Glucocorticoid repression of human with-no-lysine (K) kinase-4 gene expression is mediated by the negative response elements in the promoter

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Abstract

With-no-lysine (K) kinase-4 (WNK4) is a serine/threonine kinase that plays an essential role in the regulation of fluid and electrolyte homeostasis. The effects of glucocorticoids, key physiological regulators, on the *WNK4* gene expression are still unknown. Here, we used dexamethasone (Dex) to treat the human embryo kidney 293 (HEK293) cells and found a decrease of human *WNK4* (*hWNK4*) mRNA level by northern blot and real-time quantitative PCR. After an *hWNK4* transcriptional initiation site was located by 5' rapid amplification of cDNA end assay, a series of 5'-deleted *hWNK4* promoter-luciferase constructs were generated by PCR. Transfection of these constructs in COS-7 and HEK293 cells revealed that Dex inhibited the *hWNK4* transcriptional activity in glucocorticoid receptor (GR)-dependent pattern. Two negative glucocorticoid response elements (nGREs) were identified at -285 and -337 of the *hWNK4* gene promoter and the GR binding activity to them was increased by Dex as shown by electrophoretic mobility shift assay and chromatin immunoprecipitation. In summary, these data demonstrated that *hWNK4* was a new glucocorticoid-regulated gene whose expression was inhibited through the interaction of GR with nGREs in the promoter region.

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Introduction

Glucocorticoids are secreted by the adrenal glands and play an important role in metabolism, maintenance of homeostasis, cell proliferation, and differentiation (Buckingham 2006). As medication, glucocorticoids are widely prescribed for anti-inflammatory and immunosuppressive therapy, administered to pregnant individuals to accelerate fetal pulmonary development, and used as a component in many chemotherapy regimens for some cancers. However, long-term use of glucocorticoids has been limited by the adverse effects, including endocrine, metabolic, musculoskeletal, gastrointestinal, dermatological, and fluid and electrolyte perturbations, in addition to the development of glucocorticoid resistance. These physiological and pharmacological effects as well as the undesired side effects of glucocorticoids are hypothesized due to the regulation of gene transcription in different types of cells.

It is well known that glucocorticoids increase water, sodium, and chloride reabsorption as well as potassium and calcium excretion both inside and outside of the kidney to modulate fluid and electrolyte balance. For example, prolonged glucocorticoid administration often leads to electrolyte disturbance and hypertension, and

the prenatal administration increases the possibilities of sodium retention and hypertension in later life (Ortiz *et al.* 2003, Dickinson *et al.* 2007). In order to demonstrate the molecular basis of glucocorticoid-mediated fluid and electrolyte balance, it is necessary to find the effects of glucocorticoids on target molecules involved in electrolyte homeostasis in epithelia.

With-no-lysine (K) kinase-4 (WNK4) is a novel serine/threonine kinase lacking a characteristic lysine in the ATP-docking site and plays an important role in regulation of ion transport process. Human *WNK4* (*hWNK4*) gene, when mutated, causes pseudohypoaldosteronism type II (OMIM no. 145260) featuring hypertension and hyperkalemia due to excessive renal sodium chloride and potassium retention (Wilson *et al.* 2001). The discovery of mutations in the *hWNK4* gene has generated considerable interests in its function and the molecular mechanism leading to electrolyte disturbance and hypertension. Recent studies have shown that *WNK4* is expressed in multiple chloride-transporting epithelia and predominantly expressed in the distal convoluted tubule, connecting tubule, and collecting duct. It is subcellularly localized near tight junctions, along lateral membranes, and within the cytoplasm. Experimental

observations demonstrated that WNK4 is a natural inhibitor of diverse epithelial transporters. It inhibits the NaCl cotransporter and the renal outer medullary potassium channel activities via a kinase-independent mechanism, and increases paracellular chloride flux by phosphorylation of multiple claudins (Kahle *et al.* 2003, 2004, Yamauchi *et al.* 2004). Based on its anatomical and functional characters, WNK4 is proposed to regulate transcellular and paracellular ion flux across diverse epithelia and act as a molecular switch for electrolyte homeostasis. As mentioned above, most of the studies focused on the function of *WNK4* gene, while little is known about its upstream regulator. In this current study, we aim to identify whether *WNK4* is one of the targets for glucocorticoid-mediated electrolyte balance by investigating the effects of glucocorticoids on the *hWNK4* expression and the mechanisms.

