

Regulation of Na-K-ATPase gene expression by hyperoxia in MDCK cells

CHRISTINE H. WENDT,¹ HOWARD TOWLE,¹ RENU SHARMA,¹ SARA DUVICK,¹ KIYOSHI KAWAKAMI,² GREGORY GICK,³ AND DAVID H. INGBAR¹

¹University of Minnesota Medical School, Minneapolis, Minnesota 55455;

³State University of New York, Brooklyn, New York 11203-2012; and

²Jichi Medical School, Minamikawachi 329-0498, Japan

Wendt, Christine H., Howard Towle, Renu Sharma, Sara Duvick, Kiyoshi Kawakami, Gregory Gick, and David H. Ingbar. Regulation of Na-K-ATPase gene expression by hyperoxia in MDCK cells. *Am. J. Physiol.* 274 (*Cell Physiol.* 43): C356–C364, 1998.—Na-K-ATPase plays a central role in a variety of physiological processes, including ion transport and regulation of cell volume. Our previous data showed that hyperoxia increased the expression of Na-K-ATPase α_1 and β_1 mRNA in lung type II cells. We similarly show that hyperoxia ($\geq 95\%$ O₂ for 24–48 h) increased steady-state mRNA levels in both Na-K-ATPase subunits in Madin-Darby canine kidney (MDCK) cells. The mechanism of gene regulation by hyperoxia was assessed. Stability of the Na-K-ATPase mRNA levels of both subunits was unchanged in hyperoxia-exposed MDCK cells. To determine whether gene transcription was augmented by hyperoxia, MDCK cells were transfected with a β_1 -subunit promoter-reporter construct. Transfection with the wild-type promoter (β_1 -817) revealed a 1.9 ± 0.2 -fold increase in promoter activity. Transfection with 5' deletion constructs identified a 61-base pair (bp) region between –102 and –41 that was necessary for this increase in promoter activity by hyperoxia. Incorporation of this 61-bp region into a minimal promoter (mouse mammary tumor virus) similarly increased promoter activity 2.3-fold in the presence of hyperoxia. This increase in promoter activity was not seen when MDCK cells were incubated with various concentrations of hydrogen peroxide. In summary, hyperoxia increased Na-K-ATPase β_1 -subunit mRNA steady-state level due to increased transcription in MDCK cells. A region necessary for this hyperoxic effect on β_1 transcription is located between base pairs –102 and –41 on the promoter.

ribonucleic acid stability; transcription; transfection; sodium pump; oxidants; Madin-Darby canine kidney cells

MAINTAINING INTRACELLULAR ionic gradients and concentrations is key for cellular survival. During oxidant injury, intracellular ion concentrations can be disturbed by the oxidation of regulatory proteins and the disruption of cellular membranes. The upregulation of cellular mechanisms that maintain intracellular ionic balance may serve to counteract this effect. Oxidant injury has a complex effect on Na transport, depending on cell and oxidant type and the duration of the exposure. In some systems, oxidant injury upregulates both the Na channel and the Na-K-ATPase; both are important in cellular ionic homeostasis (3, 12, 15, 25, 27, 32a, 36, 41).

The Na-K-ATPase plays a central role in a variety of physiological processes, including transepithelial ion transport, regulation of cell volume, Na-coupled uptake of metabolic substrates (glucose, amino acids), and the propagation of the action potential of muscle and nerve (34). The Na pump utilizes 10–30% of cellular ATP to

actively transport Na and K ions across the cell membrane and maintain the transmembrane Na gradient. Although present to some extent in all cells, Na-K-ATPase is present in high density on the basolateral membranes of many epithelial cells specialized for Na transport, such as renal tubular epithelial cells. The Na-K-ATPase is a heterodimer of α - and β -subunits, with several isoforms described for each subunit. The major isoforms expressed in most epithelial cells are the α_1 - and β_1 -isoforms. The α -subunit contains the catalytic site, whereas the β -subunit appears to be required for plasma membrane targeting (13).

Expression of the Na-K-ATPase mRNA isoforms is regulated in a tissue-specific and developmental fashion (16, 22). In addition, Na pump transcription can be upregulated two- to fourfold by changes in ion concentrations (24), and gene expression is increased to similar degrees by glucocorticoids, aldosterone, and thyroid hormone (3,5,3'-triiodothyronine) in certain tissues (9, 31). A Na pump-specific positive transcription regulatory element has been characterized in the proximal promoter region of the α_1 -subunit (20). The 5' flanking regions of both α_1 and β_1 -subunits have several potential regulatory sites that may bind known transcription factors (20, 31). These include putative SP1, antioxidant responsive element (ARE), and nuclear factor- κ B (NF κ B) sites and glucocorticoid response elements. However, the functional activity of these sites remains undefined (23).

Recently, the roles of oxygen or oxidants in regulating gene expression have been recognized as important in both prokaryotic and eukaryotic systems, but the regulatory mechanisms involved are not well understood (28). Many of the genes regulated by hyperoxia or oxidants are involved in the homeostatic antioxidant response (14). Oxygen tension and oxidants also can influence the expression of genes not involved directly in the antioxidant response, such as surfactant apoprotein A, α -tubulin, β -actin, and the Na-K-ATPase (14, 15, 27). Although these proteins are not directly involved in the antioxidant process, they may play a key role in the maintenance of cell homeostasis in the face of hyperoxic or oxidant injury.

Our investigations have focused on oxidant regulation of Na-K-ATPase. We found that hyperoxia increased the steady-state levels of Na-K-ATPase mRNA in Madin-Darby canine kidney (MDCK) cells but did not alter the mRNA half-life of either subunit. The increase in β_1 mRNA resulted from increased β_1 transcription as demonstrated by transfecting cells with a promoter-reporter construct and measuring promoter activity. Deletion mutants identified a 61-base pair (bp)

region of the β_1 promoter between -102 and -41 bp upstream of the transcription initiation site that is necessary for the hyperoxic upregulation of promoter activity.

