NF-κB Inhibits Sodium Transport via Down-regulation of SGK1 in Renal Collecting Duct Principal Cells*

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Tubulointerstitial inflammation is a common feature of renal diseases. We have investigated the relationship between inflammation and Na⁺ transport in the collecting duct (CD) using the mCCDcl1 and mpkCDDcl4 principal cell models. Lipopolysaccharide (LPS) decreased basal and aldosterone-stimulated amiloride-sensitive transepithelial current in a time-dependent manner. This effect was associated with a decrease in serum and glucocorticoid-regulated kinase 1 (SGK1) mRNA and protein levels followed by a decrease in epithelial sodium channel (ENaC) α-subunit mRNA levels. The LPS-induced decrease in SGK1 expression was confirmed in isolated rat CD. This decreased expression of either SGK1 or the ENaC α-subunit was not due to enhanced degradation of mRNA. In contrast, LPS inhibited transcriptional activity of the SGK1 promoter measured by luciferase-reporter gene assay. The effect of LPS was not mediated by inhibition of mineralocorticoid or glucocorticoid receptor, because expression of both receptors was unchanged and blockade of either receptor by spironolactone or RU486, respectively, did not prevent the down-regulation of SGK1. The effect of LPS was mediated by the canonical NF-κB pathway, as overexpression of a constitutively active mutant, IKKβ (inhibitor of nuclear factor κB kinase-β) decreased SGK1 mRNA levels, and knockdown of p65 NF-κB subunit by small interfering RNA increased SGK1 mRNA levels. Chromatin immunoprecipitation showed that LPS increased p65 binding to two NF-κB sites along the SGK1 promoter. In conclusion, we show that activation of the NF-κB pathway down-regulates SGK1 expression, which might lead to decreased ENaC α-subunit expression, ultimately resulting in decreased Na⁺ transport.

Critical regulation of Na⁺ and water reabsorption occurring in the collecting duct (CD) is handled mainly by systemic hormones such as aldosterone and vasopressin, aldosterone being the major regulator of sodium (Na⁺) transport in this segment (1–3). Depending on its concentration, aldosterone either binds to the mineralocorticoid receptor (MR) alone or to both the MR and glucocorticoid receptors (GR) (4). Activated receptors translocate into the nucleus and bind to specific promoter sequences leading to increased transcription of early aldosterone-regulated genes, such as serum and glucocorticoid-regulated kinase 1 (SGK1) (5) and glucocorticoid-induced leucine zipper (GILZ) (6). Protein kinase SGK1 is a key player in regulating Na⁺ transport in the CD. It increases apical plasma membrane expression and activity of the apical epithelial sodium channel (ENaC) by reducing NEDD4 ubiquitin ligase-dependent internalization (7). SGK1 additionally activates the basolateral Na⁺,K-ATPase (8–10) with a resulting increase of vectorial Na⁺ transport. In addition to these short term effects, SGK1 exerts long term effects illustrated by the recently demonstrated relief of transcriptional inhibition of the ENaC α-subunit (11). GILZ is another regulatory protein that has been proposed to activate ENaC in the CD (6). Finally, MR and GR, both of which bind to glucocorticoid-responsive elements, directly stimulate transcription of Na⁺ transporter subunits such as ENaC (12) and Na⁺K-ATPase (13) α-subunits.

Tubulointerstitial inflammation is frequently observed in renal diseases and can be associated with clinical alterations of Na⁺ handling by the CD. For example, ENaC expression is decreased in CD of septic and post-obstructive kidney experimental models (14, 15). Furthermore, inappropriate salt loss is a hallmark of acute or chronic interstitial nephritis. The NF-κB pathway (reviewed in Refs. 16 and 17), a major proinflammatory signaling pathway, is locally activated in a large number of renal inflammatory diseases (18, 19). It can be divided into two major signaling pathways: the canonical and alternative (non-canonical) pathways. The canonical pathway is the major NF-κB pathway in most cells and is activated by various stimuli including proinflammatory cytokines and bacterial products such as lipopolysaccharide (LPS). This pathway is dependent on the nuclear factor κB kinase-β (IKKβ), which phosphorylates IkBα protein and induces its degradation by the proteasome, leading to the release of p65-p50 NF-κB heterodimers. These

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‡ The abbreviations used are: CD, collecting duct; CCD, cortical collecting duct; mCCD, mouse CCD; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; SGK1, serum and glucocorticoid-regulated kinase 1; GILZ, glucocorticoid-induced leucine zipper; ENaC, epithelial sodium channel; LPS, lipopolysaccharide; IKKβ, inhibitor of nuclear factor κB kinase-β; RNAi, RNA interference; TNF, tumor necrosis factor; IL1β, interleukin-1β; MAPK, mitogen-activated protein kinase; ChiP, chromatin immunoprecipitation.
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dimers then translocate into the nucleus and, after phosphorylation and acetylation of p65 and possible cofactor binding, selectively increase or suppress gene transcription. The alternative NF-κB pathway is triggered by various factors such as CD40R, LTβR, and BAFFR and is strictly dependent on protein kinase IKKα activation, leading to regulated cleavage of p100 NF-κB subunit and generation of p52-RelB NF-κB heterodimers. This noncanonical pathway is involved mainly in the maintenance and development of lymphoid organs. In addition to these two pathways, many other NF-κB dimers are formed, such as p50, p65, cRel, and p52 homodimers, or cRel-p65 heterodimers. Among these, p50-p50 and p52-p52 NF-κB homodimers lack a transactivation domain and therefore behave as transcriptional repressors in the absence of the Bcl3 cofactor (reviewed in Refs. 16 and 17).

