheric (142 mmHg) oxygen tensions can mediate changes in transcriptional activity of regions of the α ENaC promoter in A549 cells, a model for adult human alveolar epithelial cells. We have also investigated whether activation of the redox-sensitive transcription factor NF- κ B is involved in such processes.

EXPERIMENTAL

Cell culture

A549 cells were purchased from A.T.C.C. and maintained in Keighn's modification of Ham's F12 medium (Life Technologies, Paisley, Scotland, U.K.) supplemented with 10% (v/v) fetal bovine serum (Immune Systems, Paignton, Devon, U.K.) and antibiotics (penicillin/streptomycin; Life Technologies). Cells were maintained at 37 °C in humidified atmospheric air + 5% CO_2 ('incubator atmospheric'; $P_{O_2} \approx 142$ mmHg) or in 3% O_2 /89% N_2 /5% CO_2 ('fetal'; $P_{O_2} \approx 23$ mmHg). All treatments

and cell manipulations were performed using medium and buffers equilibrated to the respective P_{O_2} in which the cells were maintained. Cells were treated with 5 mM sulphasalazine (Sigma, Poole, Dorset, U.K.) suspended in tissue-culture medium for 2 h prior to exposing to changes in P_{O_2} .

Western blots and reverse transcriptase (RT)-PCR

For analysis of α ENaC and β -actin abundance, protein was prepared from A549 cells by scraping cells into an ice-cold solution of tissue lysis buffer [100 mM Tris, pH 6.8, 1 mM EDTA, 10% (v/v) glycerol and 10 μ l $\cdot$ ml $^{-1}$ protease-inhibitor cocktail (Sigma)]. Cells were disrupted by Dounce homogenization and subjected to centrifugation (5 min, 250 g) to remove large debris and nuclei. Analysis of NF- κ B proteins was carried out using nuclear protein extracts from A549 cells (see below). The supernatants from each preparation were heated to 94 °C for 5 min in the presence of 100 mM dithiothreitol and 2% (w/v) SDS to denature the proteins. Finally, 0.1% (w/v) Bromophenol Blue was added prior to loading the gels. Samples containing 40 μ g of protein were subjected to electrophoresis on SDS/polyacrylamide gels. Fractionated proteins were transferred to nitrocellulose membranes by semi-dry blotting and immunostained with anti- α ENaC antiserum (a kind gift from C.C. Canessa, Yale University, New Haven, CT, U.S.A.), anti- β -actin antiserum (Sigma) or antisera against NF- κ B subunits p50 or p65 (anti-NF- κ B p50 or anti-NF- κ B p65; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) using standard techniques. Immunostained proteins were visualized by enhanced chemiluminescence (ECL; Amersham Bioscience, Little Chalfont, Bucks., U.K.) and exposure to autoradiographic film.

RT-PCR was carried out on RNA extracted from A549 cells using the Trizol method (Sigma). Briefly, 2 μ g of RNA was reversed transcribed using 1 unit of avian myeloblastosis virus (AMV) RT and 100 nM oligo-dT $_{15}$ primer in AMV-RT buffer (Promega, Chilworth, Southampton, U.K.). Amplification of α ENaC mRNA was carried out using primers directed against a region of the human α ENaC gene similar to that described previously for guinea-pig [6] (sense primer, 5'-ACCCGATG-TATGGAACTGCT-3'; antisense primer, 5'-GCTGCCAG-GTTGGACAGGAG-3') or β -actin (Clontech, Basingstoke, Hants., U.K.) in a PCR reaction mix [75 mM Tris, pH 9.0, 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 1% Tween-20, 250 nM of each primer and 0.2 units of AGS gold *Taq* polymerase (Hybaid, Ashford, Middx, U.K.)] and a cycling protocol of 94 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min for 40 cycles. PCR products were fractionated on 1% agarose gels and visualized by ethidium bromide staining and UV fluorescence.

