MUTATED *KCNJ5* ACTIVATES THE ACUTE AND CHRONIC REGULATORY STEPS IN ALDOSTERONE PRODUCTION

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Abstract

Somatic and germline mutations in the inward rectifying K^+ channel (*KCNJ5*) are a common cause of primary aldosteronism (PA) in aldosterone producing adenoma and familial hyperaldosteronism type III, respectively. Dysregulation of adrenal cell calcium signaling represents one mechanism for mutated KCNJ5 stimulation of aldosterone synthase (*CYP11B2*) expression and aldosterone production. However, the mechanisms stimulating acute and chronic production of aldosterone by mutant *KCNJ5* have not been fully characterized. Herein, we defined the effects of the T158A *KCNJ5* mutation *(KCNJ5^{T158A})* on acute and chronic regulation of aldosterone production using an adrenal cell line with a doxycycline inducible *KCNJ5^{T158A}* gene (HAC15-TRE-*KCNJ5^{T158A}*). Doxycycline incubation caused a time-dependent increase in *KCNJ5^{T158A}* and *CYP11B2* mRNA and protein levels. Electrophysiological analyses confirm the loss of inward rectification and increased Na^+ permeability in *KCNJ5^{T158A}*-expressing cells. *KCNJ5^{T158A}* expression also led to activation of *CYP11B2* transcriptional regulators, NURR1 and ATF2. Acutely, *KCNJ5^{T158A}* stimulated the expression of total and phosphorylated steroidogenic acute regulatory protein (StAR). *KCNJ5^{T158A}* expression increased synthesis of aldosterone and the hybrid steroids 18-hydroxycortisol and 18-oxocortisol, measured with liquid chromatography-tandem mass spectrometry (LC-MS/MS). All of these stimulatory effects of *KCNJ5^{T158A}* were inhibited by the L-type Ca^{2+} channel blocker, verapamil. Overall, *KCNJ5^{T158A}* increases *CYP11B2* expression and production of aldosterone, corticosterone and hybrid steroids by

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upregulating both acute and chronic regulatory events in aldosterone production, and verapamil blocks KCNJ5^{T158A}-mediated pathways leading to aldosterone production.

Keywords

KCNJ5 mutations; primary aldosteronism (PA); adrenal; aldosterone

INTRODUCTION

In normal physiology, aldosterone production is mainly regulated by the renin-angiotensin-aldosterone system and plasma potassium levels. Primary aldosteronism (PA) is characterized by renin-independent aldosterone excess and represents the most common cause of secondary hypertension. The main causes of PA are aldosterone producing adenomas (APA) and bilateral adrenal hyperplasia. In 2011, Choi and colleagues reported mutations in the selectivity filter of the inward-rectifying K$^+$ channel *KCNJ5* in 35 % of APA (Choi, et al. 2011). In the past few years, several other mutations localized in or near the *KCNJ5* selectivity filter have been described, causing a change in permeability from only K$^+$ ions to allow influx of Na$^+$ ions (Akerstrom, et al. 2012; Akerstrom, et al. 2015; Boulkroun, et al. 2013; Charmandari, et al. 2012; Choi et al. 2011; Dutta, et al. 2014; Hardege, et al. 2015; Kuppusamy, et al. 2014; Lenzini, et al. 2015; Monticone, et al. 2015; Monticone, et al. 2012; Monticone, et al. 2013; Mulatero, et al. 2012b; Murthy, et al. 2012; Murthy, et al. 2014; Scholl and Lifton 2013; Scholl, et al. 2012; Thiel, et al. 2015; Williams, et al. 2013; Zennaro and Jeunemaitre 2011). The Na$^+$ influx triggers the activation of voltage-gated Ca^{2+} channels and downstream signaling pathways, which leads to elevated expression of aldosterone synthase (*CYP11B2*) and aldosterone production in basal and angiotensin II treated conditions (Hardege et al. 2015; Kuppusamy et al. 2014; Monticone et al. 2015; Monticone et al. 2012; Monticone et al. 2013; Mulatero et al. 2012b; Murthy et al. 2014; Oki, et al. 2012; Wang, et al. 2015; Williams et al. 2013; Williams, et al. 2014). The use of L-type Ca^{2+} channel blockers, such as nifedipine or verapamil, have been shown to block mutated *KCNJ5*-mediated aldosterone production *in vitro* (Kuppusamy et al. 2014; Monticone et al. 2012; Monticone et al. 2013; Oki et al. 2012; Tauber, et al. 2014). However, the details regarding the effects of KCNJ5^{T158A} on signaling events downstream of intracellular Ca^{2+} have not been well defined.

Most in vitro studies of mutant *KCNJ5* have used strategies with constitutive transgene expression in adrenocortical cells. These studies have provided useful findings regarding the effects of mutated *KCNJ5* on membrane potential, *CYP11B2* and *NURR1* transcription, and aldosterone production. Other studies have used transient methods of expression (such as electroporation), which by itself can be toxic to cells, requiring longer recovery period for cells, in addition to potentially activating stress-mediated signaling. Therefore, this strategy makes it difficult to define the acute actions of mutant KCNJ5 on adrenal cell function.

To overcome the toxic effects of transfections, we developed a doxycycline-inducible system for *KCNJ5* harboring the T158A mutation (*KCNJ5^{T158A}, encoding the mutant channel*

$Kir3.4^{T158A}$) in order to define the effect of $KCNJ5^{T158A}$ on acute and chronic events leading to aldosterone production.

