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Authors

Wang, Ying

Wang, Jian

Yang, Rong

et al.

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ORIGINAL ARTICLE

Decreased 11β-Hydroxysteroid Dehydrogenase Type 2 Expression in the Kidney May Contribute to Nicotine/Smoking-Induced Blood Pressure Elevation in Mice

Ying Wang,* Jian Wang,* Rong Yang,* Piwen Wang, Rene Porche, Samuel Kim, Kabirullah Lutfy, Limei Liu, Theodore C. Friedman, Meisheng Jiang, Yanjun Liu

ABSTRACT: Chronic nicotine exposure significantly increases hypertensive risk in smokers, but the underlying mechanisms are poorly understood. In the kidneys, 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) catalyzes the conversion from active into inactive glucocorticoids and plays a pivotal role in the regulation of blood pressure. We hypothesized that nicotine-induced blood pressure elevation is in part mediated by change in renal 11β-HSD2 leading to higher MR (mineralocorticoid receptor) occupancy. Here, we show that nicotine exposure markedly decreased the expression and activity of renal 11β-HSD2 and increased the mean systolic arterial pressure in C57BL/6J mice. Reduction of renal 11β-HSD2 expression by nicotine was correlated with the suppression of C/EBPβ (CCAAT/enhancer-binding protein-β) and activation of Akt protein kinase phosphorylation (pThr^{308}Akt/PKB) within the kidney. Conversely, nicotine-treated mice had elevated renal MR and epithelial sodium channel-α abundance. Treatment with the MR antagonist spironolactone significantly decreased the elevated mean systolic blood pressure and corrected ENaC along with inhibition of pThr^{308}Akt/PKB within the kidney in nicotine-treated mice. Suppression of Akt/PKB activation by spironolactone was accompanied by upregulation of renal C/EBPβ and amelioration of nicotine-mediated reduction of 11β-HSD2. Addition of nicotine to mouse renal cortical collecting duct M1 cells downregulated 11β-HSD2 and stimulated MR expression, and these effects are likely mediated by activation of Akt coupled inhibition of C/EBPβ. These findings suggest that nicotine-mediated suppression of 11β-HSD2 in the kidney may contribute to the development of nicotine/smoking-induced hypertension through decreasing the intrarenal deactivation of glucocorticoids. Spironolactone may prove useful in protecting against the hypertensive risks of nicotine/smoking. (***Hypertension*. 2021;77:00–00. DOI: 10.1161/HYPERTENSIONAHA.120.16458.)** • **Data Supplement**

Key Words: AKT ■ blood pressure ■ CEBP-beta ■ ENaC ■ mineralocorticoid receptor ■ nicotine ■ spironolactone

Smoking and chronic nicotine exposure often causes blood pressure (BP) elevation in smokers. Epidemiological data have demonstrated that smokers have higher BP than nonsmokers in humans.[1–3] Animal model studies have also shown that nicotine exposure elevates the mean arterial pressure in mice and rats.[4,5] Similarly, acute infusion of nicotine increased systolic BP (SBP) in healthy subjects similar to that of smoking,[6,7] whereas the cessation of exposure results in a normalization of BP.[8] These studies suggest that nicotine is responsible for the smoking-induced hypertension in smokers.

Nicotine exposure promotes the initiation and progression of hypertension in smokers, but evidence also indicates that nicotine can induce hypercortisolemia

Correspondence to: Yanjun Liu, Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Sciences, David Geffen School of Medicine at University of California, Los Angeles, CA 90059, Email dryanjunliu@hotmail.com or Limei Liu, Department of Endocrinology and Metabolism, Shanghai Jiaotong University Affiliated Sixth People's Hospital, Shanghai Diabetes Institute, China, Email lmliu@sjtu.edu.cn

*J. Wang, R. Yang, and Y. Wang contributed equally.

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ORIGINAL ARTICLE

Novelty and Significance

What Is New?

- We discovered that nicotine-induced hypertension is associated with decreased renal 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) leading to higher MR (mineralocorticoid receptor) activity by elevated glucocorticoid levels in the distal nephron, and these adverse effects are mitigated by spironolactone. We also identified that C/EBP (CCAAT/enhancer-binding protein) signaling is responsible for the nicotine-mediated alteration of 11β-HSD2 in mouse kidney. These findings clearly showed that nicotine impairs glucocorticoid inactivation and increases MR action by suppression of renal 11β-HSD2 may be a novel mechanism to account for the process of smoking/nicotine-induced blood pressure (BP) elevation.

What Is Relevant?

- Nicotine increases the risk of hypertension in smokers: however, the possible influence of renal 11β-HSD2 on the process of nicotine/smoking-induced BP elevation is unclear. 11β-HSD2 inactivates active glucocorticoids to protect normal MR activity that is essential for renal electrolytic balance and BP control. Here, the evidences are presented for that nicotine disturbed Akt signaling associated C/EBPβ, a key transcription activator of 11β-HSD2, and this association responds to nicotine exposure in the kidney, thus disorders renal function and elevates BP.

Summary

Our findings illustrate that nicotine decreased renal 11β-HSD2 contribute to increase in BP, mainly as a consequence of greater occupation of MR by glucocorticoids in the distal nephron. Our results also revealed that desirable effects of spironolactone in reducing BP in nicotine-treated mice may be associated with alteration of renal 11β-HSD2 through pThr^{308}Akt/PKB signaling mediated mechanism. Our findings suggest that tissue-specific manipulation of renal 11β-HSD2 and MR may be helpful for the treatment of nicotine-mediated hypertension and other cardiovascular risks in smokers or second-hand smokers.

Nonstandard Abbreviations and Acronyms

11β-HSD2	11β-hydroxysteroid dehydrogenase type 2
BP	blood pressure
C/EBPβ	CCAAT/enhancer-binding protein-β
ENaCα	epithelial sodium channel-α
MR	mineralocorticoid receptor
SBP	systolic BP

in humans as well as in rodents.[9–11] Patients with glucocorticoid excess (Cushing syndrome) often exhibit hypertension.[12,13] These studies implicate that elevated glucocorticoid tone may be a critical but missing link between nicotine/smoking and BP elevation. The actions of glucocorticoids on target tissues not only depend on the blood glucocorticoids levels but also are tightly regulated by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2). 11β-HSD1 is an endoplasmic ER-resident enzyme, which catalyzes the conversion of inert cortisone (in humans) and 11-dehydrocorticosterone (in rodents) to active cortisol and corticosterone in liver and fats, and thus amplifies local glucocorticoid action.[14,15] Conversely, 11β-HSD2 is largely expressed in the distal nephron of the kidneys, colon, placenta, and vascular walls, the site of mineralocorticoid action.[16] 11β-HSD2 acts exclusively as an NAD$^+$-dependent dehydrogenase working in the opposite direction to inactivate cortisol into cortisone and thereby preventing the MR (mineralocorticoid receptor) activation by glucocorticoids.[17,18] 11β-HSD2 thus provides a protective role for aldosterone to bind the MR in the distal nephron to regulate electrolytic balance and BP. Pharmacological inhibition or lacking 11β-HSD2 results in glucocorticoid occupation of renal MR and leads to Na^+ retention and hypertension.[19,20] These can be improved by MR blockers.[21,22] These studies underline the importance of renal 11β-HSD2 inhibition in the pathogenesis of hypertension.