METHODS

Cell culture and hyperoxia exposure. The low-resistance MDCK cell line was obtained from American Type Culture Collection (ATCC CCL 34). Cells were cultured on plastic tissue culture dishes and were incubated in media containing 10% fetal bovine serum (FBS; GIBCO) and 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2.5 μ g/ml amphotericin B (GIBCO) in Eagle's minimum essential medium (MEM) with Earle's salts (GIBCO). Cells were cultured with 5% CO₂-95% air at 37°C before experiments. Cells at ~ 40 –50% confluence were exposed to hyperoxia by placing the plates in a humidified, sealed chamber (Billups) that was flushed with 5% CO₂-95% O₂ at 5 l/min for 5–10 min each day. The chamber was placed in the incubator at 37°C for various time intervals. Cells continued to divide in the presence of hyperoxia and became confluent between 24 and 48 h.

RNA analysis. Total cellular RNA was extracted and isolated by the guanidinium method as previously published (2). Northern analysis was performed as previously described (27); 20 μ g of total RNA were loaded onto 1% agarose and formaldehyde gels and electrophoresed in 3-(*N*-morpholino)propanesulfonic acid buffer. The RNA was transferred to nylon membranes in 10 \times standard sodium citrate (SSC) overnight, and the membranes were heat fixed at 80°C for 2 h. Multiple photographic negatives of the ultraviolet fluorescence of the 28S and 18S RNA on the nylon membranes were obtained to normalize for loading and to assure that the exposure was within the linear range of the film. Membranes were prehybridized in 10% dextran sulfate, 50% formamide, 1% sodium dodecyl sulfate (SDS), Denhardt's solution, salmon sperm DNA, and 1 M NaCl at 42°C for 2 h. The α_1 and β_1 Na-K-ATPase probes used were full-length rat cDNAs (gifts of E. Benz, Johns Hopkins University). The probes were multiprime labeled (Promega) with ³²P, and 1 $\times 10^6$ counts/ml of solution was added to the hybridization solution overnight at 42°C as previously described (27). The membranes were washed two times in each of the following conditions: 5 min at room temperature with 2 \times SSC; 20 min at 50°C with 2 \times SSC-1% SDS; and 1 h at 68°C with 0.1 \times SSC-0.1% SDS. The membranes then were placed on film for 24–72 h. The RNA integrated optical density (IOD) was determined using Densitometry Image software. The β_1 mRNA had two transcripts on Northern analysis. Both transcripts were included in the IOD measurements for the β_1 -subunit. The RNA densitometry values were normalized to the 18S and 28S RNA densitometry value from the film negative from ethidium bromide-stained RNA fixed to nylon membranes (6). This method has been reliable for normalization and eliminates the issue of variability of certain housekeeping genes, such as actin, to hyperoxia and also eliminates the need for a second hybridization (6). Because 18S and 28S represent >90% of the RNA, apart from variations in loading, large changes in the rRNA would need to be seen to influence normalization. All experiments were performed at least in triplicate.

RNA stability. Stability of Na-K-ATPase mRNA was measured as described by Chambers et al. (4). To inhibit mRNA synthesis, cells were treated with 10 μ g/ml actinomycin D for various time intervals during the final portion of 24 h of incubation in either hyperoxia or room air (controls). Twenty-four hours of hyperoxia was chosen, since the maximal increase in steady-state mRNA levels was seen at this time.

Preliminary data showed that <20% of the original Na-K-ATPase mRNA was present after 8 h of actinomycin D; therefore, cells were treated for 2, 4, 6, and 8 h with actinomycin D at the final portion of the 24 h of incubation. Total RNA was isolated, probed, and analyzed at the end of the designated time intervals using the method described above. To calculate the half-life of each subunit in both normoxia and hyperoxia, the densitometry (IOD) from each time point was divided by that at *time 0* in that specific (normoxic or hyperoxic) condition and plotted on a log scale using previously published methods (4). Therefore, mRNA levels for each condition were designated 100% at *time 0* for all conditions to enable half-life determination, although hyperoxia-exposed cells had higher initial mRNA levels. Chambers et al. (4) reported superinduction of Na-K-ATPase mRNA within the first 2 h of actinomycin D treatment. To eliminate any possible influence of superinduction on our half-life determinations, half-lives were calculated with and without *time 0*. Because there was no change in half-lives between normoxia and hyperoxia by both methods, we reported the half-lives using *time 0*.

DNA transfection experiments. The Na-K-ATPase β_1 promoter-reporter constructs consisted of the 5' promoter region upstream from the transcription start site plus 151 bp of the first exon linked to a promoterless firefly luciferase expression vector (pXP1-luc), as previously reported (32). Briefly, an *EcoR I/Pvu II* restriction fragment within the 5' end of the Na-K-ATPase β_1 gene (-817 to $+151$ bp) was inserted into a promoterless firefly luciferase expression vector (pXP1) to yield a hybrid gene designated β_1 -817. The two 5' deletion mutants included 102 and 41 bp upstream from the transcription start site (hybrids were designated β_1 -102 and β_1 -41, respectively; Fig. 1). These mutants were generated by exonuclease III digestion of β_1 -817 as previously described, and each of these clones was sequenced to confirm the appropriate sequence and orientation (32).