MATERIALS AND METHODS

Cell Culture

Human adrenocortical carcinoma (HAC15) cells were cultured as described earlier (Monticone et al. 2012; Monticone et al. 2013; Parmar, et al. 2008; Wang, et al. 2012). The pLenti-CMV-rtTA3-Hygro vector was used to produce a lentivirus to generate the HAC15-CMV-rtTA3 cell line expressing the reverse tetracycline-controlled transactivator 3 (rtTA3) (Campeau, et al. 2009). These cells were further transduced with a lentivirus expressing $KCNJ5$ cDNA harboring the T158A mutation ($KCNJ5^{T158A}$) under the CMV promoter containing a Tet Operator element (TO), to generate the HAC15-TRE-$KCNJ5^{T158A}$ cells. Incubation with doxycycline prevents rtTA3 from physically binding to the TO, thus permitting expression of $KCNJ5^{T158A}$. The HAC15-CMV-rtTA3-hygro and HAC15-TRE-$KCNJ5^{T158A}$ cells were grown in Dulbecco's Modified Eagle's/Ham's F-12 medium (DMEM/F-12) containing 5% Cosmic calf serum (CCS, Hyclone, Logon UT), 1% ITS (Gibco) and antibiotics (penicillin-streptomycin and gentamicin). HAC15-TRE-$KCNJ5^{T158A}$ cells were further selected with 10 μg/mL puromycin. For experiments, cells were plated at a density of 50,000 cells/well in a 48-well dish (for studies involving gene expression analyses) or at 1 million cells/well in a 6-well dish (for nuclear protein isolation) for 48 h. After incubation in low-serum medium (DMEM/F-12 containing 0.1% CCS and antibiotics) for 24 h, $KCNJ5^{T158A}$ induction was initiated with 1 μg/mL doxycycline for the indicated times. For LC-MS/MS measurement of steroids, cells were incubated in doxycycline for 60 h. Inhibitor studies involved treatment of cells with 10 μM verapamil for 30 min prior to and during the course of doxycycline treatment.

For electrophysiological studies, HAC15-TRE- $KCNJ5^{T158A}$ cells treated with doxycycline for 60 h were plated on circular coverslips in 12-well plates at a density of 200,000 cells/well for 8–10 hours. During patching, the coverslips were rinsed in normal Tyrode's solution prior to patching. Due to previous observations that endogenous Kir3.4 is non-functional in HAC15 cells (Kienitz, et al. 2015), cells were transduced with lentiviruses for constitutive expression of wild-type $KCNJ5$ ($KCNJ5^{WT}$, encoding channel $Kir3.4^{WT}$) and selected with for antibiotic resistance for blasticidin. These cells over-expressing $KCNJ5^{WT}$ were used for the purpose of measuring control currents.

Viral transduction

Cells were plated for 24 h and treated with appropriate amounts of virus in growth medium devoid of any antibiotics and containing 8 μg/mL polybrene. Cells were centrifuged at 1200 rpm for 2 h and incubated overnight to attain maximal transduction efficiency (Wu, et al. 2009). Recovery was performed with the addition of 2× growth medium devoid of any antibiotics/polybrene. After an additional 48 h, cells were sub-cultured and maintained in normal growth medium with 10 μg/mL puromycin.

RNA isolation and real-time quantitative PCR (RT-qPCR)

Gene expression analyses were performed using Taqman primer-probes (to make a total of 900 nM of each primer and 400 nM probe per reaction; Life Technologies, Carlsbad, CA) and Kapa Probe fast qPCR kit master mix ABI Prism (Kapa Biosystems, Boston MA). Negative controls consisted of nuclease-free water in place of cDNA. The PCR program, consisting of 40 amplification cycles, was performed following the manufacturer's recommendations. Normalization of gene expression within each sample was performed by using the respective expression levels of *PPIA* (cyclophilin A) to calculate the Ct. Relative increase in gene expression was calculated using the $2^{-\Delta Ct}$ method.

LC-MS/MS measurements of steroids

A mixture of 60 μL aliquot of medium, internal standards and 3% isopropyl alcohol was loaded on supported liquid extraction plates (Novum, Phenomenex) and subsequently eluted with 2 mL of methyl-t-butyl ether. The organic phase was concentrated under nitrogen. The dried extract was reconstituted with 60 μL of methanol/deionized water (1:1) and transferred to a 0.25-mL vial insert. Samples (10 μL) were injected via autosampler and resolved with an Agilent 1290 binary pump HPLC on a Kinetex 50×2.1 mm, 3μm particle size biphenyl column (Phenomenex) using gradient elution with 0.25 mmol/L ammonium fluoride and methanol. The column effluent was directed into the source of an Agilent 6490 triple quadrupole mass spectrometer using electrospray ionization in positive ionization mode and analyzed using multiple reaction monitoring mode. Quantitation was accomplished by comparing ion currents for the monitored ions with 13-point quadratic external calibration curves (r^2 was minimum 0.995) and corrected for specimen dilution and recovery of internal standards using ChemStation and MassHunter software (Agilent). The retention times for steroids and respective internal standards are provided in Supplemental Table 2. Steroid levels were normalized to cellular protein and expressed as fold over basal.

Electrophysiology

Ion currents were recorded in whole-cell patch-clamp configuration using a MultiClamp 700B amplifier and Digidata 1440A digitizer (Molecular Devices, Sunnyvale, CA). Patch pipettes had resistances of 6–10 MΩ when filled with intracellular pipette solution and placed in extracellular solution. The external solution consisted of a simple HEPES-based Tyrode's solution (140 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$ and 1.8 mM $MgCl_2$). The internal pipette solution contained 95 mM K-gluconate, 15 mM KCl, 15 mM NaCl, 0.726 mM $CaCl_2$, 3 mM EGTA and 2.38 mM $MgCl_2$, 3 mM K_2-ATP, 0.025 mM K_2-GTP, 0.5 mM GTP-γ-S (pH 7.2 adjusted with KOH). In order to confirm Na^+ permeability of mutated KCNJ5 channels, NaCl in the external bath solution was replaced by choline chloride. Barium chloride (1 mM) was used to inhibit K^+ conductance. Data acquisition and analysis were performed using pCLAMP software (ver.10.3; Molecular Devices, Sunnyvale, CA). Current amplitudes were divided by cell capacitance (Cm) and expressed as current densities (pA/pF) to normalize for variable cell sizes.

