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Oishi Paul April 8, 2020

IL6 Mediated Activation of the Mineralocorticoid Receptor via Rac1 and Reactive Oxygen Species (ROS) in Hypertension

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Abstract

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By Oishi Paul

HTN is an inflammatory disease characterized by increased sodium (Na+) reabsorption and pro-inflammatory cytokines, like interleukin 6 (IL6). Although studies show a reduction in blood pressure with mineralocorticoid receptor (MR) antagonists, aldosterone (primary MR ligand) levels are not increased. This implies an alternate MR activation pathway. Herein, we investigate the L-NAME/High-Salt salt-sensitive hypertension (SS-HTN) mouse model. We note a ~2-fold increase in renal IL6 mRNA transcripts and increased expression of two primary Na+ transporters, epithelial sodium channel (ENaC) and sodium-chloride cotransporter (NCC). We have shown that IL6 activates the MR *in vitro*, and that Rac inhibition reduces mineralocorticoid response element-mediated transcriptional activation. We have also observed that IL6 increases reactive oxygen species (ROS) production, a key factor in HTN. We hypothesized that IL6 activates the MR through Rac1 and ROS generation, increasing ENaC activity. Since our data show that IL6 activates the MR and ROS production, we tested if Rac1 affects IL6-mediated MR nuclear translocation and ROS production in murine distal convoluted tubule cells (mDCT15). IL6-mediated MR translocation decreased after Rac1 knockdown. Given the reduction in MR activation, we next investigated the role of Rac1 in IL6 mediated ENaC activity. We used a voltohmmeter and measured ENaC current in mDCT15 cells. We observed a significant increase in ENaC current following IL6 treatment but noted that transfection of dominant-negative Rac1 inhibited IL6-induced ENaC current. For the role of Rac1 in IL6 mediated ROS production, Rac1 was inhibited in mDCT15 cells and treated with IL6 [100ng/mL]. Cells were stained with dihydroethidium and we visualized ROS production with confocal microscopy. Our data show that Rac1 knockdown inhibits IL6-induced ROS. Since IL6 cannot directly activate Rac1 pathway, we hypothesized that IL6 increases phosphorylation of extracellular signal-regulated kinase (ERK). We treated mDCT15 cells with IL6 and performed a Western Blot analysis. We found IL6 led to a four-fold increase in pERK2 and 0.77 increase in pERK1, while total ERK stayed constant. Together, our data support an alternative mechanism for MR and ENaC activation, in an aldosterone-independent manner. This novel research further adds to the growing literature on the relationship between hypertension and inflammation.

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Introduction and Background

Hypertension (HTN)

Hypertension (HTN) is a primary cause of cardiovascular disease and affects over 1 billion people around the world today [1]. HTN is characterized by an elevation of reactive oxygen species (ROS) and abnormal increases in sodium (Na^+) reabsorption. During the progression of HTN, one mechanism for increased ROS production is via NADPH oxidase [2]. In the kidney, ROS generation promotes sodium reabsorption, decreases glomerular filtration and leads to tissue damage [3].

The thiazide-sensitive sodium chloride-cotransporter (NCC) and epithelial sodium channel (ENaC) are two renal sodium transporters that are responsible for salt regulation and systemic blood pressure [4]. NCC is expressed throughout the distal convoluted tubule (DCT) with some expressed in the late DCT (DCT2) along with ENaC. ENaC is also expressed in the connecting tubule (CNT) and the cortical collecting duct (CCD). Together, these parts of the nephron make up the aldosterone-sensitive distal nephron (ASDN). Generally, the mineralocorticoid receptor (MR) can be activated by both glucocorticoids and mineralocorticoids. However, in the ASDN, the MR preferentially binds to aldosterone. These segments express the enzyme 11-beta-hydroxysteroid dehydrogenase (11B-HSD2). 11B-HSD2 oxidizes the glucocorticoid, cortisol, to cortisone, which cannot bind the mineralocorticoid receptor (MR). The mineralocorticoid receptor (MR) and aldosterone pathway is one of the primary mediators of the renal sodium reabsorption pathway in the late distal nephron [5]. The importance of the MR, and the overactivation of the MR during HTN, can be seen with

the clinical use of MR antagonists, spironolactone and eplerenone. When spironolactone is used as an additive treatment for resistant hypertensive patients, as studied in the PATHWAY-2 trial, there is a reduction in blood pressure and end-organ damage [6]. Interestingly, many of these HTN individuals do not exhibit an increased level of aldosterone. This may indicate an alternative mechanism for MR activation and MR-mediated sodium reabsorption in the late distal nephron.

Inflammation and Hypertension

The link between HTN and inflammation has been studied extensively over the years; however, there has been little conclusive evidence until recently. Seminal studies by Harrison and colleagues demonstrated a role for immune cells in the kidney, specifically T-cells and macrophages, in the development of HTN in animal models [7,8]. Additionally, in investigating a mechanism, other studies have shown that increased Na^+ load itself (as compared to the osmolyte control mannitol) can activate immune cells, specifically dendritic cells (DCs) and T-cells [7,9]. T-cell activation can lead to renal and vascular inflammation and increases in blood pressure [8]. Additionally, when activated by a stressor, dendritic cells (DC) release pro-inflammatory cytokines. Cytokines are pleiotropic proteins released primarily by immune cells and can interact with other cells [10]. One pro-inflammatory cytokine that is independently increased during HTN, not only in experimental animal models, but in hypertensive patients- is the cytokine interleukin-6 (IL6) [11].

IL6

IL6 is a small glycoprotein that acts as a pleiotropic cytokine that is often produced in response to infections and tissue injuries. IL6 drives many immune responses, including stimulation of immunoglobulin production and activation of T-cells. These responses are critical for initiating immune responses and the resolution of disease; however, these same cytokines can contribute to other deleterious effects. Cytokines, such as IL6, can contribute to endothelial dysfunction and vascular hypertrophy [12]. The vasculature is responsive to IL6 via classical IL6 signaling involving a membrane bound IL6 receptor (IL6R) and a membrane bound glycoprotein (gp130). Increased plasma levels of IL6 are correlated with increased blood pressure and plasma angiotensin 2 (AngII) levels [12,13]. Other studies have also suggested that IL6 can affect the expression and activity of endothelial nitric oxide synthase (eNOS) and NADPH oxidase, thus affecting nitric oxide (NO) production and contributing to increased oxidative stress [13]. The expression of the IL6R is normally very low and is expressed in only a few types of cells, thus IL6 signaling is usually low. However, in patients with septic shock, high IL6 levels have been shown to be associated with the progression of multiple organ failure [14]. The binding of IL6/IL6R to gp130 initiates many intracellular signaling pathways, including the Janus Kinase (Jak)-Signal Transducer and Activator of Transcription (STAT) pathways. JAK/STAT pathways can be activated by vascular stress factors, such as AngII, oxidative stress, and IL6 signaling [13]. Many cytokines use the JAK/STAT signaling pathway to transmit extracellular signals to the nucleus [13]. In the

context of this paper, once STAT molecules are phosphorylated, they can move into the nucleus and mediate transcription of IL6-dependent genes.