MDCK cells were plated at a density of 8×10^6 cells/35-mm plate in MEM with Earle's salts and 10% FBS. On *day 2* of culture, cells were transfected with Lipofectin (GIBCO) and the β_1 promoter-reporter construct was isolated from maxipreps (Qiagen). Prior studies to optimize DNA and lipofectin concentrations determined the optimal condition to be 1 μ g DNA and 60 μ l lipofectin per 35-mm plate. Lipofection was carried out using the manufacturer's recommendation (GIBCO) for a total of 4 h in serum-free and antibiotic-free MEM with Earle's salts. After lipofection, the cells were incubated for 48 h in MEM with Earle's salts media plus 10% FBS in normoxia or hyperoxia. In four experiments, cells were cotransfected with 0.3 μ g of cytomegalovirus (CMV) β -galactosidase. Cells were lysed and assayed for luciferase activity (Luciferase Assay System; Promega) or β -galactosidase activity (Clontech) in a luminometer (LB 9501; Berthold), and protein concentration was determined by the

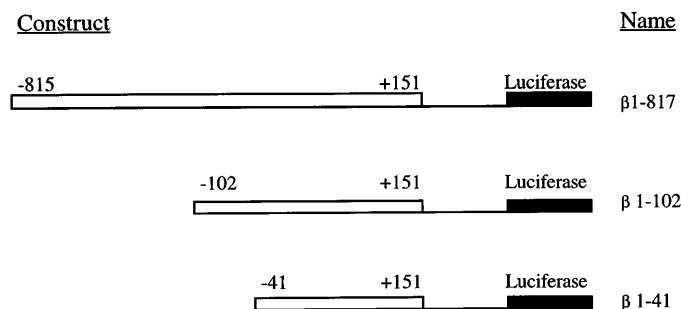


Fig. 1. Na-K-ATPase β_1 wild-type and mutant promoter constructs.

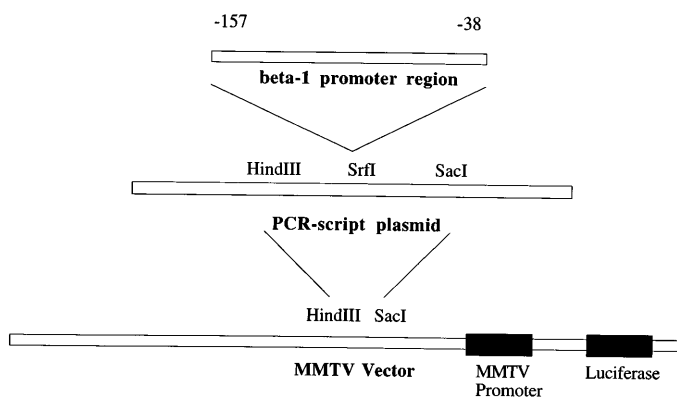


Fig. 2. Subcloning of the 119-base pair (bp) oligonucleotide of the β_1 promoter into the minimal promoter mouse mammary tumor virus (MMTV). PCR, polymerase chain reaction.

bicinchoninic acid system of Pierce. Relative luciferase activity was normalized to either β -galactosidase activity or protein concentration and in some experiments to both. The results from the four experiments with cotransfection of β -galactosidase demonstrated identical normalization to either β -galactosidase activity or protein concentration; therefore, the remainder of the experiments were normalized to protein. During our experiments, MDCK cells grew to near confluence in hyperoxia; however, overall cell number and total protein were less in hyperoxia. This decrease in cell number was corrected for by normalizing the luciferase values to either β -galactosidase activity or protein concentration. All normalized promoter activity was reported as a percent activity over control. The control is designated as the full-length promoter (β_1 -817) in normoxia.

Plasmid constructions. To confirm that the region from -102 to -41 was necessary for hyperoxic induction, an oligonucleotide spanning this region was subcloned into a minimal promoter to determine whether this region could upregulate a nonspecific promoter in the presence of hyperoxia. A 119-bp oligonucleotide was synthesized that spanned from -157 to -38 using polymerase chain reaction (PCR) amplification (5'-CTAGCCTAGCCGGCTCCTTTGTGCCGGC-CCCACGCCCGCCCTTCGGGCTCAGGCCCGCCTTCTC-GGCACCGGGATTGGCCTGCGGTGCCGCCGGTAGGGG-GAGCTACGGATGGTGGAG-3') and was subcloned into a luciferase vector containing a minimal promoter of the mouse mammary tumor virus (MMTV; ATCC 37582) (Fig. 2). The minimal promoter consisted of 109 bp of the 5' proximal promoter of the MMTV, which was not upregulated by hyperoxia. PCR amplification reactions were performed in a DNA thermal cycler (Perkin-Elmer) for 30 cycles of a three-step program (*step 1*, 1-min incubation at 96°C; *step 2*, 1-min incubation at 60°C; *step 3*, 1-min incubation at 72°C) using 2.5 units of *Pyrococcus furiosus* (Pfu) (Stratagene) and 120 ng of *EcoR* I linearized plasmid containing the -817 bp promoter (-817 to +151, pXP1-luc). The reactions contained 10 μ l of 10 \times PCR buffer (Stratagene) and 20 pmol of each primer (5' primer, 22-bp oligonucleotide from -157 to -136; 3' primer, 23-bp oligonucleotide spanning -60 to -38). A gel-purified PCR fragment was cloned into the *Srf*I site of a PCR-script plasmid (Stratagene). After *Escherichia coli* transformation, plasmid DNA was isolated and digested with *Hind* III and *Sac* I to produce a 190-bp oligonucleotide that contained the 119-bp oligonucleotide and was subcloned into *Hind* III/*Sac* I-digested MMTV-luc plasmid. Plasmids were isolated using the Qiagen maxi-prep and designated MMTV- β_1 19.

Data analysis. Results are means \pm SE of three to seven experiments. Paired evaluations for the Northern analysis and hyperoxia transfection experiments were made for experimental and control conditions within each experiment, and significance was determined by Student's *t*-test. For the transfection experiments performed with multiple concentrations of hydrogen peroxide, an analysis of variance with a priori comparisons was performed. The post hoc test was accomplished using pairwise comparisons with a Mann-Whitney *U* and a Bonferroni adjustment. Statistical significance was set at $P < 0.05$, and trends were present with $0.05 < P < 0.10$.