RESULTS

Doxycycline causes time-dependent induction of $KCNJ5^{T158A}$

The HAC15-TRE-$KCNJ5^{T158A}$ cell model was generated with doxycycline-regulated $KCNJ5^{T158A}$ transgene expression using lentiviral transduction. HAC15-TRE-$KCNJ5^{T158A}$ cells were treated with 1 μg/mL doxycycline for the indicated times to induce $KCNJ5^{T158A}$ expression. $KCNJ5^{T158A}$ exhibited a time-dependent increase in mRNA (Figure 1, *Panel A*) and protein (Figure 1, *Panel B*). Transcript expression increased after 6 h of doxycycline incubation, peaked at 36 h (increasing by ~ 40-fold) and plateaued thereafter. Western analysis indicated an increase in KCNJ5^{T158A} protein levels. Semi-quantitative densitometric analysis indicated that KCNJ5^{T158A} protein increased approximately 5-fold to 10-fold between 24 h to 60 h. Electrophysiological analyses indicated that expression of Kir3.4^{T158A} channel by doxycycline incubation also revealed increased conductance to Na$^+$ in addition to K$^+$, as indicated by sensitivity to both barium and choline. In contrast, in cells constitutively expressing the Kir3.4^{WT} channel, channel conductance was sensitive to barium but not choline (Figure 2, Panels A–C). The Kir3.4^{T158A} channel also showed a loss of the inward rectification property compared to the Kir3.4^{WT} channel (Figure 2, Panels B and C).

$KCNJ5^{T158A}$ increased *CYP11B2* expression and aldosterone production, which were reversed by blockade of calcium channels

The temporal effects of doxycycline (1 μg/mL) on *CYP11B2* expression and aldosterone production in HAC15-TRE-$KCNJ5^{T158A}$ cells were investigated. The data show that, along with increased $KCNJ5^{T158A}$ expression, there was a time-dependent stimulation in *CYP11B2* mRNA and protein levels (Figure 3 *Panels A and B*). Specifically, transcript expression peaked at 36 h and plateaued thereafter (Figure 3 *Panel A*). CYP11B2 protein levels doubled every 12 h after 24 h doxycycline incubation, attaining a 5-fold increase over basal at 48 h and almost 10-fold over basal at 60 h. Treatment with the agonist angiotensin II (Ang II) served as a positive control and led to an increase in *CYP11B2* mRNA (by ~60-fold over basal) and protein (by 8-fold) (Figure 3, *Panels A and B*). Aldosterone levels also exhibited a time-dependent increase in response to Ang II and $KCNJ5^{T158A}$ expression (Figure 3, Panel C). Ang II stimulatory effects were observed as early as 24 h, while $KCNJ5^{T158A}$-induced aldosterone increase was detected at 48 h. At 60 h Ang II or doxycycline treatment, aldosterone levels were elevated (normalized to protein) by over 4-fold and 10-fold (compared to basal), respectively (Figure 2, *Panel D*). Finally, treatment with the L-type Ca^{2+} channel blocker verapamil (1, 3, 10 and 30 μM) blocked the stimulatory effects of Kir3.4^{T158A}- and Ang II-induced aldosterone production in a concentration-dependent manner (Figure 3, Panel D). Although the highest inhibitory effect on aldosterone was observed using 30 μM verapamil, this dose also exhibited some cytotoxic effects. Therefore, the most efficient dose for verapamil was 10 μM, which inhibited *Kir3.4^{T158A}*- and Ang II-stimulated aldosterone production by approximately 65% and 70%, respectively. Treatment with 10 μM verapamil also abrogated *Kir3.4^{T158A}*- and Ang II-induced *CYP11B2* mRNA and protein expression (by approximately 85% and 70%, respectively) (Figure 3, *Panels E and F*).

Effect of *KCNJ5^T158A* on adrenal steroidogenesis

To better define the effects of *KCNJ5^T158A* expression on adrenal steroidogenesis, cells were incubated in doxycycline for 60 h, and LC-MS/MS was performed to analyze the steroids in the pathway leading to aldosterone and cortisol production, as well as the hybrid steroids 18-hydroxycortisol (18OHF) and 18-oxocortisol (18oxoF). Figure 4, Panel A provides a schematic representation of the steroidogenic pathways for the synthesis of glucocorticoids and mineralocorticoids. *KCNJ5^T158A* expression led to an increase in the production of aldosterone, as well as aldosterone precursors 11-deoxycorticosterone (DOC) and corticosterone, and cortisol (Figure 4, *Panel B*). No changes in progesterone, 17-hydroxyprogesterone and 11-deoxycortisol were observed. In addition, *KCNJ5^T158A* increased the production of the hybrid steroids 18OHF and 18oxoF by over 6-fold.

Effect of *KCNJ5^T158A* on transcriptional events that regulate *CYP11B2* expression

The transcription factors NURR1 and ATF2 are the main regulators of *CYP11B2* promoter activation and are activated by intracellular calcium levels (Bassett, et al. 2004a; Bassett, et al. 2000; Hattangady, et al. 2012; Nogueira, et al. 2009). We, therefore, investigated the effect of *KCNJ5^T158A* on the expression of *NURR1* and the phosphorylation of ATF2. Analyses for *NURR1* transcript levels indicate a time-dependent increase upon *KCNJ5^T158A* induction, with *NURR1* mRNA peaking at 8-fold over basal at 36 h and plateauing thereafter (Figure 5, *Panel A*). An increase in nuclear-localized NURR1 was also observed at 48 h of *KCNJ5^T158A* activation by doxycycline treatment (Figure 5, *Panel B*). Stimulation of *NURR1* mRNA and protein was inhibited by pre-treatment of cells with verapamil (10 μM) by approximately 70% (Figure 5, *Panel B*). Treatment with Ang II as a positive control similarly elevated *NURR1* transcript and protein by 5-fold at 60 h of incubation. Verapamil pre-treatment inhibited the Ang II-mediated effects by approximately 50%. To define the effect of *KCNJ5^T158A* on the activation of ATF2 (a member of the cAMP-response element-binding protein (CREB) family), nuclear protein from cells incubated with doxycycline or Ang II in the presence or absence of verapamil was assessed for phosphorylation. Both, Ang II and doxycycline treatment increased phosphorylation of ATF2 (normalized to total ATF2 levels) by ~ 5-fold and 4-fold, respectively (Figure 5, *Panel C*). These effects were reversible by calcium channel blockade with verapamil, which inhibited doxycycline and Ang II effects by 50% and 30%, respectively. Overall, these data indicate that *KCNJ5^T158A*-mediated calcium dysregulation leads to the activation of transcriptional events needed for *CYP11B2* transcription.