IL6 and Hypertension

Our previous studies have shown that IL6 infusion (16ng/hr, 72 hrs) can decrease total urinary volume excretion and sodium excretion. This observation led us to investigate the IL6 signaling pathway in the context of hypertension. As mentioned earlier, the effectiveness of spironolactone (MR antagonist) in lowering blood pressure suggests an overactivation of the MR in hypertension. Additionally, we have shown that IL6 can activate the MR in the late distal nephron. These studies have been performed both *in vitro* using cell lines corresponding with the late distal nephron (DCT2, mDCT15 cell line) and *in vivo* studies. The mDCT15 cell line is currently one of the best available cell culture models for the late distal convoluted tubule (DCT2), expressing many native cell signaling molecules and receptors [4]. Additionally, we have noted that IL6 infusion increases renal ENaC and NCC expression, suggesting IL6 can increase sodium reabsorption via MR activation. The mechanism by which IL6 activates the MR in this context, however, is unclear.

Reactive Oxygen Species

Hypertension is associated with an elevation in reactive oxygen species (ROS) [3]. In living cells, ROS is naturally produced through at least two pathways, specifically by

mitochondria during oxidative phosphorylation and by the enzyme NADPH oxidase (NOX). Mitochondrial ROS are generally neutralized by ROS degrading enzymes within the mitochondria (such as mitochondrial superoxide dismutase). But in pathological circumstances, large excesses of mitochondrial ROS can lead to apoptosis, which is unlikely in this context. However, NOX-generated ROS can lead to a disturbance in the normal redox state of tissues that can lead to oxidative stress (making this pathway more relevant to this study). Superoxide (O_2^-), a ROS derived from an oxygen radical, which is produced in response to appropriate engagement of membrane receptors by NADPH oxidase [15]. Active NADPH is a ubiquitous source of superoxide. ROS generation can facilitate oxidative modification of biological macromolecules-- including nucleic acids, sugars, lipids and protein. This can result in DNA damage and inactivation of key enzymes [16]. In the kidneys, ROS promotes salt absorption, decreases glomerular filtration, and leads to tissue damage [3].

Ras-related C3 botulinum toxin substrate 1 (Rac1)

Studies have shown that Rac1 (Rho GTPase) promotes the assembly of NADPH oxidase complex [15]. Rac1 is required to anchor cytosolic p67 subunit to the membrane for the assembly and activation of NADPH oxidase, leading to superoxide formation [17]. Novel research findings show that Rac1 may act as an upstream regulator of the MR and serve as a determinant for salt sensitivity and blood pressure [18]. However, the way Rac1 becomes activated in this context is unclear.

Our preliminary data suggest a mechanism for IL6 mediated MR and ENaC activation. Interestingly, we observed that Rac inhibition (EHT drug) reduces IL6 mediated mineralocorticoid response element (MRE) mediated transcriptional activation, which is a downstream effect of MR activation. Additionally, we have also observed that IL6 increases NCC activity in our cell culture model for the DCT2. However, co-treatment with IL6 and a Rac inhibitor reduces this increased sodium transport. Thus, given the previous literature and our own findings, we will test the hypothesis that Rac1 affects IL6 mediated MR activation and ENaC activity through ROS production in the late distal nephron.

Methods

Salt-Sensitive Animal Hypertension Model

Mice were given an oral nitric oxide synthase (NOS) inhibitor L-NAME (0.5mg/mL) in their drinking water for two weeks, as described in *Itani et al* [19]. After a washout period (1 week), we gave the mice a high salt diet (4% NaCl) for two weeks. The mice were placed in metabolic cages before and after being placed on a high salt diet. During this time, their urine volume was noted. Additionally, using tail-cuff methods, the systolic blood pressure was noted for both groups. After 3-4 days in metabolic cages, the renal cortex was harvested. The total protein was isolated for western blot analysis and q-PCR. Renal ENaC alpha and pNCC/NCC expression were determined and q-PCR was used to quantify IL6 mRNA transcripts.

Cell Culture and Treatments

For cell culture experiments, we used a mammalian model for the DCT2 (mDCT15 cells) [20]. mDCT15 cells were plated on cell culture dishes and grown in growth media containing a 50:50 mix of DMEM/F12, 5% heat-inactivated fetal bovine serum (FBS) and 1% Penicillin/Streptomycin. Cells were incubated at 37°C, 5% CO_2. All transfections (5µg) were done at 50-70% confluence, with *X-fect* protocol.

Epithelial Volt-Ohm meter Measurements (EVOM)

mDCT15 cells were grown in a confluent monolayer on trans-well cell culture plates in media. Cells were transfected with Rac1 expression vectors (wildtype, knock down, and overexpression) and washed 4-6 hours later. After transfections, we measured

amiloride-sensitive sodium transport, or ENaC activity, using an epithelial voltohmmeter (EVOM) before and after treatment. The treatments were as follows: aldosterone (100nM), IL6 (100ng/mL), SC144 (gp130 inhibitor [2µmol]), spironolactone (MR antagonist, Spiro [10nM]). To ensure that this increase in ENaC activity was MR dependent, we transfected for Rac1 overexpression and treat cells with MR antagonist (Spiro) + IL6. Data were analyzed as fold changes from baseline measurements. To confirm that current reflected ENaC channels only, cells were treated with amiloride and currents measured reflected those of empty trans-wells.

Western Blot

mDCT15 cells grown in cell culture dishes and then treated with IL6 (100ng/mL, 30 min). After incubation, cells were lysed with Western lysis buffer (80 µl/well). Western lysis buffer was prepared on ice with 1% protease inhibitor, 1% phosphatase inhibitor, 1% EDTA, 97% M-PER. With total protein lysate, BCA was performed. Total protein lysate (40-60µg, boiled for 10 minutes) was electrophoresed on a 4-15% mini-protean TGX (Bio-Rad) protein gel and transferred onto a nitrocellulose membrane (Bio-Rad). Non-specific binding sites were blocked with either 5% dry milk and Tris-buffered saline solution with tween (TBST, 0.1%) or 5% w/v BSA and TBST (0.1%) for 1hr. The following primary antibodies were used (overnight, 4°C): Rac1 [1:500], pNCC [1:3000], ENaCα [1:5000], ERK [1:1000], pERK[1:1000], GAPDH [1:20,000], Beta Actin [1:6000]. Blots were washed 3x with TBST and 1x with TBS. Then membranes were probed with either fluorescent goat anti-mouse or goat anti-rabbit secondary antibodies (IRDye680)

for 1 hr. Blots were washed once more (3x TBST, 1x TBS). Membranes were then imaged using Licor Imaging Systems and intensity of signal was analyzed with ImageJ.

MR EGFP

mDCT15 cells were co-transfected (60-70% confluence, X-fect) with an EGFP-tagged MR and Rac1 expression vectors. We used our expression vectors for Rac1 to knock down the Rac1 gene or transfect for wild-type Rac1. Both groups were washed and treated with IL6 [100ng/mL] for 30 min. Cells were then washed, fixed with 4% paraformaldehyde (PFA), and mounted on slides with 4′,6-diamidino-2-phenylindole (DAPI, nuclear counterstain). The cells were imaged using confocal microscopy on an Olympus Fluoview 1000 confocal microscope at 40x magnification (with oil).