RESULTS

Effects of hyperoxia on Na-K-ATPase steady-state mRNA levels. Previous studies have shown that hyperoxia increased the steady-state levels of Na-K-ATPase α_1 and β_1 mRNA in intact rat lung and primary cultures of rat alveolar epithelial cells (2, 3, 15, 27). We hypothesized that hyperoxia can increase Na-K-ATPase mRNA steady-state levels in MDCK cells and that this occurs by an increase in transcription. The canine kidney epithelial cell line (MDCK) has abundant Na-K-ATPase mRNA and protein and has increased ion and fluid transport in the presence of hyperoxia (8). MDCK cells were exposed to normoxia or hyperoxia (95% O₂-5% CO₂) for 24 and 48 h. Northern analysis of total RNA revealed that hyperoxia increased α_1 and β_1 mRNA in MDCK cells 3.4 \pm 1.2- and 5.2 \pm 1.1-fold, respectively, over control conditions at 24 h (Fig. 3). The increase in the α_1 mRNA steady-state levels showed a statistical trend but was not statistically significant ($P < 0.10$). The increased mRNA levels in MDCK cells remained elevated 3.4-fold at 48 h for the β_1 -subunit but decreased to a 1.4-fold elevation in the α_1 -subunit compared with normoxic controls (Fig. 3).

Effects of hyperoxia on Na-K-ATPase mRNA stability. Hyperoxia can increase the stability of specific mRNAs in certain tissues, such as catalase in the lung (5). To determine whether the increase in Na-K-ATPase steady-state level of mRNA was due to an increase in mRNA stability, hyperoxic and normoxic MDCK cells were incubated in actinomycin D to inhibit RNA synthesis, and Na-K-ATPase mRNA half-lives were measured. Half-lives were measured using previously published methods, by normalizing each time point by the zero time point for that specific condition (normoxia or hyperoxia). The IOD at various time points was plotted on a log scale, and the half-lives were calculated. Because the observed half-life of Na-K-ATPase was relatively short, it was unlikely that the inhibition of the synthesis of potential regulatory proteins influenced the Na-K-ATPase half-life. Superinduction in the first hour of actinomycin D treatment, which has been described by others (4), was not seen, since our first time point occurred at 2 h of treatment. Half-life determinations did not identify any differences between normoxia or hyperoxia of either the α_1 or β_1 mRNA subunits (Fig. 4) with or without time point zero to eliminate any potential effect of superinduction. The mRNA half-lives of the α_1 -subunit were 4.4 h ($r = 0.96$) and 4.7 h ($r = 0.90$) in normoxia and hyperoxia,

