Nuclear Factor κB Mediates Suppression of Canonical Transient Receptor Potential 6 Expression by Reactive Oxygen Species and Protein Kinase C in Kidney Cells

Yanxia Wang§, Min Ding‡, Sarika Chaudhari‡, Yanfeng Ding§, Joseph Yuan§, Dorota Stankowska§, Shaqing He§, Raghu Krishnamoorthy§, Joseph T. Cunningham§, and Rong Ma†

Background: TRPC6 expression in glomerular cells is suppressed by ROS through a PKC mechanism.

Results: Activation and inhibition of NF-κB could mimic and inhibit the ROS/PKC effect on TRPC6 expression, respectively.

Conclusion: NF-κB mediates the inhibitory effect of ROS/PKC on TRPC6 expression in mesangial cells.

Significance: This study delineated a molecular mechanism for regulation of TRPC6 at the transcriptional level.

This study was carried out to explore the molecular mechanism for down-regulation of TRPC6 expression in the reactive oxygen species (ROS)/PKC signaling in kidney cells. In cultured human mesangial cells, H2O2 and TNF-α inhibited TRPC6 mRNA expression in a time-dependent manner. Inhibition of NF-κB reversed both H2O2- and phorbol 12-myristate 13-acetate (PMA)-induced decrease in TRPC6 protein expression. Activation of NF-κB by knocking down IkBa using siRNA could mimic the suppressive effect of ROS/PKC on TRPC6. A Ca2⁺ imaging study showed that activation and inhibition of NF-κB significantly decreased and increased the TRPC6-mediated Ca²⁺ entry, respectively. Further experiments showed that PMA, but not its inactive analog 4α-phorbol 12, 13-didecanoate (4α-PDD), caused phosphorylation of IkBa and stimulated the nuclear translocation of NF-κB p50 and p65 subunits. The PMA-dependent IkBa phosphorylation was significantly inhibited by G6976. Electrophoretic mobility shift assay revealed that PMA stimulated DNA binding activity of NF-κB. Furthermore, specific knockdown of p65, but not p50, prevented an H2O2 inhibitory effect on TRPC6 protein expression, suggesting p65 as a predominant NF-κB subunit repressing TRPC6. In agreement with a major role of p65, chromatin immunoprecipitation assays showed that PMA treatment induced p65 binding to the TRPC6 promoter. Moreover, PMA treatment increased the association of p65 with histone deacetylase (HDAC) and decreased histone acetylation at the TRPC6 promoter. Consistently, knockdown of HDAC2 by siRNA or inhibition of HDAC with trichostatin A prevented a H2O2-induced decrease in TRPC6 mRNA and protein expressions, respectively. Taken together, our findings imply an important role of NF-κB in a negative regulation of TRPC6 expression at the gene transcription level in kidney cells.

TRPC6 belongs to the TRPC2 family, which is composed of seven members, named TRPC1–7 (1). All TRPCs are Ca²⁺-conductive non-selective cation channels and are globally expressed with implications to a variety of cellular functions from gene expression to cell proliferation (2). Over the past few years, we and others have demonstrated that TRPC6 was involved in vascular tone (3), diabetic kidney disease (4, 5), idiopathic pulmonary hypertension (6, 7), focal segmental glomerulosclerosis (8, 9), and cardiac hypertrophy (10). A multifunctional TRPC6 channel is tightly gated/regulated by multifactorial pathways to maintain cellular homeostasis. In the long history of exploring how TRPC6 channel is controlled, tremendous efforts have been made to uncover the acute mechanisms for regulation of the channels. These include membrane receptor activation (11), intracellular Ca²⁺ store depletion (12), stretch, membrane lipids (13), ROS (3, 14, 15), protein kinases (16–18), and trafficking (14, 19). However, how TRPC6 channel is regulated at the transcriptional level remains unknown. Our recent studies demonstrated that ROS acutely (in minutes) activated TRPC6 channels in vascular smooth muscle cells (3) but suppressed TRPC6 channel expression with prolonged exposure (in hours) in renal glomerular MCs (5). Because many diseases such as diabetes and hypertension are characterized with oxidative stress with chronic progression (20–23), identification of mechanisms for chronic regulation of TRPC6 channels by ROS has clear clinical significance.

Glomerular MCs sit between glomerular capillary loops and maintain the structural architecture of the capillary networks. These cells play important roles in mesangial matrix homeostasis, regulation of glomerular filtration rate, and phagocytosis of apoptotic cells in glomerulus (24–26). MC dysfunction is closely associated with several glomerular diseases, such as diabetic nephropathy (27–29). Previous studies from our group

* This work was supported, in whole or in part, by National Institutes of Health Grant 5 R01 DK079968-01A2 (NIDDK; to R. M.). This work was also supported by American Heart Association South Central Affiliate Grant-in-aid 13GRTT550013 (to R. M.).
† To whom correspondence should be addressed: 3500 Camp Bowie Blvd., Dept. of Integrative Physiology, University of North Texas Health Science Center, Fort Worth, TX 76107. Tel.: 817-735-2516; Fax: 817-735-5084; E-mail: rong.ma@unthsc.edu.

The abbreviations used are: TRPC, transient receptor potential; NF-κB, nuclear factor κB; ROS, reactive oxygen species; PMA, phorbol 12-myristate 13-acetate; 4α-PDD, 4α-phorbol 12, 13-didecanoate; HDAC, histone deacetylase; MC, mesangial cell; IDT, integrated density value; CNAP1, chromosome condensation-related SMC-associated protein 1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide.
demonstrated that TRPC6 was expressed in MCs (30) and regulated MC function. The abundance of TRPC6 protein was significantly reduced in human MCs with chronic treatment with ROS via a PKC mechanism (5). However, the detailed molecular mechanism downstream of ROS/PKC is unknown. In the present study we showed evidence that NF-κB was the mediator linking ROS/PKC to TRPC6 expression by repressing TRPC6 gene transcription.

**EXPERIMENTAL PROCEDURES**

**MC Culture and Transient Transfection**—Human MCs belong to Clonetics™ renal MC system and were purchased from Lonza (Walkersville, MD). MCs were cultured in low glucose (5.6 mM) DMEM medium (Invitrogen) supplemented with 25 mM HEPES, 4 mM glutamine, 1.0 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20% FBS. Cells were growth-arrested with 0.5% FBS medium during treatments. Only subpassages less than nine generations were used this study.

All siRNA oligonucleotides, including scramble control sequences, were transiently transfected into MCs using DharmaFECT 2 transfection reagent (Thermo Scientific, Rockford, IL) following the protocols provided by the manufacturer and described in Ref. 14. Seventy-two hours after transfection, cells were either harvested for Western blot or used for a fura-2 study.

3-[(4,5-Dimethylthiazol-2-yl)-2-5 Diphenyl Tetrazolium Bromide (MTT) Assay—The MTT assay was conducted to evaluate cell viability in the presence of H$_2$O$_2$. MCs were plated in one 96-well with $\sim 30 \times 10^3$/100 μl/well 1 day before the assay. Cells were treated with 10 μl of the 12 mM MTT (EMD Millipore, Billerica, MA) stock solution and were incubated at 37 °C for 4 h. The medium was then removed, and 50 μl of DMSO was added to each well. After incubation at 37 °C for 10 min, absorbance at 540 nm with a reference wavelength of 570 nm was measured.