Reactive Oxygen Species

mDCT15 cells were transfected with Rac1 expression vectors. Specifically, we transfected with Rac1 dominant-negative (Rac1 knockdown vector), wild-type Rac1, and constitutively active Rac1. As a transfection control, we transfected pcDNA3 for the sham group. All groups were treated with IL6. Then cells were stained with dihydroethidium (DHE; ROS reporter) and using confocal microscopy (Olympus Fluoview 1000), we visualized ROS production. Live cells were imaged at 20x magnification with the following lasers: Alexa Fluor 488 and Alexa Fluor 633. Lasers were determined based the autofluorescence of mDCT15 cells and excitation/emission (nm) of DHE.

Results

Specific Aim 1: *Determine if L-NAME model of salt-sensitive hypertension in vivo has increased IL6 mRNA and increased expression of late distal nephron sodium transporters*. A paper published by Itani et al. (2016) demonstrated that administration of L-NAME and high salt diet (4%) led to increased systolic blood pressure [19]. Our data also support these observations (Figure 1). We also noted similar urine volume among control and L-NAME groups before the mice were placed on a high salt diet. However, after the first day of high salt diet (4% NaCl), there was a distinct difference in urine output. While both groups were fed a high-salt diet, the L-NAME treated mice had lower urine excretion (Figure 2). It was previously unknown exactly how this drug increases systolic blood pressure, especially given the wash-out period following L-NAME treatment. The Itani et al. (2016) paper also showed that the L-NAME/high-salt model showed increased levels of renal memory T cells and increased macrophage and dendritic cell surface expression. In this research, we observed that L-NAME treated mice had nearly two-times higher renal IL6 mRNA transcripts, in comparison to control (Figure 3). Additionally, we show that L-NAME treated mice had significantly higher renal ENaCα expression and higher pNCC/NCC expression (Figure 4). This suggests that increased renal IL6 and expression of sodium transporters may partially explain for the salt-sensitive hypertension that these L-NAME treated mice experience upon high salt diet consumption. These data support the role of IL6 in hypertension.

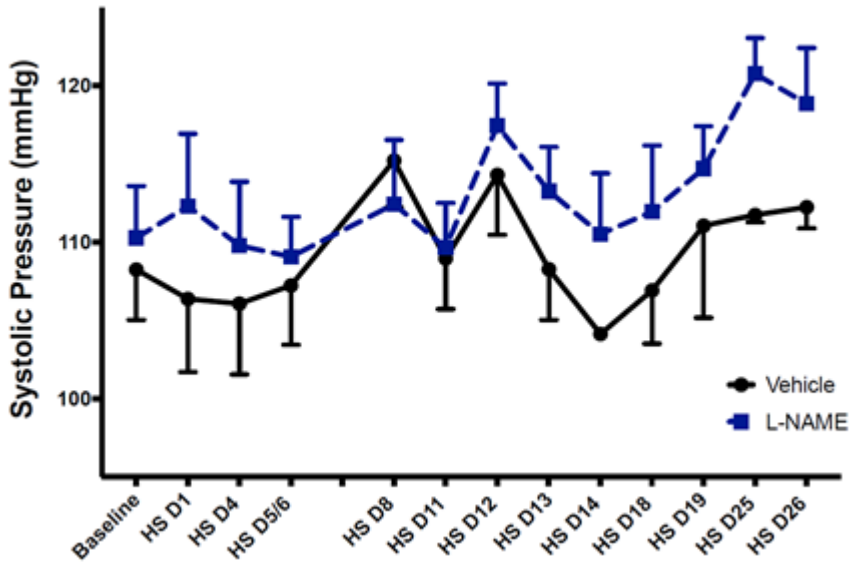

Figure 1: *L-NAME and High Salt Diet increases systemic blood pressure in vivo*. Systolic blood pressure measurements were obtained via tail cuff methods for L-NAME and vehicle groups of mice. A training period was implemented, and blood pressure measurements were averaged among ten trials (each day). *Data are expressed as mean±SEM; n=2-7.*

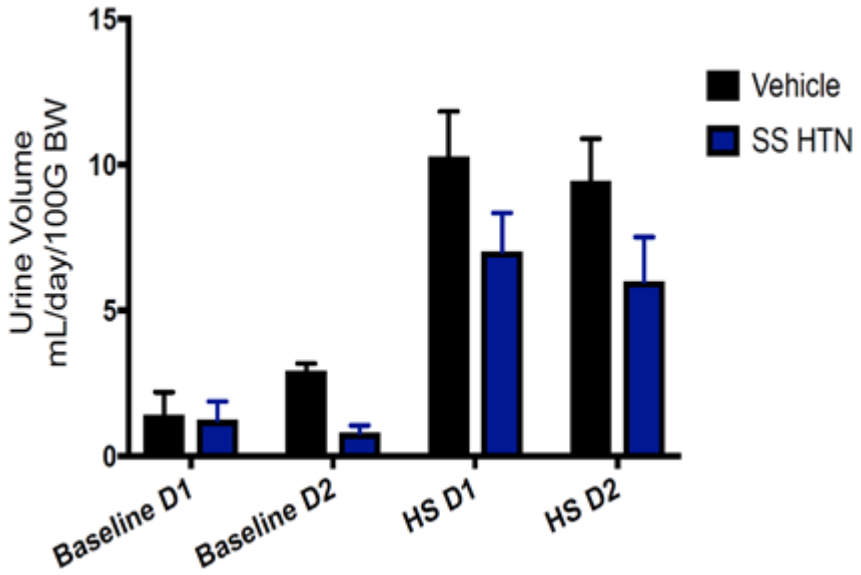

Figure 2: ***L-NAME treatment decreases urine volume excretion upon high salt diet consumption.*** Urine output measurements were taken from mice before and after the start of high salt diet (4% NaCl). Urine volume was normalized to body weight (per 100g). *Data are expressed as mean±SEM; n=3.*

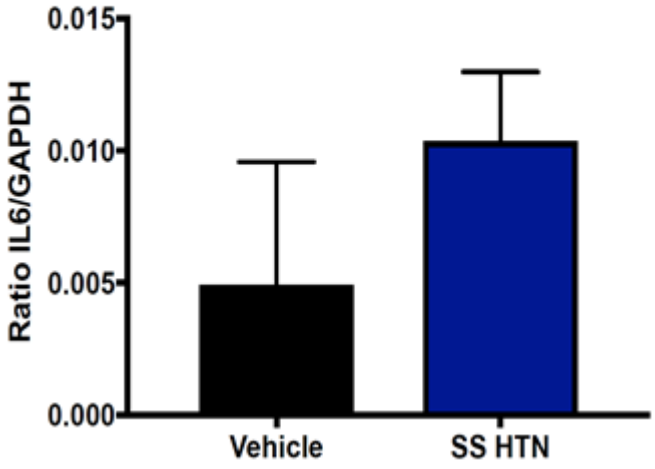

Figure 3: L-NAME treatment and High Salt Diet increases Interleukin 6 (IL6) mRNA transcript levels. SS-HTN group was treated with LNAME, while vehicle group had standard water. Quantitative PCR (qPCR) was used to determine IL6 mRNA transcripts, normalized to GAPDH. *Data are represented as mean±SEM; n=3-12.*