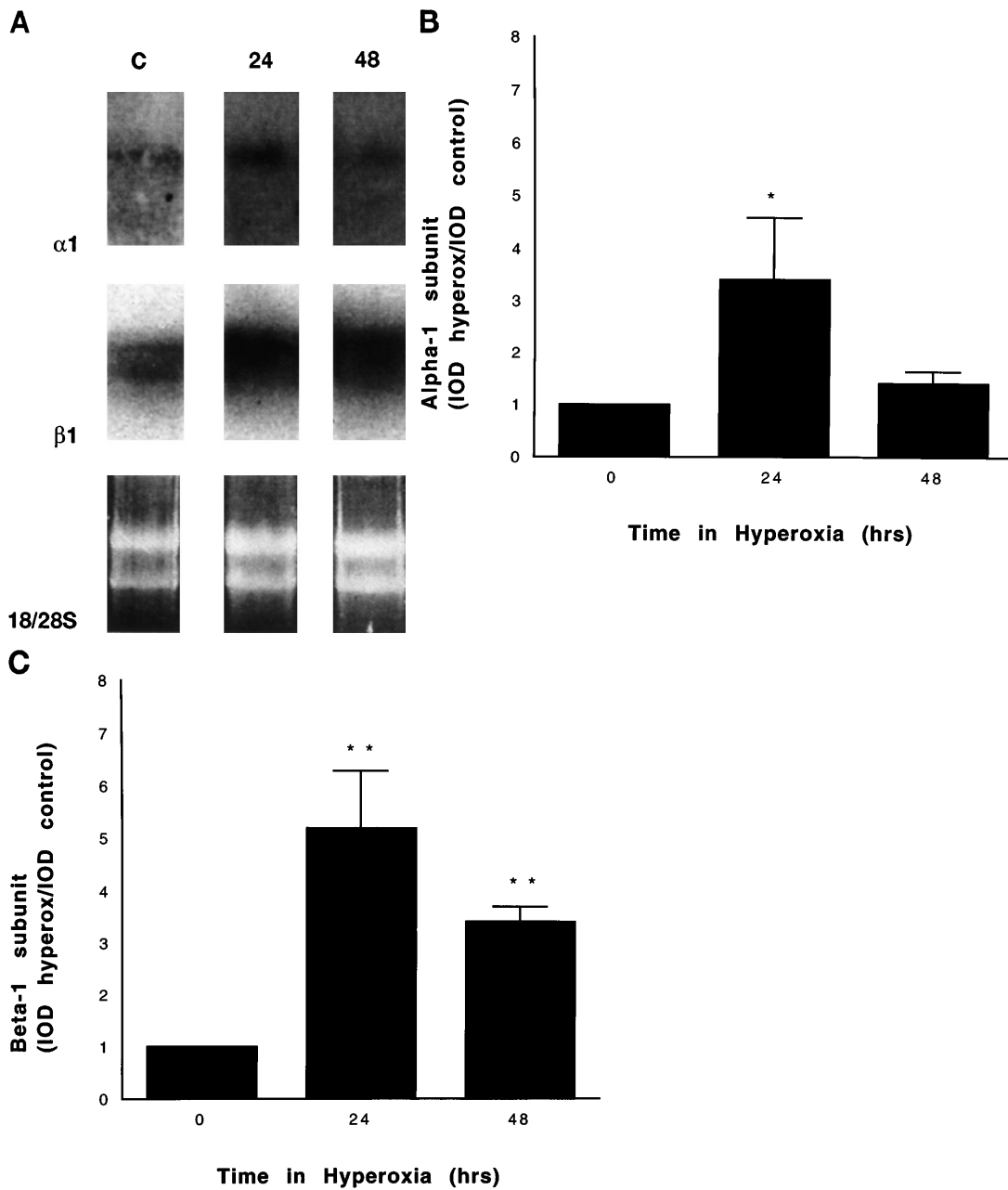


Fig. 3. Hyperoxia increases the mRNA steady-state level of Na-K-ATPase α_1 and β_1 in Madin-Darby canine kidney (MDCK) cells. Cells were exposed to hyperoxia for 0 [control (C)], 24, or 48 h. A: representative autoradiogram of a Northern blot probed for α_1 and β_1 Na-K-ATPase subunit mRNAs. Cells were exposed to hyperoxia for 0 (C), 24, and 48 h. Each lane was loaded with 20 μ g of RNA. B and C: quantitative scanning densitometry of 3 Northern blot autoradiograms of α_1 -subunit (B) and β_1 -subunit (C). Each data point represents mean \pm SE of values from 3 separate experiments and is a ratio of the integrated optical density (IOD) of the signal from hyperoxia-exposed cells to that of control cells (0 h hyperoxia). * $P < 0.1$, ** $P < 0.05$, compared with RNA values from control cells.

respectively. Similarly, the β_1 -subunit mRNA half-lives were 5.9 h ($r = 0.78$) in normoxia and 6.2 h ($r = 0.80$) in hyperoxia. Thus hyperoxia did not increase the stability of either the α_1 or β_1 mRNAs, suggesting that the increased steady-state mRNA levels were due to increased transcription.

Effects of hyperoxia on Na-K-ATPase β_1 gene transcription. We studied the transcription of the β_1 -subunit since it had a greater and more sustained increase in steady-state levels of mRNA compared with the α_1 -

subunit. To determine whether the increase in Na-K-ATPase β_1 mRNA was due to increased transcription, MDCK cells were transiently transfected with an expression vector construct of the β_1 promoter linked to a luciferase reporter gene. Transfection with the β_1 -817 promoter construct revealed a 1.9 ± 0.2 -fold increase in luciferase activity in the presence of hyperoxia compared with normoxia (Table 1).

To localize the region(s) in the promoter responsible for this hyperoxic increase in transcription, MDCK

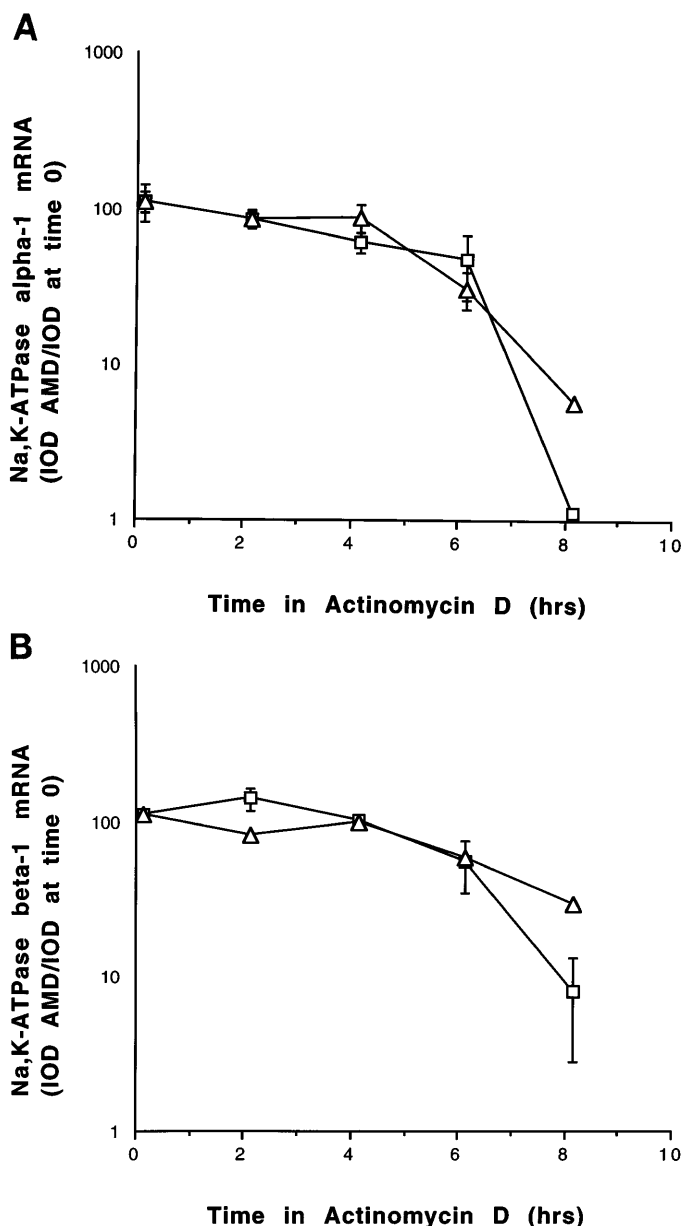


Fig. 4. Effect of hyperoxia on Na,K-ATPase α_1 -subunit (A) and β_1 -subunit (B) mRNA half-lives. Cells were exposed to hyperoxia for 48 h. Each data point represents mean \pm SE of values from 3 separate experiments. All time points were normalized to *time 0* for that given condition (hyperoxia or normoxia) and plotted on a log scale; therefore, all *time 0* points start at 100%. Calculated half-lives for α_1 -subunit were 4.4 h ($r = 0.96$; normoxia) and 4.7 h ($r = 0.90$; hyperoxia) and for β_1 -subunit were 5.9 h ($r = 0.78$; normoxia) and 6.2 h ($r = 0.80$; hyperoxia). There is no significant difference between the half-lives for each subunit comparing hyperoxia to normoxia. AMD, actinomycin D; \square , normoxia; \triangle , hyperoxia.