Materials and methods

Cell culture and RNA isolation

Human embryo kidney 293 (HEK293) and African green monkey kidney (COS-7) cells were maintained in Dulbecco's Modified Eagle's Medium (GIBCO) with 10% fetal bovine serum, 100 unit/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. To examine the effect of dexamethasone (Dex; Sigma-Aldrich) on the *hWNK4* gene expression, HEK293 cultures were switched to serum-free media and then exposed to 1 µM Dex or 1 µM Dex with 10 µM RU486 (Sigma-Aldrich) for 24 h. Total RNA was extracted from these cells using TRIzol reagent (Invitrogen).

Northern blotting analysis

Total RNA (20 µg) was fractionated in a 1.2% agarose-formaldehyde gel and then transferred onto a nylon membrane Hybond-N (Amersham Biosciences). A 321 bp antisense cRNA from *hWNK4* cDNA was labeled, hybridized, and detected by CDP-Star using DIG Northern starter kit (Roche) according to the manufacturer's instructions. Signals of hybridization bands were detected on X-ray film and quantified by densitometric analyses. After exposure to film (5–60 min), the membrane was stripped and then rehybridized with the β -actin probe for normalization.

Reverse transcription and real-time quantitative PCR

Total RNA (1 µg) was reversely transcribed with random primers using reverse transcription reagent kit (Promega) following the manufacturer's protocol. Real-time quantitative PCR was performed in an ABI Prism 7500

Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using a FAM-labeled probe, 5'-FAM-CTTCCTGCTCCCGCCGCCGC-Eclipse-3', and a pair of primers specific to the *hWNK4* transcript giving a 144 bp product, forward 5'-GTGAAGGCTGCGGAAGACTC-3' and reverse 5'-CTGGGTCTCCATGTCCTCCTT-3'. The 25 µl reaction mixtures contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 µM dNTPs, 1.25 U *Taq* polymerase, 40 ng template cDNA, and 150 nM probe and primers (400 nM for each). All PCR reagents were from TaKaRa Biotech (Dalian, China). The reaction condition was 95 °C for 3 min followed by 40 cycles at 95 °C for 15 s and 60 °C for 50 s. As an internal control, β -actin was measured under the same conditions in real-time quantitative PCR with a FAM-labeled probe, 5' FAM-CCAGGGCGTGATGGTGGG-CAT-Eclipse 3', and a pair of primers specific for β -actin transcript giving a 155 bp product, forward 5'-CCGTCTTCCCCTCCATCG-3' and reverse 5'-GTCCCAGTTGGTGACGATGC-3'. Cycle threshold values (*C_t*) were analyzed by the SDS1.4 software (Applied Biosystems) and relative quantification of *hWNK4* expression was determined using the comparative *C_t* method (ABI Prism 7500, SDS User Bulletin; Applied Biosystems). Each sample was amplified in triplicate to obtain average *C_t* value. Reaction without cDNA templates was used as negative control. Results were expressed as relative *hWNK4* expression compared with the untreated control that was taken as 100%.

5' rapid amplification of cDNA end (5'-RACE) assay

Human Kidney Marathon-Ready cDNA amplification kit (Clontech) was used according to the supplier's instructions. Forward primers were a 27-mer adaptor primer (AP1) and a 23-mer nested AP2. Reverse primers were gene-specific primers (GSP) of the *hWNK4* gene exon 1 including outer primer (GSP1) and inner primer (GSP2): 5'-ACGCGCGGAGTCTTCCGCAGCCT-3' and 5'-CTGGGACATGAGGACGGTGGTCT-3'. The first-round PCR was performed using template Marathon-Ready cDNA and primers AP1 and GSP1. PCR product was diluted for the second-round PCR using primers AP2 and GSP2. The PCR products were analyzed by agarose gel electrophoresis. DNA from major band was cloned into the pMD18-T vector (TaKaRa Biotech) and then sequenced.