Nicotine exposure has been shown to decrease placental 11β-HSD2 mRNA levels and elevate circulating glucocorticoid levels in pregnant rats,[23] implying that disturbance of tissue-specific glucocorticoid metabolism may contribute to nicotine/smoking-induced adverse outcomes. The transcription factors C/EBPs (CCAAT/enhancer-binding proteins) are transcriptional regulators, and several C/EBP binding sites have been found in the promoter region of the 11β-HSD2 gene.[24,25] Moreover, the transcriptional levels of C/EBPβ have been reported to be decreased in the airway epithelium of smokers.[26] However, whether nicotine might modulate renal 11β-HSD2 and exert adverse effects on electrolyte balance and BP regulation have not been explored.

Here, we examined the impact of nicotine on prereceptor inactivating metabolism of glucocorticoids and BP regulation by analyzing the expression and activity of renal 11β-HSD2 and determined the effect of an MR antagonist spironolactone on nicotine-induced metabolic phenotype and on the expression of renal target

gene encoding key molecules that regulate electrolyte balance in C57BL/6J mice. We also tested whether transcriptional factor C/EBP signaling is responsible for the nicotine alteration of 11β-HSD2 in cultured mouse renal cells.

MATERIALS AND METHODS

The authors declare that the detailed methods are available within the article and in the Data Supplement. The data that support the findings of this study are available from the corresponding author on request.

Animals and Treatment

Ten-week-old C57BL/6J male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in an SPF facility on a 12:12-hour light/dark cycle. Animals had free access to water and standard-salt (0.35% sodium) diet. Mice were given nicotine (100 μg/mL; Sigma-Aldrich, St Louis, MO) in a 2% saccharine solution, or 2% saccharine solution in drinking water for 12 weeks.[5,27] Concurrently, mice were randomly assigned to receive either the MR antagonist spironolactone (Sigma-Aldrich, St Louis, MO) injected daily (50 mg/kg, SC),[28] the ENaC antagonist amiloride (20 mg/L; Sigma-Aldrich, St Louis, MO) in the drinking water or vehicle until the end of the 12-week nicotine treatment. All animal experiments were approved by the Institutional Animal Care and Use Committees of the Charles R. Drew University and Tongji University and were performed in accordance with the recommendation of the American Veterinary Medical Association.

Statistical Analysis

All values are expressed as the mean±SEM. The data of distribution and comparisons between 2 groups were performed by unpaired Student *t* test. To compare multiple groups, 1- or 2-way ANOVA was used. The post hoc Tukey test was performed to reveal significant differences between groups. $P<0.05$ was considered statistically significant.

RESULTS

Nicotine Exposure Leads to BP Elevation and Decreases Renal 11β-HSD2 Expression

Initially, we tested the impact of nicotine on BP, metabolic parameters, and renal 11β-HSD2. The average concentrations of plasma nicotine in mice after oral administration of nicotine (Table) were comparable to habitual smokers.[29] The mean SBP was not changed by nicotine exposure during the first 4 weeks. However, after nicotine exposure for 8 weeks, the SBP was 17% higher than that of controls ($P<0.05$). At 12 weeks, the SBP values were elevated further to 142±7.8 mm Hg, 28% higher in mice treated with nicotine than controls (111±6.2 mm Hg; Figure 1A), with no change in diastolic BP (data not shown). Moreover, the mean arterial pressure measured directly through the carotid artery also showed a significant increase in nicotine-treated mice (132±14.4 versus 98±8.6 mm Hg). Blood glucose and corticosterone levels were increased, whereas serum renin levels were decreased in mice on nicotine. However, body weight, serum aldosterone, creatinine, urine volume, and urinary albumin/creatinine ratio were not different from controls (Table). In contrast, renal 11β-HSD2 mRNA levels were decreased in nicotine-treated mice compared with controls (Figure 1B). Similarly, renal 11β-HSD2 protein levels were decreased by 1.9-fold in nicotine-treated mice (Figure 1C). Moreover, the change in renal 11β-HSD2 activity was negatively correlated with SBP in mice on nicotine (Figure 1E and 1F). Furthermore, the urinary corticosterone/11-dehydrocorticosterone ratio was increased by 2.5-fold in nicotine-treated mice versus controls (Figure 1D).

Nicotine Modulates C/EBP Expression and Activates Akt/PKB Signaling in the Kidney

We next analyzed the expression of renal C/EBPα and C/EBPβ, key transcriptional regulators of 11β-HSD2 expression.[24,25] In parallel with the decrease in 11β-HSD2 mRNA levels, renal C/EBPβ mRNA and protein expression were decreased with reduction of pSer^{105}C/EBPβ content by 2.4-fold in nicotine-treated mice and returned to those of control mice after MR antagonist spironolactone treatment (Figure 2A and 2B), whereas C/EBPα mRNA expression, a weak activator of 11β-HSD2,[24] remained relatively stable or unchanged (Figure 2A). In contrast, renal pThr^{308}Akt content was markedly increased without changes in total Akt protein levels after nicotine exposure and normalized by spironolactone treatment (Figure 2C). In addition, mice treated with nicotine also showed increased renal pSer^{473}Akt levels that were restored to those of control mice by spironolactone (data not shown).

Nicotine Stimulates Renal MR and ENaC Expression and Disturbs Electrolytic Balance

As shown in Figure 2D and 2E, renal MR mRNA and protein levels were markedly increased by nicotine and were attenuated by spironolactone treatment, whereas renal GR abundance was unaltered (data not shown). The results of immunohistochemistry analysis showed that nicotine-induced upregulation of MR expression (Figure 2F) with downregulation of 11β-HSD2 in the distal nephron of mice (Figure 2F), which were ameliorated by spironolactone. In line with the MR expression, renal epithelial sodium channel-α (ENaCα) mRNA and protein expression were elevated, along with increased the expression levels of cleaved ENaCα in nicotine-treated mice (Figure 3A and 3B), which were normalized by spironolactone. Double immunofluorescence staining identifying MR and 11β-HSD2 expression colocalized

Table. The Effects of Nicotine and Spironolactone on Metabolic Parameters

N=6–8/group	Veh	Veh+SP	Nic	Nic+SP
Body weight, g (n=8)	31.6±2.9	30.7±3.2	29.8±4.0	32.1±3.8
Nicotine, ng/mL (n=6)	0	0	29.0±10*	27.6±13
Corticosterone, ng/mL (n=8)	108±16	113±15	196±29*	152±37
Aldosterone, pg/mL (n=8)	147±25	131±22	169±24	138±13
Renin, ng/mL (n=8)	445±32	461±33	240±28*	490±44†
Blood glucose, mg/dL (n=6)	117±8.2	124±8.5	144±14‡	120±12§
Serum Cre, mg/dL (n=6)	0.44±0.04	0.38±0.20	0.52±0.06	0.41±0.07
Urine volume, mL/24 h (n=8)	0.92±0.14	0.97±0.20	1.08±0.32	0.95±0.13
Urine albumin/Cre, mg/mg (n=8)	0.89±0.09	0.87±0.16	1.04±0.26	1.08±0.22

Data are mean±SEM for 6–8 mice in each group. Cre indicates creatinine; Nic, nicotine-treated mice; Nic+SP, nicotine and spironolactone-treated mice; Veh + SP, vehicle- and spironolactone-treated mice; and Veh, vehicle-treated mice.