Molecular cross-talk between transport signaling and the NF-κB pathway has recently been described. Indeed SGK1, in addition to its major role in transport regulation, has been shown to interact with the canonical NF-κB pathway. SGK1 physically associates with IKKβ leading to activation of the NF-κB pathway in cultured cells (20). Conversely, GILZ functions as an anti-inflammatory factor, inhibiting the NF-κB pathway (21, 22). Finally, a mutual antagonism between the GR and the NF-κB pathway has been well described (23). This led us to hypothesize that a cross-talk exists between the NF-κB pathway and Na+ transport. Our aim was first to test the hypothesis that activation of the NF-κB pathway alters Na+ transport in CD principal cells and then to investigate the molecular pathway involved in this effect. For this purpose, we used both mCCDcl1 and mpkCCDcl4 cells, which display conserved major electrical and regulatory properties of CD principal cells, i.e. high electrical resistance and response to corticosteroids and vasopressin (4, 24). In this work we demonstrate that: 1) basal and aldosterone-stimulated amiloride-sensitive electrogenic ion transport and SGK1 expression are significantly decreased by proinflammatory stimuli independently of MR or GR inhibition; and 2) the canonical NF-κB pathway directly down-regulates SGK1 expression, which may participate in reducing ENaC α-subunit expression, leading altogether to a decrease in sodium transport.

EXPERIMENTAL PROCEDURES

Chemicals—Highly purified LPS from Escherichia coli O55:B5 (Calbiochem) was prepared as a 5 mg/ml stock solution in water and stored at −20 °C. Aldosterone, spironolactone, corticosterone, and RU486 (Sigma) were prepared as 10 mM stock solutions in 100% ethanol or DMSO and stored at −20 °C.

Cell Culture—mCCDcl1 cells (passages 20–32) and mpkCCDcl4 cells (passages 20–34) were seeded on permeable filters (Transwell®, Corning Costar, Cambridge, MA), grown to confluence in culture medium supplemented with 2% fetal calf serum (4), and then maintained in serum- and hormone-deprived medium for 48 h before performing experiments.

Isolated Rat Kidney Tubules—Male Sprague-Dawley rats (200–250 g body weight; Charles River, Saint Germain de l’Arbresle, France) were anesthetized with pentobarbital (5 mg/100 g body weight, intraperitoneal), and the left kidney was perfused with incubation solution (120 mM NaCl, 5 mM KCl, 4 mM NaHCO3, 1 mM CaCl2, 1 mM MgSO4, 0.2 mM NaH2PO4, 0.15 mM Na2HPO4, 5 mM glucose, 10 mM lactate, 1 mM pyruvate, 4 mM essential and nonessential amino acids, 0.03 mM vitamins, 20 mM HEPES, 0.1% bovine serum albumin, pH 7.45) containing 250 μl of collagenase (Blendszyme 2; Hoffmann-La Roche) to hydrolyze interstitial tissue. Afterward, the kidney was removed, sliced into small pyramids, and incubated for 20 min at 30 °C in an oxygenated (95% O2 and 5% CO2) incubation solution containing 100 μl of collagenase. Single cortical collecting ducts (CCD) were isolated by microdissection in ice-cold oxygenated incubation solution and stored in ice-cold medium until used. Each experimental group was constituted by pools of 50 CCDs from the same animal.

Electrophysiology—As described previously (9), the equivalent short-circuit current was calculated according to Ohm’s law from the values of transepithelial potential difference and resistance measured with a Millicell (Millipore) device. These measurements were not performed in transiently transfected cells because the electrical resistance of the monolayer could be measured only 6–7 days post-transfection, whereas expression of the transgene and the efficacy of small interfering RNA persisted at best for 3–4 days.

Transfection—Transfection was performed by electroporating cells as described previously (25). Briefly, 4 × 106 cells were transferred to electroporation cuvettes (Gene Pulser cuvette, 0.4 cm, Bio-Rad) along with 400 μl of culture medium supplemented with 10% serum and 1.2 mmol of RNA interference (RNAi) or 8 pmol of plasmid containing cDNA encoding for enhanced green fluorescent protein, constitutively active IKKβ, β-galactosidase, p105, or p50. Cells were electroporated (300 mV, 960 picofarads) using a Bio-Rad Gene Pulser, seeded at a density that allows confluence without division, and allowed to recover for 24 h in culture medium containing 10% serum. Transfected cells were then maintained in serum-free, hormone-deprived medium for another 24 h for RNAi or 48 h for plasmids. RNAi were designed using an Invitrogen design tool. RNAi targeting sense primers were: p65, 5′-GCACCUGCAUGUUGAUGCUGAUGAATT-3′, and scramble RNA, 5′-GCA-GUGAUGUGAUGCUACCGAATT-3′; cRel, 5′-GCU-GAGUACCUAGCAUGGAAATT-3′, and scramble RNA, 5′-CAGCUGUCGCCUCACCACCAUGU-3′; p105/p50, 5′-GGGAGGAGAUAAUACCCUUCUGATT-3′, and scramble RNA, 5′-CAGCUGUGAUCUACCAACACUCUAU-3′; SGK1, 5′-GGGUGUCCUGAUGAGAUCCUATT-3′, and scramble RNA, 5′-CGACACUCCCAU-UCCUCAGGAATT-3′. Constitutively active IKKβ, obtained by mutating Ser-176 and Ser-180 residues to Glu (26), was a generous gift from Dr S. Gosh (Yale University School of Medicine, New Haven, CT), and pRSV NF-κB (p50) and p105 were generous gifts from Dr N. Perkins (University of Dundee, Scotland, UK).