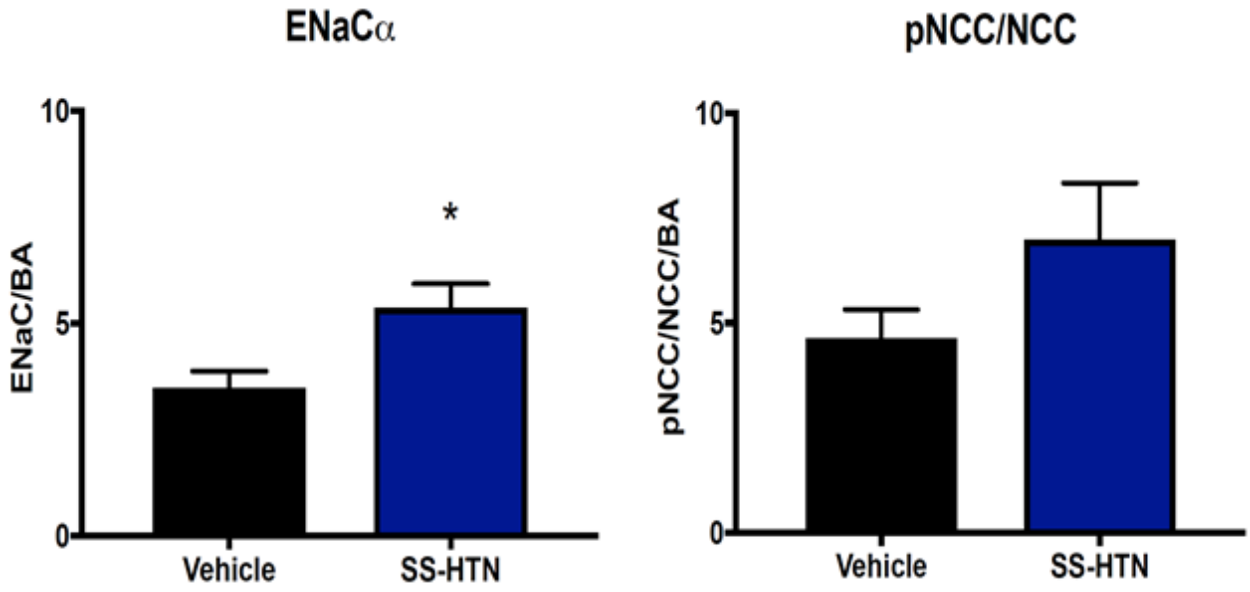

Figure 4: L-NAME treatment and High Salt Diet increases ENaC*α* and pNCC/NCC expression in renal cortex *in vivo.* Samples were made from kidney cortex lysate from mice treated with L-NAME (SS-HTN group) or standard water (vehicle). Total protein was used for western blot analysis, and data are normalized to beta-actin. *Data are represented as mean±SEM; n=4-5, *p<0.05 Student's t-test.* Error bars represent ±SEM.

Specific Aim 2: *IL6 mediated MR activation via Rac1 in mDCT15 cells.*

Previous data from our laboratory have shown that IL6 can activate the MR in mDCT15 cells. However, the specific mechanism was previously unknown. Given that we had observed that Rac inhibition reduced mineralocorticoid receptor element (MRE) activation (downstream MR activation target), we assumed the Rac GTPase molecule had a role in MR activation. With the co-transfection of eGFP-tagged MR vectors and Rac1 expression vectors, we noted reduced MR nuclear translocation when Rac1 was knocked-down in mDCT15 cells (Figures 5a and 5b). Despite IL6 treatment (100 ng/mL) Image 5b shows more “background” green, which is indicative of the eGFP-MR that remained in the cytoplasm, or inactive MR. Images were captured using confocal microscopy (40x magnification). Figures 5a and 5b depict representative images for each treatment group.

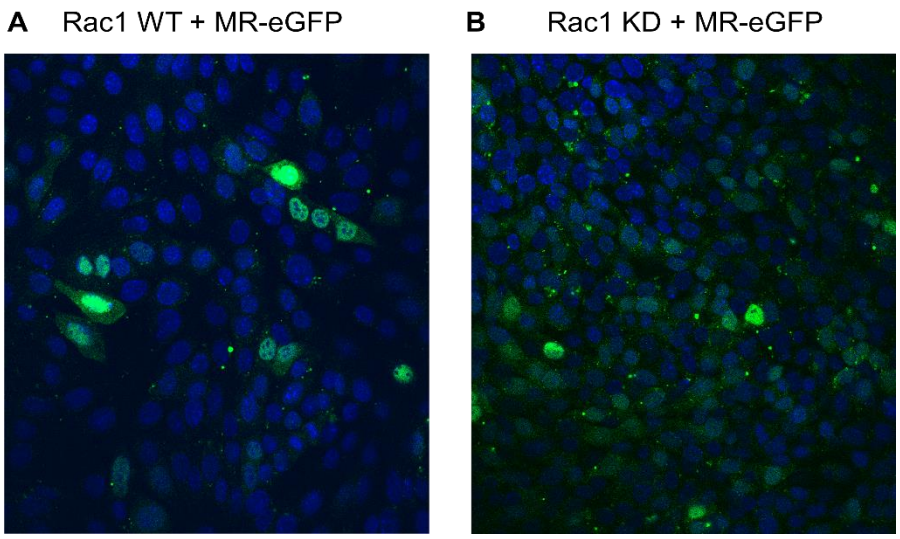

***Figure 5a (left): IL6 mediated MR Nuclear Translocation with wild-type Rac1 expression vector*.** mDCT15 cells were co-transfected with GFP-tagged MR and Rac1 wild-type expression vector. Cells were treated with IL6 prior to fixing onto slides for confocal microscopy. Cells were stained with DAPI, a nuclear counterstain (blue). Cytosolic to nuclear translocation of MR is indicative of MR activation, as illustrated by green fluorescence. Magnification: 40x (with oil)

Figure 5b (right): ***Knockdown of Rac1 reduces IL6 mediated MR Nuclear Translocation****.* mDCT15 cells were co-transfected with GFP-tagged MR and Rac1 dominant-negative expression vector. Cells were treated with IL6 prior to fixing onto slides for confocal microscopy. Cells were stained with DAPI, a nuclear counterstain (blue). Cytosolic to nuclear translocation of MR is indicative of MR activation, as illustrated by green fluorescence. Magnification: 40x (with oil)

Specific Aim 3: *IL6 mediated ENaC activation via Rac1 in mDCT15 cells.*

Previous data in the laboratory have shown that IL6 increases thiazide-sensitive sodium transport, or NCC activity, in mDCT15 cells. However, the role of IL6 on amiloride-sensitive sodium transport, or ENaC activity, in mDCT15 cells had been previously unknown. Using EVOM, we measured changes in ENaC current with treatment of aldosterone (100nM) or IL6 (100ng/mL). As shown in Figure 6, IL6 increased ENaC activity, even more than the native MR ligand, aldosterone. Specificity of IL6 activation pathway was confirmed with co-treatment of IL6 and SC144 (gp130 inhibitor, subunit on IL6 receptor). With SC144, IL6 mediated ENaC activation was completely inhibited. This suggests that this IL6 mediated sodium transport is specific to IL6 signaling. Given the reduction in IL6 mediated MR activation seen with the knockdown of Rac1, we became interested in the role of Rac1 in this context. Therefore, we used Rac1 expression vectors and treated cells with IL6. As shown in Figure 6, we observed a significant increase in ENaC current following IL6 treatment (12.53-fold change/baseline). However, DN Rac1 led to the reduction of ENaC activation, despite IL6 treatment (-1.16-fold change/baseline). This suggests the role of Rac1 in IL6 mediated ENaC activation in the late DCT2. Additionally, when cells were transfected with a constitutively active (CA) Rac1 and treated with IL6, Spironolactone (Spiro, MR antagonist) completely blocked ENaC activity. This suggests that MR activation is required for ENaC activity, in this context.