Quantitative Real Time-RT-PCR—The total RNA was isolated from cultured human MCs using a PerfectPure RNA culture cell kit (5 Prime, Inc., Hamburg, Germany) following the manufacturer’s protocol. Human TRPC6 primers (forward, GCCAATGAGCATCTGGAAAT; reverse, TGGAGTCACAAGTTACATA) were synthesized by IDT (Coralville, Iowa), iScript cDNA synthesis kit (Bio-Rad) was used for RT reactions with 1.0 μg of total RNA in a final volume of 20 μl following the manufacturer’s reaction protocol. Real time PCR used 0.2 μg of RT product, 100 nm primer, and was performed using iQ SYBR Green supermix (Bio-Rad) in a final volume of 20 μl. The PCR mix was denatured at 95 °C for 10 min followed by 45 cycles of melting at 95 °C for 15 s, annealing at 57 °C for 10 s, and elongation at 72 °C for 15 s. After amplification, a melting curve analysis from 65 to 95 °C with a heating rate of 0.02 °C/s with a continuous fluorescence acquisition was made. The assay was run on a C1000™ Thermal Cycler (Bio-Rad). The average C$_t$ (threshold cycle) of fluorescence units was used to analyze the mRNA levels. The TRPC6 mRNA levels were normalized by β-actin mRNA levels. Quantification was calculated as follows: mRNA levels $= 2^{ΔΔCt}$, where ΔC$_t$ = C$_t$TRPC6 - C$_t$actin, Δ(ΔC$_t$) = ΔC$_{t,T}$ - ΔC$_{t,0}$, where ΔC$_{t,T}$ represents ΔC$_t$ at different time points of H$_2$O$_2$ treatment, and ΔC$_{t,0}$ represents ΔC$_t$ without H$_2$O$_2$ treatment.

**Preparation of Nuclear Extracts**—Preparation of nuclear extracts from human MCs was performed using Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific) following the manufacturer’s protocol. The extracts were stored at −80 °C until use.

Western Blots—As described in our previous publication (5). Briefly, the whole cell lysates or nuclear extracts were fractionated by 10% SDS-PAGE, transferred to PVDF membranes, and probed with primary TRPC6, actin, phospho-IκBα (Ser-32), IκBα, p50, p65, and lamin A/C antibodies. Bound antibodies were visualized with Super Signal West Femto (all proteins except actin) or Pico (for actin) Luminol/Enhancer Solution (Thermo Scientific). The specific protein bands were visualized and captured using an AlphaEase FC Imaging System (Alpha Innotech, San Leandro, CA). The IDT of each band was measured by drawing a rectangle outlining the band using AlphaEase FC software with auto background subtraction. If a protein had double bands, a total IDT by summation of each band IDT was used. The expression levels of TRPC6, phospho-IκBα, and IκBα proteins were quantified by normalization of the IDTs of those protein bands to that of actin bands on the same blot. In Fig. 10, the expression levels of nuclear p50 and p65 proteins were normalized to lamin A/C.

**Coimmunoprecipitation of Nuclear Proteins**—Nuclear extracts (50 μg) from human MCs with various treatments (Fig. 11A) were incubated with 2 μg of p65 primary antibody to pull down the nuclear p65 protein following the protocol described in our previous publication (31). The precipitated proteins were resolved by regular Western blot and probed with a HDAC2 antibody. A nuclear protein, lamin A/C, from 10 μg of the corresponding nuclear extracts was used as an input.

**Fluorescent Immunocytochemistry**—Human MCs were plated on 22 × 22–1 mm glass coverslips. Cells with or without pretreatments as indicated in Fig. 7C were fixed with 4% paraformaldehyde for 15 min at room temperature. After being washed with PBS, the cells were then incubated with ice-cold acetone at −20 °C for 10 min. After 30 min of incubation with blocking buffer, the cells were incubated with either p50 or p65 primary antibody at 1:400 and 1:100, respectively, in PBS plus 10% donkey serum and 0.2% Triton X-100 at 4 °C overnight. After three washes with PBS, the cells were then incubated with goat anti-rabbit secondary antibodies conjugated with Alexa Fluor 568 (for p50) or with Alexa Fluor 488 (for p65) (Invitrogen) at a concentration of 1:500 for 1 h at room temperature. DAPI (Invitrogen) was used for staining nuclei. For a fluorescent staining control, equal amounts of rabbit IgG were used instead of the primary antibodies. Localization of NF-κB was delineated as follows: gene transcription.
DNA labeling kit (Thermo Scientific). Nuclear extracts (5 μg of protein) were incubated in 1× binding buffer containing 2.5% glycerol, 50 ng/μl poly(dI-dC), 5 mM MgCl₂, 0.05% Nonidet P-40, and 4 pmol of biotin-labeled oligonucleotide in a total volume of 20 μl at room temperature for 20 min. The reaction mixture was then subjected to electrophoresis in a 6% polyacrylamide gel using 0.5 × Tris borate EDTA as the running buffer. For competition studies, nuclear extracts were incubated with a 50-fold molar excess of unlabeled oligonucleotide. After transfer to nylon membrane at 100 V for 30 min at 4 °C, the membrane was then cross-linked for 60 s in a UV cross-linker. The binding complexes were then developed using Chemiluminescent Nucleic Acid Detection Module from Thermo Scientific following the manufacturer’s protocol.

ChIP Assay—After MCs were treated as indicated in Figs. 10 and 11B, cells were cross-linked by 1% formaldehyde for 10 min at room temperature. Glycine was then added at a final concentration of 0.125 M to neutralize formaldehyde. After two washes with PBS, cells were scraped and collected by centrifugation (750 × g). Cells were then resuspended in lysis buffer 1 (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% Nonidet P-40, 0.25% Triton X-100, and a protease inhibitor mixture) and incubated at 4 °C for 10 min. Nuclei were then isolated by centrifugation (1350 × g) and lysed in lysis buffer 2 (10 mM Tris-HCl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, and a protease inhibitor mixture). The pelleted chromatin was then resuspended in lysis buffer 3 (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-lauroylsarcosine, and proteinase inhibitor). After sonication, a 1/10 volume of 10% Triton X-100 was added to the lysates followed by centrifugation at 20,000 × g at 4 °C for 10 min, and the supernatants were incubated with 2 μg of p65 antibody (Abcam, Cambridge, MA) or a control rabbit IgG (Cell Signaling Technology, Danvers, MA) overnight at 4 °C. After immunoprecipitation, the samples were incubated with 25 μl of Magnetic A/G beads (Thermo Scientific) for 2 h at room temperature, and the immune complexes were collected by Magnetic Stand and washed 4 times with radioimmunoprecipitation assay buffer (10 mM Tris-HCl, 0.25 mM LiCl, 0.5% Nonidet P-40, and 0.5% sodium deoxycholate, pH 7.5) followed by 2 washes with Tris-EDTA buffer with 50 mM NaCl. Then 50 μl of 0.1% Chelex 100 were added to the washed beads pellets, and the samples were boiled for 10 min at 100 °C. The pellets were then incubated with RNase for 1 h and proteinase K for 30 min at 55 °C. The supernatants were collected after maximum speed centrifugation (20,000 × g) at room temperature and subjected to quantitative real-time PCR or regular PCR. A volume of 2 μl of immunoprecipitated chromatin from the cells with different treatments was analyzed by real-time PCR. The PCR was performed in duplicate using iQ SYBR Green Supermix reagents (Bio-Rad). Real-time PCR conditions used were 95 °C for 3 min and 40 cycles with 95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s. Melting curves were measured in the PCR machine between 60 and 95 °C with a resolution of 0.5 °C.