Electrophoretic mobility-shift assays (EMSA)

Nuclear extracts were prepared from $\approx 1 \times 10^7$ A549 cells. After washing twice in ice-cold PBS, cells were collected and pelleted by centrifugation at 450 g for 5 min. The cell pellet was resuspended in 400 μ l of buffer A (10 mM Tris, pH 7.8, 2 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$ and 0.5 mM dithiothreitol) supplemented with 10 μ l $\cdot$ ml $^{-1}$ protease-inhibitor cocktail and incubated on ice for 5 min. Cells were lysed by the addition of 0.4% (v/v) Nonidet P-40 on ice for 5 min and the nuclei pelleted by centrifugation at 4500 g for 5 min at 4 °C. The nuclei were resuspended in buffer B [20 mM Tris, pH 7.8, 2 mM EDTA, 420 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol and 20% (v/v) glycerol] and protease-inhibitor cocktail as above. Nuclei were lysed by gentle agitation for 30 min at 4 °C. After centrifugation at 10000 g for 5 min at 4 °C, the nuclear extract was collected and stored at –70 °C. Protein concentration was

determined by the Folin and Cocteau method (Sigma) using BSA as a standard. EMSAs were carried out using double-stranded oligonucleotide probes containing a common consensus binding site for NF- κ B (underlined), 5'-AGTTGAGGGG-ACTTTCCCAGCT-3', or the human α ENaC NF- κ B consensus sequence, 5'-ACACTTGGGACTCCCCCCTT-3'. The probes were end-labelled with [γ - 32 P]ATP and 1 pmol (with a radioactivity of $\approx 2 \times 10^4$ c.p.m.) was incubated with 10 μ g of nuclear extract in 40 μ l of DNA-binding buffer [20 mM Hepes, 1 mM MgCl₂, 4% Ficoll and 2 μ g of poly(dI-dC)] for 30 min at room temperature. The DNA-protein complexes were resolved from free oligonucleotides by electrophoresis using 4% polyacrylamide gels (acrylamide/bisacrylamide, 60:1) run at 125 V for 30 min. Gels were then dried and exposed to autoradiographic film (Kodak, Hemel Hempstead, Herts., U.K.) at -70° with an intensifying screen. Control experiments were carried out using a mutant double-stranded oligonucleotide, 5'-AGTTGA-GGAACTTTCCAGCT-3', where the consensus binding site for NF- κ B was disrupted (underlined). Nuclear extracts were also pre-bound with antibodies directed against p50 and p65 (Santa Cruz Biotechnology) prior to incubation with labelled DNA.

α ENaC-luciferase reporter gene constructs

All α ENaC promoter constructs were engineered from genomic DNA by the use of PCR to amplify selected regions of the published 5'-flanking region (GenBank accession number HSU81961) [9]. Genomic DNA was isolated from A549 cells using Tri-Reagent (Sigma) following the manufacturer's protocol. PCR was performed using 1 μ g of genomic DNA in a reaction mix similar to that described above and subjected to a cycling regimen of 94 $^\circ$ C for 3 min, 58 $^\circ$ C for 1 min and 72 $^\circ$ C for 1 min for 30 cycles. PCR products were resolved on 1% agarose gels and the DNA excised and cleaned using QIAEX (Qiagen, Crawley, West Sussex, U.K.). Products (100 ng) were directionally subcloned into the promoter-less luciferase vector pGL3basic (Promega) by subjecting them to restriction-enzyme digestion (see below) and ligation with T4 ligase (Promega) into 0.5 μ g of correspondingly digested pGL3basic vector. Ligated plasmid constructs were fully sequenced using an ABI automated sequencer to confirm identity with the published sequence and suitable plasmids were prepared for transfection using the Endo-free maxi kit (Qiagen). For simplicity, the nucleotide positions (in bp) marking the extent of each construct described below refer to the sequence HSU81961. The exon arrangement is as described by Thomas et al. [15] and is shown in Figure 1. pGL3E0.8: PCR amplification of the human α ENaC promoter region using oligonucleotide primers corresponding to positions 1306–1325 (primer 1) and 2194–2218 (primer 2) were subcloned into pGL3basic as described previously [9]. This produced a region of the α ENaC promoter arising at position 1443 in exon 1A (1389–2061), containing the NF- κ B consensus sequence, the 3' transcriptional initiation site (TIS) of exon 1B and minimal promoter region P2, fused with the luciferase gene at position 2158 within exon 1B (2116–2728). pGL3E1.4: primers corresponding to positions 1–25 (primer 3) and 1424–1443 (primer 4) were designed to include *KpnI* and *XhoI* sites at the 5' and 3' ends of each primer, respectively, to amplify a 5'-flanking region containing the GRE, the 5' TIS of exon 1A and minimal promoter region P1 fused with the luciferase gene at position 1443 within exon 1A. pGL3E2.2: this extensive 5'-flanking region was amplified using primers 3 and 2. The construct encompasses a region containing the GRE, the 5' exon 1A TIS and the 3' exon 1B TIS, and is fused with the luciferase gene at position 2218

within exon 1B. pGL3E1.9: this construct lacks extreme 5' sequences and the 3' exon 1B TIS of the human α ENaC gene, but includes the GRE, the 5' exon 1A TIS and the NF- κ B consensus sequence and is fused with the luciferase gene at position 2050 at the end of exon 1A. Both pGL3E1.9 and pGL3E0.8 were kind gifts from Dr Christie Thomas (University of Iowa, Iowa City, IA, U.S.A.). The NF- κ B promoter construct containing three NF- κ B consensus sequences in tandem cloned into pGL3pro (Promega) was a kind gift from Dr Zonglin Wu (St. George's Hospital Medical School, London, U.K.).