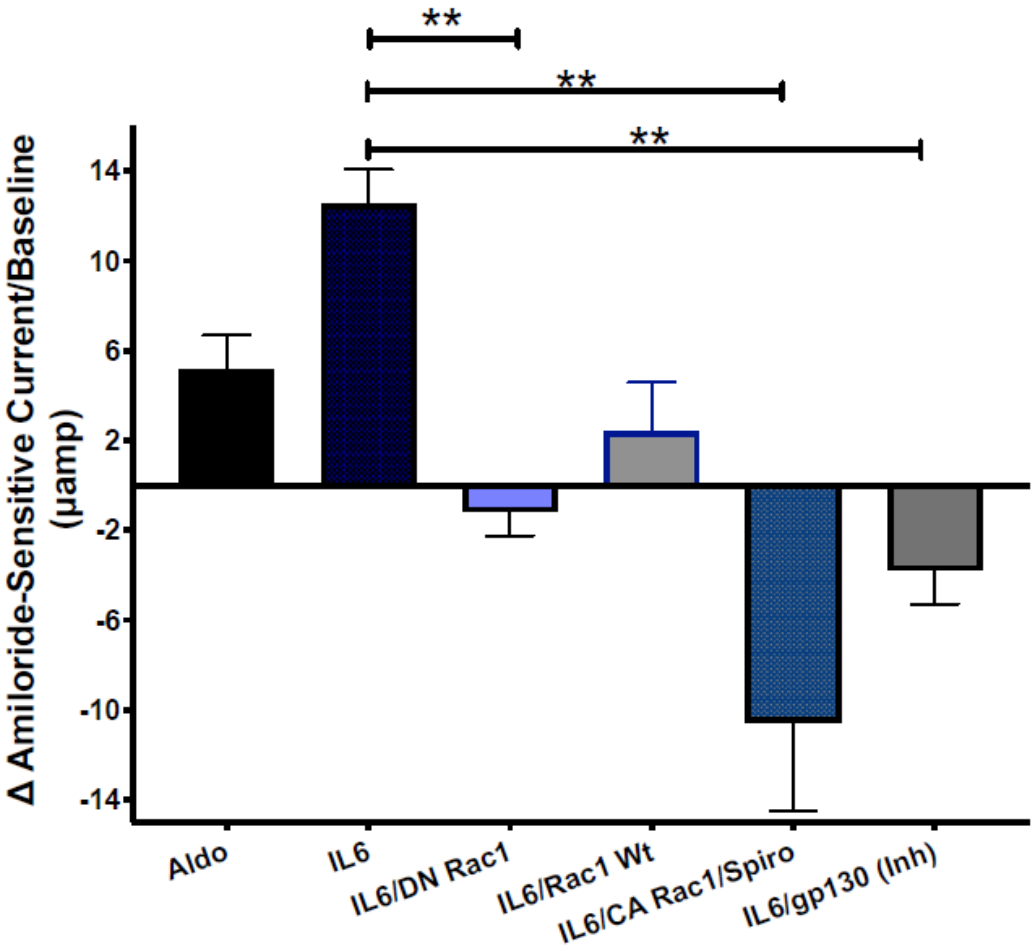

Figure 6: Rac1 knock-down inhibits IL6 mediated ENaC activation in mDCT15 cell line. Current was determined using EVOM and calculated with transepithelial electrical resistance and voltage measurements in cell culture model for DCT2 (mDCT15). mDCT15 cells were transfected with Rac1 vectors. Changes in current were compared to baseline measurements. Cells were treated for 1 hr. Data are expressed as mean ± SEM, n=4-15, *p<0.05 ANOVA, Kruskal-Wallis. Error bars represent ±SEM. Lines among groups indicate significant differences among different treatment groups.

Specific Aim 4: *IL6 mediated ROS production via Rac1 in mDCT15 cells*

Multiple studies have shown that MR activation leads to ENaC activation, such as Loffling et al. (2016) [21. Our data have shown that IL6 can activate the MR, but it seems unlikely that ENaC activation occurs solely through MR activation. Staruschenko et al. (2013) have shown that NADPH oxidase (NOX) and the generation of reactive oxygen species can directly activate ENaC [22]. Additionally, they also showed that aldosterone increases reactive oxygen species, such as superoxide. Studies conducted by Yin et al. (2011) suggest that ROS can activate phosphoinositide-3-kinase (P13K), which leads to the phosphorylation of PDK/SGK1 [23]. SGK1 prevents degradation of ENaC subunits from the plasma membrane [24]. By preventing degradation, this prolongs ENaC mediated sodium transport. Our preliminary laboratory data have shown that IL6 can increase ROS as well. Like our previous questions, we wanted to investigate the role of Rac1 in this context. As illustrated in Figure 7, transfection of Rac1 dominant-negative (Rac DN) to knock down Rac1 significantly reduces ROS production (1093±63 vs. 524±38, $p<0.001$), even with the presence of IL6 (100ng/mL). This suggests that Rac1 is vital for IL6 mediated ROS generation. These novel findings may further elucidate how MR activation and increased ROS production can activate ENaC in the late distal nephron.