Effect of *KCNJ5^T158A* on acute regulatory events in aldosterone production

To further characterize the role of acute events in *KCNJ5^T158A*-mediated events, we investigated the effects on steroidogenic acute regulatory protein (StAR), an established acute regulator of aldosterone synthesis. The regulation of *STAR* transcription as well as the post-translational modification of StAR, were determined. Figure 6, *Panel A* shows a representative western blot from three experiments examining phosphorylated StAR, total StAR and GAPDH (as a protein loading control). After 24 h of *KCNJ5^T158A* transgene expression by doxycycline incubation, phospho-StAR was elevated by approximately 4-fold ($p<0.05$) after normalization to GAPDH (Figure 6, *Panel B*). A 3-fold ($p<0.05$) increase in

total StAR was observed following *KCNJ5*[T158A] expression (Figure 6, *Panel C*). Pretreatment with verapamil (10 μM) inhibited *KCNJ5*[T158A]-mediated stimulatory effects on total and phospho-StAR. Initial time-response curves for total and phospho-StAR (data not shown) indicated that *KCNJ5*[T158A] increased phospho-StAR at 24 h. Increases in total StAR were observed starting 6 h post doxycycline treatment.

DISCUSSION

Herein, we defined the acute and chronic events involved in *KCNJ5*[T158A]–induced aldosterone production. We developed a cell line with a doxycycline-inducible construct allowing for conditional expression of *KCNJ5*[T158A], a mutation which occurs both as a germline mutation in familial hyperaldosteronism type III and as a somatic mutation in APA (Mulatero et al. 2012b; Oki et al. 2012). Transient or stable constitutive expression of mutated KCNJ5 causes increases in aldosterone production and expression of *CYP11B2*, through cell depolarization and resultant activation of calcium-dependent kinases (Monticone et al. 2012; Monticone et al. 2013; Oki et al. 2012; Williams et al. 2013). The specific advantage of the inducible expression of *KCNJ5*[T158A] is the ability to investigate the temporal events of molecular responses. Characterization of the cell line demonstrated time-dependent increases in *KCNJ5*[T158A] transgene mRNA and protein expression leading to increased *CYP11B2* transcript and protein levels, as well as aldosterone production, while these effects were absent in control treated wild-type HAC15 cells (Supplemental Figure S1.). In summary, the cell line represents a model to study molecular pathophysiological changes following the initial event of *KCNJ5* gene mutation.

The *KCNJ5*[T158A] mutation showed effects on the traditional steroids from both the mineralocorticoid and glucocorticoid pathways, as well as on the hybrid steroids, 18OHF and 18oxoF, which are frequently elevated in PA (Mulatero, et al. 2012a; Oki et al. 2012). The largest stimulatory effects were observed in 18OHF and 18oxoF, most likely mirroring increased CYP11B2 activity. Increases in aldosterone and its precursors, DOC and corticosterone were also observed. *KCNJ5*[T158A] expression resulted in a modest increase in cortisol production, in agreement with previous studies that demonstrate a modest increased in constitutive *KCNJ5*[T158A]-mediated *CYP11B1* transcript levels (Oki et al. 2012). In addition, due to increased *CYP11B2* expression, cortisol was further converted into hybrid steroids, 18OHF and 18oxoF by *CYP11B2*. Verapamil inhibited the *KCNJ5*[T158A] effects on aldosterone, 18OHF and 18oxoF synthesis, but not on corticosterone and DOC. Thus, stimulatory effects of *KCNJ5*[T158A] as well as the inhibitory effects of verapamil appear to be specific to *CYP11B2*.

From a molecular perspective, the physiological regulation of aldosterone production in response to Ang II or elevated serum potassium involves two key regulatory steps. The acute rate-limiting step includes the activation of StAR (occurring within seconds to minutes of agonist treatment). StAR regulates the transport of cholesterol from the outer to the inner mitochondrial membrane. The chronic limiting step involves CYP11B2 mediated conversion of DOC to aldosterone, which is mainly controlled on the transcriptional level. Ang II and K^+ lead to the expression of NURR1, as well as the activation by phosphorylation of members of the CREB family, including ATF2 (Bassett, et al. 2004c; Bassett et al. 2004a;

Hattangady et al. 2012; Nogueira et al. 2009; Xing, et al. 2011). Both of these steps are regulated by elevated intracellular calcium and the activation of calcium-calmodulin kinases (CaMKs) (Pezzi, et al. 1996; Pezzi, et al. 1997). These kinases activate the CREB family and NURR1 expression, which then translocate to the nucleus and bind the *CYP11B2* promoter to initiate its transcription. *In vitro* studies have shown that mutated *KCNJ5*-mediated aldosterone excess in PA involves elevation of intracellular calcium. Additionally, our studies are the first to show increased nuclear protein levels of phosphorylated ATF2 after induction of *KCNJ5^{T158A}*. These results are in agreement with recent findings in APA, which support the activation of calcium-calmodulin kinases I (CaMK I) and elevated nuclear localization of active, phosphorylated CREB (Sackmann, et al. 2011).