Transfections and luciferase assays

A549 cells were plated out into 12-well tissue-culture plates at 1×10^5 cells/well and continually maintained at a PO_2 of 142 or 23 mmHg. The following day cells were washed twice in PO_2 -buffered Dulbecco's modified Eagle's medium (DMEM) and co-transfected for 5 h with 0.5 μ g of pSV- β -galactosidase (transfection control; Promega) and 1 μ g of the α ENaC promoter construct, pGL3basic (promoter-less control) or pGL3control (constitutive promoter control) using 4 μ l of Lipofectamine (Life Technologies) in 200 μ l of DMEM/well. Following transfection, 1 ml of PO_2 -buffered DMEM/10% fetal calf serum was added to each well and the cells incubated overnight. After transfection (24 h) the cells were either left at the PO_2 at which they had been transfected or shifted from 23 to 142 mmHg for 24 h. Pre-treatment with sulphasalazine was carried out 2 h prior to shifting cells between PO_2 values.

To analyse the luciferase activity generated in the cells, plates were removed from the incubator and placed immediately on ice. Cells were washed twice with ice-cold PBS and incubated at room temperature in 100 μ l of lysis buffer (Promega) per well for 20 min. Cells and lysis buffer were then scraped from the wells into ice-cold Eppendorf tubes, vortex-mixed for 15 s and centrifuged at 10000 g for 2 min at 4 $^\circ$ C. For luciferase assays, 20 μ l of cell lysis supernatant was pipetted into the wells of a white 96-well assay plate (Thermo Labsystems, Ashford, Kent, U.K.) at room temperature. Using a luminometer, 100 μ l of luminescent substrate (Promega) was injected into each well, mixed with the supernatant and the luminescence measured for 10 s. The β -galactosidase assays required 30 μ l of supernatant to be pre-incubated with 196 μ l of buffer and 4 μ l of substrate (Luminescent β -gal assay kit; Clontech) for 1 h at room temperature prior to direct measurement of luminescence for 10 s as described above.

Luciferase and β -galactosidase activity are a measure of transcriptional activity of the reporter constructs. Transfection efficiency was controlled by comparison with β -galactosidase activity, and the luciferase activity of the constructs was normalized to the promoter-less control pGL3 basic. pGL3NF- κ B was normalized to pGL3pro. We found that there was little change in transfection efficiency within samples. However, in the cells subjected to changes in PO_2 we found that there were transient PO_2 -induced changes in the level of the promoter-driven constructs pSV- β -galactosidase and pGL3control used as controls ($P < 0.05$, $n = 6$). There were no such detectable changes in the promoter-less control pGL3basic. Thus to control for non-specific effects of PO_2 , data are expressed as relative luciferase activity (ENaC promoter construct/ β -galactosidase activity) – (pGL3basic/ β -galactosidase).

Statistics

All data are presented as means $\pm$ S.E.M. The value n refers to the number of independent experiments. Statistical analysis of mean values was carried out using Student's paired or un-

paired *t* tests. A *P* value of < 0.05 was considered statistically significant.

RESULTS

α ENaC protein and mRNA abundance

In order to confirm the presence of α ENaC mRNA and protein in A549 cells, Western blots were carried out using protein extracted from A549 cells maintained at PO_2 of 23 mmHg (fetal) or 142 mmHg (atmospheric). Two protein bands of ≈ 97 and ≈ 116 kDa were immunostained with the α ENaC antiserum. As the unglycosylated form of α ENaC is generally considered to be ≈ 97 kDa the larger immunostained band may represent a glycosylated form of the protein and is similar in size to α ENaC protein in A549 cells described by Wodopia et al. [12]. α ENaC protein was more abundant in cells maintained at a PO_2 of 142 mmHg than in cells maintained at 23 mmHg when compared with levels of β -actin (2.9 ± 0.6 -fold). These data suggest that there is a PO_2 -associated increase in the protein abundance of α ENaC in A549 cells (Figure 2A). RT-PCR amplification of a 761 bp product also confirmed the presence of α ENaC mRNA in the A549 cells. Although not fully quantitative, comparison with β -actin mRNA levels suggested that the abundance of amplified product was 1.5–2.0-fold higher from RNA derived from cells maintained at a PO_2 of 142 mmHg than at 23 mmHg (Figure 2B).