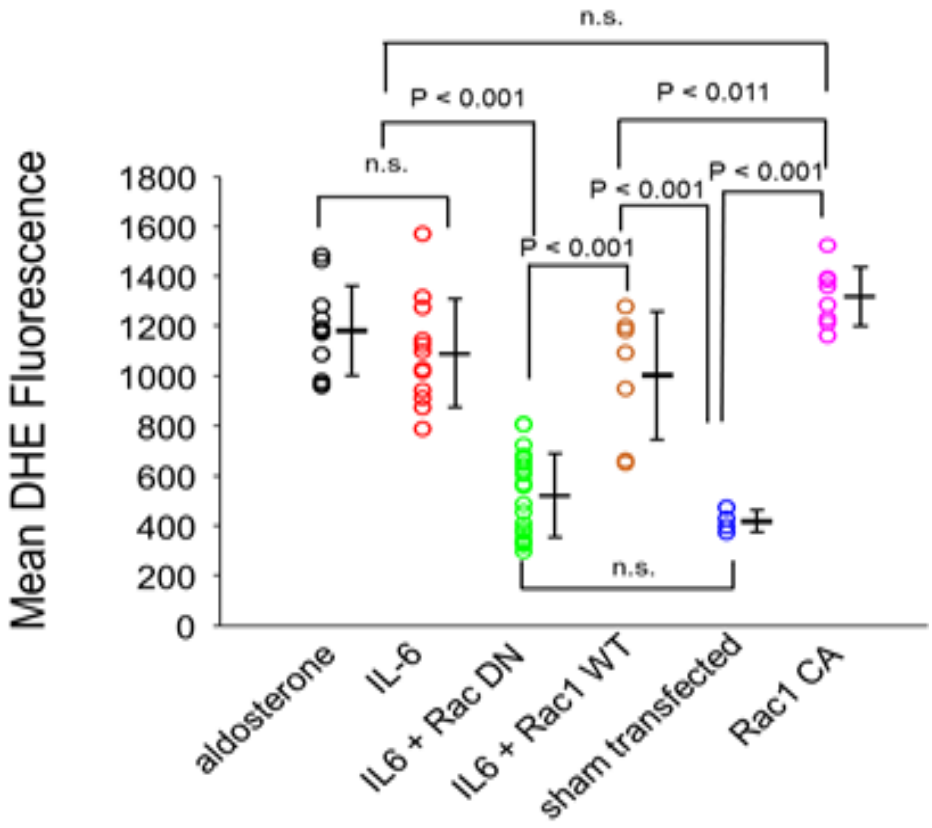

Figure 7: Rac1 knockdown reduces IL6 mediated reactive oxygen species (ROS) generation in mDCT15 cells. mDCT15 cells were transfected with Rac1 DN and treated with IL6. Effects of various Rac1 expressions in IL6 treated cells (100ng/mL) on ROS generation was measured by imaging dihydroethidium (DHE, superoxide reporter, 30 min). Mean DHE fluorescence was measured using confocal microscopy. Results are mean± SEM, n= 4-5 experiments, p-values as noted among treatment groups. “n.s” indicates non-significant differences. Error bars represent ±SEM. *One Way Analysis of Variance test.*

Specific Aim 5: *Determine if IL6 leads to activation of pERK in mDCT15 cells*

We wanted to investigate how IL6 can activate the Rac1 GTPase. ERK1/2 is an important signaling molecule that activates Rac1 via phosphorylation [25]. Rac1 activation has previously been shown to be inhibited by an Extracellular Signal-Regulated Kinase (ERK1/2) inhibitor in human mesothelial cells (MSTO-211H) [25]. Additionally, other studies have found that IL6 induced a temporary activation of ERK in neuroblastoma (SHSY5Y) cell line [26]. This experiment aimed to determine if IL6 (100ng/mL) can activate ERK1/2 in mDCT15 cells, which could then activate Rac1 in this signaling mechanism. As illustrated in Figure 8, when mDCT15 cells were treated with IL6 (30 min), there was an increase in phosphorylated ERK expression. This suggests that when IL6 is present, ERK1/2 is phosphorylated (or activated). For pERK1, IL6 increased protein expression to a greater extent than in the untreated group (3274±1477 vs. 1850±1132, n=4). Similarly, IL6 also increased pERK2 expression (11455±2690 vs. 2857±1063, n=4). Additionally, after IL6 treatment, there was a greater difference in activation of ERK2 than ERK1 (11455±2690 vs. 3274±1477, n=4). Despite IL6 treatment, however, total ERK expression did not substantially change. This finding suggests a signaling mechanism that connects IL6 signaling to Rac1 activation, ultimately leading to ROS production and MR activation, thus, mediating sodium reabsorption through ENaC.

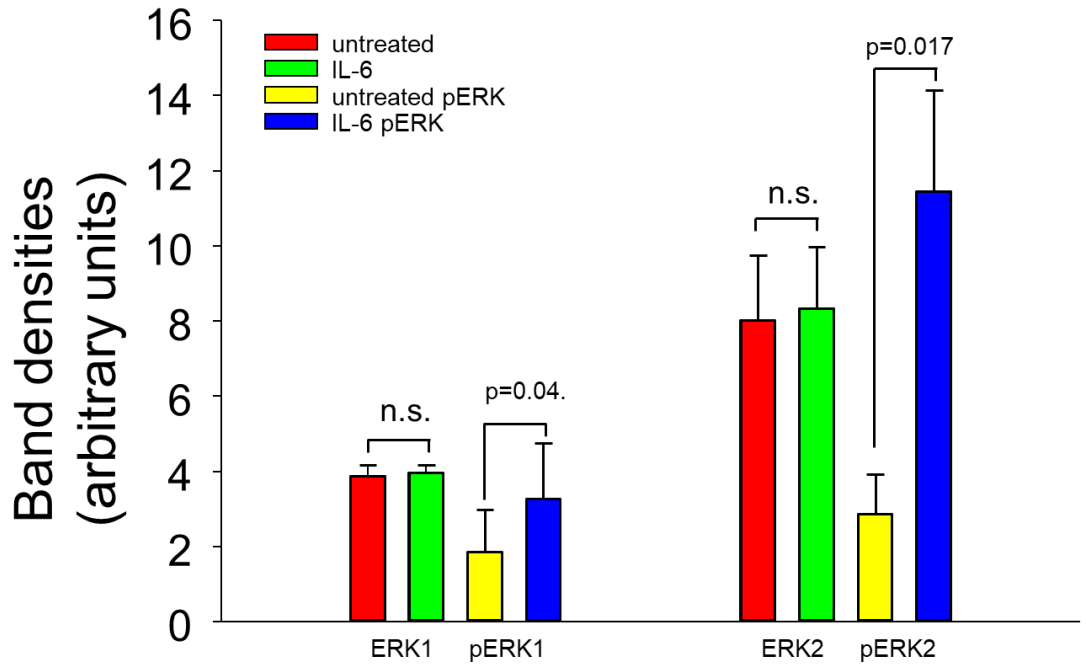

Figure 8: IL6 *increases phosphorylated ERK (pERK) expression in mDCT15 cells*. Total cell lysate of mDCT15 cells was isolated then used for a Western Blot analysis with ERK and pERK primary antibodies. Cells were treated with IL6 for seven, fifteen, and thirty minutes. Fluorescence was measured at thirty-minute treatment using ImageJ program. Y-axis values are illustrated as arbitrary units (in thousands). Error bars represent mean ±SEM. *Paired Student T-test,* p-values as indicated, n=4.

Discussion

Many hypertensive patients respond well to mineralocorticoid receptor (MR) blockers, such as spironolactone, suggesting overactivation of the MR. But many HTN patients do not have increased levels of the primary MR ligand, aldosterone. This suggests an alternative mechanism for MR activation and MR-mediated Na^+ reabsorption. Understanding these alternative mechanisms for MR transactivation is important to help develop specific therapies for treating cardiovascular and renal diseases. Excessive sodium load, as would be associated with high MR activity, has been shown to activate antigen-presenting cells (APCs) and T-cells, implicating inflammation in hypertension. These T-cells release cytokines, such as IL6, that have been shown to promote vascular and renal dysfunction and damage [27]. Given that hypertension (HTN) is now recognized as an inflammatory illness, these data provide new insight into the inflammatory pathways that may lead to the dysregulation of sodium reabsorption in the late distal nephron.

In this study, we present novel findings for IL-6 signaling in salt-sensitive hypertension. While previous data in the laboratory have shown that the proinflammatory cytokine IL6 can activate the MR in the late distal nephron *in vitro*, the L-NAME SS-HTN model further supports the role of IL6. Given the physiological changes noted in L-NAME treated mice, the data suggest this is an appropriate model to study salt-sensitive hypertension. Within this model, we noted nearly twice as much IL6 mRNA and increased renal ENaC and pNCC expression, in comparison to a control group.