Fluorescence Measurement of [Ca²⁺]i—Measurements of [Ca²⁺]i in MCs using fura-2 were performed using dual excitation wavelength fluorescence microscopy. MCs grown on a coverslip (22 × 22 mm) were loaded with fura-2 by incubation for 50 min at room temperature in the dark in physiological saline solution containing 2 μM acetoxyethyl ester of fura-2 (fura-2/AM), 0.09 g/dl DMSO, and 0.018 g/dl Pluronic F-127 (Invitrogen) followed by washing 3 times. The cells were then incubated with fura-2-free physiological saline solution for an additional 20 min. The coverslip was then placed in a perfusion chamber (Warner, Model RC-20H) mounted on the stage of a Nikon Diaphot inverted microscope. Fura-2 fluorescence was monitored by a ratio technique (excitation at 340 and 380 nm, emission at 510 nm) using NIS Elements AR™ software (Nikon Instruments Inc., Melville, NY) at room temperature. [Ca²⁺]i was calculated using the software following the manufacturer’s instructions. Calibrations were performed in vivo at the end of each experiment, and conditions of high [Ca²⁺]i were achieved by the addition of 5 μM ionomycin, whereas conditions of low [Ca²⁺]i were obtained by the addition of 5 mM EGTA.

Materials and Reagents—The siRNA oligonucleotides against human IκBα, p50, and p65 were designed and synthesized by Cell Signaling Technology. The control scramble siRNA (Non-targeting siRNA #1, D-001810-01-20) was purchased from Thermo Scientific. InSolution™ NF-κB activation inhibitor and helenalin were purchased from Calbiochem. Rabbit anti-IκBα and rabbit anti-phospho-IκBα (Ser32) antibodies were purchased from Cell Signaling Technology. Mouse anti-p50, rabbit anti-p65, and rabbit anti-Lamin A/C antibodies were purchased from Abcam. Rabbit anti-acetyl histone H3 antibody was purchased from Millipore. DAPI was purchased from Invitrogen. Protease Inhibitor Mixture tablets were obtained from Roche Applied Science. All other primary antibodies and chemicals were purchased from Sigma.

Statistical Analysis—Data are reported as the means ± S.E. One-way analysis of variance plus Student-Newman-Keuls post hoc analysis and Student’s unpaired t test were used to analyze the differences among multiple groups and between two groups, respectively. p < 0.05 was considered statistically significant. Statistical analysis was performed using SigmaStat (Jandel Scientific, San Rafael, CA).

RESULTS

TRPC6 mRNA Level Was Decreased by H₂O₂ in MCs—We have previously demonstrated that H₂O₂ reduced the abundance of TRPC6 protein in human MCs through a PKC mech-
anism (5). To determine whether the H\textsubscript{2}O\textsubscript{2} effect was at a transcriptional or translational level, we conducted quantitative real-time RT-PCR using samples from human MCs either without H\textsubscript{2}O\textsubscript{2} treatment or with H\textsubscript{2}O\textsubscript{2} treatment for various time periods. As shown in Fig. 1, H\textsubscript{2}O\textsubscript{2} induced a time-dependent decrease in TRPC6 mRNA steady-state levels, and a significant decrease was observed after 2 h of treatment (Fig. 1). These data suggest that H\textsubscript{2}O\textsubscript{2} inhibited TRPC6 expression by suppression of TRPC6 gene.

NF-κB-mediated H\textsubscript{2}O\textsubscript{2} and PKC-induced Reduction of TRPC6 Protein Expression in MCs—NF-κB is a well-known transcription factor downstream of both ROS and PKC (32–35). To determine if this was also the case in suppression of TRPC6 expression by the cascade of H\textsubscript{2}O\textsubscript{2}/PKC, we assessed TRPC6 protein expression in MCs treated with H\textsubscript{2}O\textsubscript{2} or phorbol 12-myristate 13-acetate (PMA) in the presence and absence of NF-κB inhibition. As shown in Fig. 2, A and C, H\textsubscript{2}O\textsubscript{2} at 200 μM for 6 h significantly decreased TRPC6 protein abundance, and this inhibitory effect was completely prevented by NF-κB activation inhibitor (10 μM) but not by its vehicle (DMSO). H\textsubscript{2}O\textsubscript{2} treatment did not affect MC viability assessed by MTT assay (data not shown). Consistent with our previous study (5), PMA, but not its inactive analog 4α-PDD significantly decreased TRPC6 expression (Fig. 2, Ba and Da). The PMA effect was also abolished by NF-κB inhibition with NF-κB activation inhibitor (10 μM) and Helenalin (1 μM) (Fig. 2, Bb and...
Suppression of TRPC6 Expression by NF-κB

These results suggest NF-κB is a downstream molecule of ROS and PKC in the suppression of TRPC6 protein expression.

Activation of NF-κB Decreased TRPC6 Protein Expression in MCs—The major form of NF-κB is a heterodimer of the p50 and p65/RelA subunits that are localized to the cytoplasm as a latent form (36). In unstimulated cells, the p50 and p65/RelA complex is retained in the cytoplasm by association with the inhibitor of NF-κB, IkBα (36). Thus, removal of the inhibitory IkBα is required for NF-κB activation. To determine if NF-κB negatively regulated TRPC6 expression, we activated endogenous NF-κB in human MCs by knocking down the prototypical IkBα, using siRNA. Compared with the MCs without transfection and with transfection with scramble siRNA, the IkBα expression level in the MCs transfected with siRNA against human IkBα reduced significantly (Fig. 3). Corresponding to this decrease, the abundance of TRPC6 protein decreased significantly (Fig. 3). These data suggest that direct activation of NF-κB could mimic ROS and PKC effect on TRPC6 expression and, thus, support the idea that NF-κB is a component in the TRPC6 regulation cascade.