Figure 2 PO_2 -induced rise in α ENaC protein and mRNA levels in A549 cells

(A) Representative Western blot of 40 μ g of protein extracted from A549 cells maintained at a PO_2 of 23 or 142 mmHg immunostained with anti α ENaC and β -actin antisera. The sizes of the respective immunostained proteins are shown to the left. (B) α ENaC mRNA in A549 cells. Representative agarose gel of α ENaC (left-hand panel) and β -actin (right-hand panel) PCR products amplified by RT-PCR from A549 cells maintained at 23 or 142 mmHg. The predicted sizes of the α ENaC and β -actin PCR products in bp are shown.

Figure 3 NF- κ B protein and binding activity in A549 cells

Representative Western blots of NF- κ B p50 recombinant protein (5 μ g) and nuclear protein (40 μ g) extracted from A549 cells maintained at a PO_2 of 23 or 142 mmHg immunostained with (A) anti-NF- κ B p50 and (B) anti-NF- κ B p65 antisera. The positions of protein size markers are shown to the left. (C–E) EMSAs using 10 μ g of nuclear protein extracted from A549 cells maintained at 23 or 142 mmHg or shifted from 23 to 142 mmHg for 24 h using (C) NF- κ B missense oligonucleotides (electronic image), (D) α ENaC NF- κ B consensus sequence oligonucleotide (electronic image) and (E) a common NF- κ B consensus oligonucleotide pre-incubated with (+) and without (–) NF- κ B p65 antiserum (autoradiograph image). (F) Electronic imager quantification of shifted bands from three independent gels using α ENaC NF- κ B consensus sequence oligonucleotides expressed graphically as c.p.m./1000. **P* < 0.05 , significantly different from 23 mmHg.

NF- κ B identity and binding activity

To elucidate the effect of PO_2 on activation of NF- κ B in A549 cells, we investigated the isoform identity of NF- κ B, its binding

Figure 4 P_{O_2} -evoked transactivation of a NF- κ B-luciferase reporter construct in A549 cells

(A) Relative luciferase activity of pGLNF- κ Bpro reporter construct in A549 cells maintained at 23 or 142 mmHg or shifted from 23 to 142 mmHg for 24 h. The identity and a schematic diagram of the construct is shown above the graph. (B) Relative luciferase activity of the NF- κ B-luciferase reporter construct in A549 cells maintained at 23 mmHg or shifted from 23 to 142 mmHg for 24 h after pre-treatment of A549 cells with 5 mM sulphasalazine, a specific inhibitor of NF- κ B activation. * $P < 0.05$ significantly different from relative luciferase activity at 23 mmHg; † $P < 0.05$ significantly different from relative luciferase activity in 142 mmHg.

activity under different P_{O_2} regimens and the ability of P_{O_2} to modulate NF- κ B transactivation of a NF- κ B-luciferase reporter construct (pGLNF- κ Bpro). Immunostained products of NF- κ B p65 but not p50 were detected on Western blots of nuclear protein extracted from A549 cells maintained in fetal (23 mmHg) or incubator atmospheric (142 mmHg) P_{O_2} (Figures 3A and 3B). EMSAs showed that NF- κ B specifically bound and inhibited the mobility of both the α ENaC (Figure 3D) and a common NF- κ B consensus sequence (Figure 3E), but not the missense consensus sequence (Figure 3C). A non-specifically bound product was also seen on gels using the common NF- κ B consensus sequence (Figure 3E). There was a significant rise in NF- κ B-binding activity in cells shifted from a P_{O_2} of 23 to 142 mmHg for 24 h ($P < 0.05$, $n = 3$). There was also a small apparent increase in NF- κ B-binding activity in cells maintained in atmospheric P_{O_2} (142 mmHg) compared with fetal P_{O_2} (23 mmHg), but significance could not be attributed to these results ($P = 0.09$, $n = 5$; Figure 3F). Pre-incubation with p65 antiserum of nuclear extracts from cells incubated at 142 mmHg suppressed NF- κ B-binding activity but had little effect on extracts from cells maintained at 23 mmHg. These data suggest that NF- κ B p65 is present in A549 cells and that its binding activity is increased with a shift in P_{O_2} from 23 to 142 mmHg.