Real-time PCR Analysis—RNA extraction, reverse transcription, and real-time PCR analysis were performed as described previously (25). Primers used for detection of mouse acidic ribosomal phosphoprotein P0 were 5′-ATCTCCAGGAGGCCATCTG-3′ and 5′-GTTCAGCATGTTCAGCAGTG-3′; for rat acidic ribosomal phosphoprotein P0, 5′-CCTCTCCCTC-GGGCTGATC-3′ and 5′-GGGCTGTAGATGCTGCCATT-3′.
3′; for mouse αENaC, 5′-CTGCACTGTGACCCCTTAACG-3′ and 5′-CTGAGGAACACACGACCACTTCTG-3′; for mouse Na,K-ATPase α-subunit, 5′-TCTCTCACTTCCACCAA-CAA-3′ and 5′-TTTGGGCTAGTACGATTTG-3′; for mouse SGK1, 5′-CCCAACTCCCGGTTTCTGAC-3′ and 5′-CGGTCGTCTATGAGAAGACA-3′; for rat SGK1, 5′-GGGACAAGCCGCTCCATTCTGCT-3′ and 5′-CCGTCAGCTTGAGAG-3′; for mouse p65, 5′-CGTTGTTGCTATTGCAGA-3′ and 5′-GGTGGCCCTGATAAGA-3′; for mouse IκBα, 5′-CGGAGGAACGAAGACTGTTT-3′ and 5′-TTACCTGACAAAATGACTTCCA-3′; for mouse p65, 5′-GGGATCCATGATCTGGTTTCTGAGCA-3′ and 5′-GTCCATTCTGAGT-3′; for mouse c-Rel, 5′-CCAGGCCGAAGCTGAACTTTGGA-3′ and 5′-GTGGGGTGTAGTGCCCTTCAAC-3′; and for human p105/p50 (Santa Cruz Biotechnology), c-Rel, and p65 (Cell Signaling Technology). GR and PKA catalytic subunit were detected by monoclonal antibody 907 (1/2000) (a kind gift from Dr. C. Christie P. Thomas, University of Iowa), and pRL vector (Promega, Madison, WI), containing the promoterless Renilla luciferase (Promega). Cells were co-transfected with both plasmids, and luciferase activity was measured using the Dual Luciferase assay system (Promega) according to the manufacturer’s instructions. The light produced was measured using a Lumat LB 9507 luminometer (EG&G Berthold). The ratio of firefly/Renilla luciferase activity was calculated to normalize results with respect to the efficiency of transfection.

**Statistics**—Results are given as the mean ± S.E. from n independent experiments. Each experiment was performed on cells from the same passage. Data are expressed as mean ± S.E. Comparisons between two groups were performed by unpaired Student’s t test if not stated otherwise. Multiple group analyses were done using ANOVA and Tukey’s test for post hoc analysis.

**RESULTS**

Proinflammatory Stimuli Inhibit Amiloride-sensitive Electrogenic Sodium Transport and Alter SGK1 Expression in CD Cells—We assessed the effect of LPS, a classical activator of the NF-κB pathway, on electrogenic ion transport in mCCDcl1 and mpkCDcl4 cells, a second differentiated mouse CD principal cell line (24). mCCDcl1 cells were treated or not with 100 ng/ml LPS in

**Chromatin Immunoprecipitation (ChIP) Assay—**A ChIP assay was performed as described previously (29). Briefly, after stimulation cells were fixed rapidly in ice-cold cross-linking solution (1% formaldehyde, 9 mM NaCl, 4.5 mM HEPES, pH 8) before cell lysis (0.5% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfon fluoride, 50 mM NaF, 1 mM orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM Tris, pH 8) and isolation of nuclei by centrifugation. Nuclei were then lysed (1% Triton X-100, 0.5% SDS, 0.5% Sarkosyl, 0.5 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfon fluoride, 50 mM NaF, 1 mM orthovanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM Tris, pH 8), and pelleted chromatin was resuspended (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8) before fragmentation (<1 kb) by sonication. For immunoprecipitation, chromatin extracts (100 μg) were preclared for 1 h with protein A-Sepharose beads (Amersham Biosciences) and salmon sperm DNA in radioimmunoprecipitation assay buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 140 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8) and then incubated overnight at 4 °C with polyclonal anti-p65 antibody (Santa Cruz Biotechnology). Immune complexes were precipitated with protein A-Sepharose beads for 3 h at 4 °C. Reversal of cross-linking was achieved by overnight incubation at 65 °C with proteinase K and 1% SDS. DNA was extracted with phenol-chloroform and precipitated with ethanol supplemented with 0.3 M sodium acetate (pH 5.2) and 100 μg of glycogen in a final volume of 1.5 ml. Real-time PCR was performed as described above. Primers flanking the proximal NF-κB site (−2488) of the SGK1 promoter were 5′-TCAGGACAGGAACTATTGAG-3′ and 5′-TCAGGAAAGCTATTGAG-3′ and those flanking the distal NF-κB site (−1975) were 5′-AGTTATGAGAATCTTCTCAG-3′ and 5′-CTTGGTGCTCCTGAGTTG-3′. Results were expressed as fold of control (base-line condition). Control experiments were performed either without antibodies (mock) or without genomic DNA fragments (radioimmunoprecipitation assay buffer).