StAR regulates the movement of cholesterol from the outer to the inner mitochondrial membrane. StAR activity is regulated at the level of transcription and translation, as well as by post-translational modification. The transcriptional and translational regulation of *StAR* controls the level of expression of the 37 kDa StAR protein, while phosphorylation of StAR is crucial for its activity (Alberta, et al. 1989; Clark and Stocco 1997; Clark, et al. 1994; Estabrook and Rainey 1996; Krueger and Orme-Johnson 1983). Increased intracellular calcium levels, following Ang II treatment, cause increased StAR activation in the H295R cell line (Clark, et al. 1995; Nanba, et al. 2015; Olala, et al. 2014) as well as primary cultures of bovine adrenocortical cells (Betancourt-Calle, et al. 2001; Yamazaki, et al. 2006). In agreement with these studies, *KCNJ5^{T158A}* expression increased expression and phosphorylation of StAR. Finally, maximum *KCNJ5^{T158A}*-induced activation of StAR was observed at 24 h (earliest time point for StAR phosphorylation, data not shown), mirroring the fact that transcription of the transgene and translation of StAR protein following doxycycline treatment are necessary. Ang II treatment led to StAR activation as early as 30 min (data not shown).

We also evaluated the effects of constitutively expressed *KCNJ5^{T158A}* in primary cultures of human adrenal fasciculata-reticularis (ZF/ZR) (Supplemental figure S2). While a dose-dependent increase in *KCNJ5^{T158A}* mRNA was observed, no increase in *CYP11B2* mRNA or aldosterone production was obtained. A positive control consisting of ZF/ZR cells treated with ACTH did yield increased *CYP11B2* mRNA expression and aldosterone production. Importantly, the ZF/ZR cells were also unresponsive to Ang II or K$^+$. These results, along with reports from other laboratories describing a ZF-like phenotype in APA harboring *KCNJ5* mutations (Azizan, et al. 2012), may be the cause that mutations in *KCNJ5* alone in ZF cells do not sufficiently lead to cause autonomous aldosterone production. The inherent challenge herein is the ability to obtain Ang II-responsive ZG cells, as normal adrenal glands generally lack a distinct *CYP11B2*-expressing ZG, but rather harbor a few, scattered *CYP11B2*-expressing cell clusters (Nishimoto, et al. 2010).