Plasmid construction

The luciferase reporter plasmids used in this study were derived from pGL3-Basic (Promega) lacking eukaryotic promoter and enhancer. The PCR fragment comprising -484 to +62 of the *hWNK4* gene was generated using human genomic DNA with forward primer

5'-CACTGACCTCTCCGTTCCGGC-3' and reverse primer 5'-GACATGAGGACGGTGGTCTC-3'. This PCR product was cloned into pMD18-T vector and subcloned into the unique *KpnI*/*HindIII* sites of pGL3-Basic vector to generate a reporter construct p484-luc. A series of 5'-deleted constructs were derived from the p484-luc by PCR. The forward primers were 5'-CGCACAAACAGGTT-3' (−337), 5'-TTTGCTCACTCTTAGTGCGG-3' (−325), 5'-CACTCAGTTCTGG-3' (−285), 5'-TGGCCTCAGAGT-GAGACT-3' (−275), 5'-GAAAGAAGGGGACGC-3' (−241), 5'-GGCGGTGACTAAGGTGAG-3' (−216), and 5'-AGCGAGTCCGTCTGTCAG-3' (−52). The reverse primer was the same one as used in the construction of the p484-luc. All constructs were confirmed by sequencing with no coding frame shift in the luciferase gene. For transient transfection, the plasmids were prepared using Mid Pre plasmid kits (Qiagen).

Transient transfection and luciferase assays

HEK293 or COS-7 cells were seeded in 24-well plates, grown to 80–90% confluence, and transfected by Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with 1 µg reporter construct, with or without 0.5 µg expression vector coding for human glucocorticoid receptor (GR; pRShGR α). Cotransfection of 0.01 µg pRL-TK (Promega), a plasmid encoding for Renilla luciferase, was performed to normalize transfection efficiencies. After 24 h, cells were treated with Dex (1 µM) in the presence or absence of RU486 (10 µM) and incubated for 24 h. Cells were then harvested and luciferase activities were measured using the Dual-Luciferase Reporter Assay system (Promega) on Lumat LB9507 luminometer (Bethold Technologies, Bad Wildbad, Germany).

Nuclear extracts preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts from HEK293 cells with or without Dex treatment were prepared and EMSA was carried out as previously described (Zhao *et al.* 1999). Briefly, oligonucleotides corresponding to the putative glucocorticoid response motifs at the *hWNK4* promoter (−285: 5'-CACTCAGTTCTGG-3' and −337: 5'-CGCACAAACAGGTT-3') and mutant oligonucleotides with underlined mutated bases (5'-CACTTAATTATGG-3' and 5'-CGCAATAACAAGTT-3') were synthesized and annealed. glucocorticoid response element (GRE) consensus oligonucleotides were purchased from Promega and anti-GR antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Probes were end radiolabeled with [γ -³²P]ATP (Furui Biotechnology, Beijing, China) using T4 polynucleotide kinase (TaKaRa Biotech). Binding reactions were

performed in a volume of 30 µl containing ~5000 cpm labeled probe, 12 µg nuclear extracts, 1 µg/µl poly(dI-dC), and 1× binding buffer (10 mM HEPES, 50 mM KCl, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM dithiothreitol) on ice for 30 min. For competition assays, 100- or 500-fold molar excess of the unlabeled double-stranded DNA was added to the reaction mixture. For supershift assays, the anti-GR antibody was incubated for 30 min at room temperature before the probe was added. Reaction mixtures were loaded onto an 8% polyacrylamide gel and run at 150 V in 0.375×tris-borate-EDTA (TBE) for 4 h. The gel was dried under vacuum and autoradiographed.