NF-κB Activity Influenced TRPC6-mediated Ca2+ Entry in MCs—We next carried out Ca2+ imaging study to examine the functional consequence of NF-κB effect on TRPC6 protein expression. Hyperforin has been used by us and others to specifically activate TRPC6 channels (3, 37, 38). In the present study we used hyperforin to stimulate Ca2+ entry via TRPC6 channels in MCs with and without activation or inhibition of NF-κB. Ca2+ entry response was evaluated using a classical Ca2+ omission-addition protocol (3). In the first line of experiments, TRPC6-mediated Ca2+ influx was assessed in MCs with and without knocking down IkBα. In untransfected cells, re-addition of Ca2+ itself elevated the intracellular Ca2+ concentration ([Ca2+]i) by 66.9 ± 6.4 nm. Hyperforin (10 μM) significantly increased the Ca2+ response to 197.2 ± 48.1 nm (Fig. 4B). However, activation of NF-κB by siRNA against IkBα abolished the hyperforin-enhanced Ca2+ influx, but scramble transfection did not (Fig. 4A and B). In the second line of experiments, we examined if inactivation of NF-κB could augment TRPC6-mediated Ca2+ entry. In MCs growth-arrested for 6 h, hyperforin treatment evoked a rise of [Ca2+]i by 103.5 ± 20.9 nm upon Ca2+ re-addition into the bath. With H2O2 treatment (200 μM for 6 h in FBS free medium), the hyperforin response was significantly attenuated. Pretreatment of the cells with NF-κB activation inhibitor reversed the H2O2 inhibitory effect (i.e., [Ca2+]i by 103.5 ± 20.9 nm upon Ca2+ re-addition into the bath. With H2O2 treatment (200 μM for 6 h in FBS free medium), the hyperforin response was significantly attenuated. Pretreatment of the cells with NF-κB activation inhibitor reversed the H2O2 inhibitory effect (Fig. 4C and D). Furthermore, NF-κB inhibitor itself also significantly increased basal Ca2+ entry (without hyperforin stimulation) upon Ca2+ re-addition (Fig. 4E). These fura-2 data provided functional evidence for a negative regulation of TRPC6 channels by NF-κB.

PMA Phosphorylated IkBα in Human MCs—If NF-κB mediated a ROS/PKC-induced decrease in TRPC6 expression, then activation of PKC is expected to activate NF-κB. It has been

**FIGURE 3. A Western blot shows the effect of knockdown of IkBα on TRPC6 protein expression in human MCs. A, a representative experiment shows TRPC6 protein expression in MCs without transfection (UT) or transfected with siRNA against human IkBα (siRNA-IkBα) or with scramble sequence (Scramble).** Actin was used as a loading control. B and C, summary data show TRPC6 (B) and IkBα (C) expression levels (normalized to actin) in the groups indicated in A. * represents a significant difference as compared with both UT and Scramble. n indicates the number of independent experiments.
Suppression of TRPC6 Expression by NF-κB

**FIGURE 4.** Effect of NF-κB activity on TRPC6-mediated Ca\(^{2+}\) entry in human MCs. A, representative traces show \([\text{Ca}^{2+}]_i\) in untransfected (UTran) MCs with or without hyperforin (Hy) treatment or in the scramble siRNA (Scr-Hy)- or IκBα siRNA (siRNA-Hy)-transfected MCs treated with hyperforin. B, shown are summarized \([\text{Ca}^{2+}]_i\) responses from the experiments presented in A. *, \(p < 0.01\), compared with untransfected. †, \(p < 0.05\), compared with all other groups. C, shown are representative \([\text{Ca}^{2+}]_i\) traces in MCs treated with hyperforin (Hy) with or without prior incubation with \(\text{H}_2\text{O}_2\) (200 \(\mu\text{M}\) for 6 h) in the presence or absence of InSolution™ NF-κB activation inhibitor (NF-κB Inh, 10 \(\mu\text{M}\)). Cells with or without \(\text{H}_2\text{O}_2\) treatment were growth-arrested for 6 h before hyperforin stimulation. In both A and C, hyperforin (10 \(\mu\text{M}\)) was applied 30 min before experiments and was included in the bathing solution throughout the experiments. \([\text{Ca}^{2+}]_j\) indicates the Ca\(^{2+}\) concentration in bathing solution. All the traces were smoothed using the 2D smooth function of SigmaPlot program. D, summarized \([\text{Ca}^{2+}]_i\) responses from experiments are presented in C. *, \(p < 0.01\), compared with both Hy and \(\text{H}_2\text{O}_2\)-Hy. E, \(\Delta[\text{Ca}^{2+}]_i\), was the difference in \([\text{Ca}^{2+}]_i\) before and after the addition of 2 mM Ca\(^{2+}\) to the bath. The numbers inside the parentheses represent the number of cells analyzed.

**FIGURE 5.** A Western blot shows a time-dependent expression of phosphorylated IκBα (P-IκBα) and total IκBα (T-IκBα) in human MCs treated with 1 \(\mu\text{M}\) PMA in serum-free medium. A, representative experiments are shown. Actin was used as a loading control. B, shown are quantifications of P-IκBα (85a) and T-IκBα (85b) by normalization to actin for the experiments indicated in A. The value at time 0 was taken as 1. * denotes a significant difference, compared with time 0. \(n\) indicates the number of independent experiments. C, a representative Western blot shows the PMA (1 \(\mu\text{M}\) for 0.5 h) and 4α-PDD (1 \(\mu\text{M}\) for 0.5 h) effect on P-IκBα and T-IκBα expression in human MCs. Actin was used as a loading control.
known that phosphorylation of, and subsequent degradation of IκBα in response to stimuli is the mechanism for NF-κB activation (39–41). We thus assessed whether the phosphorylation of IκBα occurred in MCs upon PMA treatment. As shown in Fig. 5, PMA treatment (1 μM) resulted in phosphorylation of IκBα. A significant increase occurred within 30 min and was sustained as long as 4 h (Fig. 5, A and Bα). Furthermore, PMA induced a time-dependent reduction of the total IκBα (Fig. 5, A and Bb). The PMA effect was specific because 4α-PDD did not evoke phosphorylation and degradation of IκBα (Fig. 5C).

Gö6976 Inhibited PMA-induced IκBα Phosphorylation—Our previous study demonstrated that the PKC isoform suppressing TRPC6 expression was PKCa (5). If NF-κB mediated the PKC effect on TRPC6, inhibition of PKCa should depress the PKC-dependent IκBα phosphorylation. As shown in Fig. 6, treatment of MCs with 1 μM PMA for 30 min markedly increased the level of phosphorylated IκBα, whereas pretreatment of the cells with 300 nM Gö6976 (a PKCa and βI inhibitor), but not DMSO (vehicle), significantly prevented IκBα from being phosphorylated (Fig. 6).

**PMA Stimulated Nuclear Translocation of NF-κB**—After release from its inhibitor IκBα, NF-κB translocates from the cytoplasm to the nucleus for regulation of its target gene transcription (36). Using a Western blot assay on the cell nuclear extracts, we were able to detect a significant increase in the expression levels of p50 and p65, two major subunits of NF-κB in MCs treated with 1 μM PMA. Consistent with IκBα phosphorylation, the nuclear translocation of both p50 and p65 occurred within 30 min after PMA treatment and continued for at least 4 h, the longest time period we observed in this study (Fig. 7, Aa–c). However, the total amounts of both p50 and p65 proteins were not altered by the PMA treatments (Fig. 7, Ba–c). Immunofluorescence staining also showed the nuclear translocation of NF-κB in response to PMA but not to 4α-PDD (Fig. 7C).

**PMA Stimulated DNA Binding Activity of NF-κB**—To detect if the PKC-stimulated nuclear translocation of NF-κB can be translated to promotion of DNA binding of the transcription factor, EMSA was performed using a commercial probe specific for NF-κB binding (Santa Cruz). Nuclear extracts from unstimulated MCs produced a detectable NF-κB-DNA complex (lane 2 in Fig. 8A). When MCs were treated with PMA (1 μM for 60 min), the formation of the complex was significantly enhanced (lane 4 in Fig. 8A and B), an effect similar to a known NF-κB activator, TNF-α (lane 8 in Fig. 8A). The PMA effect was specific because the same concentration of 4α-PDD did not increase the complex formation (lane 6 in Fig. 8A and B). The formation of the NF-κB-DNA complex was dramatically attenuated by a 50-fold excess of the same unlabeled NF-κB oligonucleotide in the nuclear extracts from all groups of MCs (unstimulated, PMA-treated, 4α-PDD-treated, and TNF-α-treated, corresponding to lanes 3, 5, 7 and 9 in Fig. 8A, respectively), indicating that the complex formation was specific.