cells were transfected with two 5' deletion mutants (β_1 -102 and β_1 -41 bp). Promoter sequence analysis showed putative binding sites for redox-sensitive transcription factors on the β_1 promoter. Two consensus sequences for the ARE were located at -790 and -434 . In addition, there was a consensus sequence for NF κ B at -796 . If hyperoxia acted through these putative sites, then neither deletion mutant would have increased its promoter activity with hyperoxia (Fig. 5). In

Table 1. Effect of hyperoxia on Na,K-ATPase β_1 -subunit promoter activity

Construct 5' Base Pair	Normoxia	Hyperoxia	Hyperoxia/Normoxia
β_1 -817	100	190 \pm 21.2	1.9 \pm 0.2*
β_1 -102	62.1 \pm 15.0	120 \pm 35.6	1.9 \pm 0.3†
β_1 -41	2.7 \pm 1.9	1.4 \pm 0.7	0.8 \pm 0.1

Values are means \pm SE from 3–8 experiments. Luciferase activity was normalized to either cytomegalovirus (CMV)- β -galactosidase activity or protein concentration and is reported as a percent activity over control. Control is designated as the wild-type promoter (-817 construct) in normoxia. SE is calculated as SD of the percent activities divided by the square root of the number of experiments. Ratio of hyperoxia/normoxia was derived for each individual experiment, and SE was calculated as SD of the ratios divided by square root of the number of experiments. * $P < 0.02$; † $P < 0.05$.

normoxic MDCK cells, the basal promoter activity decreased as the 5' promoter size decreased (Table 1). The β_1 -102 deletion construct had 62.1% of the promoter activity of the wild-type construct, and the β_1 -41 deletion construct had 2.7% activity. In the presence of hyperoxia, promoter activity was induced 1.9 \pm 0.3-fold in the β_1 -102 deletion mutant, similar to the induction seen in the wild-type construct, even though this deletion construct eliminated the putative ARE and NF κ B sites. In contrast, the β_1 -41 deletion mutant had no inducible promoter activity with hyperoxia (Table 1). Thus the region between -41 and -102 both contributed to basal activity of the promoter and was necessary for the increase in promoter activity by hyperoxia. This result also indicated that the hyperoxic effect did not require the putative ARE and NF κ B sites.

Induction by hyperoxia in a minimal promoter construct. Transfection experiments with 5' deletion constructs of the β_1 promoter identified a 61-bp region (-102 to -41) that was necessary for the induction in promoter activity by hyperoxia. To confirm that the 61-bp β_1 promoter region identified (-102 to -41) was specific for hyperoxia induction, a 119-bp oligonucleotide spanning this region (-157 to -38) was subcloned into a luciferase vector containing the minimal promoter of the MMTV (designated MMTV- β 119). The MMTV plasmid consisted of 119 bp of the proximal promoter of the MMTV in a luciferase vector. Transfection experiments with the MMTV plasmid alone revealed minimal promoter activity that was not inducible by hyperoxia, whereas transfection with MMTV- β 119 showed an increase in promoter activity in normoxia that was induced an additional 2.3 \pm 0.2-fold in the presence of hyperoxia. This induction by hyperoxia was identical to that seen in the native Na,K-ATPase

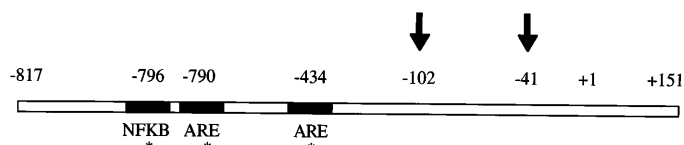


Fig. 5. Na,K-ATPase β_1 promoter constructs and putative regulatory sites. Arrows, deletion constructs; * putative regulatory sites; ARE, antioxidant responsive element.

promoter, confirming that this region is specific and sufficient for the hyperoxia induction.

Effects of hydrogen peroxide on Na-K-ATPase gene transcription. There are multiple mechanisms by which hyperoxia could affect gene expression, including specific oxidants and partial pressure of oxygen or indirectly through cellular injury. Because hyperoxia can generate reactive oxygen species, including hydrogen peroxide, one possibility was that hyperoxia induced gene transcription through the effect of hydrogen peroxide on the β_1 promoter. Hydrogen peroxide has high cellular permeability and activates the ARE in other genes (33). To test this possibility, we transfected MDCK cells with the β_1 -817 construct in the presence of hydrogen peroxide for 48 h at concentrations known to activate the ARE in other cell systems and measured promoter activity. At low concentrations of hydrogen peroxide, cells proliferated and became confluent, whereas cellular injury was evident at higher concentrations (50 μ M), since cells were observed to be 50–60% confluent after 48 h of hydrogen peroxide exposure. Hydrogen peroxide did not increase promoter activity in cells transfected with the β_1 -817 at any concentration (Fig. 6), even after correcting for total protein, which decreased with the higher concentrations of hydrogen peroxide. There was a slight increase in promoter activity at the 10 μ M dose; however, this was not statistically significant and was much less than the hyperoxic induction. Therefore, the increase in β_1 promoter activity in hyperoxia does not appear to be mediated via hydrogen peroxide. This lack of hydrogen peroxide response, along with the 5' deletion studies,

indicated that the two putative ARE consensus sites were not responsible for the hyperoxic response.