In summary, this study has defined the effects of *KCNJ5^{T158A}* on 1) acute events (including StAR phosphorylation) and 2) chronic transcriptional events (including induction and activation of ATF2 and NURR1) 3) adrenal steroidogenesis in HAC15 cells and 4) confirmed the ability of verapamil to block *KCNJ5^{T158A}* activation of aldosterone production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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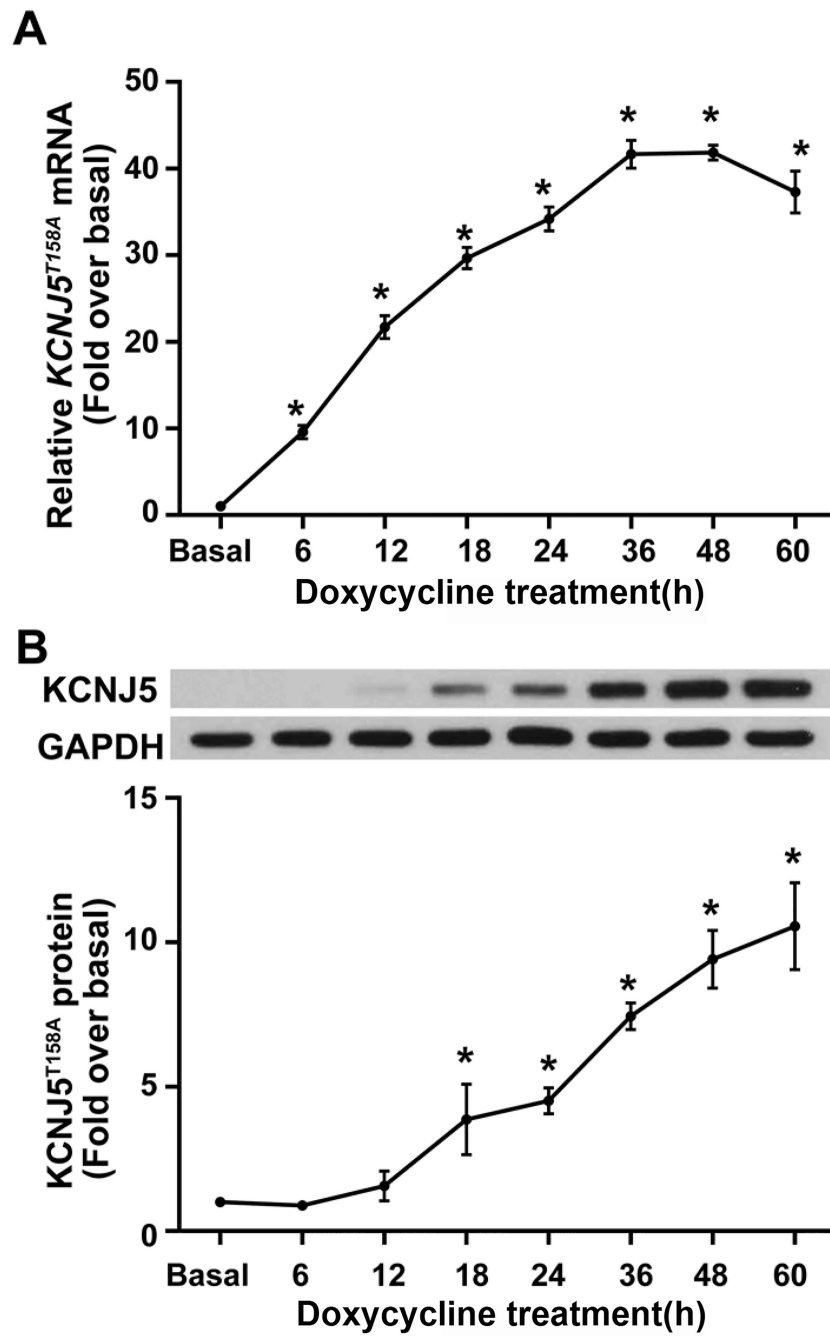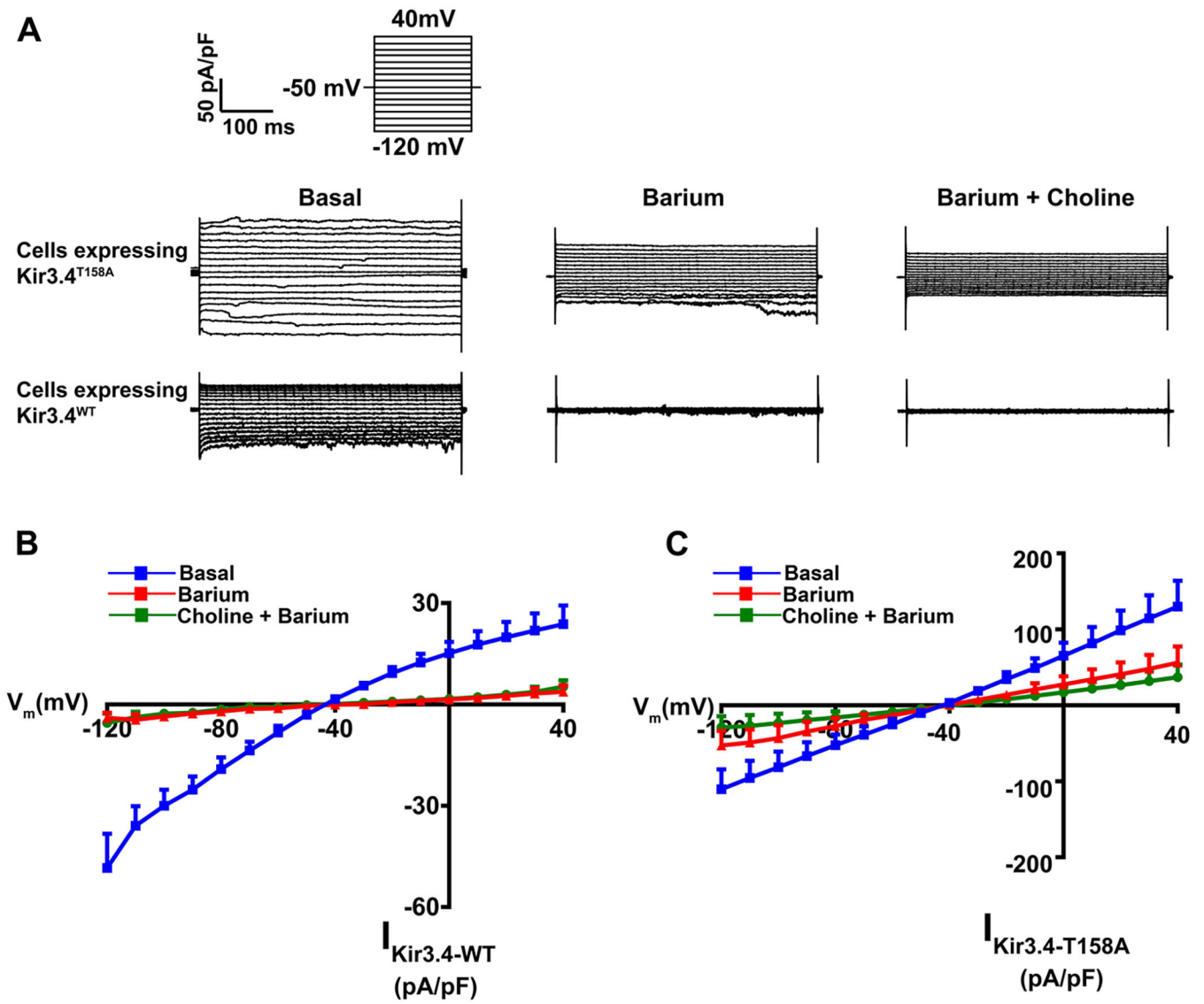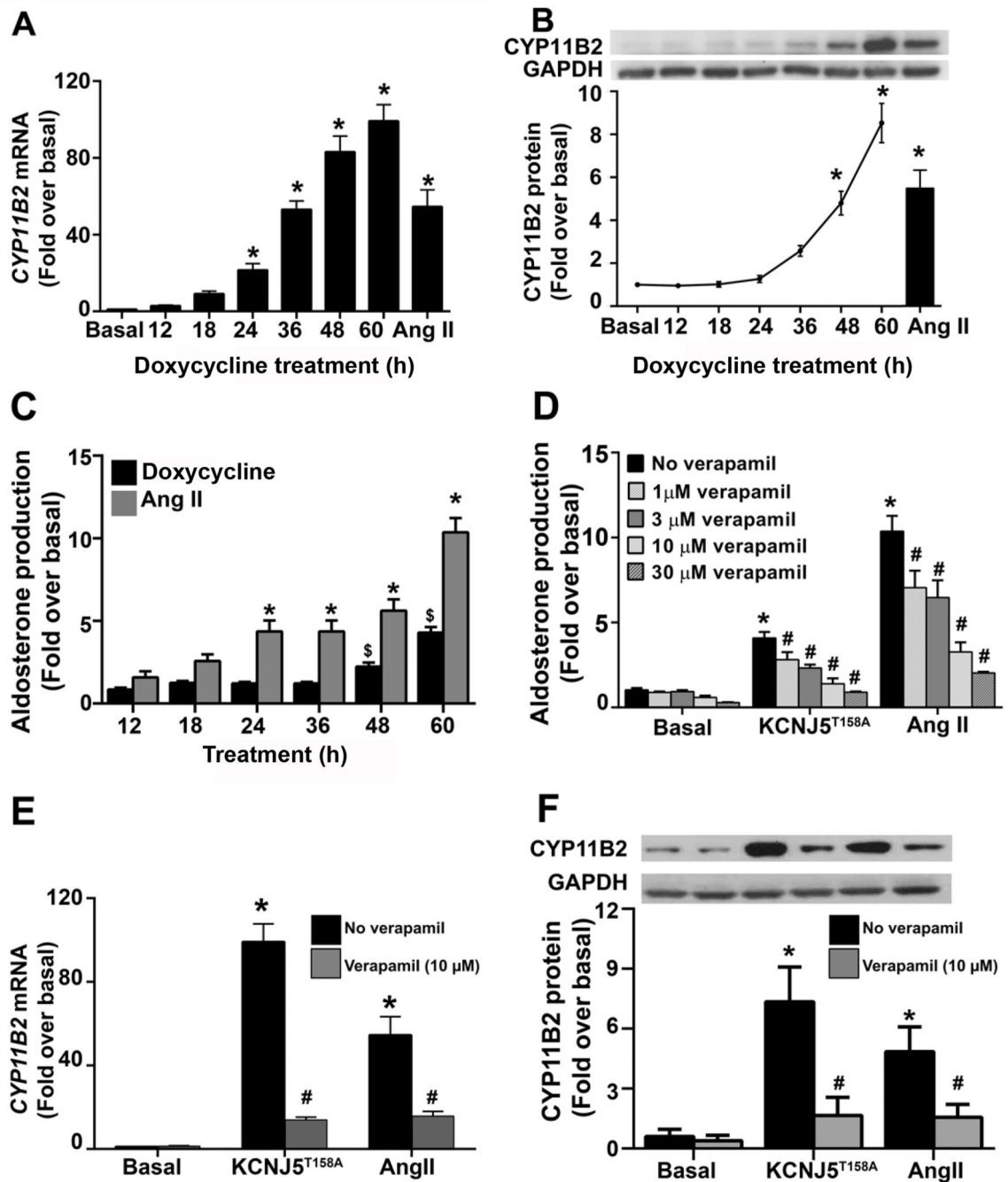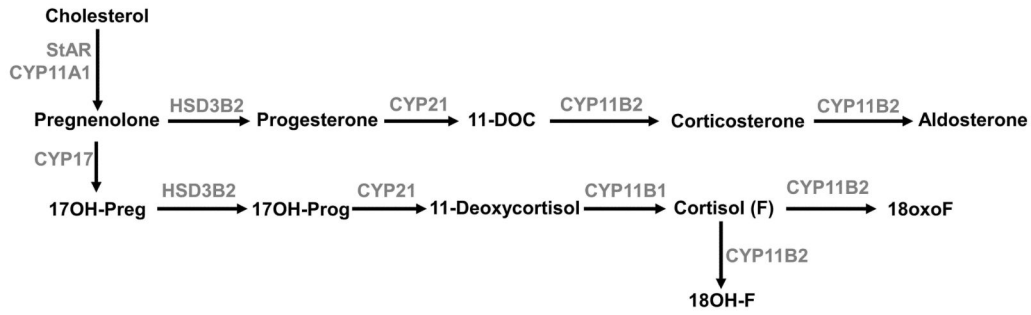
Figure 1.