**p65 Was the Predominant NF-κB Subunit Mediating H₂O₂ Effect on TRPC6 Expression**—If NF-κB is a mediator for H₂O₂ inhibition on TRPC6 expression, then activation of NF-κB should mimic the H₂O₂ effect. Because TNF-α is a well known NF-κB activator and we have shown that TNF-α promoted...
NF-κB-DNA binding in MCs (Fig. 8A), we then further examined if TNF-α could suppress TRPC6 gene transcription. Similar to H$_2$O$_2$, TNF-α (20 ng/ml) evoked a time-dependent inhibition on TRPC6 mRNA expression. The time frames of the effect for both H$_2$O$_2$ and TNF-α were the same, and 2 h were required for a significant inhibition (Fig. 9A).

To determine if both p50 and p65 or only one of the two subunits were involved in the suppression of TRPC6 transcrip-
tion in H2O2/PKC signaling pathway, we individually knocked down the NF-κB subunits using siRNA specifically against human p50 or p65. As shown in Fig. 9, B and C, H2O2 treatment (200 μM for 6 h) significantly reduced the abundance of TRPC6 protein. Knockdown of p65 not only reversed the H2O2 effect but also significantly increased the expression level of TRPC6 compared with untransfected cells or scramble siRNA-transfected cells (Fig. 9, B and D), suggesting that the endogenous p65 might have a tonic effect on TRPC6 transcription. However, knockdown of p50 did not affect the inhibition of H2O2 on TRPC6 protein expression (Fig. 9, B and D). A Western blot showed that the knockdown efficiencies of siRNA p50 and p65 were comparable (Fig. 9C). These results suggest that p65 is the subunit mediating the H2O2 effect on TRPC6 protein expression in MCs.

PMA Induced p65 Binding to the TRPC6 Promoter in Human MCs—Consistent with a role of p65 in down-regulation of TRPC6 expression in ROS/PKC signaling, ChIP assays showed that PMA stimulated p65, but not a control IgG, binding to the TRPC6 promoter in human MCs (Fig. 10, A and B). As a positive control, PMA also stimulated p65 binding to the promoter region of IκB (Fig. 10, A and C). However, PMA did not stimulate p65 binding to a region of genomic DNA between the GAPDH gene and the
Suppression of TRPC6 Expression by NF-κB

FIGURE 10. PMA induced p65 binding to the TRPC6 promoter in human MCs. Cells were with (PMA) or without (UT) stimulation with 1 μM PMA for 60 min, and ChIP analyses were performed using an anti-p65 antibody. Promoter copy number was quantified by quantitative real-time PCR in duplicate using a specific primer that amplifies an NF-κB binding site in the TRPC6 or IkBa promoter. Normal rabbit IgG was used as a negative control for the specificity of immunoprecipitation. A 174-bp genomic region flanking the GAPDH and the chromosome condensation-related SMC-associated protein (CNAP1) gene, which does not have an NF-κB binding site, was used as a negative control for the specificity of NF-κB binding to TRPC6 gene. As a positive control, aliquots (1/10 of immunoprecipitates) of chromatin fragments obtained before immunoprecipitation were also subjected to PCR analysis (Input). A, a representative experiment shows the NF-κB p65 binding to the TRPC6 promoter. Normal rabbit IgG was used as a negative control for the specificity of NF-κB binding to the TRPC6 promoter. B–D, shown are summary data from four independent experiments presented in A. Data are expressed as p65-fold enrichment by normalizing p65-TRPC6 binding (B) or p65-IkBa binding (C) or p65-GAPDH binding (D) to their corresponding input chromatin. In B and C, * denotes p < 0.05, compared with UT.

chromosome condensation-related SMC-associated protein (CNAP1) gene, in which there is no transcription factor binding site (Fig. 10, A and D). These results suggest that the p65-TRPC6 binding in response to PMA stimulation was specific.

Repression of TRPC6 Transcription by ROS/PKC/p65 Required Histone Deacetylases—Because NF-κB p65 has been reported to recruit histone deacetylase (HDAC) to repress its target gene expression (42, 43), we evaluated the possibility that histone deacetylation was a downstream mechanism for inhibition of TRPC6 gene transcription in the ROS/PKC/p65 signaling pathway. In agreement with this speculation, co-immunoprecipitation experiments revealed that PMA, but not 4α-PDD, increased associations of NF-κB p65 with HDAC2, a major endogenous HDAC in MCs (44, 45) (Fig. 11A). Further ChIP assays were conducted to examine histone H3 acetylation at the TRPC6 promoter in response to PMA treatment. As shown in Fig. 11B, treatment with PMA, but not 4α-PDD, resulted in a strong decrease in histone acetylation at the TRPC6 promoter. Furthermore, specific knockdown of HDAC2 by siRNA significantly prevented a H2O2-induced decrease in TRPC6 mRNA expression (Figs. 1 and 11C). Consistently, pretreatment of MCs with HDAC inhibitor, trichostatin A (TSA) reversed H2O2-induced decrease in TRPC6 protein expression (Fig. 11, D and E). These results suggest that HDACs mediated the repressive effect of ROS/PKC/NF-κB p65 on TRPC6.

DISCUSSION

Although acute regulation of TRPC6 channel has been well documented (3, 11, 13, 15–19, 46, 47), a long term controlling mechanism, particularly at a transcriptional level, remains unknown to a large extent. We have previously described a ROS/PKC pathway for negative regulation of TRPC6 protein expression in kidney MCs (5). The present study provided evidence that NF-κB is a key molecule downstream of ROS/PKC in the cascade of TRPC6 gene regulation in kidney cells. The evidence includes the following: 1) NF-κB inhibition significantly attenuated H2O2 and/or PKC activation-induced decrease in TRPC6 protein expression, 2) NF-κB activation could mimic the H2O2/PKC effect on TRPC6 expression, 3) PKC could activate NF-κB, stimulate its nuclear translocation, and further its binding to the TRPC6 promoter, and finally 4) activation and inhibition of NF-κB significantly suppressed and enhanced TRPC6-mediated Ca2+ entry, respectively. NF-κB is a transcription factor that participates in a wide range of cellular responses, such as inflammation and proliferation when a cell is insulted by pathogenic stimuli (43, 48, 49). Like NF-κB, TRPC6 is also involved in inflammatory responses (50, 51), and TRPC6-associated Ca2+ entry is also associated with cell proliferation (2, 52, 53). Thus, the repression of TRPC6 gene by NF-κB could provide a protective mechanism or brake mechanism to prevent the occurrence of vicious reaction chains when the cells are continuously exposed to noxious stimuli like oxidative stress observed in many diseases.
Suppression of TRPC6 Expression by NF-κB