DISCUSSION

Oxidant injury can oxidize regulatory proteins, disrupt the cell membrane, and subsequently perturb intracellular ion concentrations. Upregulation of cellular mechanisms to maintain Na and ion concentrations would be essential to preserve cellular and organ homeostasis. Determining the effects of oxidant injury on the Na-K-ATPase is of major importance, since the Na-K-ATPase, along with the Na channel, are key proteins in maintaining intracellular Na homeostasis and in vectorial Na transport. Previous studies have shown that hyperoxia and other oxidants can upregulate the Na-K-ATPase and the Na channel (2, 12, 15, 25, 39).

The effects of oxidant injury on the Na-K-ATPase are complex. Hyperoxia increased Na-K-ATPase mRNA steady-state levels consistently in type II cells and MDCK cells (3, 7, 12, 27). It is unclear whether this was a direct effect of oxygen tension or was due to the production of reactive oxygen species (ROS). The effects of oxidant injury on Na-K-ATPase protein concentrations and activity likely depend on the type and concentration of oxidant species, cell or tissue type, duration of injury, and available antioxidant defenses. As an example, subacute hyperoxia increased Na-K-ATPase protein concentrations and pump activity in alveolar epithelial cells, whereas higher concentrations of sustained hyperoxia suppressed protein concentration, which then increased in recovery (3, 27, 30). Duration of exposure may also be important, since short durations of hyperbaric oxygen increased activity in both cerebrocortical membranes and alveolar epithelial cells (12, 42). Na-K-ATPase activity has a variable response to reactive oxygen species, depending on the specific oxygen species and cell type. Cardiac myocytes decrease Na-K-ATPase activity in the presence of xanthine/xanthine oxidase, whereas hydrogen peroxide increases activity in vascular endothelial cells (8, 39).

The functional significance of the increased pump transcription with hyperoxia is uncertain. Our preliminary data (not shown) did not demonstrate an increase in Na-K-ATPase protein concentrations in MDCK cells exposed to 24–48 h of hyperoxia. However, this may be due to a suppression of translation or increased protein degradation by oxidants, as previously reported for the isolated Na pump (38). The increased transcription could play an important role in maintaining normal protein levels. Alternatively, the increase in Na-K-ATPase mRNA may prime the cell with available mRNA ready for protein synthesis once the ROS are cleared, similar to the results seen in alveolar epithelial cells.

Oxidant stress or hyperoxia increases the expression of multiple genes in many different tissues. Many genes regulated by hyperoxia or oxidants are involved in the antioxidant response. These genes include the tissue inhibitor of metalloproteinases in mesenchymal cells and manganese superoxide dismutase and catalase in

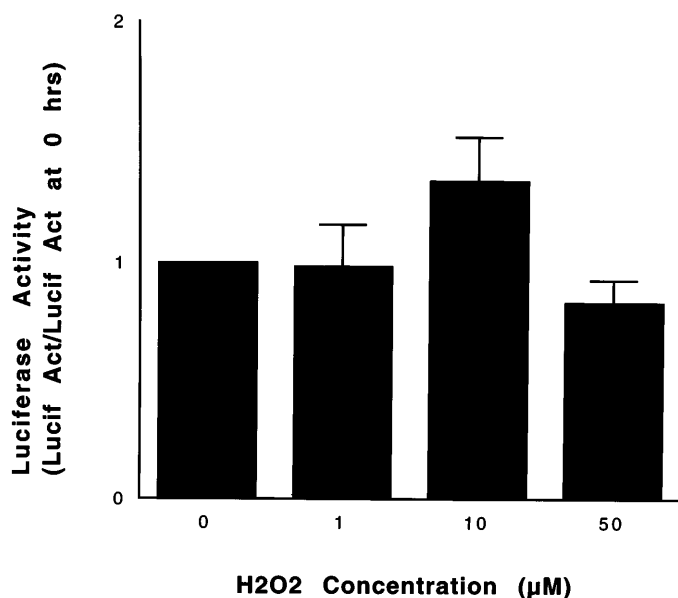


Fig. 6. Effect of hydrogen peroxide on Na-K-ATPase β_1 promoter activity. Cells were transfected with the β_1 -817 construct and exposed to various concentrations of hydrogen peroxide for 48 h. Cells were harvested and luciferase activity was measured. Data are reported as times increase over control, i.e., Na-K-ATPase promoter activity in absence of hydrogen peroxide. Each data point represents mean \pm SE of values from 5 separate experiments. There is no statistical significance between the various concentrations of hydrogen peroxide by analysis of variance.

kidney and lung epithelial cells (14). The magnitude of the effect of hyperoxia or oxidants on gene expression typically is three- to sixfold for antioxidant enzymes (14), in contrast to larger increases for heat shock protein genes and other stress response genes (14). Other classes of genes not involved in the antioxidant process, but necessary for cellular and organ homeostasis, such as the Na-K-ATPase or surfactant, are upregulated by hyperoxia or oxidants as well. The magnitude of gene induction in these genes tends to be similar to that of antioxidant enzyme genes and less than the induction of stress response genes.

We previously reported that hyperoxia increased the steady-state level of Na-K-ATPase mRNA in rat lung *in vivo* (27) and rat alveolar epithelial cells *in vitro* (2, 3) three- to fivefold. Similarly, we now demonstrate that this increased gene expression also occurred in MDCK cells using a model of *in vitro* hyperoxia. These renal tubular epithelial cells are responsible for vectorial Na transport and have abundant Na-K-ATPase (7). In addition, hyperoxia upregulated fluid transport in MDCK cells as indicated by the formation of domes in the presence of hyperoxia (7). We believe this degree of gene upregulation is physiologically significant because 1) it is similar to the extent of upregulation observed for some antioxidant enzymes upregulated by oxidants or hyperoxia (33), 2) it is similar to the degree of upregulation in Na-K-ATPase gene expression in other physiological states such as changes in intracellular ion concentration or hormonal stimulation (9, 32), and 3) the relatively high basal gene expression of Na-K-ATPase suggests that a small change in gene expression may induce relatively large changes in steady-state protein levels, although this may not occur until the cell is recovering from the oxidant stress.