*$P<0.01$ vs vehicle-treated mice.

†$P=0.008$ vs nicotine-treated mice.

‡$P=0.038$ vs vehicle-treated mice.

§$P<0.05$ vs nicotine-treated mice.

with distal tubular marker Peanut Agglutinin (Figure S1 in the Data Supplement). However, renal ENaCγ abundance was unchanged, but its cleaved protein content was increased by nicotine and reversed by spironolactone (Figure 3A and 3C). Similarly, renal Na^+-Cl^- cotransporter mRNA and protein expression levels were slightly increased with elevated T53-phosphorylated Na^+-Cl^- cotransporter content by nicotine and were decreased by spironolactone (Figure S2). In addition, nicotine treatment had no significant effect on renal ENaCβ and Na^+-K^+-Cl^- cotransporter 2 abundance compared with controls (Figure S2). In contrast, mice treated with nicotine had decreased urinary Na^+ content (Figure 3D) and increased plasma Na^+ levels compared with controls and were restored to those of control mice by spironolactone (Figure 3F). Moreover, urinary K^+ excretion was increased (Figure 3D) and the urinary Na^+/K^+ ratio (Figure 3E) was decreased with slight reduction in serum K^+ levels after nicotine exposure (Figure 3F), which were normalized by spironolactone treatment.

Spironolactone Attenuates the Progression of Nicotine-Induced BP Elevation

As expected, spironolactone treatment prevented the increases in the mean SBP (Figure 1A) and normalized the high blood glucose and low renin levels in nicotine-treated mice (Table). However, spironolactone had no significant effects on serum aldosterone or corticosterone levels and had no effects on weight, urine volume, creatinine, and urinary albumin/creatinine ratio (Table). In contrast, spironolactone treatment rescued the nicotine-induced decreases in the expression and activity of renal 11β-HSD2 (Figure 1B, 1C, and 1E) and decreased the high urine corticosterone/11-dehydrocorticosterone ratio to that of control mice (Figure 1D).

To further verify the MR target gene ENaC involvement in the development of nicotine-induced BP elevation, nicotine-treated mice were also given ENaC blocker amiloride treatment. Consequently, amiloride treatment increased urinary Na^+/K^+ ratio (Figure S3B) and significantly reduced the increased SBP in nicotine-treated mice compared with vehicle-treated controls (Figure S3A).

Spironolactone Modulates Nicotine-Induced Alterations of 11β-HSD2 and MR Gene Expression via Inhibition of $pThr^{308}$ Akt Activation in cortical collecting duct M1 Cells

To confirm our in vivo observations, we studied the effect of nicotine on 11β-HSD2 and C/EBPβ in culture CCD M1 cells. Treatment of CCD M1 cells with increasing concentrations of nicotine (10^{-9}–10^{-4} mol/L) for 24 hours led to concentration-dependent decrease in 11β-HSD2 mRNA levels and protein expression (Figure 4A and 4B). In contrast, MR protein expression was increased by 1.3- to 1.9-fold over controls (Figure 4B). However, CCD M1 cells treated with both spironolactone (10^{-6} mol/L) and nicotine (10^{-6} mol/L, which is similar to that seen in smokers), failed to decrease 11β-HSD2 activity (Figure 4C), and did not affect MR and ENaCα abundance (Figure 4D and 4F) in these cells. Also, nicotine increased $pThr^{308}$Akt expression but decreased C/EBPβ mRNA and protein levels. These effects of nicotine in these cells were attenuated by spironolactone (Figure 4D, 4E, and 4F).

Repression of 11β-HSD2 by Nicotine Involves Activation of Akt Kinase via Modulation of C/EBPβ

Because nicotine exerts its cellular adverse effects through activation of Akt/PKB,[30,31] we tested if inhibition of Akt kinase could block the nicotine repression of 11β-HSD2. Treatment of CCD M1 cells with nicotine (10^{-6} mol/L) in the presence of Akt inhibitor VIII