An earlier study by Yu et al. (6) reported that NF-κB promoted TRPC6 gene transcription in pulmonary artery smooth muscle cells from idiopathic pulmonary arterial hypertension patients harboring the −254C→G single-nucleotide polymorphism. This single nucleotide mutation in TRPC6 gene promoter creates an additional NF-κB binding site that confers the stimulatory effect of NF-κB. The opposite effect of NF-κB observed in the present study suggests that the native and mutation-generated NF-κB binding consensuses may function differently in regulation of TRPC6 promoter activity. Indeed, in addition to multiple NF-κB binding sites, the binding sites for many other transcription factors are also present in the promoter region of TRPC6. It is possible that the particular −254G-generated new NF-κB binding site in idiopathic pulmonary arterial hypertension patients may facilitate interactions of various transcription factors (e.g. NFAT, AP-1) to promote TRPC6 gene transcription (54). This is supported by the data from the same study of Yu et al. (6) in which TRPC6 expression was not significantly altered by NF-κB activation in the pulmonary artery smooth muscle cells without the additional binding site. Another possibility for the difference between Yu et al. (6) and our studies might be simply due to a cell type-specific effect of NF-κB. For instance, NF-κB represses anti-apoptotic gene expression in U-2 OS human osteosarcoma cells (55) but stimulates anti-apoptotic genes in HeLa human carcinoma cells (56) in response to the chemotherapeutic drugs (daunorubicin/doxorubicin). Although the mechanism for the cell type-specific effect is not clear, the nature of co-factors that are required for NF-κB function in different types of cells might play a role. It is possible that in kidney MCs, repressive co-factors are predominant, whereas in the pulmonary artery smooth muscle cells, a strong interaction between NF-κB and its co-activators may play a major role.

NF-κB stimulates or represses target gene transcription differently depending on the nature of the bound NF-κB homo- or heterodimer (36). In general, heterodimers of p65/p50 are transcriptional activators, whereas the homodimers of p50/p50 act as transcriptional repressors (57–59). In the present study, knockdown of p65, but not p50, prevented a H2O2-induced decrease in TRPC6 mRNA levels (Fig. 9, B and D). We thereby infer that although ROS/PKC stimulates the nuclear translocation of both p50 and p65 subunits in MCs, it is a p65 subunit that

FIGURE 11. A, a coimmunoprecipitation assay shows an increase in association of nuclear p65 with HDAC by activation of PKC. Human MCs were stimulated with 1 μM PMA or 4α-PDD or 20 ng/ml TNF-α for 1 h. Nuclear extracts (50 μg) were immunoprecipitated (IP) with 2 μg of p65 antibody and immunoblotted (IB) with HDAC antibody. Lamin A/C from 10 μg of the nuclear extracts was used as input. TNF-α and 4α-PDD were used as a positive and a negative control for PMA, respectively. The image was representative from three independent experiments. B, ChIP assays, showing PMA but not 4α-PDD stimulation, induced histone H3 deacetylation at the TRPC6 promoter. Human MCs were without stimulation (UT) or were stimulated with 1 μM PMA or 4α-PDD for 1 h, and ChIP assays were performed using an antibody directed to acetylated histone H3 (lysines 9 and 14). The presence of acetylated histone H3 was detected by PCR. Normal rabbit IgG was used as a negative control for the specificity of the antibody. GAPDH and input were described in Fig. 10. The images were representative ones from three separate experiments. C, quantitative real time RT-PCR shows the effect of knockdown of HDAC2 by siRNA on H2O2-induced decrease in TRPC6 mRNA in human MCs. UT, MCs without transfection and without H2O2 treatment. * denotes p < 0.05 versus both UT and siRNA-HDAC group. n indicates the number of independent experiments. D, representative immunoblots show TRPC6 protein expressions in MCs without H2O2 treatment (UT) or treated with H2O2 (200 μM for 6 h) with or without prior incubation with trichostatin A (TSA, 3.3 μM for 24 h) or DMSO (vehicle control). Actin was used as a loading control. E, shown are summary data from the experiments presented in D. TRPC6 protein expression levels were normalized to actin. * denotes p < 0.05, versus both UT and trichostatin A + H2O2, n indicates the number of independent experiments.
represses TRPC6 gene transcription. Although most known actions of NF-κB p65 subunit involve induction of gene transcription, it may also actively repress gene expression (42, 43, 55, 60). Several mechanisms are involved in the p65-dependent gene repression. Recently, the importance of HDAC activity has become apparent. Histone deacetylation mediated by HDAC generally leads to transcriptional repression. NF-κB, primarily the p65 subunit, can bind HDAC, recruit the enzymes to its target gene, and consequently change p65 activity from induction to repression of transcription (42, 43, 60, 61). For instance, HDAC activity is required for p65-dependent repression of the peroxisome proliferator-activated receptor δ target gene in human keratinocytes (61), of the Klotho gene in mouse kidney cells (43), and of anti-apoptotic genes in human osteosarcoma cells (55). The present study suggests that histone deacetylation may also be a mechanism for the p65-mediated TRPC6 gene repression in MCs because 1) activation of PKC increased associations of p65 with HDAC and decreased histone acetylation at the TRPC6 promoter (Fig. 11, A and B), and 2) biological and pharmacological inhibition of HDAC prevented a H₂O₂-induced decrease in TRPC6 mRNA and protein expressions, respectively (Fig. 11, C–E). HDACs are expressed in MCs and regulate MC function by regulating transcription of several genes (62–64). Because TRPC6 participates in MC contractile function, and dysfunction of TRPC6 channels may contribute to diabetic hyperfiltration (5), our findings on the HDACs-controlled TRPC6 expression may provide a means of treating glomerular diseases at gene transcriptional level.

In addition to NF-κB, other transcription factors may also act on the TRPC6 gene as either an activator or a repressor. By searching transcription factor consensus sequences in TRPC6 promoter region, we found multiple potential binding sites for a variety of candidate transcription factors within up to ~2000 nucleotides from TRPC6 transcription start site, including AP-1 and SP-1. It is possible that some of these factors may also regulate the TRPC6 gene through a mechanism independent of NF-κB. Alternatively, one or more the transcription factors (activators) interact with NF-κB in the TRPC6 promoter region and consequently lose their capability of gene transactivation. Indeed, antagonistic and synergistic interactions between NF-κB and other promoter-bound transcription factors have been previously described. For instance, in lung epithelial cells p65 interacts with SP1, a potent transcription activator, to produce transcription repression of the Bmp4 gene (65). Whether the similar mechanism also exists in MCs needs further investigation.

In summary, the present study provides a molecular mechanism for ROS/PKC-regulated TRPC6 channels at the gene transcriptional level. The diagram in Fig. 12 schematically describes a contribution of NF-κB to this regulatory pathway. Given that the maintenance of cell homeostasis is life-span work and development of many diseases is a long and progres-
Suppression of TRPC6 Expression by NF-κB

cosive course, understanding the mechanisms for chronic regulation of TRPC6 channel has important physiological and pathological significance.