To determine whether the increase in Na-K-ATPase mRNA steady-state levels in MDCK cells was due to transcriptional or posttranscriptional regulation, we measured the mRNA half-lives of the Na-K-ATPase subunits in normoxia and hyperoxia. The mRNA half-lives of some genes increased in the presence of hyperoxia, such as catalase (5); however, hyperoxia did not change the half-lives of either Na-K-ATPase subunit mRNA in our studies. To determine whether the increased mRNA levels were due to transcription, we transfected MDCK cells with a β_1 -subunit promoter-reporter construct and measured promoter activity in normoxia and hyperoxia. Our studies focused on the β_1 promoter, since it had a greater and more sustained increase in steady-state levels of mRNA. Transfection experiments demonstrated an almost twofold increase in promoter activity in our wild-type construct containing 817 bp upstream of the transcription initiation site. With the use of deletion mutants of the β_1 promoter, we localized a 61-bp region between -102 and -41 that was necessary for this hyperoxia effect.

Transcription of many genes in prokaryotes and eukaryotes can be induced by oxygen partial pressure or ROS. The mechanism most clearly was described for the oxy-R regulon in *E. coli*. In this system, the oxidation of the oxy-R protein caused a conformational

change of this protein, while constitutively bound to DNA, which increased transcription of several antioxidant enzyme genes (35). In eukaryotes, oxygen tension regulated the erythropoietin gene expression via the hypoxia-inducible factor-1 transcription factor (10). The best-characterized eukaryotic system with oxidant regulation of gene expression is the ARE (33). Transcription of the glutathione *S*-reductase γ subunit and NADPH-quinone reductase genes were upregulated by hydrogen peroxide and other aromatic organic redox compounds through an active ARE (33). Several other eukaryotic transcription factors are functionally altered by the redox state, including AP-1 and NF κ B, and play a role in the gene induction by reactive oxygen species (26). NF κ B was activated by ROS in many cell types, including the kidney and the lung, and has been linked to the upregulation of nitric oxide synthase expression and the induction of lung inflammation (1).

Relatively little is known about the regulation of transcription of the Na-K-ATPase subunits. Current knowledge of the β_1 promoter region is summarized in Table 2. There are several putative sites for redox regulation of the β_1 -subunit gene, since the promoter contains two partial consensus sequences for ARE and one for NF κ B. We localized an area necessary for the regulation by hyperoxia to a 61-bp region between -41 and -102 bp. This region is distinct from the location of the putative consensus sequences for an ARE (-790 and -434) and NF κ B (-796). To test directly whether the ARE consensus sequence was active, the impact of hydrogen peroxide exposure was assessed, since hydrogen peroxide activated the ARE in other cell systems (33). In our system, hydrogen peroxide did not activate transcription of the β_1 -817 construct containing the putative ARE sites and our identified hyperoxia site (-102 to -41). Although there was a small increase in promoter activity at 10 μ M hydrogen peroxide, this was not statistically significant or was not as large as the hyperoxia effect. Therefore, the induction by hyperoxia is not mediated through hydrogen peroxide or through the putative ARE sites.

Table 2. Potential transcription factor binding sites in promoter region of rat Na-K-ATPase β_1 gene

Promoter Region	Consensus Sequence
-788, -758, -700, -594, -589, -350	Glucocorticoid response elements*
-589, -443	Thyroid hormone response elements*
-810, -709, -382	Serum-induced transcriptional activation sites*
-785, -718, -715, -434, -315	Calcium-induced transcriptional activation sites*
-605, -496, -244, -122	GC boxes
-790, -434	ARE
-796	NF κ B
-100, -122, -65	SP1*
-31	TATA box

Numbers indicate the distance of the potential element from the transcription initiation start site. ARE, antioxidant responsive element. *Partial consensus sequences.

Sequence homology analysis of the 61-bp hyperoxic regulatory region (-41 to -102) did not identify other consensus sequences with possible oxygen or oxidant sensitivity, such as AP-1 or NF κ B. This 61-bp promoter region is very GC rich and has several partial consensus sequences for the SP1 transcription factor. SP1 is a common transcription factor that serves as a regulatory site for basal promoter activity. SP1 transcription factors can be involved in oxidant gene regulation, since they contain thiol groups that are redox susceptible when the factor is in its unbound state (19). In addition, they may serve as a transcription cofactor for sites of promoter induction (17). Each putative SP1 site identified on this 61-bp β_1 promoter region has a consensus for five of the six core bases but much less homology for the two flanking bases on each side of the core. These SP1 sites may represent possible primary redox regulatory sites for hyperoxia, acting as a transcription cofactor for hyperoxia, or the induction by hyperoxia may act through a unique, previously undefined mechanism.

The Na-K-ATPase is an important protein for vectorial ion and fluid transport and for maintaining cellular homeostasis, especially in the face of injury. Models of renal reperfusion have identified oxidant-induced upregulation of several genes, such as superoxide dismutase, that may be protective to further oxidant injury (40). The upregulation of kidney Na-K-ATPase during oxidant injury may play a role in preserving cellular homeostasis and in maintaining normal Na balance. In this model system, we demonstrated that hyperoxia increased Na-K-ATPase gene expression and transcription of the β_1 -subunit and localized one region of the β_1 promoter required for this response. Future identification of the transcription factors and core sequence involved in this induction by hyperoxia will add to our growing knowledge of oxygen and oxidant effects on gene transcription.

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Address for reprint requests: C. H. Wendt, Box 276 University of Minnesota Heath Center, 420 Delaware St. SE, Minneapolis, MN 55455.

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