Chromatin immunoprecipitation (ChIP) assay

HEK293 cells were treated with or without Dex for 24 h. Formaldehyde (1%) was added to the medium and incubated for 20 min at 37 °C. Cells were harvested, resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris-HCl (pH 8.1)), incubated at 4 °C for 10 min, and then sonicated to generate approximate 100–300 bp DNA fragments. One-third of the lysate was used as DNA input control. The remaining two-thirds were diluted tenfold with ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1 mM EDTA, 10 mM Tris-HCl, 150 mM NaCl) followed by incubation with specific anti-GR antibody or with nonimmune rabbit immuno-globulin G (IgG) as a negative control at 4 °C overnight. Immunoprecipitated complexes were collected by protein A/G-agarose beads, washed, and incubated at room temperature for 20 min. Cross-linking of protein–DNA complexes was reversed at 65 °C for 5 h. Then, DNA was extracted with phenol/chloroform. PCR was carried out in 25 µl for 30 cycles using the primers specific for the *hWNK4* promoter. The set of primers was as follows: forward 5'-CACTGACCTCTCCGTTCCGGC-3' (−484) and reverse 5'-CGAGCAGCCCCTCACCTTAG-3' (−189) for a 296 bp fragment, forward 5'-CACTGACCTCTCCGTTCCGGC-3' (−484) and reverse 5'-ggagcatcctccgcactaa-3' (−295) for a 190 bp fragment, and forward 5'-TCCCCTCAGTTCTGGCCTCA-3' (−288) and reverse 5'-CGAGCAGCCCCTCACCTTAG-3' (−189) for a 100 bp fragment. PCRs using neuronal nitric oxide synthase (*nNOS*) gene-specific promoter primers (forward 5'-ATGTGGAAGACAGCATAGACC-3' and reverse 5'-AGAGGCAAAGAGGAAAACCACCA-3') to generate a 189 bp fragment without GR binding were used as a negative control. All PCR signals stained with ethidium bromide in 1.5% agarose gels were quantified by densitometric analyses to calculate the amount of PCR products. The values were normalized to that of input and shown as a percentage of untreated controls, which were taken as 100% for each group. All *in vivo* ChIP assays were

repeated at least thrice with similar results and a representative result was shown.

Densitometric analyses and statistical analysis

All densitometric analyses were performed using LabWorks Image Acquisition and Analysis Software (Media Cybernetics, UVP Inc., Cambridge, UK). Data were shown as mean $\pm$ S.E.M. from at least three independent experiments. Differences between quantitative datasets were analyzed by one-way ANOVA or *t*-test as appropriate and $P < 0.05$ was considered significant.

Results

Suppression of the *hWNK4* mRNA by glucocorticoids in HEK293 cells

To assess whether glucocorticoids are implicated in modulation of the *hWNK4* expression, we initially detected the *hWNK4* mRNA level in HEK293 cells

expressing endogenous GR (Oakley *et al.* 1999). Both northern blot and real-time quantitative PCR showed that the *hWNK4* mRNA level decreased to 28–35% of the untreated control after treatment of 1 μ M Dex for 24 h and was restored by concomitant treatment with RU486, a GR antagonist (Fig. 1). These indicated that glucocorticoids suppressed the expression of *hWNK4* mRNA via GR.

Cloning and structural analysis of 5'-flanking region of the *hWNK4* gene

To determine the transcriptional initiation site of *hWNK4*, we performed 5'-RACE analysis to map the transcriptional initiation. With a primer binding to exon 1 and human kidney Marathon-Ready cDNA, we obtained a 442 bp band; furthermore, a 102 bp band was obtained by nested PCR. After cloning and sequencing the PCR products, we located a transcriptional initiation site at 21 bp upstream of the translation initiation ATG in the *hWNK4* gene (Fig. 2). Then, we characterized the *hWNK4* gene promoter. A 600 bp

Figure 1 Effect of Dex on *hWNK4* mRNA expression level in HEK293 cells. HEK293 cells were exposed to 1 μ M Dex with or without 10 μ M RU486 for 24 h. (A) Northern blot analysis was used to determine the expression of *hWNK4* mRNA. The *hWNK4* mRNA levels were quantitated by densitometric analysis, normalized to β -actin. Results are expressed as a percentage of untreated control that is taken as 100%. The bar is mean $\pm$ S.E.M. from three independent experiments (* $P < 0.01$ compared with untreated control; # $P < 0.01$ compared with Dex alone). Representative northern blot of *hWNK4* and β -actin are shown in the lower panel. (B) Real-time PCR was used to determine the expression of *hWNK4* mRNA using the comparative C_t method. β -actin was used for normalization. Results are expressed as a percentage of untreated control that is taken as 100%. The bar is mean $\pm$ S.E.M. from three independent experiments. (* $P < 0.01$ compared with untreated control; # $P < 0.01$ compared with Dex alone).

Figure 2 Determination of transcription initiation site of the *hWNK4* gene by 5'-RACE. A product of the first-round PCR was the 442 bp band and nested PCR product was the 102 bp band. M, 100 bp DNA ladder.