While prior data from the Wynne Laboratory have shown that IL6 activates MR and ENaC, the data here implicate ERK/Rac1 and ROS generation in this process. We observed that Rac1 knockdown in mDCT15 cells led to reduced MR nuclear translocation (Figures 5a, 5b). Since this translocation is a primary step in MR activation, this suggests a reduction in MR activation. Furthermore, our data also show the role of Rac1 in IL6 mediated ENaC activation and ROS generation (Figures 6,7). Together, these data suggest that Rac1 activity affects downstream IL6-mediated responses in the DCT2, including MR, ROS production, and ENaC activation. In our IL6-Rac1-MR-ENaC signaling pathway, we hypothesized that IL6 activates ERK to mediate Rac1 activation. The data (as illustrated in Figure 8) support this hypothesis, as IL6 increases pERK expression in mDCT15 cells. IL6 increased both pERK1 and pERK2 expression, while ERK expression remained constant. A schematic has been added to this paper to summarize the results from our study and put them into the context of IL6 signaling in the DCT2 (Figure 9).

In addition to our own preliminary data on IL6 mediated increase in ROS production, a study conducted by Schrader et al. (2007) observed that Ang-II-induced increases in vascular superoxide were absent in IL6 deficient mice [28]. Collectively, these findings provide strong evidence that IL6 mediates superoxide generation. The data in this paper pertaining to the role of Rac1 support this current literature on superoxide production. The GTPase Rac1 is one of the cytosolic components that aid in the assembly of the NADPH oxidase complex, a key step in the progression of hypertension.

In summary, many studies have shown that excessive sodium can activate immune cells, such as antigen-presenting cells (APC) and dendritic cells. However, it has remained unknown as to why certain individuals become more salt-sensitive than others. Even without increased aldosterone, our cell culture experiments support the role of increased IL6 signaling in salt-sensitive hypertension. Our current data support IL6 signaling increasing pERK expression as a preliminary step to Rac1 and NADPH oxidase activation, ultimately producing superoxide and other ROS. Rac1 and ROS generation in IL6 mediated MR and ENaC activation may provide further insight on the pathogenesis of salt-sensitive hypertension. This may indicate how MR overactivation in the context of hypertension may occur in an aldosterone-independent manner. The data suggest that IL6 leads to extreme changes in the DCT2, especially in the context of sodium transport and oxidative injury. This novel research further elaborates on the relationship between hypertension and inflammation. This would provide further specific targets for designing therapeutics for treating hypertension.

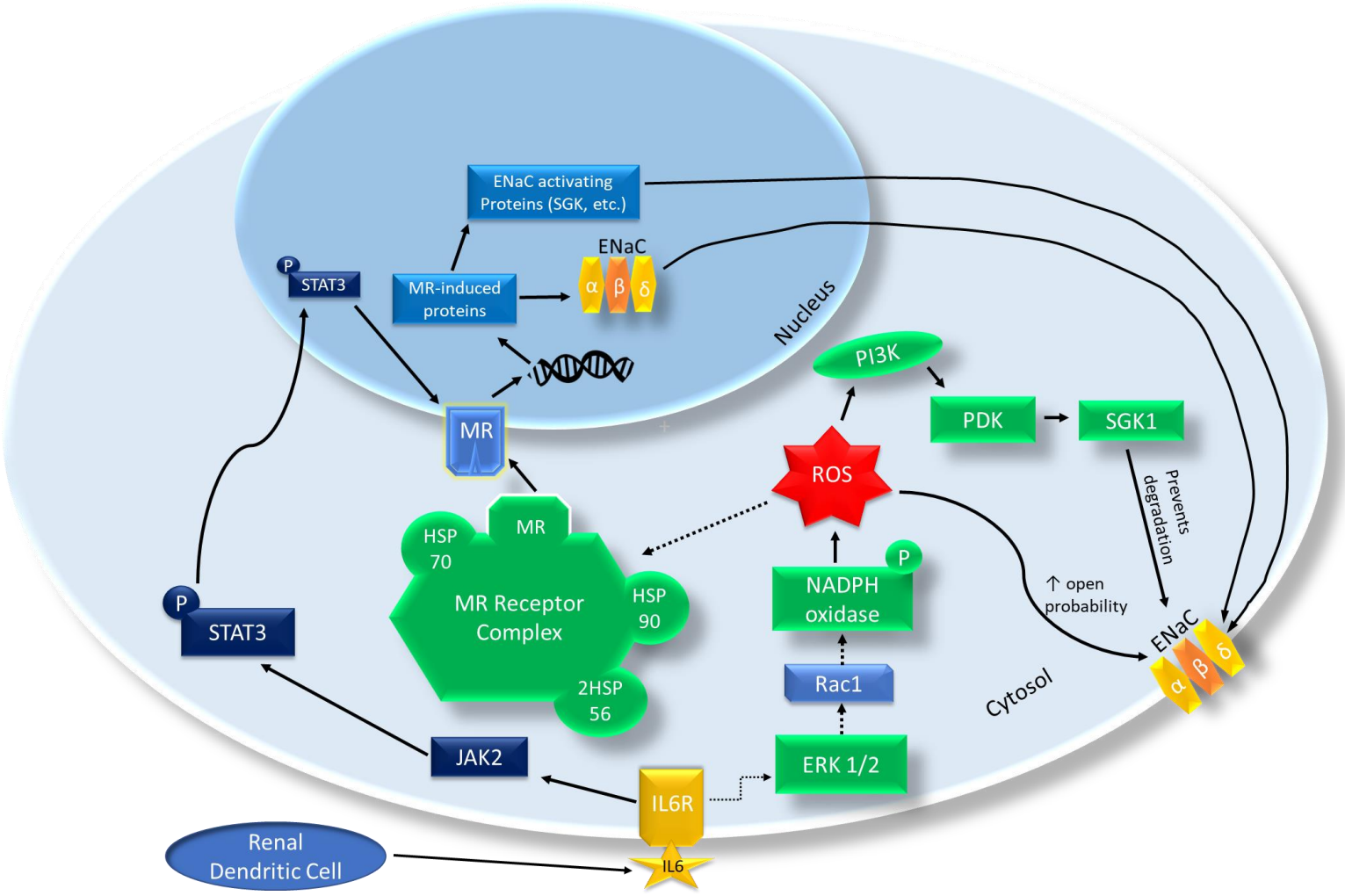

Figure 9: ***Proposed IL-6 Signaling Schematic in mDCT15 cells*****.** Dotted lines indicate correlational relationships and solid lines refer to causal relationships.