Luciferase Reporter Gene Assay—Luciferase plasmid constructs used for transfection were the previously described (30) pGL3 Basic, containing 3000 bp of the proximal 5′-flanking region of human sgk1 upstream of exon 1 upstream of the firefly (Photinus pyralis) luciferase coding sequence (a kind gift of Dr. C. Christine P. Thomas, University of Iowa), and pRL vector (Promega, Madison, WI), containing the Renilla (Renilla reniformis) luciferase coding sequence downstream of a constitutively active cytomegalovirus promoter (CMV-1; Promega). Cells were co-transfected with both plasmids, and luciferase activity was measured using the Dual Luciferase assay system (Promega) according to the manufacturer’s instructions. The light produced was measured using a Lumat LB 9507 luminometer (EG&G Berthold). The ratio of firefly/Renilla luciferase activity was calculated to normalize results with respect to the efficiency of transfection.
the absence or presence of $10^{-6}$ M aldosterone for 6 h (Fig. 1A). The results show that LPS significantly decreased the transepithelial equivalent short-circuit current ($p < 0.05$) (Fig. 1A) both in the absence and presence of aldosterone. It should be mentioned however that the equivalent short-circuit current remained stimulated by aldosterone in the presence of LPS. The addition of $10^{-5}$ M amiloride to the apical compartment abolished the transepithelial current, indicating that vectorial Na$^+$ transport from the apical to the basolateral compartment accounts for the measured current (data not shown). Fig. 1C shows that LPS induced a time-dependent inhibition of electrolytic sodium transport in mpkCCD$_{14}$ cells. The decrease in transepithelial current was seen together with an LPS-dependent increase of transepithelial resistance in both cell types (data not shown) indicating the absence of cell damage or alteration of tight junction permeability. This increase in transepithelial resistance was most likely because of decreased sodium conductance.

Therefore, LPS inhibits vectorial Na$^+$ transport under both basal and aldosterone-stimulated conditions in different types of cultured CD principal cells.

**In vivo** CD cells are exposed to both mineralo- and glucocorticoids. 11β-Hydroxysteroid dehydrogenase-2 protects CD cells from occupancy of the MR and thereby stimulation of sodium transport by glucocorticoids. Activation of the NF-$\kappa$B pathway has recently been demonstrated to decrease 11β-hydroxysteroid dehydrogenase-2 expression (31) potentially leading to MR occupancy by glucocorticoids and stimulation of sodium transport. We confirmed the decrease in 11β-hydroxysteroid dehydrogenase-2 mRNA levels in mCCD$_{14}$ cells treated for 6 h with 100 ng/ml LPS (as percent of control ± S.E.: 50 ± 9, $n = 4$, $p < 0.05$). We therefore assessed the effect of physiological doses of mineralo- and glucocorticoids on electrolytic sodium transport by mCCD$_{14}$ cells. In the presence of $10^{-7}$ M corticosterone and $10^{-5}$ M aldosterone, LPS partially prevented the increase in electrolytic current in mCCD$_{14}$ cells (Fig. 1B). These results show that under conditions mimicking the physiological situation, LPS inhibits CD transepithelial sodium transport.

Because LPS inhibits the amiloride-sensitive transepithelial current (mostly, if not exclusively, consisting of ENaC-mediated Na$^+$ transport (4)), we assessed mRNA expression levels of genes encoding regulators of CD Na$^+$ transport including early aldosterone-induced genes such as SGK1 and GILZ. Fig. 2A shows that SGK1 expression levels decreased by two-thirds (as
percent of control ± S.E.: 100 versus 33 ± 5; p < 0.05) in cells incubated with 100 ng/ml LPS for 6 h. In the presence of 10^{-6} M aldosterone, LPS induced a relative reduction of the increase of SGK1 mRNA (as percent of control ± S.E.: 1094 ± 250 versus 409 ± 37; p < 0.05). In contrast, basal and aldosterone-induced GILZ1 and -2 (32) mRNA levels were not significantly altered by LPS (Fig. 2B). It is well established that aldosterone increases the expression levels of ENaC and Na,K-ATPase α-subunits mRNA in the CD (33, 34). Our results show that mRNA expression levels of the ENaC α-subunit was decreased after 6 h of exposure to 100 ng/ml LPS (Fig. 2D) under basal conditions (as percent of control ± S.E.: α-ENaC: 100 versus 52 ± 9, n = 4, p < 0.05). Under aldosterone-stimulated conditions, LPS reduced the increase in ENaC α-subunit mRNA (α-ENaC: 333 ± 25 versus 188 ± 51, n = 4, p < 0.05). Fig. 2C shows that expression of the Na,K-ATPase α-subunit decreased significantly only in the presence of aldosterone (142 ± 11 versus 110 ± 10, n = 7, p < 0.05). These effects persisted for at least 24 h (data not shown). Finally, we observed a nearly 10-fold increase of IκBα mRNA expression in response to LPS (data not shown), indicating that LPS activates the canonical NF-κB pathway in CD principal cells. All together, our results indicate that LPS modulates the expression levels of some, but not all, aldosterone-dependent genes.