We also investigated whether such a P_{O_2} -evoked rise in NF- κ B-binding activity could transactivate an NF- κ B-driven luciferase reporter gene. A549 cells were transfected with the pGLNF- κ Bpro construct or pGL3pro (control). The level of luciferase activity of the constructs was determined in cells either maintained at a P_{O_2} of 23 or 142 mmHg or shifted from 23 to 142 mmHg for 24 h. The presence of the simian virus 40 promoter (pro) in pGLNF- κ Bpro induced high levels of relative luciferase activity that were similar in cells maintained at 23 mmHg (4107 ± 640 arbitrary units) or 142 mmHg (4425 ± 443 arbitrary units). Shifting cells from 23 to 142 mmHg for 24 h evoked a significant rise (7543 ± 343 arbitrary units) in relative luciferase activity of the pGLNF- κ Bpro construct compared with cells maintained at 23 or 142 mmHg ($P < 0.01$, $n = 4$; Figure 4A). To confirm that this effect was mediated by activation of NF- κ B,

cells were maintained at a P_{O_2} of 23 mmHg or shifted from 23 to 142 mmHg after pre-treatment with 5 mM sulphasalazine, a specific blocker of NF- κ B activation. Under these conditions no significant change in relative luciferase activity was seen between the shifted cells and those maintained at a P_{O_2} of 23 mmHg ($P = 0.9$, $n = 4$; Figure 4B), suggesting that NF- κ B activation is critical for transactivation of the luciferase construct. These data suggest that exposing A549 cells to a transient shift in P_{O_2} from fetal (23 mmHg) to atmospheric (142 mmHg) levels for 24 h transactivates reporter-gene expression via activation of NF- κ B and binding to NF- κ B consensus sequences.

α ENaC-luciferase promoter constructs

Four α ENaC 5'-flanking region-luciferase reporter gene plasmids were constructed (see the Experimental section). pGL3E2.2 contained an extensive 5'-flanking region with 5' and 3' TIS for exons 1A and 1B respectively, the GRE and NF- κ B consensus sequences [15]. pGL3E0.8 contained an identical 3' region to pGL3E2.2 but lacked the 5' TIS of exon 1A and preceding 5' sequences. pGL3E1.9 was identical to pGL3E2.2 but lacked extreme 5' sequences, the 3' TIS of exon 1B, a 3' intron and a splice site [9,15]. pGL3E1.4 contained 5' regions common to pGL3E2.2 and pGL3E1.9, but lacked sequences 3' of the exon 1A TIS, including the NF- κ B consensus binding sequence (Figure 5A). All the α ENaC constructs were transfected into paired batches of A549 cells maintained at a P_{O_2} of 23 or 142 mmHg. The relative luciferase activity of each promoter construct was compared 24 h post-transfection. All of the constructs showed levels of relative luciferase activity that were significantly higher than the promoter-less vector pGL3basic ($P < 0.05$, $n = 4$). The construct pGL3E2.2 exhibited the lowest relative luciferase activity (5 ± 1.3 -fold higher than pGL3basic at 142 mmHg). This level of activity was higher than that which we have described previously in FDLE cells [8] but is consistent with the work of Sayegh et al. [9]. pGL3E2.2 also exhibited a small but significantly higher level of activity in cells maintained at a P_{O_2} of 142 mmHg compared with those maintained at 23 mmHg (9 ± 2

Figure 5 Human α ENaC-luciferase reporter constructs

(A) Regions of the 5'-flanking sequence of α ENaC were linked to luciferase reporter gene sequences. The length and identity of each construct used in this study are shown beneath a schematic representation of the 5'-flanking region of the α ENaC gene. The GRE is shown as a shaded box, NF- κ B as a black box and the TISs for exons 1A and 1B are shown as filled arrows. The positions of several other transcription-factor consensus sequences are shown as open boxes (but are not fully inclusive) and the minimal promoter regions P1 and P2 are shown as open boxes immediately below the diagram. Some restriction-enzyme sites are also marked. C/EBP, CCAAT-enhancer-binding protein; PEA3, polyomavirus enhancer activator 3. (B) Relative luciferase activity of reporter constructs shown in (A) in A549 cells maintained at a P_{O_2} of 23 mmHg (filled bars) or 142 mmHg (open bars), 24 h post-transfection. * $P < 0.05$ and ** $P < 0.01$, significantly different from relative luciferase activity in 23 mmHg.