5'-flanking sequence (GenBank AC016889) was analyzed for transcription factor binding sites with Match (public version 1.0) search (Kel *et al.* 2003). Results showed that the promoter exhibited no TATA-box motif, while a putative initiator element, which was thought to be strongly required for the transcription initiator activity of TATA-less promoter, was found at position -1 (CTACCCT). It conformed well to a

consensus initiator sequence PyPyANA/TPyPy except for the underlined C. We also found several potential transcription factor binding sites in this region, such as GR, Sp1, AP-2 α , and C/EBP (Fig. 3).

Repression of *hWNK4* promoter activity through GR

To confirm the suppression of *hWNK4* mRNA level by glucocorticoids through GR, we transfected p484-luc (an *hWNK4* promoter luciferase reporter construct spanning -484 to +62) with or without GR expression vector in COS-7 cells that do not contain endogenous GR (Oakley *et al.* 1999) and therefore provide a null background for GR expression. Luciferase assay showed that in the context of GR, Dex treatment reached the maximal suppression of the promoter activity (Fig. 4A). Furthermore, Dex-suppressed *hWNK4* promoter activity in a dose-dependent pattern (Fig. 4B). These results suggested that the suppression of the *hWNK4* promoter activity by glucocorticoids was in a GR-dependent pattern and the 484 bp upstream sequence of the *hWNK4* gene was sufficient to confer the suppression of *hWNK4* gene transcription by glucocorticoids.

Characteristics of the basal activity of the *hWNK4* promoter

To delineate potential transcriptional regulatory elements, a series of luciferase reporter constructs containing 5'-flanking deletions of the *hWNK4* promoter

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caaggtggcc tggaggggat ggatgaccgg gctctcgggt tgggggaggg acttatcctg -541
gggagtacac acccattccc tatgcagacc agagggacaa caacgtcaga ctttccact -481
AP-2
gacctctccg ttgagcctgg ggcctactccg gacagttagg aggggggtgt gctgccttat -421
AP-2α
ctggggtgtg caggagggtg gtgacctccc cctaagtaat cccccactag ggtccgggtg -361
tcgttctctc ctgccctcct ccgcgcacaa acagggttgc tcactcttag tgcgggagga -301
GR2 C/EBP
tgctccgcgg tctcdcactc agttctggcc tcagagttag actgcccgcg ggggctcggg -241
GRI
aaagaagggg acgcggctgg gcggggcggt gactaagggt aggggctgct cgcgggatcc -181
SP-1
gcaccgcggg agcagctcgg agggggcgcc ctggggggca agggcgggcc gcggggcgcc -121
SP-1 SP-1
gacggggggc gggccaggcc gagctggggg ctggggccgc agccgctcag ccggagcgca -61
SP-1
ggcacccag cgagtcgctc tgtagggccg cctcctctcc ggccgtctga ttttctaccc -1
└─┬─
ttcggcgccc tgccttctct catgttgcca tccccggcca cgagaccac cgtcctcatg +60
M L A S P A T E T T V L M
tccagactg aggcgcacct gccctgcgg cccccgcctc ctcttggcac cgcggggcag +120
S Q T E A D L A L R P P P P L G T A G Q

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Figure 3 Nucleotide sequence of the 5'-flanking region of *hWNK4* gene (GenBank AC016889). Potential transcription factor binding motifs are boxed including the two glucocorticoid receptor elements (GR1 and GR2). The transcription initiation site is indicated by a bent arrow.

Figure 4 Promoter activity of p484-luc (an *hWNK4* promoter luciferase reporter construct spanning -484 to +62) in COS-7 cells. (A) The p484-luc construct was cotransfected with (+) or without (-) GR vector into COS-7 cells in absence (-) or presence (+) of 1 μM Dex. (B) COS-7 cells were cotransfected with p484-luc construct and GR vector and treated with Dex at the indicated concentrations for 24 h. Luciferase activities were measured by a luminometer (Bethold), normalized to *Renilla* luciferase activities. Results are expressed as the fold of pGL3-Basic vector that is taken as 1. The bar is mean ± S.E.M. from three independent experiments in duplicate for each construct (* $P < 0.05$ compared with control; # $P < 0.01$ compared with control).

were generated (Fig. 5A) and transiently transfected into HEK293 cells. Luciferase assay of the basal promoter activity showed that there were multiple *cis*-acting elements essential for the promoter activity. A region between -216 and -52 was necessary for optimal activity. The deletion of p337-luc construct to -325 and p285-luc construct to -275 resulted in the increased promoter activity, implying that 12 bp from -337 to -325 and 10 bp from -285 to -275 were negative regulatory elements. In addition, three regions -484 to -337, -325 to -285, and -275 to -52 played positive regulatory role in the transcription of *hWNK4* gene (Fig. 5B).