Figure 2.

Figure 3.

A. Steroidogenic pathway for mineraocorticoids and glucocorticoids
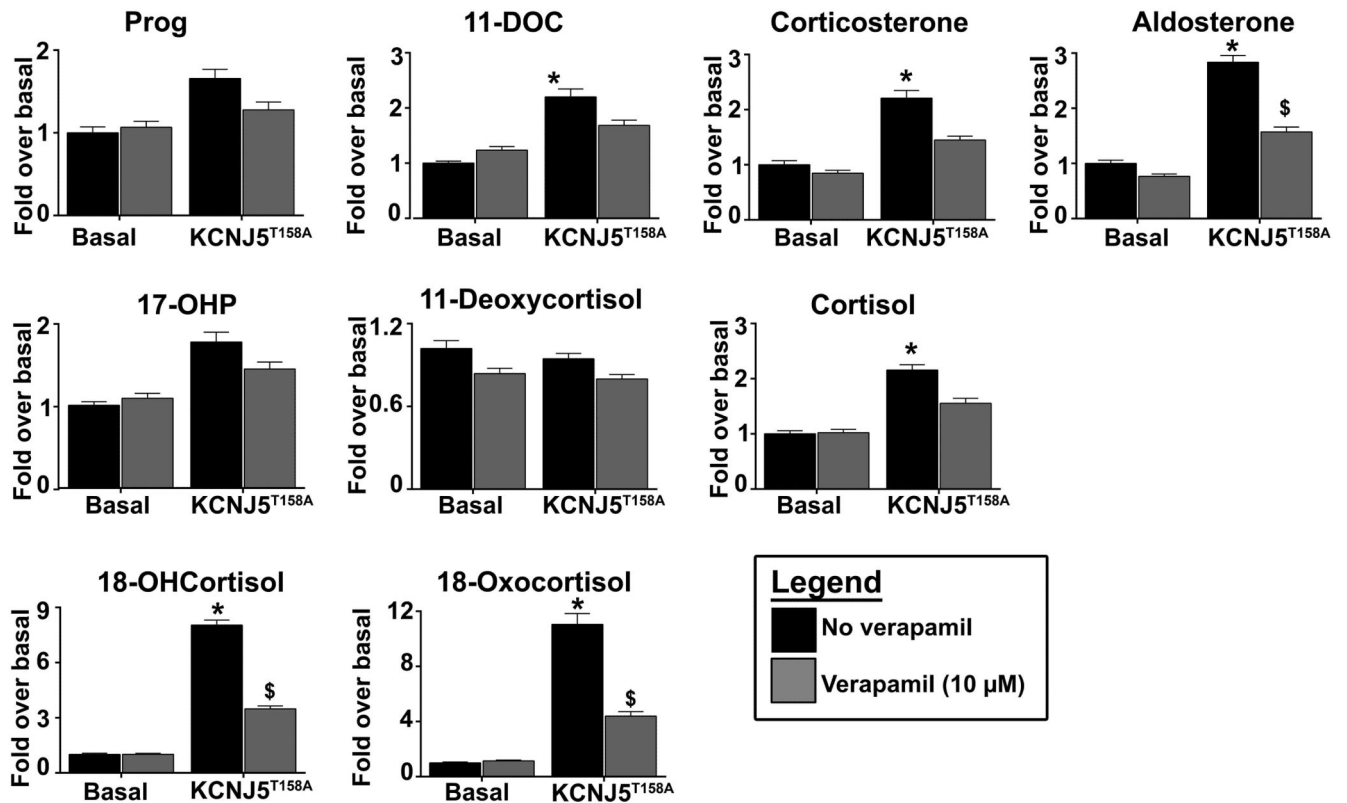

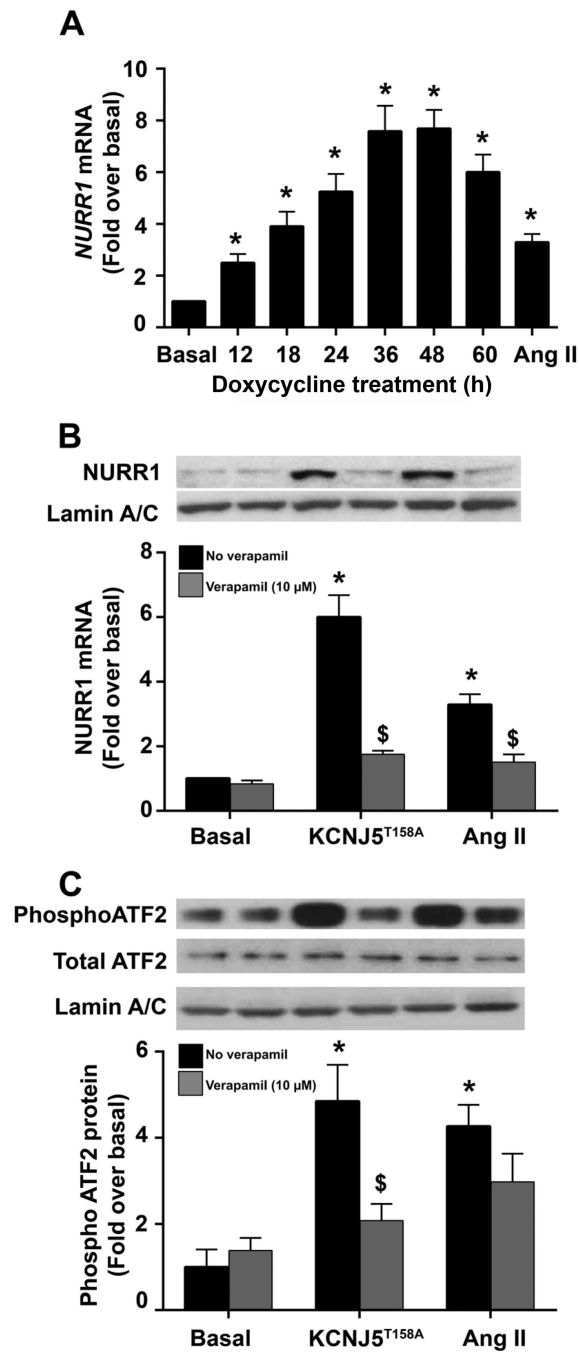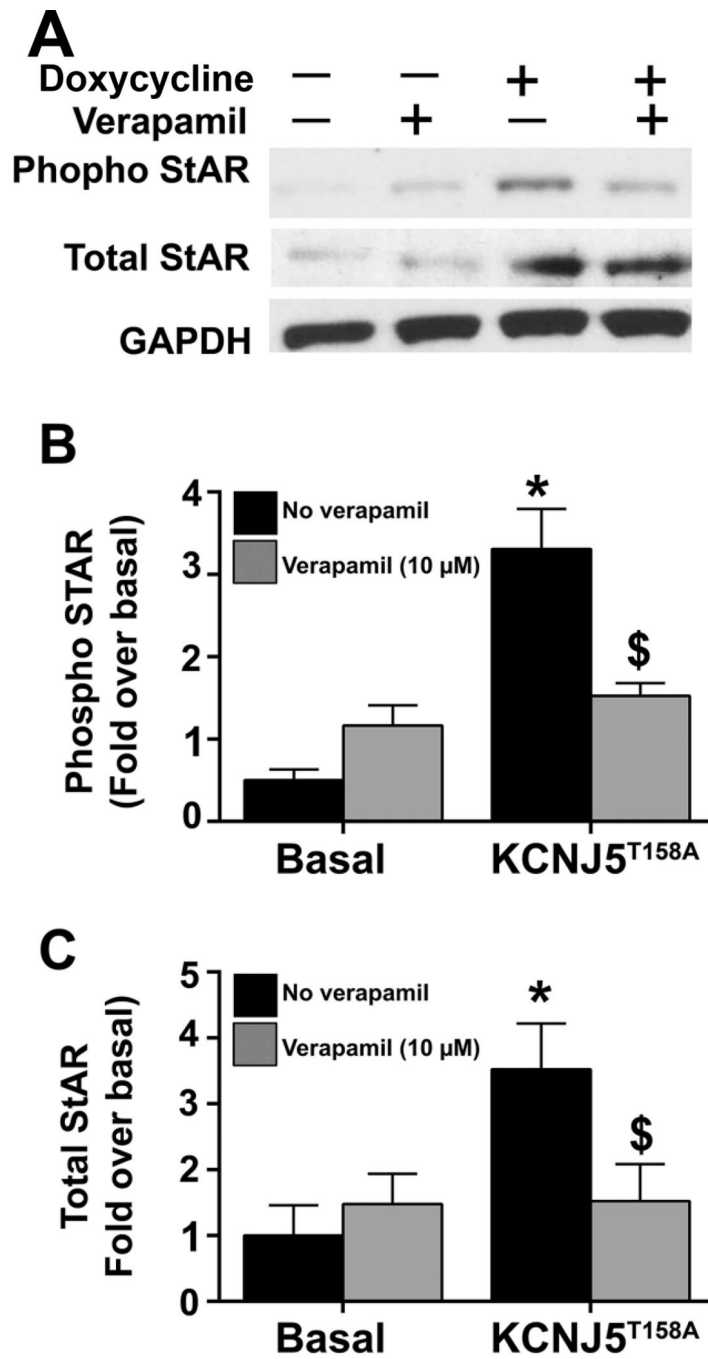## B. Effect of KCNJ5^{T158A} on steroid production

Figure 4.

A

B

C

Figure 5.

Figure 6.