and 3 ± 1 respectively, $P < 0.05$, $n = 7$). The construct pGL3E0.8, which contained a 3' region identical with pGL3E2.2, exhibited an overall higher level of relative luciferase activity that was also significantly higher in cells maintained at 142 mmHg than at 23 mmHg (43 ± 15 and 17 ± 2 respectively, $P < 0.05$, $n = 12$). In contrast with pGL3E2.2 and pGL3E0.8, pGL3E1.9 exhibited a striking difference in its response to P_{O_2} . In this construct the effect of P_{O_2} on transcriptional activity was reversed. There was significantly lower luciferase activity in cells maintained at 142 mmHg than at 23 mmHg (19 ± 5 and 109 ± 29 respectively, $P < 0.01$, $n = 12$). Although pGL3E1.4 exhibited a high level of promotional activity (37 ± 18 -fold compared with pGL3 basic at 142 mmHg), no significant difference in luciferase activity could be determined between the different P_{O_2} regimens ($P = 0.3$, $n = 8$; Figure 5B). Taken together, these data suggest that sequences deleted in pGL3E1.9, including the 3' end of the promoter region and the NF- κ B consensus sequence, are crucial in determining the direction and response of the α ENaC promoter to changes in P_{O_2} .

As transiently shifting cells from 23 to 142 mmHg increased NF- κ B-binding activity, the effect of NF- κ B activation on the luciferase activity of the α ENaC promoter constructs, pGL3E2.2,

pGL3E1.9 and pGL3E0.8, was investigated. These constructs all contain the NF- κ B consensus binding sequence. Transiently shifting cells from 23 to 142 mmHg for 24 h had no significant effect on the relative luciferase activity of pGL3E2.2 when compared with cells maintained at 23 mmHg ($P = 0.35$, $n = 4$) but evoked a small significant increase in relative luciferase activity of pGL3E0.8 (17 ± 2 to 24 ± 2 , $n = 12$, $P < 0.05$; Figures 6A and 6C). However, the relative luciferase activities of pGL3E2.2 and pGL3E0.8 remained ≈ 2 -fold higher in cells maintained in incubator atmospheric P_{O_2} (142 mmHg; 7 ± 2 , $n = 11$, $P < 0.05$, and 43 ± 15 , $n = 6$, $P < 0.05$) compared with cells maintained at 23 mmHg (3 ± 1 , $n = 11$, and 17 ± 2 , $n = 12$ respectively). After pre-treatment of cells with 5 mM sulphasalazine, the shift-induced rise in activity of pGL3E0.8 was not significant (an effect similar to that described for pGLNF- κ B pro), suggesting weak transactivation of this construct by NF- κ B. However, the activity of pGL3E0.8 and pGL3E2.2, in cells maintained at 142 mmHg, was unaffected. Levels remained significantly higher (68 ± 16 , $n = 8$, $P < 0.01$, and 7 ± 1 , $n = 4$, $P < 0.05$) when compared with 23 mmHg (21 ± 6 , $n = 8$, and 3 ± 1 , $n = 4$; Figures 6B and 6D), inferring that NF- κ B activation alone did not mediate this P_{O_2} -evoked effect.

Figure 6 P_{O_2} -evoked increase in transcriptional activity of α ENaC-luciferase reporter constructs

(A, C and E) Relative luciferase activity of the α ENaC-luciferase constructs pGL3E2.2, pGL3E0.8 and pGL3E1.9 in A549 cells maintained at 23 or 142 mmHg or shifted from 23 to 142 mmHg for 24 h. The identity and a schematic diagram of the relevant construct are shown above each graph. (B, D and F) Relative luciferase activity of the transfected construct under the same conditions described for (A), (C) and (E) with (solid bars) and without (open bars) pre-treatment with 5 mM sulphasalazine. * $P < 0.05$ and ** $P < 0.01$, significantly different from 23 mmHg; † $P < 0.05$, significantly different from relative luciferase activity in the absence of sulphasalazine.

In contrast, pGL3E1.9 exhibited a significant decrease in relative luciferase activity when cells were shifted from 23 to 142 mmHg for 24 h (50 ± 3 , $P = 0.001$, $n = 4$). The relative luciferase activity was further decreased in cells maintained at 142 mmHg (24 ± 7 , $P < 0.01$, $n = 12$) when compared with cells maintained at a P_{O_2} of 23 mmHg (111 ± 28 , $n = 12$). At a P_{O_2} of 23 mmHg, pre-treatment of cells with sulphasalazine had no effect on the relative luciferase activity of the construct compared

with untreated cells (185 ± 5 and 177 ± 18 , respectively, $n = 4$, $P = 0.8$; Figure 6E). However, sulphasalazine significantly reversed the oxygen-induced suppression of luciferase activity in cells shifted from 23 to 142 mmHg (from 104 ± 11 to 166 ± 32 , $n = 4$, $P < 0.01$) and those maintained at 142 mmHg (from 54 ± 7 to 168 ± 11 , $n = 4$, $P < 0.001$; Figure 6F). These data suggest that NF- κ B activation plays a critical role in the P_{O_2} -mediated suppression of promoter activity of this construct.