Glucocorticoid-related elements in the *hWNK4* promoter

Our attention is focused on the association of the negative elements with the Dex-reduced promoter activity. Significant decrease of promoter activity was observed in p484-luc, p337-luc, and p285-luc constructs, but not in p325-luc and p275-luc. Furthermore, the Dex-reduced activity was effectively abolished by RU486, a GR antagonist, confirming that these effects required GR binding (Fig. 5C). These observations suggested that negative GREs (nGREs) were located at positions -285 (nGRE1) and -337 (nGRE2) in the *hWNK4* promoter.

Identification of two functional nGREs in the *hWNK4* promoter

To identify whether GR binding to the putative binding sites (-285 and -337), we synthesized nGRE1 and nGRE2 oligonucleotides and performed EMSA with HEK293 nuclear extracts. Both nGRE1 and nGRE2

probes formed a strong complex with nuclear extracts that were competed by 100- or 500-fold molar excess of unlabeled nGRE1 and nGRE2 respectively (Fig. 6A), but not by 500-fold excess of unlabeled nGRE1 and nGRE2 mutant (in Fig. 6B and C). The GRE consensus binding was also competed by 100-fold molar excess of unlabeled nGRE1 or nGRE2 probe (Fig. 6A). Further studies showed that their binding activities were blocked by anti-GR antibody, but not by IgG control (Fig. 6B and C) and enhanced with Dex-treated nuclear extracts (Fig. 6D and E).

To confirm the results of EMSA, we performed *in vivo* ChIP assay. After chromatin from Dex-treated and -untreated cells was immunoprecipitated with anti-GR antibody, we amplified -484 to -189 region containing both nGRE1 and nGRE2, -484 to -295 region containing nGRE2, and -288 to -189 region containing nGRE1 and obtained 296, 190, and 100 bp products respectively (Fig. 7A). All bindings were specific, as both immunoprecipitation with nonimmune IgG and with an anti-p65 antibody (data not shown) did not show *hWNK4* promoter fragments. Moreover, when nuclear extracts were immunoprecipitated with the anti-GR antibody, no fragment was detected by PCRs using *nNOS*-specific primers, which was used to identify a nuclear-factor kappaB (NF-κB) binding site previously (Li *et al.* 2007; Fig. 7B). The amount of PCR products can reflect the level of GR binding in ChIP. Therefore, we quantified the PCR products and found that the amounts of 296, 190, and 100 bp products in Dex-treated group were higher compared with those in the Dex-untreated group (Fig. 7C). These indicated that nGRE1 and nGRE2 were located at position -285 and -337 of the *hWNK4* promoter and Dex treatment resulted in a significant increase of GR binding to both sites.

Figure 5 Identification of the *cis*-elements required for Dex-mediated *hWNK4* transcription. (A) Schematic representation of a series of 5'-deleted *hWNK4* promoter luciferase constructs. The transcription initiation site is indicated by a bent arrow. Numbers indicate base pairs from transcription start site. (B) The basal activity of the corresponding constructs in HEK293 cells. Luciferase activities were measured by a luminometer (Bethold) normalized to *Renilla* luciferase activities. Results are expressed as the fold of pGL3-Basic vector that is taken as 1. The bar is mean $\pm$ S.E.M. from three independent experiments in duplicate for each construct. (C) The comparison of the effects of Dex or Dex with RU486 treatment on the activity of 5'-deleted *hWNK4* promoter/luciferase constructs in HEK293 cells. HEK293 cells were transfected with the indicated constructs and treated with (the black bar) or without (the white bar) 1 μ M Dex or 1 μ M Dex combined with 10 μ M RU486 (the grey bar) for 24 h. Luciferase activities were measured by a luminometer (Bethold), normalized to *Renilla* luciferase activities. Results are expressed as a percentage of the untreated control that is taken as 100%. The bar is mean $\pm$ S.E.M. from three independent experiments in duplicate for each construct (* P <0.05 compared with control; # P <0.05 compared with Dex alone).