To further characterize the effect of LPS on SGK1 expression, we performed a dose dependence curve. Fig. 3A shows that in mCCDcl1 cells pretreated for 24 h with 10^{-6} M aldosterone, the addition of LPS for 6 h produced a dose-dependent effect with a threshold observed at 1 ng/ml and a maximal effect obtained at 10 ng/ml. To determine the specificity of SGK1 down-regulation, we measured the expression levels of SGK1 after 24 h of incubation with 100 ng/ml LPS or with other activators of the NF-κB pathway, namely TNFα and IL1β. Down-regulated SGK1 mRNA expression persisted for 24 h with a magnitude similar to that observed in cells treated with LPS, TNFα, or IL1β both in the absence and the presence of 10^{-6} M aldosterone (Fig. 3B). These observations indicate that various activators of the canonical NF-κB pathway have similar effects on SGK1 expression. Finally, we also observed down-regulated SGK1 expression induced by LPS in both mpkCCDcl4 cells (Fig. 3C) and isolated rat CCDs (Fig. 3D). Our results are therefore consistent with a physiological control of SGK1 expression by the canonical NF-κB pathway.

Decreased SGK1 Expression Is Followed by a Decrease in ENaC α-Subunit Expression and Occurs Independently of Increased mRNA Degradation in Response to LPS—To better characterize the regulation of SGK1 and ENaC α-subunit mRNA by LPS, we performed time course experiments. SGK1 mRNA expression was rapidly down-regulated by LPS; hence a significant decrease in SGK1 mRNA levels was already observed after 30 min (Fig. 4A). In contrast, ENaC α-subunit mRNA expression was significantly decreased only after 4 h (Fig. 4B). These observations suggest that SGK1 is an early target of LPS, whereas ENaC α-subunit expression is secondarily altered. We further assessed whether degradation of both mRNA species was enhanced by LPS treatment. We pretreated cells with 10^{-6} M aldosterone with or without 100 ng/ml LPS for 2 (SGK1) or 4 h (ENaC α-subunit) to enhance transcription of
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LPS does not enhance SGK1 or ENaC α-subunit mRNA degradation but may rather decrease transcriptional activity of both genes.

Because we hypothesized that SGK1 transcription is decreased by LPS, we studied the effect of LPS treatment on activity of a proximal fragment of the human SGK1 promoter (30). The luciferase reporter gene assay showed that the human SGK1 promoter fragment is constitutively active in mCCDcl1 cells. SGK1 promoter activity was inhibited by about 40% by 100 ng/ml LPS applied for either 3 or 6 h in the presence of 10^{-6} M aldosterone. In the absence of aldosterone pretreatment, the decrease in luciferase activity was not significant, due to biological variability (data not shown). These results strongly suggest that LPS inhibits human SGK1 gene transcription.

Given the early effect of LPS on SGK1 mRNA, we hypothesized that SGK1 down-regulation may in turn modulate ENaC α-subunit expression. To assess this hypothesis, ENaC α-subunit mRNA levels were measured after SGK1 knockdown by RNAi in mCCDcl1 cells. Cells were treated with 10^{-6} M aldosterone to induce high levels of SGK1 expression. Transfection of mCCDcl1 cells with RNAi targeting SGK1 decreased its mRNA expression to 57 ± 3% (p < 0.05) (Fig. 4F) of levels observed in cells transfected with scramble RNAi. Western blotting analysis showed a decrease of SGK1 protein expression in response to RNAi (Fig. 4F). SGK1 knockdown resulted in decreased ENaC α-subunit mRNA expression, which was significantly different from control values (as percent of scramble ± S.E.: 100 versus 74 ± 4%, p < 0.05) (Fig. 4F). These results suggest that SGK1 mRNA is a primary target of LPS and that decreased SGK1 expression may in turn participate in reducing ENaC α-subunit mRNA levels.

LPS-induced Down-regulation of SGK1 Expression Is Not Mediated by Decreased MR and GR Activity—To determine whether aldosterone influences the effect of LPS on SGK1 expression, we studied the effect of addition of 100 ng/ml LPS in both genes before the addition of 5 × 10^{-6} M actinomycin D to inhibit de novo transcription. The results show that LPS did not increase the rate of SGK1 or ENaC α-subunit mRNA degradation. Indeed, the slope of mRNA decay was not steeper for SGK1 (Fig. 4C) or α-ENaC (Fig. 4D). These results indicate that

![Image](https://example.com/image.png)