REFERENCES

1. Clapham, D. E. (2003) TRP channels as cellular sensors. Nature 426, 517–524

2. Abramowitz, J., and Birnbaumer, L. (2009) Physiology and pathophysiology of canonical transient receptor potential channels. FASEB J. 23, 297–328

3. Ding, Y., Winters, A., Ding, M., Graham, S., Akopova, I., Mualem, S., Wang, Y., Hong, J. H., Gryczynski, Z., Yang, S. H., Birnbaumer, L., and Ma, R. (2011) Reactive oxygen species-mediated TRPC6 activation in vascular myocytes, a mechanism for vasoconstrictor-regulated vascular tone. J. Biol. Chem. 286, 31799–31809

4. Graham, S., Ding, M., Sours-Brothers, S., Yorio, T., Ma, J. X., and Ma, R. (2007) Down-regulation of TRPC6 protein expression by high glucose, a possible mechanism for the impaired C\textsuperscript{a+}\textsuperscript{2+} signaling in glomerular mesangial cells. Am. J. Physiol. Renal Physiol. 293, F1381–F1390

5. Graham, S., Gorin, Y., Abboud, H. E., Ding, M., Lee, D. Y., Shi, H., Ding, Y., and Ma, R. (2011) Abundance of TRPC6 protein in glomerular mesangial cells is decreased by ROS and PKC in diabetes. Am. J. Physiol. Cell Physiol. 301, C304–C315

6. Yu, Y., Keller, S. H., Remillard, C. V., Safrina, O., Nicholson, A., Zhang, S. L., Jiang, W., Vangala, N., Landsberg, J. W., Wang, J. Y., Thistlethwaite, P. A., Channin, R. N., Robbins, I. M., Loyd, J. E., Ghofrani, H. A., Grimminger, F., Schermuly, R. T., Cahan, M. D., Rubin, L. J., and Yuan, J. X. (2009) A functional single-nucleotide polymorphism in the TRPC6 gene promoter associated with idiopathic pulmonary arterial hypertension. Circulation 119, 2313–2322

7. Yu, Y., Fantozzi, I., Remillard, C. V., Landsberg, J. W., Chachkes, N., Platoshyn, O., Tigno, D. D., Thistlethwaite, P. A., Rubin, L. J., and Yuan, J. X. (2004) Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. Proc. Natl. Acad. Sci. U.S.A. 101, 13861–13866

8. Winn, M. P., Conlon, P. L., Lynn, K. L., Farrington, M. K., Creazzo, T., Hawkins, A. F., Daskalakis, N., Kwan, S. Y., Ebersviller, S., Burchette, J. L., Hawkins, A. F., and Winn, M. P. (2007) Enhanced expression of transient receptor potential channels in idiopathic pulmonary arterial hypertension. J. Biol. Chem. 282, 1446–1457

9. Winn, M. P., Daskalakis, N., Spurney, R. F., and Middleton, J. P. (2006) NF-κB for lung ischaemia-reperfusion induced oedema in mice.

10. Onohara, N., Nishida, M., Inoue, R., Kobayashi, H., Sumimoto, H., Sato, Y., Mizuno, N. (1999) Molecular cloning and characterization of rat TRPC6 channel has important physiological and pathological significance.

11. Estacion, M., Li, S., Sinkins, W. G., Gosling, M., Bahra, P., Poll, C., West−

12. Mizuno, N. (1999) Molecular cloning and characterization of rat TRPC6 channel has important physiological and pathological significance.

13. Hofmann, T., Obukhov, A. G., Schaefer, M., Harteneck, C., Gudermann, T., Ghofrani, H. A., and Dietrich, A. (2012) Activation of TRPC6 channels is essential for lung ischaemia-reperfusion induced oedema in mice. Nat. Commun. 3, 649

14. Shen, B., Kwan, H. Y., Ma, X., Wong, C. O., Du, J., Huang, Y., and Yao, X. (2011) cAMP activates TRPC6 channels via the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)-mitogen-activated protein kinase (MEK)-ERK1/2 signaling pathway. J. Biol. Chem. 286, 19439–19445

15. Monet, M., Francouer, N., and Boulay, G. (2012) Down-regulation of phosphoinositide 3-kinase and PTEN in the mechanism of activation of TRPC6 in vascular smooth muscle cells. J. Biol. Chem. 287, 17672–17681

16. Bousquet, S. M., Monet, M., and Boulay, G. (2010) Protein kinase C-depen−

17. Weissmann, N., Sydykov, A., Kwan, H. Y., Ma, X., Wong, C. O., Du, J., Huang, Y., and Yao, X. (2011) cAMP activates TRPC6 channels via the phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)-mitogen-activated protein kinase (MEK)-ERK1/2 signaling pathway. J. Biol. Chem. 286, 19439–19445

18. Bousquet, S. M., Monet, M., and Boulay, G. (2010) Protein kinase C-depen−

19. Tay, D. Y., Akagi, S., Sun, L., Nayak, B., Xie, P., Wada, J., Chung, S. S., and Danesh, F. R. (2005) Cell biology of diabetic kidney disease. Nephron. Exp. Nephrol. 101, e100–e110

20. Sours, B. D., Jiang, D., Zhou, W., and Ma, R. (2006) Expression and localization of canonical transient receptor potential (TRPC) proteins in human glomerular mesangial cells. Am. J. Physiol. Renal Physiol. 290, F1507–F1515

21. Sours-Brothers, S., Ding, M., Graham, S., and Ma, R. (2009) Interaction between TRPC1/TRPC4 assembly and STIM1 contributes to store-operated Ca\textsuperscript{2+} entry in mesangial cells. Exp. Biol. Med. 234, 637–648

22. Canty, T. G., Boyle, E. M., Farr, A., Morgan, E. N., Verrier, E. D., and Pohlman, T. H. (1999) Oxidative stress induces NF-κB nuclear translocation without degradation of IκBα. Circulation 100, Suppl. II, 361–364

23. Shi, X. Z., Lindholm, P. F., and Sarna, S. K. (2003) NF-κB activation by oxidative stress and inflammation suppresses contractility in colonic circular smooth muscle cells. Gastroenterology 124, 1369–1380

24. Tsai, K. L., Chu, T. H., Tsai, M. H., Chen, H. Y., and Ou, H. C. (2012) Vinorelbine-induced oxidative injury in human endothelial cells mediated by AMPK/PKC/NADPH/NF-κB pathways. Cell Biochem. Biophys. 62, 467–479

25. Huang, C. Y., Lan, J. L., Chen, Y. D., Yang, C. Y., Chen, Y. M., Li, J. P., Huang, C. Y., Liu, P. E., Wang, X., and Tan, T. H. (2011) The kinase GLK controls autophagy and NF-κB signaling by activating the kinase PKC-θ in T cells. Nat. Immunol. 12, 1113–1118

26. Nishikori, M. (2005) Classical and alternative NF-κB activation pathways and their roles in lymphoid malignancies. J. Clin. Exp. Hematopathol. 45, 15–24

27. Müller, M., Essin, K., Hill, K., Beschmann, H., Rubant, S., Schemp, C. M., Gollasch, M., Boehncke, W. H., Harteneck, C., Müller, W. E., and Leuner, K. (2008) Specific TRPC6 channel activation, a novel approach to stimulate keratinocyte differentiation. J. Biol. Chem. 283, 33942–33954