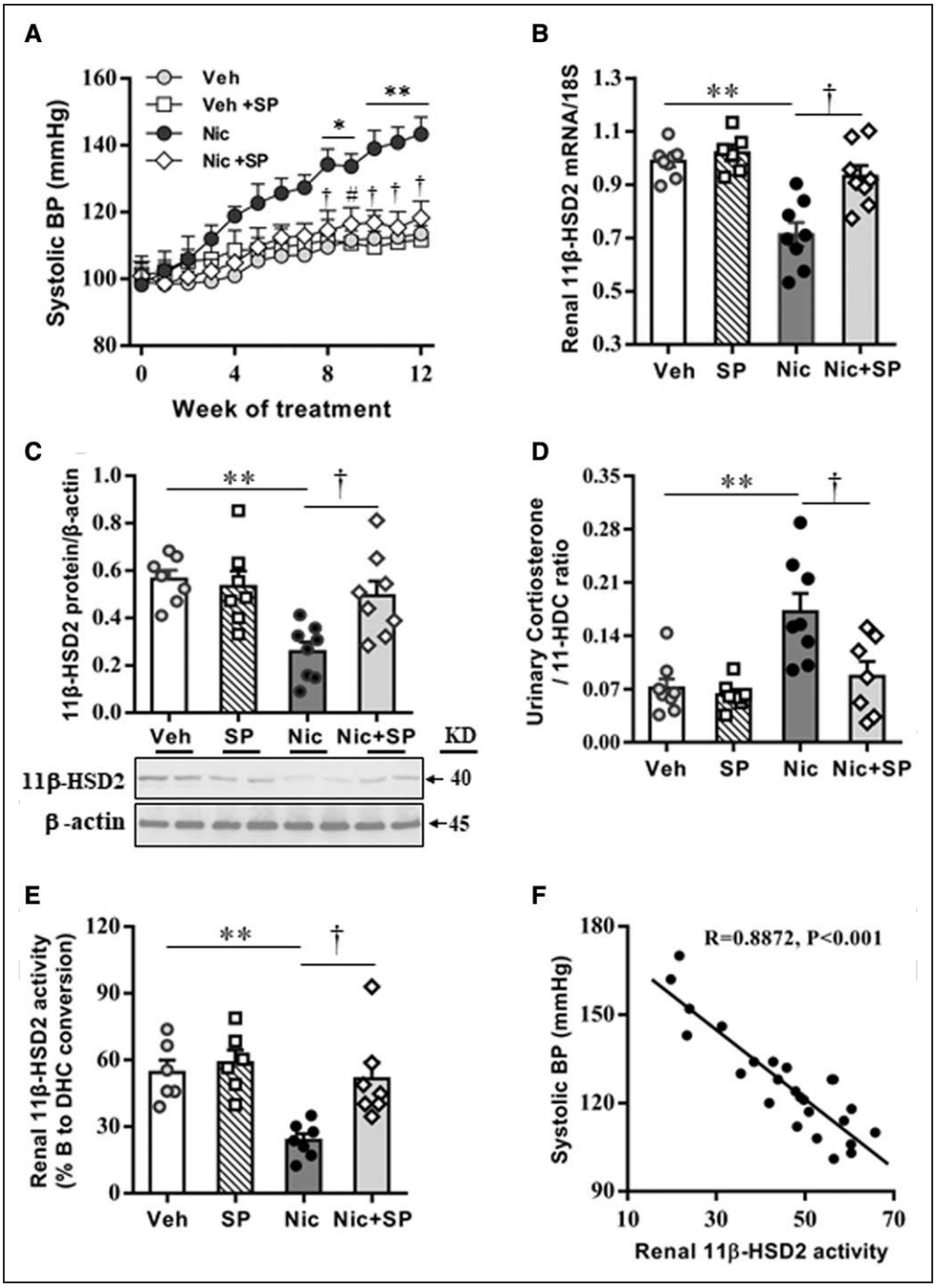

Figure 1. Nicotine exposure elevation of blood pressure and suppression of renal 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) expression and activity in C57BL/6J male mice.

A, Time course of systolic blood pressure (BP) in mice. **B**, Relative expression of renal 11β-HSD2 mRNA levels was normalized to 18S. **C**, Relative expression of 11β-HSD2 protein levels was done relative to the amount of β-actin. **D**, The ratio of corticosterone/11-dehydrocorticosterone (11-DHC) in 24-h urine samples of each group mice. **E**, The enzyme activity is expressed as percentage conversion of [^{3}H] corticosterone (**B**) to [^{3}H] 11-DHC. **F**, Correlation between 11β-HSD2 activity and systolic BP. Data are presented as mean±SEM (n=6–8) mice. **P*<0.05 and ***P*<0.01 vs vehicle-treated mice; #*P*<0.05 vs. nicotine-treated mice; †*P*<0.01 vs nicotine-treated mice.

(10^{-6} mol/L) failed to suppress 11β-HSD2 and did not induce changes in MR mRNA expression in comparison with vehicle-treated cells (Figure 5A) with inactivation of pThr^{308}Akt expression (Figure 5B), suggesting that activation of Akt is required for the nicotine-mediated decrease of 11β-HSD2.

Since the activation of Akt has emerged as a negative regulator of C/EBPβ expression,[24,32,33] we examined if inhibition of Akt kinase can affect the nicotine-mediated reduction of C/EBPβ. Coincubation of cells with both Akt inhibitor and nicotine blunted the nicotine-induced suppression of C/EBPβ protein expression (Figure 5C). Moreover,

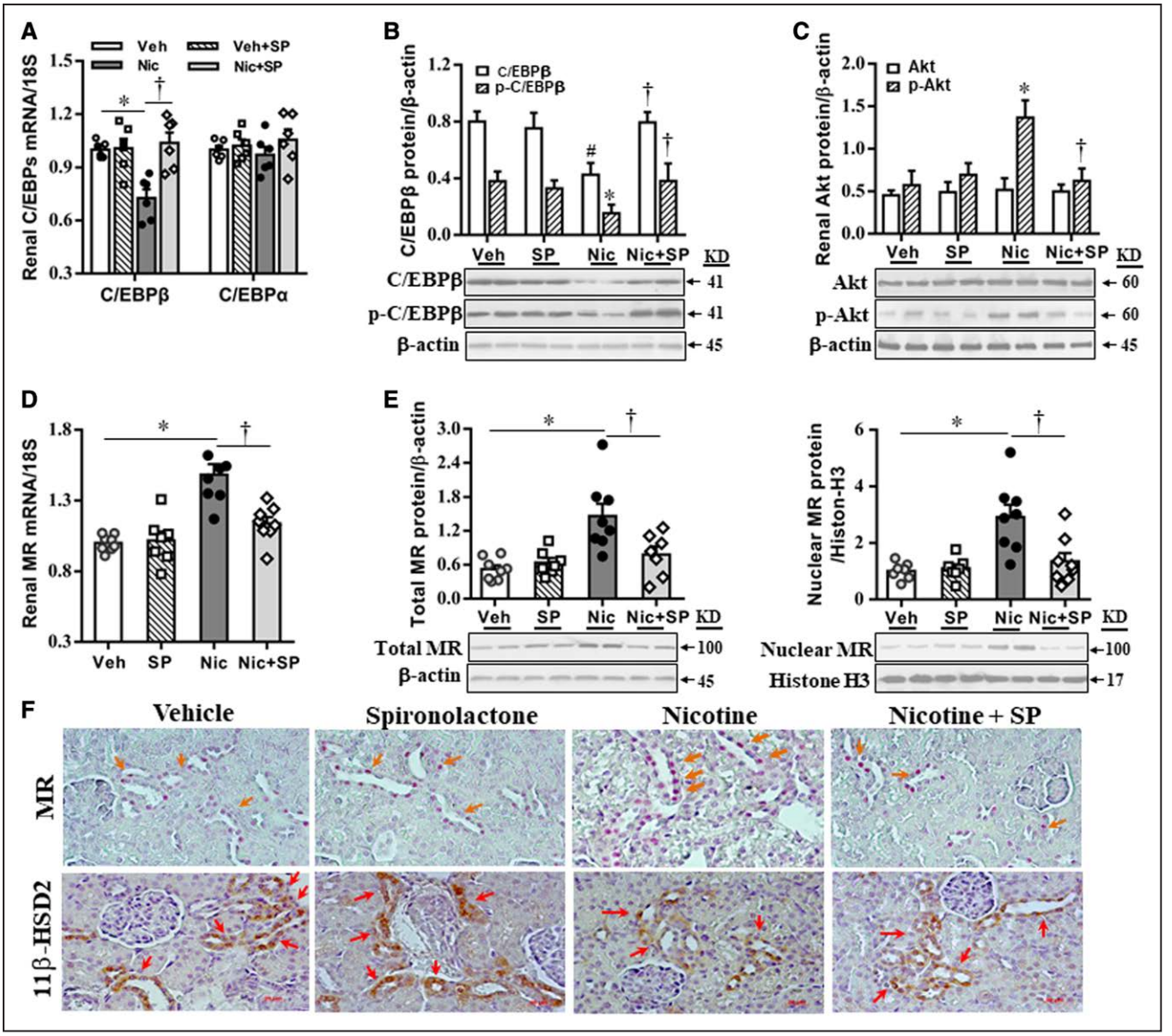

Figure 2. Alterations of C/EBPβ (CCAAT/enhancer-binding protein-β), Akt kinase, MR (mineralocorticoid receptor), and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) expression in the kidney from vehicle- and nicotine-exposed mice after spironolactone or vehicle treatment.