**FIGURE 4.** SGK1 down-regulation is followed by a reduction of ENaC α-subunit expression, and mRNA degradation is not enhanced by LPS in mCCDcl1 cells. A and B, confluent mCCDcl1 cells grown on polycarbonate filters were incubated for 30, 60, or 90 min or 2, 4, or 6 h at 37 °C in the presence or absence of 100 ng/ml LPS (filled bars). Total RNA was extracted and reverse-transcribed, and real-time PCR was performed with primers specific for SGK1 (A) and ENaC α-subunit (B). Results are expressed as -fold of control values (untreated cells). Bars are means ± S.E. from four independent experiments. *, p < 0.05 compared with untreated cells (control (Ctl)). C and D, confluent mCCDcl1 cells were preincubated with or without 100 ng/ml LPS for 2 h (SGK1 (C)) or 4 h (ENaC α-subunit (D)). Cells were then treated with 5 × 10^{-6} M actinomycin D without (solid line) or with (dashed line) 100 ng/ml LPS. Total RNA was extracted and reverse-transcribed, and real-time PCR was performed with primers specific for SGK1 (C) and ENaC α-subunit (D). E, mCCD cells were transfected with a plasmid encoding the first 3000 bp of the human SGK1 promoter fused to the firefly luciferase coding sequence together with a control plasmid encoding for Renilla luciferase. Luciferase activity was measured as described under "Experimental Procedures." Cells were treated with 10^{-6} M aldosterone for 24 h in the absence (Ctl) or presence of LPS stimulation for 3 (LPS 3h) or 6 additional h (LPS 6h). Results were calculated as a ratio of firefly/Renilla luciferase activity and expressed as -fold of control values. Bars are means ± S.E. from seven independent experiments. *, p < 0.05 compared with control values. F, mCCDcl1 cells were transiently transfected by electroporation with scramble RNAi (Scr) (open bars) or SGK1 RNAi (Si) (filled bars), seeded on filters, grown for 24 h, and incubated for another 24 h in hormone- and serum-deprived medium prior to an additional 6 h of incubation in the presence of 10^{-6} M aldosterone (Aldo). Total protein was extracted and separated by 10% SDS-PAGE prior to Western blotting using an anti-SGK1 antibody. A representative experiment of four total experiments is shown (inset). Total RNA was extracted and reverse-transcribed, and real-time PCR was performed with primers specific for SGK1 (right panel) or ENaC α-subunit (left panel). Results are expressed as -fold of control. *, p < 0.05 compared with control cells.
the presence of increasing concentrations of aldosterone. We observed that SGK1 mRNA expression was decreased whatever the dose of aldosterone used (Fig. 5A). Similarly, SGK1 protein expression decreased to ~50% independently of the dose of aldosterone used (Fig. 5, B and C). A similar down-regulation of SGK1 protein expression by LPS was observed in cells co-treated with $10^{-7}$ M corticosterone and $10^{-8}$ M aldosterone (data not shown).

Like native CD principal cells, mCCD$_{el}$ cells express both MR and GR, and either or both receptors can be stimulated depending on aldosterone concentration (4). We investigated which pathway mediates the down-regulation of SGK1 in the presence of proinflammatory stimuli. We therefore assessed whether LPS directly alters MR and/or GR abundance or activity leading to changes in gene transcription. We first assessed total protein expression of both receptors in mCCD$_{el}$ cells treated or not with LPS. The results show that neither MR (Fig. 6A, top row, and B, left panel) nor GR (Fig. 6A, middle row, and B, right panel) protein expression was decreased after 6 h of LPS stimulation. To investigate a possible alteration of receptor activity despite unchanged expression, specific pharmacological inhibitors were tested. Spironolactone ($10^{-6}$ M), a competitive MR inhibitor, was expected to block the effect produced by low concentrations of aldosterone ($10^{-8}$ M), whereas RU486 ($10^{-5}$ M), a competitive GR inhibitor, was expected to block the effect produced by high concentrations of aldosterone ($10^{-6}$ M) as shown previously in mCCD$_{el}$ cells (4). As expected, spironolactone mainly antagonized the increase of SGK1 expression induced by $10^{-8}$ M aldosterone (as percent of control ± S.E.: 324 ± 32 versus 106 ± 9 in the presence of spironolactone, $p < 0.05$), whereas it only partially prevented the effect induced by $10^{-6}$ M aldosterone (1755 ± 311 versus 1399 ± 288, $p = 0.42$) (Fig. 6C). However, SGK1 mRNA accumulation in response to either $10^{-8}$ or $10^{-6}$ M aldosterone was attenuated by LPS to a similar extent (50–70%) in the presence or absence of spironolactone (Fig. 6C). As expected, RU486 mainly inhibited the effect of $10^{-6}$ M aldosterone (as percent of control ± S.E.: 2019 ± 738 without RU versus 176 ± 20 with RU, $p < 0.05$), whereas it did not alter the effect of $10^{-8}$ M aldosterone (Fig. 6D). Fig. 6D shows that despite an efficient GR blockade by RU486, LPS still reduced the relative aldosterone-induced SGK1 expression. Altogether, our results indicate that the effect of LPS on SGK1 expression cannot be explained by alterations of MR and GR activity alone.

Activation of the Canonical NF-κB Pathway Mediates the Inhibitory Effect of LPS on SGK1 mRNA Expression—To confirm the regulation of SGK1 by specific NF-κB pathway activation, we used mCCD$_{el}$ cells transfected with a constitutively active IKKβ mutant. Cell transfection resulted in a 5-fold increase of IKKβ mRNA expression (Fig. 7A). TNFα mRNA expression, taken as a marker of IKKβ activity, significantly increased by 6–8-fold in cells transfected with constitutively active IKKβ as compared with control cells (Fig. 7B). The results depicted in Fig. 7C indicate that expression of constitutively active IKKβ alone down-regulated SGK1 mRNA expression in the absence of LPS or cytokine treatment.