References

1. Go, A. S., Mozaffarian, D., Roger, V. L., Benjamin, E. J., Berry, J. D., Borden, W. B. Stroke Statistics, S. (2013). Heart disease and stroke statistics--2013 update: a report from the American Heart Association. *Circulation, 127*(1), e6-e245. doi:10.1161/CIR.0b013e31828124ad
2. Wilcox, C. (2002). Reactive Oxygen Species: Roles in Blood Pressure and Kidney Function. *Current Science, Curent Hypertension Reports*(4), 160-166
3. Lassegue, B., & Griendling, K. K. (2004). Reactive oxygen species in hypertension; An update. *Am J Hypertens, 17*(9), 852-860. doi:10.1016/j.amjhyper.2004.02.004
4. Wynne, B. M., Mistry, A. C., Al-Khalili, O., Mallick, R., Theilig, F., Eaton, D. C., & Hoover, R. S. (2017). Aldosterone Modulates the Association between NCC and ENaC. *Sci Rep, 7*(1), 4149. doi:10.1038/s41598-017-03510-5
5. Eaton, D. C., Malik, B., Saxena, N. C., Al-Khalili, O. K., & Yue, G. (2001). Mechanisms of aldosterone's action on epithelial Na + transport. *J Membr Biol, 184*(3), 313-319. doi:10.1007/s00232-001-0098-x
6. Williams, B., MacDonald, T. M., Morant, S., Webb, D. J., Sever, P., McInnes, G., Brown, M. J. (2015). Spironolactone versus placebo, bisoprolol, and doxazosin to determine the optimal treatment for drug-resistant hypertension (PATHWAY-2): a randomised, double-blind, crossover trial. *The Lancet, 386*(10008), 2059-2068. doi:10.1016/s0140-6736(15)00257-3
7. Barbaro, N. R., Foss, J. D., Kryshtal, D. O., Tsyba, N., Kumaresan, S., Harrison, D.G., Xiao, L., Kirabo, A. (2017). Dendritic Cell Amiloride-Sensitive Channels Knollmann, B.C., Mediate Sodium-Induced Inflammation and Hypertension. *Cell Rep, 21*(4), 1009-1020. doi:10.1016/j.celrep.2017.10.002
8. Harrison, D. G., Vinh, A., H., L., & Madhur, M. S. (2010). Role of the adaptive immune system in hypertension. *Current Opinion in Pharmacology*(10), 203-207. doi:10.1016\j.coph.2010.01.006
9. Foss, J. D., Kirabo, A., & Harrison, D. G. (2017). Do high-salt microenvironments drive hypertensive inflammation? *Am J Physiol Regul Integr Comp Physiol, 312*(1), R1-R4. doi:10.1152/ajpregu.00414.2016
10. Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. *Int Anesthesiol Clin, 45*(2), 27-37. doi:10.1097/AIA.0b013e318034194e
11. Lee, D. L., Leite, R., Fleming, C., Pollock, J. S., Webb, R. C., & Brands, M. W. (2004). Hypertensive response to acute stress is attenuated in interleukin-6 knockout mice. *Hypertension, 44*(3), 259-263. doi:10.1161/01.HYP.0000139913.56461.fb
12. Didion, S. P. (2017). Cellular and oxidative mechanisms associated with Interleukin-6 signaling in the vasculature. *Int J Mol Sci, 18*(12). doi:10.3390/ijms18122563
13. Grote, K., Luchtefeld, M., & Schieffer, B. (2005). JANUS under stress--role of JAK/STAT signaling pathway in vascular diseases. *Vascul Pharmacol, 43*(5), 357-363. doi:10.1016/j.vph.2005.08.021

14. Geppert, A., Steiner, A., Zorn, G., Delle-Karth, G., Koreny, M., Haumer, M., Heinz, G. (2002). Multiple organ failure in patients with cardiogenic shock is is associated with high plasma levels of interleukin-6. *Crit Care Med, 30*, 1987-1994. doi:10.1097/01.CCM.0000026730.19872.33
15. Pick, E. (2014). Role of the Rho GTPase Rac in the activation of the phagocyte NADPH oxidase: outsourcing a key task. *Small GTPases, 5*, e27952. doi:10.4161/sgtp.27952
16. Hordijk, P. L. (2006). Regulation of NADPH oxidases: the role of Rac proteins. *Circ Res, 98*(4), 453-462. doi:10.1161/01.RES.0000204727.46710.5e
17. Zhu, H., Shan, L., & Peng, T. (2009). Rac1 mediates sex difference in cardiac tumor necrosis factor-alpha expression via NADPH oxidase-ERK1/2/p38 MAPK pathway in endotoxemia. *J Mol Cell Cardiol, 47*(2), 264-274. doi:10.1016/j.yjmcc.2009.05.002
18. Fujita, T. (2014). Mechanism of salt-sensitive hypertension: focus on adrenal and sympathetic nervous systems. *J Am Soc Nephrol, 25*(6), 1148-1155. doi:10.1681/ASN.2013121258
19. Itani, H. A., Xiao, L., Saleh, M. A., Wu, J., Pilkinton, M. A., Dale, B. L., Harrison, D. G. (2016). CD70 exacerbates blood pressure elevation and renal damage in response to repeated hypertensive stimuli. *Circ Res, 118*(8), 1233-1243. doi:10.1161/CIRCRESAHA.115.308111
20. Ko, B., Mistry, A. C., Hanson, L., Mallick, R., Cooke, L. L., Hack, B. K., . . . Hoover, R. S. (2012). A new model of the distal convoluted tubule. *Am J Physiol Renal Physiol, 303*(5), F700-710. doi:10.1152/ajprenal.00139.2012
21. Czogalla, J., Vohra, T., Penton, D., Kirschmann, M., Craigie, E., & Loffing, J. (2016). The mineralocorticoid receptor (MR) regulates ENaC but not NCC in mice with random MR deletion. *Pflugers Arch, 468*(5), 849-858. doi:10.1007/s00424-016-1798-5
22. Ilatovskaya, D. V., Pavlov, T. S., Levchenko, V., & Staruschenko, A. (2013). ROS production as a common mechanism of ENaC regulation by EGF, insulin, and IGF-1. *Am J Physiol Cell Physiol, 304*(1), C102-111. doi:10.1152/ajpcell.00231.2012
23. Qi, S., Xin, Y., Guo, Y., Diao, Y., Kou, X., Luo, L., & Yin, Z. (2012). Ampelopsin reduces endotoxic inflammation via repressing ROS-mediated activation of PI3K/Akt/NF-kappaB signaling pathways. *Int Immunopharmacol, 12*(1), 278-287. doi:10.1016/j.intimp.2011.12.001
24. Lang, F., & Pearce, D. (2016). Regulation of the epithelial Na+ channel by the mTORC2/SGK1 pathway. *Nephrol Dial Transplant, 31*(2), 200-205. doi:10.1093/ndt/gfv270
25. Niba, E. T., Nagaya, H., Kanno, T., Tsuchiya, A., Gotoh, A., Tabata, C., Nishizaki, T. (2013). Crosstalk between PI3 kinase/PDK1/Akt/Rac1 and Ras/Raf/MEK/ERK pathways downstream PDGF receptor. *Cell Physiol Biochem, 31*(6), 905-913. doi:10.1159/000350108

26. Li, X. Z., Sui, C. Y., Chen, Q., Chen, X. P., Zhang, H., & Zhou, X. P. (2013). Promotion of autophagy at the maturation step by IL-6 is associated with the sustained mitogen-activated protein kinase/extracellular signal-regulated kinase activity. *Mol Cell Biochem, 380*(1-2), 219-227. doi:10.1007/s11010-013-1676-9
27. McMaster, W. G., Kirabo, A., Madhur, M. S., & Harrison, D. G. (2015). Inflammation, immunity, and hypertensive end-organ damage. *Circ Res, 116*(6), 1022-1033. doi:10.1161/CIRCRESAHA.116.303697
28. Schrader, L. I., Kinzenbaw, D. A., Johnson, A. W., Faraci, F. M., & Didion, S. P. (2007). IL-6 deficiency protects against angiotensin II induced endothelial dysfunction and hypertrophy. *Arterioscler Thromb Vasc Biol, 27*(12), 2576-2581. doi:10.1161/ATVBAHA.107.153080