DISCUSSION

Using several promoter constructs that comprise different 5'-flanking regions of the α ENaC gene, we have shown that sequences of the α ENaC promoter are responsive to changes in P_{O_2} . It was apparent that a region (1433–2159) encompassing the NF- κ B consensus sequence and 3' TIS of exon 1B are required for P_{O_2} -evoked enhancement of transcriptional activity. Constructs containing these features (pGL3E2.2 and pGL3E0.8) exhibited a low luciferase activity in A549 cells that increased $\approx$ 2-fold with a prolonged increase in P_{O_2} from 23 to 142 mmHg, similar to our previous findings in FDLE cells [7,8]. Such changes in transcriptional activity potentially underlie P_{O_2} -evoked changes in cellular α ENaC mRNA and protein levels. Although not fully quantified, our data suggest the mRNA abundance was higher ($\approx$ 2-fold) in A549 cells maintained at atmospheric rather than fetal P_{O_2} (23 mmHg). These findings are consistent with data from rat FDLE cells exposed to similar changes in P_{O_2} [5]. Furthermore, concordant with other workers, we have also shown that α ENaC protein abundance in A549 cells is higher in cells exposed to environmental P_{O_2} (142 mmHg) compared with the relatively hypoxic fetal P_{O_2} [12].

In contrast with the findings described above, we found that further deletion of 5'-flanking sequences (including the 3' TIS for exon 1B), while retaining the 5' TIS of exon 1A and NF- κ B consensus sequence, resulted in an opposing response to changes in P_{O_2} [9,15]. The relative luciferase activity of the construct pGL3E1.9 was markedly suppressed by raising P_{O_2} . Thus we identified two P_{O_2} -mediated effects. First, one that enhanced transcription and was associated with the presence of the 3' TIS and NF- κ B consensus sequence. Secondly, a mechanism that suppressed transcription from the 5' TIS and was associated with the presence of the GRE and NF- κ B consensus sequences but the absence of the 3' TIS.

A common factor in these P_{O_2} responsive constructs was the presence of the NF- κ B consensus binding sequence. It has been reported previously by ourselves and others that the rise in α ENaC mRNA and function in FDLE cells was associated with P_{O_2} -evoked generation of reactive oxygen species and activation of the transcription factor NF- κ B [5,8,13]. Inactive NF- κ B proteins p50 and p65 exist as a complex with the NF- κ B inhibitor protein (I κ B) in the cytoplasm. Activation (i.e. by changes in P_{O_2}) results in dissociation of NF- κ B proteins from I κ B, their translocation to the nucleus and subsequent binding to consensus sequences in the promoter of a target gene (such as α ENaC) to modify transcription [17]. We have verified that the components of such a pathway are functional in A549 cells. We have shown that NF- κ B p65 (Rel A) was the predominant isoform in A549 nuclear extracts. Similarly, p65 but not p50 has also been described in rat fetal alveolar type II cells [18]. We have also presented evidence to confirm that NF- κ B can bind the human α ENaC NF- κ B consensus sequence and that binding activity was increased in A549 cell nuclear extracts subjected to a transient rise in P_{O_2} from 23 to 142 mmHg. These findings are consistent with those described in rat FDLE cells exposed to similar changes in P_{O_2} [5,8] and in A549 cells after exposure to hyperoxia ($\approx$ 142 to 722 mmHg) [19]. In parallel with the P_{O_2} -evoked rise in NF- κ B-binding activity, we have established that transactivation of a NF- κ B luciferase reporter gene could be induced in these cells. As the effect was blockable with sulphasalazine, a specific inhibitor of I κ B phosphorylation and thus NF- κ B activation [20,21], this confirms further that transcriptional enhancement was NF- κ B-mediated. Importantly, no significant changes in NF- κ B-binding activity or reporter transactivation were seen in cells maintained at 23 or 142 mmHg. This suggests that only

transient changes in P_{O_2} (24 h) had the potential to enhance gene transcription in A549 cells via NF- κ B activation alone. Moreover, this observation is consistent with the findings of Li et al. [19], who showed that the oxygen-induced nuclear translocation of activated NF- κ B in A549 cells occurred over a similar time course.