Because activation of IKKβ alone mimicked down-regulated SGK1 expression observed in response to LPS and inflammasome cytokines, and MR and GR antagonism did not appear to be responsible for this down-regulation, we studied a potential direct pathway of NF-κB in regulated SGK1 expression in mCCD$_{el}$ cells. LPS treatment induces massive nuclear translocation of p50, p65, and c-Rel NF-κB subunits in mCCD$_{el}$ cells. Therefore, we performed knockdown of p65, p105/p50, and c-Rel by RNAi to determine whether these subunits are limiting steps in the observed regulation of SGK1 expression. RNAi

3 V. Leroy, unpublished observations.
decreased p65 mRNA levels by 50–60% in the absence of LPS and by more than two-thirds in LPS-treated cells (Fig. 8A). The efficiency of p65 knockdown was further assessed by Western blotting, and the results showed that p65 protein expression was efficiently suppressed. As shown by Fig. 8B, p65 knock-
down increased SGK1 mRNA expression in the presence or absence of LPS. This indicates that the p65 subunit exerts an inhibitory effect on SGK1 mRNA accumulation. Knockdown of p105/p50 by RNAi decreased both p105 mRNA (the precursor of p50) and p50 protein expression by about two-thirds (Fig. 8C). Knockdown of p50 had no effect on SGK1 mRNA levels, (Fig. 8D). Finally silencing of c-Rel resulting in decreased mRNA, and protein expression did not alter SGK1 mRNA expression either (data not shown). These findings indicated that the p65 NF-κB subunit plays an important role in LPS-induced down-regulated SGK1 expression, probably by associating with p50 as a heterodimer.

In addition to p65-p50 heterodimers, the p50 NF-κB subunit can form homodimers in response to proinflammatory cytokines. These homodimers behave as transcriptional inhibitors, as demonstrated for 11-βHSD2 in response to TNFα (31). To test the possibility that p50 homodimers also participate in the inhibitory effect of LPS and cytokines on SGK1 expression, we transfected cells with either p105 (the precursor of p50 NF-κB subunit, which undergoes constitutive processing) or a truncated cDNA directly encoding p50. Cell transfection resulted in mRNA and p50 protein (Fig. 9, A and B) overexpression. SGK1 mRNA abundance was not altered by the presence of high levels of p50 protein. Therefore, it is unlikely that the observed effects of proinflammatory stimuli on SGK1 expression are mediated by p50 homodimers, leaving p65-p50 heterodimers as being the most likely critical factor involved and p65 as the limiting factor.

This led us to investigate a potential direct regulation of SGK1 expression by NF-κB units. Computer-assisted analysis of the mouse SGK1 promoter revealed two potential NF-κB/Rel binding sites (16), located −2488 bp (GGCGCTTCCA) and −1975 bp (CGGAATCGCC) upstream of the ATG start codon, in addition to the putative glucocorticoid-responsive element site located at −1000 kb (Fig. 10A, GRE). Importantly, putative NF-κB/Rel sites were also found at similar locations along the rat and human SGK1 promoters, indicating a conservation between species (35). To confirm that p65 binds to either or both putative NF-κB binding sites, we performed a ChIP assay using an anti-p65 antibody. ChIP analysis revealed a 4-fold increase (p < 0.05) in the PCR-amplified signal from DNA fragments from mCCD cells containing putative NF-κB binding site that were obtained from LPS-treated cells as compared with signals obtained from untreated cells (Fig. 10, B and C). NF-κB p65 subunit did not appear to significantly bind to either NF-κB binding site under basal conditions. These results indicate that the SGK1 mouse promoter contains at least two NF-κB binding sites that are occupied by p65 in response to LPS stimulation.

MAPKs are known to be activated in response to proinflammatory stimuli and may participate in controlled SGK1 expression (36). We therefore assessed the effect of specific MAPK pharmacological inhibition on down-regulated SGK1 mRNA expression induced by LPS. mCCD cells were preincubated for 1 h without or with 10−5 M UO126, an extracellular signal-regulated kinase (ERK) inhibitor, SB203580, a p38 kinase inhibitor or SP60012, a c-Jun NH2-terminal kinase (JNK) inhibitor, prior to LPS treatment. None of these inhibitors succeeded in preventing SGK1 down-regulated expression in response to LPS (data not shown).

**DISCUSSION**

The results of the present study reveal that proinflammatory cytokines and LPS antagonize electrogenic Na+ transport at least partly via NF-κB-dependent inhibition of SGK1 transcrip-
NF-κB Inhibits SGK1 Expression

tion. This may in turn participate in the regulation of ENaC activity as well as its expression.

In the highly differentiated aldosterone-responsive mCCDcl1 principal cell line (4), as well as in mpkCCD_{cl4} cells, proinflammatory stimuli decreased basal as well as gluco- and mineralocorticoid-stimulated electrogenic Na\(^+\) transport. This highlights the major role played by local factors that act in parallel with systemic hormones in the regulation of ion transport in the CD. Indeed, the observed inhibition of electrogenic vectorial Na\(^+\) transport would lead to important renal Na\(^+\) loss because the CD is the site of final adjustment of tubular Na\(^+\) handling according to the requirement of Na\(^+\) balance.