Discussion

Herein, we elucidated for the first time that glucocorticoids suppressed the *hWNK4* mRNA expression in HEK293 cells. Furthermore, we analyzed the activity of *hWNK4* promoter and identified nGREs in the 5'-flanking region of the *hWNK4* gene that functioned in glucocorticoid-mediated repression of the *hWNK4* gene expression.

Glucocorticoids are critical physiological regulators of fluid and electrolyte homeostasis. They exert their major

function by binding to the intracellular GR that acts as a ligand-activated nuclear transcription factor. Upon ligand binding, the receptor translocates to the nucleus where it interacts with GREs and regulates the transcription of specific gene in target epithelia (Fuller *et al.* 2000). The effects of glucocorticoids on epithelial transporter genes have been studied in a variety of epithelial cells and tissues (Wald *et al.* 1998, Snyder 2005). However, to date, there is no data about the modulation of the *WNK4* expression by glucocorticoids. We used Dex, a synthetic glucocorticoid that is poor substrate for 11 β -hydroxysteroid

Figure 6 Specific GR binding to nGREs of the *hWNK4* promoter by EMSA. EMSA was performed with nuclear extracts from HEK293 cells and the labeled GRE consensus oligonucleotide (*GRE Cons) or the labeled nGREs of the *hWNK4* promoter (*nGRE1 and *nGRE2). (A) Lane 1 is *nGRE2 probe without nuclear extracts. The binding complexes were competed by 100- or 500-fold molar excess of unlabeled nGRE1 or nGRE2 as indicated. NS, nonspecific binding. (B and C) *nGRE2 and *nGRE1 binding complexes were competed by 500-fold molar excess of unlabeled nGRE2 and nGRE1 respectively and blocked by anti-GR antibody, but neither competed by 500-fold molar excess of unlabeled mutant nGRE2 and nGRE1 respectively nor blocked by nonimmune IgG. (D) The Dex-treated nuclear extracts enhanced the binding affinity of *GRE Cons, *nGRE1, and *nGRE2 compared with the same amount of Dex-untreated nuclear extracts. (E–H) The relative GR binding affinity in EMSA quantified by densitometric analyses from the data in A–D. The levels of GR binding of lane 2 (A), lane 1 (B and C), and Dex-untreated controls (D) are taken as 100. The bar is mean $\pm$ S.E.M. from three independent experiments (* $P < 0.01$; # $P < 0.05$ compared with untreated control). ND, not detectable.

dehydrogenase, to determine the effects of glucocorticoids on the *hWNK4* expression in HEK293 cells. Dex decreased *hWNK4* mRNA level to 28–35% of the control after 24-h treatment; and mRNA reduction caused by Dex was effectively prevented by RU486, a synthetic GR antagonist. This long-lasting cellular response to Dex may be caused by transcriptional regulation. Therefore, we identified a transcriptional initiation site of the *hWNK4* gene and isolated the 5'-flanking region of the *hWNK4* gene to generate the fusion construct with luciferase reporter gene. The luciferase assay demonstrated that Dex inhibited the *hWNK4* transcriptional activity in GR-dependent pattern according to the decrease of the *hWNK4* mRNA expression.

To our knowledge, GR exists in most cells and acts as ligand-activated nuclear transcription factors to exert the physiological functions of glucocorticoids. Upon ligand binding, the GR translocates to the nucleus where it activates or suppresses gene transcription. In this regard, we further investigated the effect of Dex on the various portions of the *hWNK4* promoter in HEK293 cells. We found that the Dex-mediated suppression