A and **D**, Relative expression of C/EBPs and MR mRNA levels were measured by real-time polymerase chain reaction (PCR) and normalized to 18S. **B**, **C**, and **E**, Relative expression of C/EBPβ, pSer^{105}C/EBPβ (**B**), Akt, pThr^{308}Akt (**C**), total and nuclear MR protein levels (**E**) were analyzed by Western blot. Data are presented as mean±SEM (n=7–8) mice. **F**, Immunoreactive signaling of kidney sections for MR and 11β-HSD2 expression in the distal nephron (magnification ×40). #P<0.05 vs vehicle-treated controls; *P<0.01 vs vehicle-treated mice; †P<0.01 vs nicotine-treated mice.

nicotine-induced reduction of pSer^{105}C/EBPβ content was attenuated by incubation with the Akt inhibitor in these cells (Figure 5C). However, Akt inhibitor alone (10^{-6} mol/L) did not affect 11β-HSD2 and had no effect on C/EBPβ expression in CCD M1 cells in comparison with control cells.

C/EBPβ Mediates Nicotine-Induced Suppression of 11β-HSD2 Expression

Next, we investigated if enhancing C/EBPβ might modulate the effects of nicotine on 11β-HSD2 expression. As shown in Figure 5D and 5E, C/EBPβ mRNA and protein levels were markedly increased in CCD M1 cells transfected with plasmid cDNA3.1 C/EBPβ in comparison with cells transfected with plasmid control, respectively. Consequently, overexpression of C/EBPβ in cells attenuated the nicotine-mediated decreases in 11β-HSD2 mRNA and protein expression levels compared with control cells (Figure 5D and 5F), indicating that nicotine-induced suppression of 11β-HSD2 is mediated in part by reduction of C/EBPβ. In addition, activation of 11β-HSD2 activity by its overexpression reversed the nicotine-mediated upregulation of MR and ENaCα in these intact cells (data not shown).

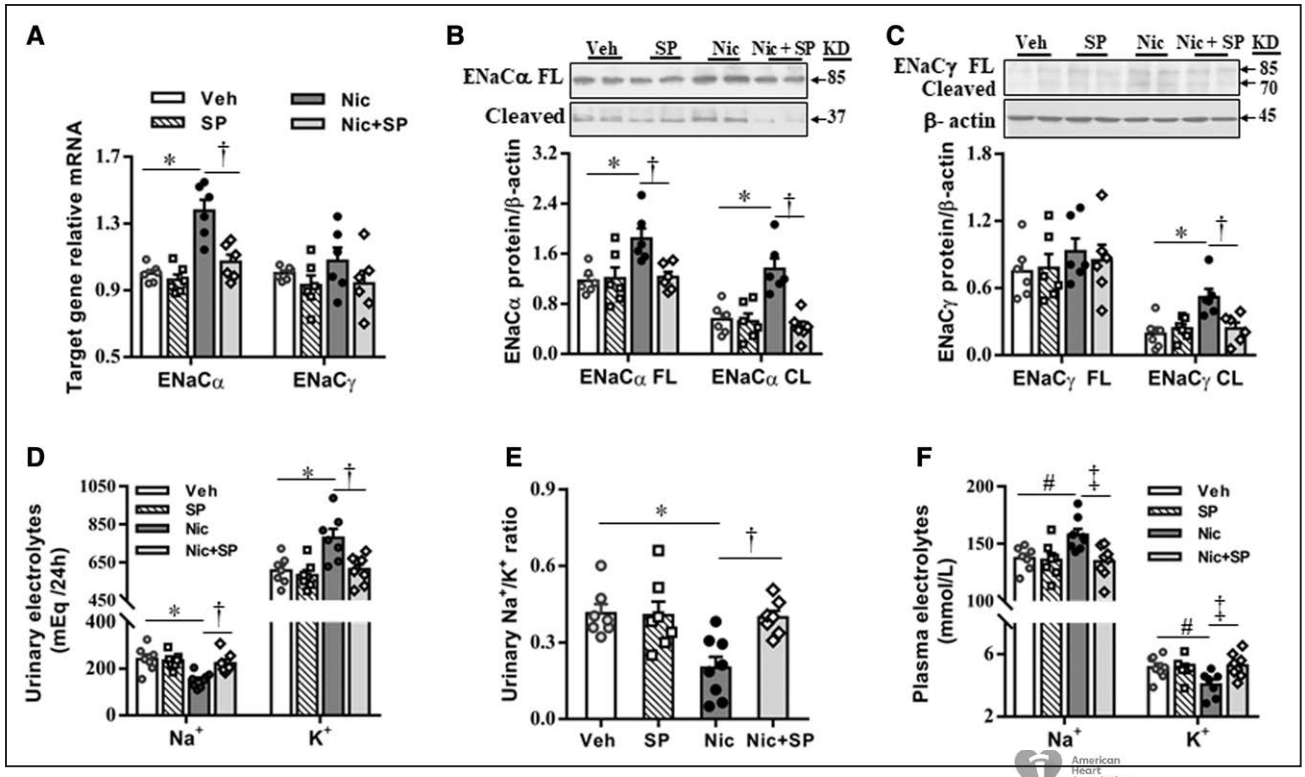

Figure 3. Effects of spironolactone on renal epithelial sodium channel (ENaC) expression and electrolytic balance in control and nicotine-exposed mice.

A, Relative renal ENaCα and ENaCγ mRNA levels were measured by real-time polymerase chain reaction (PCR) and normalized to 18S. **B** and **C**, Relative expression of full-length ENaCα (FL), cleaved ENaCα (CL), full-length ENaCγ (FL), and cleaved form (CL) protein levels were quantified and standardized to β-actin. **D** and **E**, Urinary sodium and potassium (**D**) and Na^+/K^+ ratio (**E**) were measured using flame photometry from 24-h urine samples collected by metabolic cage at the end of the experiment. **F**, Serum Na^+ and K^+ levels. Data are presented as mean±SEM (n=6–8). **P*<0.01 vs control mice; †*P*<0.01 vs nicotine-treated mice; #*P*<0.05 vs control mice; ‡*P*<0.05 vs nicotine-treated mice.

Nicotine Enhances Glucocorticoid-Induced Expression of MR and ENaCα in CCD M1 cells

To investigate if nicotine could increase glucocorticoid occupancy of MR, CCD M1 cells were treated with both nicotine and corticosterone. Treatment of cells with corticosterone (10^{-8} mol/L, similar to that seen in animal plasma levels of biologically active glucocorticoids)[34] slightly increased MR and ENaCα mRNA and protein expression levels (Figure S4A, S4C, and S4D) but did not affect 11β-HSD2 activity compared with vehicle-treated cells (Figure S4B), indicating that corticosterone is metabolized by endogenous 11β-HSD2. In contrast, treatment of cells with both nicotine (10^{-6} mol/L) and corticosterone (10^{-8} mol/L) decreased 11β-HSD2 activity and enhanced the corticosterone-induced MR and ENaCα expression compared with control cells (Figure S4), suggesting that reduction of renal 11β-HSD2 by nicotine causes occupancy of MR by glucocorticoids.