This observation may have several physiological significances. In renal epithelial cells, Na,K-ATPase-mediated extrusion of intracellular Na\(^+\) consumes an important part of cellular ATP (37). Therefore, a reduction of transport activity may provide a means of sparing cellular energy resources, which favors cell survival under conditions of metabolic stress. Moreover, as aldosterone itself has been shown recently to activate the NF-κB pathway in collecting duct cells (38), the observed effect of NF-κB activation may constitute a negative feedback loop of aldosterone activity on Na\(^+\) transport. Finally, as a consequence of our observations, under pathologic conditions, activation of the NF-κB pathway in the renal tubulointerstitium is expected to induce a need for higher systemic concentrations of aldosterone for the maintenance of similar Na\(^+\) transport activity along the CD.

The molecular basis of the inhibition of Na\(^+\) transport by proinflammatory stimuli appears to rely on the down-regulation of SGK1, a major controller of Na\(^+\) transport in CD principal cells, mostly at the transcriptional level. SGK1 mRNA expression was indeed decreased to a great extent by LPS and other activators of the NF-κB pathway such as IL1β and TNFα. Protein expression of SGK1 was also down-regulated. This is expected to decrease Na\(^+\) transport, as SGK1 is a critical modulator of ENaC and Na,K-ATPase activity at least in part via modulation of their cell surface expression (39, 40). In addition to these well described effects, recent experimental evidence indicates that SGK1 may also play a role in the long term control of Na\(^+\) transport by relieving transcriptional repression of the ENaC α-subunit gene by modulating the histone methylation state (11). The results of the present study also suggest that inhibition of SGK1 transcription participates in decreased ENaC α-subunit mRNA levels in response to NF-κB activation. Indeed, inhibition of SGK1 mRNA accumulation preceded the decrease in ENaC α-subunit mRNA expression, and knock-down of SGK1 resulted in decreased expression of ENaC α-subunit mRNA. These results highlight the central role of SGK1 transcriptional inhibition by proinflammatory factors. However, we cannot exclude an additional role in which proinflammatory factors may act directly on the ENaC α-subunit promoter.

SGK1 expression appears to be controlled by proinflammatory stimuli. We first established that decreased SGK1 mRNA levels are not due to enhanced degradation. The results of our luciferase reporter gene assay strongly suggest that inhibition of SGK1 gene transcription is the most likely mechanism to explain the observed LPS-induced down-regulation of SGK1; this down-regulation could be observed in mouse and rat tissue as well as in a human SGK1 promoter. Given the well established opposition between the GR and the NF-κB pathway (23), it was tempting to speculate that NF-κB activation modulates SGK1 expression via inhibition of MR- and/or GR-dependent signaling. This hypothesis was, however, not supported by our experimental findings, given the absence of regulation of MR and GR expression and activity by active NF-κB. A direct pathway between the NF-κB system and SGK1 regulation appears more likely, as expression of a constitutively active IKKβ mutant, the protein kinase that activates the canonical NF-κB pathway via phosphorylation of IkBα and p65 NF-κB subunit, inhibited both basal and aldosterone-induced SGK1 expression. Similarly, silencing of p65 increased SGK1 mRNA expression. The fact that silencing of p65 did not abolish the effect produced by LPS is most likely related to the persistent activation (via phosphorylation and acetylation, for example) of remaining p65 subunits by LPS. Finally, LPS induced the NF-κB p65 subunit to bind to the SGK1 promoter as demonstrated by ChIP analysis. Such decreased transcriptional activity induced by direct binding of the NF-κB p65 subunit to a target gene promoter has recently been described for other genes (41, 42). Interestingly, IKKβ has been demonstrated to interact physically with the β-subunit of ENaC, and overexpression of wild-type IKKβ increased ENaC current in the Xenopus oocyte expression system (38). Here we demonstrate that a constitutively active IKKβ mutant inhibits SGK1 transcription, which does not exclude the possibility that IKKβ also directly controls ENaC activity independently of NF-κB activation (17). In addition, stimulation of ENaC activity and inhibition of SGK1 transcription may exhibit different time courses, as we have shown that NF-κB activation by TNFα stimulates amiloride-sensitive current within minutes (43) in mpkCCD_{cl4} cells, whereas LPS inhibits the amiloride-sensitive current within hours in mCCD_{cl1} cells, as shown here.

In conclusion, we have demonstrated that the NF-κB pathway modulates Na\(^+\) transport in CD principal cells. This effect is at least partly due to inhibition of SGK1 gene transcription via binding of p65 NF-κB subunit to specific sites of the SGK1 promoter, an event that very likely regulates αENaC and Na,K-ATPase α-subunit activity and that may also participate in regulating αENaC expression. The inhibitory effect of NF-κB on Na\(^+\) transport may participate in salt wasting observed in the context of tubulointerstitial inflammation of the kidney, and it may also indirectly influence systemic aldosterone levels. Moreover, given the pleiotropic role of SGK1 at the cellular level, reduced expression of NF-κB by SGK1 may play a role in cell survival as well as in local control of inflammation.

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