occurred significantly in p285-luc and p337-luc of the *hWNK4* proximal promoter. Moreover, RU486 successfully blocked the reduction caused by Dex treatment. We subsequently identified the negative elements by EMSA and ChIP. The GR binding to negative elements was confirmed by the competition of consensus GRE and interaction with anti-GR antibody. All observations suggested that the negative elements at position –285 and –337 were responsive to glucocorticoids (nGRE1 and nGRE2). Whereas, p325-luc construct containing GRE1 showed no distinct response to Dex in luciferase assay. A possible explanation is that a positive regulatory element harbored in the region –325 to –285 and interfered in its response to Dex. We compared our nGREs with the published nGREs and proved again that nGREs, unlike GREs that share a common consensus, were different in sequence (Malkoski & Dorin 1999, Radoja *et al.* 2000, Ou *et al.* 2001). Considerable evidences indicated that the mechanisms of GR-mediated transcriptional repression involve either GR binding to a simple nGRE such as in the keratin gene (Radoja *et al.* 2000) or GR interacting with a second

Figure 7 ChIP assays with anti-GR antibody. Chromatin from Dex-treated or -untreated HEK293 cells was immunoprecipitated with anti-GR antibody or nonimmune IgG as a negative control. Input DNA corresponded to the chromatin fragments before immunoprecipitation. (A) The PCR products of *hWNK4* promoter were a 296 bp fragment containing both nGRE1 and nGRE2, a 190 and 100 bp fragment containing nGRE2 and nGRE1 respectively. M represented 100 bp DNA ladder. (B) A pair of primers corresponding to *nNOS* promoter giving a 189 bp fragment was used as a negative control. (C) The relative amount of PCR products representing the GR binding activity in ChIP was quantified by densitometric analyses, normalized to that of input. Results are expressed as a percentage of untreated controls that are taken as 100% for each group. The bar is mean $\pm$ S.E.M. from three independent experiments (* $P < 0.05$; # $P < 0.001$ compared with untreated control). ND, not detectable.

transcription factor, just like that in the osteocalcin, IL-6, and *c-fms* genes (Meyer *et al.* 1997, De Bosscher *et al.* 2000, Flick *et al.* 2002). Interaction of GR with a second transcription factor is a complex pattern including GR competing an activator binding to GRE (competitive nGRE), GR tethering a factor binding to nGRE (tethering nGRE), and both GR and a factor binding to nGRE (composite nGRE). Our EMSA showed that the *in vitro* binding was blocked by anti-GR antibody but not by AP-1 (c-jun and c-fos), NF- κ B (p65 and p50), and Oct-1 antibodies (data not shown); ChIP revealed that the binding of GR to the specific nGREs occurred in a native chromosome environment *in vitro*. However, these data could not rule out other factors interacting with nGREs of *hWNK4* promoter in transcriptional repression. Indeed, in the case of competitive nGRE and composite nGRE, GR directly binds nGRE and undoubtedly tethers additional factors. For example, the Dex-mediated repression of the pro-opiomelanocortin (*POMC*) gene expression has been initially described as direct binding of GR to a nGRE (Drouin *et al.* 1993), and subsequent studies have attributed the Dex-mediated repression of the *POMC* promoter to tethering the transcription factor Nur77 (Philips *et al.* 1997). Therefore, we speculated that a combination of these mechanisms could contribute to the glucocorticoid transrepressive effect on the *hWNK4* transcription. In addition, the glucocorticoid repression has been reported on posttranscriptional modifications in which the adenylate/uridylylate-rich elements (AREs) within

the 3'-UTR are now considered central *cis*-elements in the gene regulation (Stellato 2004). AREs interact with ARE-binding factors and mediate mRNA stability and translation. As we did not find a tandem AUUUA sequence in a TA-rich stretch of the 3'-UTR of the *hWNK4* gene, we could not deduce the potential for posttranscription regulation in glucocorticoid repression of the *hWNK4* mRNA expression.

In summary, our study demonstrates that glucocorticoids suppress *hWNK4* mRNA expression in HEK293 cells, and this suppression can be mediated by two nGREs that are not only required for GR-mediated transcriptional repression but also essential for appropriate transcription level of the *hWNK4* gene. Further studies will be necessary to identify the cofactors that participate in the glucocorticoid-mediated repression of the *hWNK4* expression. Such analyses of the *hWNK4* gene involved in the glucocorticoid regulation will facilitate the identification of responses that are desirable or undesirable for pharmacological effects such as electrolyte disturbance and hypertension. It may be feasible to produce glucocorticoid-related drugs that selectively target the inflammatory process and lead to individualized therapies.

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