DISCUSSION

Accumulating evidence suggests that nicotine exposure effects renal function and increases hypertensive and other cardiovascular disease risks. Here, we show that nicotine exposure disturbed mouse renal 11β-HSD2 and increased the mean arterial pressure that was correlated with impaired corticosterone inactivation. We found that mice exposed to nicotine had decreased renal 11β-HSD2 expression and activity and elevated SBP. In contrast, urinary corticosterone/11-dehydrocorticosterone ratio that reflects 11β-HSD2 activity was increased in the kidneys of nicotine-treated mice. Moreover, our cell studies showed that nicotine causes downregulation of 11β-HSD2 in CCD M1 cells, major sites of 11β-HSD2 expression in the kidney.[35] These data indicate that the ability of 11β-HSD2 deactivation of corticosterone to 11-dehydrocorticosterone metabolism in the distal nephron is impaired by nicotine and thus could result in elevated local corticosterone tone in the kidneys. Our findings imply that the nicotine-mediated decrease of 11β-HSD2 in the kidneys may be associated with the development of nicotine/smoking-induced BP elevation. This is in agreement with smokers having higher BP than nonsmokers[1–3] and nicotine exposure increases urine cortisol levels.[29,36] Furthermore, we observed that nicotine decreased 11β-HSD2 expression is positive correlated with downregulation of C/EBPβ in CCD M1 cells and in kidneys of mice. This is in line with that C/EBPβ

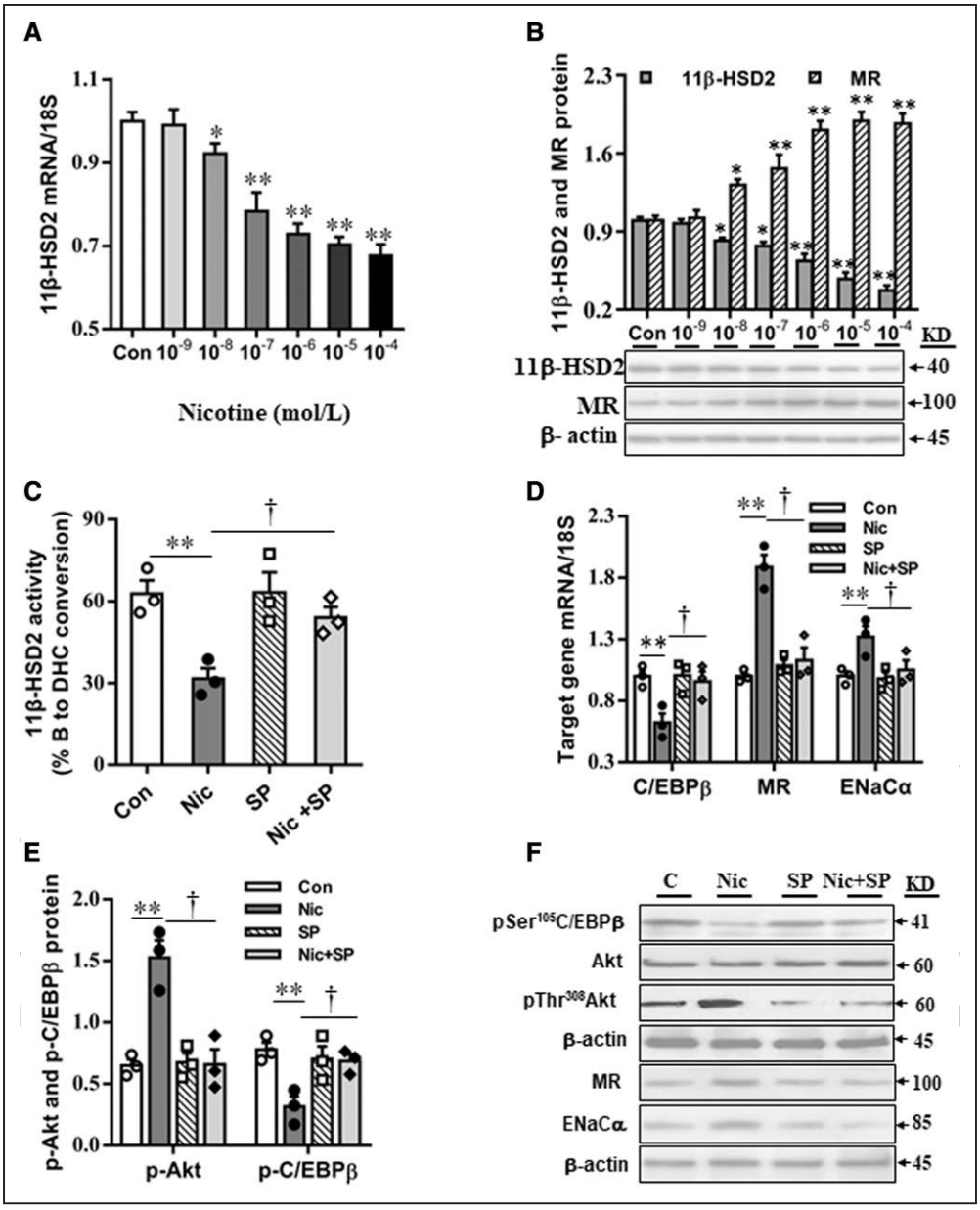

Figure 4. Nicotine downregulates 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) and C/EBPβ (CCAAT/enhancer-binding protein-β) and stimulates MR (mineralocorticoid receptor) expression in CCD M1 cells.
Cells were incubated with increasing concentration of nicotine (10^{-9}–10^{-4} mol/L) for 24 h. **A**, The mRNA expression levels of 11β-HSD2 were analyzed by real-time polymerase chain reaction (PCR) and normalized to 18S. **B**, The expression of 11β-HSD2 and MR protein levels relative to the amount of β-actin. **C**, Cells treated with nicotine (10^{-6} mol/L; Nic) in the absence or presence of spironolactone (10^{-6} mol/L; Sp) for 24 h. 11β-HSD2 activity was measured in CCD cells using corticosterone (**B**) as the substrate. **D**, Relative expression of C/EBPβ, MR, and ENaCα mRNA levels. **E** and **F**, Immunoblot analysis of pSer^{105}C/EBPβ, Akt, pThr^{308}Akt, MR, ENaCα. Data are the mean±SEM from 3 separate culture preparations. *P<0.05 and **P<0.01 vs controls; †P<0.01 vs nicotine-treated cells.

acting as a transcriptional activator of 11β-HSD2, and C/EBPβ levels were decreased in the airway epithelium of smokers.[26] Conversely, induction of C/EBPβ by its overexpression attenuated the nicotine-mediated repression of 11β-HSD2 in CCD M1 cells. Our data suggest that reduction of C/EBPβ may be responsible for nicotine-mediated repression of 11β-HSD2. Reduction of C/EBPβ could decrease its positive effects on the 11β-HSD2 gene transcription and thus contribute to decreased renal 11β-HSD2 expression linked to the process of nicotine-induced BP elevation.