28. Leuner, K., Kazanski, V., Müller, M., Essin, K., Henke, B., Gollasch, M., Harteneck, C., and Müller, W. E. (2007) Hyperforin. A key constituent of
St. John’s Wort specifically activates TRPC6 channels. FASEB J. 21, 4101–4111
39. Kretz-Remy, C., Mehlen, P., Mirault, M. E., and Arrigo, A. P. (1996) Inhibition of IkB-α phosphorylation and degradation and subsequent NF-κB activation by glutathione peroxidase overexpression. J. Cell Biol. 133, 1083–1093
40. Beg, A. A., Finco, T. S., Nantermet, P. V., and Baldwin, A. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of IκBα. A mechanism for NF-κB activation. Mol. Cell. Biol. 13, 3301–3310
41. Ghosh, S., and Baltimore, D. (1990) Activation in vitro of NF-κB by phosphorylation of its inhibitor IκB. Nature 344, 678–682
42. Hong, C. Y., Park, J. H., Seo, K. H., Kim, J. M., Im, S. Y., Lee, J. W., Choi, H. S., and Lee, K. (2003) Expression of MIS in the testis is down-regulated by tumor necrosis factor α through the negative regulation of SF-1 transactivation by NF-κB. Mol. Cell. Biol. 23, 6000–6012
43. Moreno, J. A., Izquierdo, M. C., Sanchez-Nino, M. D., Suarez-Alvarez, B., Lopez-Larrea, C., Jakubowski, A., Blanco, J., Ramirez, R., Selpas, R., Ruiz-Ortega, M., Egido, J., Ortiz, A., and Sanz, A. B. (2011) The inflammatory cytokines TWEAK and TNFα reduce renal klotho expression through NFκB. J. Am. Soc. Nephrol. 22, 1315–1325
44. Yu, Z., Zhang, W., and Kone, B. C. (2002) Histone Deacetylases augment phosphorylation of its inhibitor IκB. Nature 344, 678–682
45. Reiser, J., Polu, K. R., Möller, C. C., Kenlan, P., Altintas, M. M., Wei, C., Faul, C., Herbert, S., Villegas, I., Avila-Casado, C., McGee, M., Sugimoto, H., Brown, D., Kalluri, R., Mundel, P., Smith, P. L., Clapham, D. E., and Pollak, M. R. (2005) TRPC6 is a glomerular slit diaphragm-associated channel required for normal renal function. Nat. Genet. 37, 739–744
46. Inoue, R., Jensen, L. J., Iian, Z., Shi, J., Hai, L., Lurie, A. I., Henriksen, F. H., Salomonsson, M., Morita, H., Kawaiabayshi, Y., Mori, M., Mori, Y., and Ito, Y. (2009) Synergistic activation of vascular TRPC6 channel by receptor and mechanical stimulation via phospholipase C/diacylglycerol and phospholipase A₂/ω-hydroxylation/20-HETE pathways. Circ. Res. 104, 1399–1409
47. Kim, J. M., Cho, H. H., Lee, S. Y., Hong, C. P., Yang, J. W., Kim, Y. S., Suh, K. T., and Jung, J. S. (2012) Role of IRAK1 on TNF-induced proliferation and NF-κB activation in human bone marrow mesenchymal stem cells. Cell Physiol. Biochem. 30, 49–60
48. Gastonguay, A., Berg, T., Hauser, A. D., Schulz, N., Lorimer, E., and Williams, C. L. (2012) The role of Rac1 in the regulation of NF-κB activity, cell proliferation, and cell migration in non-small-cell lung carcinoma. Cancer Biol. Ther. 13, 647–656
49. Hamid, R., and Newman, J. H. (2009) Evidence for inflammatory signaling in idiopathic pulmonary artery hypertension. TRPC6 and nuclear factor-κB. Circulation 119, 2297–2298
50. Sel, S., Rost, B. R., Yildirim, A. O., Sel, B., Kalwa, H., Fehrenbach, H., Renz, H., Guerdemann, T., and Dietrich, A. (2008) Loss of classical transient receptor potential 6 channel reduces allergic airway response. Clin. Exp. Allergy 38, 1548–1558
51. Yu, Y., Sweeney, M., Zhang, S., Platoshyn, O., Landsberg, I., Rothman, A., and Yuan, J. X. (2003) PDGF stimulates pulmonary vascular smooth muscle cell proliferation by up-regulating TRPC6 expression. Am. J. Physiol. Cell Physiol. 284, C316–C330
52. Ge, R., Tai, Y., Sun, Y., Zhou, K., Yang, S., Cheng, T., Zou, Q., Shen, F., and Wang, Y. (2009) role of TRPC6 channels in VEGF-mediated angiogenesis. Cancer Lett. 283, 43–51
53. Kuwahara, K., Wang, Y., McAnally, J., Richardson, J. A., Basell-Duby, R., Hill, J. A., and Olson, E. N. (2006) TRPC6 fulfills a calcineurin signaling circuit during pathologic cardiac remodeling. J. Clin. Invest. 116, 3114–3126
54. Campbell, K. J., Rocha, S., and Perkins, N. D. (2004) Active repression of antiapoptotic gene expression by RelA(p65) NF-κB. Mol. Cell 13, 853–865
55. Bottero, V., Basuttil, V., Loubat, A., Magné, N., Fischel, J. L., Milano, G., and Peyron, J. F. (2001) Activation of nuclear factor κB through the IKK complex by the topoisomerase poisons SN38 and doxorubicin. A brake to apoptosis in HeLa human carcinoma cells. Cancer Res. 61, 7785–7791
56. Satou, R., Miyata, K., Katsurada, A., Navar, L. G., and Kobori, H. (2010) Tumor necrosis factor-α suppresses angiotensinogen expression through formation of a p50/p50 homodimer in human renal proximal tubular cells. Am. J. Physiol. Cell Physiol. 299, C750–C759
57. Hirano, F., Tanaka, H., Hirano, Y., Hiramoto, M., Handa, H., Makino, I., and Scheidecreit, C. (1998) Functional interference of Sp1 and NF-κB through the same DNA binding site. Mol. Cell. Biol. 18, 1266–1274
58. Höcherl, K., Schmidt, C., Kurt, B., and Bucher, M. (2010) Inhibition of NF-κB ameliorates sepsis-induced down-regulation of aquaporin-2/V₂ receptor expression and acute renal failure in vivo. Am. J. Physiol. Renal Physiol. 298, F196–F204
59. Lu, X., Farmer, P., Rubin, J., and Nanes, M. S. (2004) Integration of the NFκB p65 subunit into the vitamine D receptor transcriptional complex. Identification of p65 domains that inhibit 1,25-dihydroxyvitamin D₃-stimulated transcription. J. Cell Biochem. 92, 833–848
60. Kretz-Remy, C., Mehlen, P., Mirault, M. E., and Arrigo, A. P. (1996) Inhibition of IkB-α phosphorylation and degradation and subsequent NF-κB activation by glutathione peroxidase overexpression. J. Cell Biol. 133, 1083–1093
61. Aarenstrup, L., Flindt, E. N., Otkjaer, K., Kirkegaard, M., Andersen, J. S., and Peyron, J. F. (2001) Activation of nuclear factor κB through the IKK complex by the topoisomerase poisons SN38 and doxorubicin. A brake to apoptosis in HeLa human carcinoma cells. Cancer Res. 61, 7785–7791