Taking these findings into account, our data suggest that the P_{O_2} -evoked increase in α ENaC promoter activity of constructs pGL3E2.2 and pGL3E0.8 in A549 cells was only partially consistent with NF- κ B enhancement of transcription. A transient (24 h) P_{O_2} -evoked increase in relative luciferase activity was only seen with the construct pGL3E0.8. That the effect was abrogated by sulphasalazine potentially indicated weak transactivation by NF- κ B. Certainly, NF- κ B transactivation of pGL3E0.8 would be expected to be less than that of pGLNF- κ Bpro, because the α ENaC sequences only weakly promote transcriptional activity and support a single NF- κ B consensus sequence. In contrast, pGLNF- κ Bpro contains three tandem repeats of a NF- κ B consensus sequence linked to a strong simian virus 40 promoter [22]. However, the effect seen in pGL3E0.8 was not evident in pGL3E2.2. In addition, the highest level of transcriptional activity of both constructs was in cells maintained continuously at atmospheric P_{O_2} and this activity was not suppressed by sulphasalazine. Furthermore, in FDLE cells, transcriptional activation and increase in mRNA levels of α ENaC are generally slower (24–48 h) than activation of NF- κ B would support (see above) [5,8]. Therefore, we suggest that transient changes in NF- κ B activity alone elicit negligible enhancement of α ENaC promoter activity in A549 cells. Factors other than, or additional to, NF- κ B may be required for the long-term rise in transcriptional activity in response to increased P_{O_2} . For example, NF- κ B p65 is known to interact with a number of other transcription factors, such as c-Jun and Sp1, to co-operatively enhance transcription [17].

Nevertheless, our data show that NF- κ B activation does play an important role in the suppression of transcriptional activity from the 5' TIS of exon 1A in the construct pGL3E1.9. The P_{O_2} -evoked suppression of luciferase activity was evident after transiently shifting cells from 23 to 142 mmHg for 24 h and the effect was markedly reversed by sulphasalazine, consistent with NF- κ B activation being a critical step in this event [20,21]. That there was a further suppression of activity after prolonged exposure to raised levels of P_{O_2} and that sulphasalazine continued to reverse the effect suggests an endogenous NF- κ B activity in A549 cells maintained at 142 mmHg. However, we were unable to show significantly higher levels of NF- κ B activity in 142 mmHg compared with 23 mmHg using EMSAs (Figure 3F). How NF- κ B acts to suppress transcription is as yet unknown. It may act by binding directly to the consensus sequence in the 5'-flanking region of α ENaC or by interaction with other pathways that modulate α ENaC transcription. For example, Wang and colleagues [14] have described a pathway whereby H_2O_2 inhibited the transcriptional activity of an α ENaC construct similar to pGL3E1.9, via the GRE (Figure 1). Further work will be necessary to elucidate the mechanism by which P_{O_2} -evoked NF- κ B activation suppresses transcriptional activity of the α ENaC promoter. In particular, to determine if the NF- κ B consensus binding sequence is essential for transcriptional suppression, if the GRE is also involved and whether NF- κ B interacts with other oxidant-stress-induced pathways such as that described by Wang et al. [14,23,24].

In summary, we have found that promoter regions of the α ENaC gene exhibit changes in transcriptional activity associated with physiological changes in P_{O_2} and NF- κ B activation. P_{O_2} -evoked changes in transcriptional activity of the unmodified 5'-

flanking region closely mimicked physiological changes in lung epithelial cell α ENaC mRNA levels [5,11] (Figure 2B), protein levels [7,12] (Figure 2A) and amiloride-sensitive apical conductance (G_{Na^+}) [8]. The increase in PO_2 -evoked promoter activity was associated with a region containing the 3' TIS of exon 1B and the NF- κ B consensus sequence, but was not commensurate with activation of NF- κ B alone. The major effect of PO_2 -evoked NF- κ B activation in these cells was via a pathway that suppressed transcription from the 5' TIS of exon 1A of the α ENaC gene. Bearing these two findings in mind, we might speculate that PO_2 -evoked activation of NF- κ B could alter transcriptional start site usage and thus provide an additional regulatory mechanism by altering cellular mRNA isoforms, translational efficiency and the functional characteristics of the α ENaC protein products [15,25]. Differential regulation and shifts in multiple TIS usage have been described for a number of genes, including cyclin B1 and xanthine dehydrogenase, leading to changes in translational efficiency of the gene product [26,27]. However, further work will be required to clarify whether this is also the case with the α ENaC gene and to establish whether the transcriptional effects that we describe have physiological consequences *in vivo*.

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