In mice and humans, inhibition or lacking of 11β-HSD2 has been reported to induce Na^+ retention and hypertension.[19,20] We showed that nicotine decreased renal 11β-HSD2 and increased MR and its target gene ENaCα expression that correlates with SBP elevation. Given that aldosterone and corticosterone have a similar affinity for MR, we propose that nicotine-induced elevation in

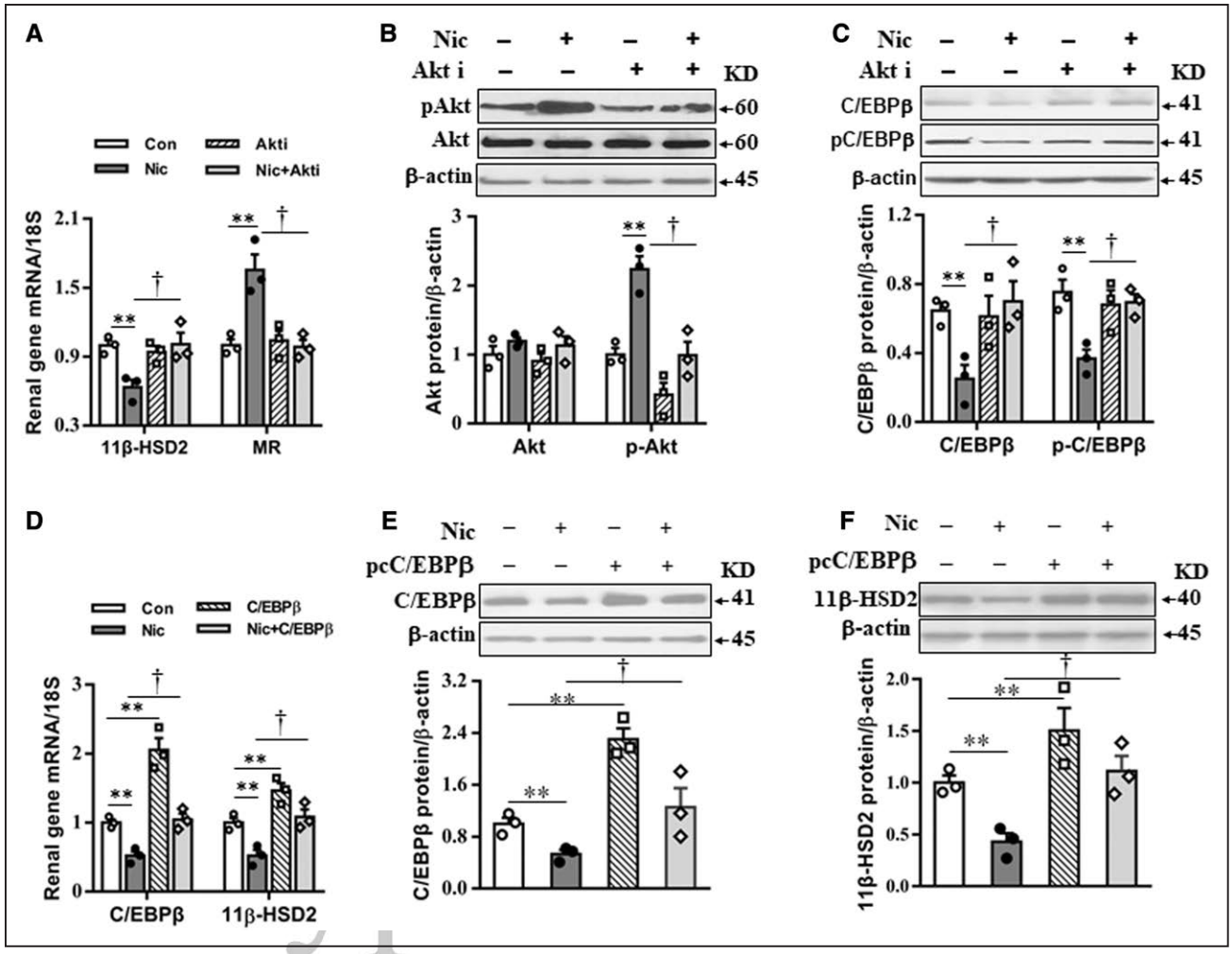

Figure 5. The effects of Akt inhibitor and C/EBPβ (CCAAT/enhancer-binding protein-β) overexpression in CCD M1 cells. Cells were incubated with nicotine (10^{-6} mol/L) in the absence or presence an Akt inhibitor VIII (10^{-6} mol/L; Akti) for 24 h. **A**, Relative alterations of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) and MR (mineralocorticoid receptor) mRNA levels. **B** and **C**, Relative expression levels of Akt, p-Akt (**B**), C/EBPβ, and p-C/EBPβ protein (**C**). **D**, Activation of C/EBPβ mRNA by its overexpression elevated 11β-HSD2 and attenuated the effects of nicotine on C/EBPβ and 11β-HSD2 expression in CCD M1 cells. Cells were transfected with either pcDNA3.1 C/EBPβ or control vector and cultured with or without nicotine for 24 h. **E** and **F**, Western blots demonstrating the impact of C/EBPβ overexpression on C/EBPβ (**E**) and 11β-HSD2 (**F**) proteins in these cells. Values are the mean±SEM from 3 separate culture preparations. ***P*<0.01 vs controls; †*P*<0.01 vs nicotine-treated cells.

corticosterone levels in the kidney after reduced 11β-HSD2 could increase renal MR expression. This is in agreement with our results demonstrating that nicotine enhanced the corticosterone-induced MR and ENaCα expression in CCD M1 cells, indicating that reduction of 11β-HSD2 by nicotine may contribute to increase MR occupancy by glucocorticoids. Moreover, we also found that the expression levels of cleaved ENaCα and ENaCγ, the major markers of ENaC activity[37] were increased in the kidneys of mice on nicotine, and this was accompanied by elevated serum Na^+ levels with decreased urinary Na^+/K^+ ratio. These data indicate that nicotine-mediated induction of renal MR and ENaC activity may contribute to abnormal Na^+ excretion and BP elevation. Induction of renal MR and ENaC by nicotine could promote aberrant urinary Na^+ reabsorption and decrease natriuresis. This would result in Na^+ retention linked to the development of hypertension. This is in agreement with nicotine decreasing urinary Na^+ excretion and increasing plasma Na^+ levels in rats[38] and in smokers.[29] Furthermore, we showed that nicotine-reduced serum renin is consistent with Na^+ retention and suppression of secretion, although did not suppress blood aldosterone levels that differ from that observed in mouse apparent mineralocorticoid excess model that exhibits decrease aldosterone levels without change in plasma glucocorticoid levels.[39] Moreover, nicotine decreased serum renin levels correlated with increased renal ENaC expression and BP in mice on normal salt diet. This differs from that seen in rats on high-salt diet in which the nicotine increased renin levels, whereas no alter renin activity in low-salt diet.[40] This is consistent with that renin secretion is negatively

regulated by renal ENaC and BP and can be modified by salt diet.[41,42] Our findings suggest that nicotine-induced BP elevation may be partly mediated through decreased 11β-HSD2 leading to higher MR occupation by unmetabolized corticosterone in the kidney. In addition, nicotine could also affect the sympathetic activity[43] endothelial function,[4] and immune fibrosis,[44] all of which are associated with the development of hypertension.

Pharmacological blockade of MR by spironolactone is known to affect several genes associated with renal function and BP and antagonizes the glucocorticoid-induced MR activation and hypertension after impaired 11β-HSD2 activity in animal models as well as in humans.[21,22,45] In the current study, we found that spironolactone treatment attenuated nicotine-induced activation of renal MR by glucocorticoids and prevented the increases in SBP. Moreover, spironolactone also reduced the renal cleaved ENaCα and ENaCγ protein contents with concomitant reversal of abnormal urinary Na^+ and K^+ levels and corrected the high serum Na^+ and the slightly low K^+ seen in nicotine-treated mice. These data indicate that spironolactone may exert its beneficial effects in protecting against the nicotine-induced hypertension by blocking the excessive MR occupancy and reducing ENaC abundance in the kidneys. This is consistent with the notion that MR antagonists can effectively block occupying renal MR by glucocorticoids and its resultant Na^+ retention and hypertension.[21,22] We also observed that ENaC blocker amiloride treatment markedly increased urinary Na^+/K^+ ratio and attenuated the elevated SBP in mice on nicotine. Our data support the possibility that the enhanced MR action in the kidney may contribute to the nicotine-induced Na^+ abnormalities and BP elevation through abnormal induction of ENaC-driven Na^+ reabsorption. In addition, spironolactone also reversed the nicotine-mediated reduction in renin levels, suggesting that there is a correction signal to suppress renin, which may be via BP or other mechanisms. However, MR is also present in other tissues, including brain, aorta, and heart, and is shown to have abundant expression in the central nervous system.[46,47] Central inhibition or deletion of 11β-HSD2 induces hypertension by activation of MR in brain, and these effects can be blocked by spironolactone in rats.[48,49] Thus, the possibility that affecting 11β-HSD2 activity and MR mediated responses in the brains and in the vasculature may cause the development of nicotine-induced hypertension, or at least the potential effects of MR antagonism in the brain and blood vessels contributing to reduction of BP in nicotine-treated mice must also be considered.

In addition, the current study also observed that inhibition of renal MR activation by spironolactone is correlated with attenuation of nicotine-induced reduction of 11β-HSD2 with upregulation of C/EBPβ in CCD M1 cells and the kidneys of mice. Moreover, overexpression of C/EBPβ exerted comparable effects to spironolactone on attenuating the nicotine-mediated repression of 11β-HSD2 in these intact cells. Induction of renal C/EBPβ could, in turn, stimulate 11β-HSD2 expression, thereby limiting intercellular availability of active glucocorticoids binding to activate MR linked to reduction of MR expression and normalized BP. Thus, the beneficial effects of spironolactone in the prevention of nicotine-induced BP elevation are not only largely due to its blockade of MR but also may be associated with the endogenous alterations of 11β-HSD2 in the kidneys. This is supported by earlier reports that spironolactone rescues the hyperglycemia-induced suppression of renal 11β-HSD2 and improves the development of hypertension in diabetic rats.[28]

Several mechanisms may be involved in the spironolactone-mediated improvement of the repression of nicotine-induced C/EBPβ and 11β-HSD2 and decreased MR activation. Indeed, recent studies have reported that spironolactone has a beneficial effect on renal cells through inhibition of Akt phosphorylation activation[50,51] that is increased by excess steroids binding to MR in VSMC and cardiomyocytes,[52,53] implying that spironolactone antagonizes the steroids occupancy of the MR that may require Akt signaling. Consistent with this notion, we found that the levels of p-Thr^{308}Akt were increased in response to the suppression of C/EBPβ and 11β-HSD2 with concomitant activation of MR in the kidneys of nicotine-treated mice. This is in agreement with nicotine increases Akt phosphorylation in VAMC and renal cells[30,31] and decreases C/EBPβ in epithelial cells.[26] Moreover, nicotinic acetylcholine receptors are expressed in the mouse kidney epithelial cells, where nicotine causes renal dysfunction via binding to nicotinic receptors and stimulation of α7 subunit of nicotinic acetylcholine receptors activates Akt signaling in the mouse kidneys.[54] This offers a potential signaling mechanism of the nicotine activation of Akt and thus reduction of C/EBPβ and subsequent suppression of 11β-HSD2 in CCD cells and in the mouse kidneys. Conversely, we observed that spironolactone inhibited the nicotine-induced activation of pThr^{308}Akt and upregulated C/EBPβ, which corresponded to elevated 11β-HSD2 and subsequently reduction of MR expression within the kidney. Thus, inhibition of pThr^{308}Akt activation by spironolactone may provide protective effects against glucocorticoid-mediated activation of MR in the kidney for preventing nicotine-induced BP elevation through alterations of C/EBPβ regulation of 11β-HSD2 expression. In addition, inactivation of pThr^{308}Akt by its inhibitor blocked the nicotine-mediated repression of C/EBPβ and rescued 11β-HSD2 in CCD M1 cells, indicating that activation of Akt signaling is responsible for nicotine-induced suppression of C/EBPβ leading to low 11β-HSD2 expression that could allow intracellular active glucocorticoids occupancy of MR in the kidney. This is in agreement with the notion that inhibition of Akt kinase

increases C/EBPβ expression in liver cells.[26,32] Our findings suggest that inhibition of pThr[308]Akt activation in kidneys may be a potential mechanism to account for the spironolactone attenuating nicotine-induced reduction of 11β-HSD2 through upregulation of C/EBPβ. Taken together, these findings support the hypothesis that spironolactone prevention of the nicotine-induced BP elevation may be exerted, in part by inhibition of Akt signaling leading to activation of C/EBPβ stimulation of 11β-HSD2.

In conclusion, our results indicate that nicotine-mediated suppression of renal 11β-HSD2 leading to greater occupying MR by glucocorticoids, and this response plays a significant role in the development of nicotine-induced hypertension. These findings suggest that modulation of renal 11β-HSD2 and MR may provide a possible therapeutic approach for the treatment of nicotine/smoking-mediated hypertension and other cardiovascular disorders in smokers.

Perspectives

Our studies demonstrate that nicotine downregulated renal 11β-HSD2, leading to increased intrarenal glucocorticoid activity and elevation of BP. Nicotine impairs glucocorticoid metabolism by repression of renal 11β-HSD2 led to higher MR occupancy, which may be an emerging mediator for smoking-induced BP elevation. Spironolactone may have potential effects in protecting against the hypertensive risks of nicotine/smoking. Future studies are required to determine if these mice would cause salt-sensitive hypertension which would provide additional insights into the mechanism underlie nicotine/smoking disorders renal function and elevated BP.

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Affiliations

Department of Pediatrics, Shanghai Tenth People's Hospital, Tongji University School of Medicine, China (Y.W., R.Y., Y.L.). Division of Endocrinology, Metabolism and Molecular Medicine, Charles R. Drew University of Medicine and Sciences, Los Angeles, CA (Y.W., J.W., P.W., R.P., S.K., K.L., T.C.F., Y.L.). David Geffen School of Medicine at University of California, Los Angeles (T.C.F., Y.L.). Department of Neonatology, The First Hospital of Jilin University, Changchun, China (J.W.). College of Pharmacy, Western University of Health Sciences, Pomona, CA (K.L.). Department of Endocrinology and Metabolism, Shanghai Jiaotong University Affiliated Sixth People's Hospital, Shanghai Diabetes Institute, China (L.L.). Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles (M.J.).

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Disclosures

None.

Supplemental Materials

Expanded Materials and Methods
Supplemental Figures
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