睾酮通过WNK1激酶调节肾脏钾离子相关通道的研究

孙宏 孙飞 刘珍*

(温州医科大学检验医学院、生命科学学院, 温州 325035)

摘要 该文探讨了睾酮通过WNK1(with-no-lysine kinase 1)激酶对肾脏钾离子相关通道的调控从而引起血压变化的机制。构建包含正常雄鼠、去势雄鼠和去势后注射睾酮雄鼠的实验模型, 通过检测表型, 运用实时荧光定量核酸扩增检测系统(Real-time quantitative polymerase chain reaction detecting system, QPCR)、免疫蛋白印迹(Western blot, WB)技术检测WNK1激酶、受WNK1介导的肾脏外髓钾通道(renal outer medullary potassium, ROMK)、大流量钙激活钾通道(large-conductance Ca^{2+}-activated K^+ channel, BK)和钠钾氯同向转运体2(Na-K-Cl cotransporter 2, NKCC2)的mRNA与蛋白表达, 同时用免疫荧光技术验证。表型结果显示, 去势雄鼠的血钾上升, 血压下降。去势雄鼠接受睾酮注射后, 血钾下降, 血压升高。QPCR和WB的结果显示, 去势雄鼠的WNK1相比于正常雄鼠均升高, 同时去势雄鼠注射睾酮之后, WNK1恢复原有表达。QPCR和WB的结果还显示, 去势雄鼠ROMK降低, BK-α和NKCC2升高, 去势雄鼠接受睾酮注射后, 三种钾离子相关通道的表达恢复正常。免疫荧光结果与QPCR和WB的结果相一致, 磷酸化WNK1的WB结果和正常雄鼠相比, 去势雄鼠磷酸化WNK1升高, 去势雄鼠接受睾酮注射后, 磷酸化WNK1降低。实验结果表明, 睾酮引起了WNK1、p-WNK1、ROMK、BK-α和NKCC2的变化。WNK1抑制剂注射实验发现, WNK1下游的肾脏离子通道ROMK表达升高, BK表达降低, 钠钾氯同向转运体NKCC2也降低, 表明WNK1可以调控ROMK、BK和NKCC2引起血压变化。这些发现表明, 睾酮能通过WNK1来调控肾脏钾离子相关通道进而影响血压。

关键词 睾酮; WNK1; ROMK; BK; NKCC2; 血压

Research on the Regulation of Potassium-Related Channels in Kidney by Testosterone through WNK1 Kinase

Sun Hong, Sun Fei, Liu Zhen*

(*School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou 325035, China*)

Abstract In this paper, the mechanism of changes in blood pressure caused by testosterone through with-no-lysine kinase 1 (WNK1) kinase regulation of potassium-related channels in kidney have been investigated. The experimental models are established on normal male mice, castrated male mice and castrated males injected with testosterone. The phenotype was measured. The protein and mRNA expressions of WNK1 kinase and renal potassium ion channels renal outer medullary potassium (ROMK), large conductance Ca^{2+}-activated K^+ (BK) and Na-K-2Cl cotransporter (NKCC2) mediated by WNK1 were detected by western blot (WB) and real-time quantitative polymerase chain reaction detecting system (QPCR) technology. Meanwhile, immunofluorescence technique was

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*通讯作者。Tel: 15168756006, E-mail: LiuzhenWMU@163.com

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*Corresponding author. Tel: +86-15168756006, E-mail: LiuzhenWMU@163.com

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used to verify the results. The phenotypic results showed that the blood potassium increase while the blood pressure decrease in castrated male mice. When the castrated male mice received testosterone, blood potassium decreased and blood pressure increased. WB and QPCR results showed that WNK1 increased in castrated male mice compared to normal male mice, and the normal expression of WNK1 was restored in castrated male mice with the testosterone injected. In the castrated male mice, the potassium channel ROMK decreased, while BK-α and NKCC2 increased. The expression of three potassium-related channels in castrated male mice are recovered after testosterone injection. Immunofluorescence results are consistent with the results of QPCR and WB. The WB results of phospho-WNK1 showed that phospho-WNK1, compared with normal male mice, elevated in ovariectomized male mice, and phospho-WNK1 decreased in castrated male mice after testosterone injection. The experimental results indicate that testosterone causes changes in WNK1, p-WNK1, ROMK, BK-α, and NKCC2. WNK1 inhibitor injection experiments showed that the expression of ROMK in the renal ion channel downstream of WNK1 increased, and the expression of BK decreased. Furthermore, the sodium-potassium chloride cotransporter 2 decreased, which indicates that WNK1 can regulate ROMK, BK and NKCC2. These findings indicate that testosterone can regulate blood potassium in renal potassium-related channels through WNK1.

Keywords testosterone; WNK1; ROMK; BK; NKCC2; blood pressure

WNK激酶是一类丝氨酸苏氨酸蛋白激酶, 是肾脏离子通道的上游调控元件, 它包括四个亚型: WNK1、WNK2、WNK3和WNK4[1]。WNK激酶的突变会导致一种以高血压为主要特征的疾病, 称为假性醛固酮减少症II型(pseudohypoaldosteronism type II, PHA-II)[2]。

Mayan等[3]研究表明, 女性PHA-II患者的高血压并发症的出现率和严重程度均明显比男性PHA-II患者低, 这表明, PHA-II患者的高血压症状在性别之间有差异。类似的血压性别差异在动物模型中也有报道。例如, 在原发性高血压大鼠中, 雌性的高血压发生时间要比雄性晚[4-6]。Calhou等[7]研究揭示了雌性WKR(wistar-kyoto rats, WKR)大鼠的血压明显比雄性大鼠低。这一现象引起了研究者们广泛的关注, 相关研究者对此展开研究并发现, 雄激素睾酮在性别血压差异中起着重要作用。研究表明, 去势雄性自发性高血压大鼠(spontaneously hypertensive rat, SHR)血压明显比正常雄鼠低, 在给去势雄性SHR大鼠注射睾酮之后, 血压相比会回升, 这表明睾酮参与了血压的性别差异调控[8-10]。更进一步的研究发现, 睾酮在维持血压平衡方面起着多重作用[11-12], 睾酮不仅可以通过调节心血管来影响血压, 还可以通过调节肾脏钠离子通道的变化来维持电解质和血压的平衡。Su等[13]研究发现, 相比于正常雄鼠, 去势雄鼠的上皮钠通道(epithelial sodium channel, ENaC)表达量会降低, 在接受睾酮注射后, ENaC又恢复原有水平, 这说明睾酮会影响肾脏钠离子通道的表达。

肾脏钾离子平衡在维持血压平衡方面同样起着重要作用[14]。研究表明, 远曲小管的钾平衡是控制血压的一个重要组成部分[14]。动物实验研究和临床证据表明, 高钾摄入会使血压降低, 钾耗竭会加剧原发性高血压[15]。钾离子相关通道主要包括ROMK[16]、BK[17]和NKCC2[18-19], 分别表达于整个远端肾小管、皮质集合管和髓袢升支粗段, 这些钾离子相关通道在钾平衡和血压的调节过程中扮演不可或缺的生理角色。ROMK[20]、BK[21-22]和NKCC2[23]的缺失会导致钾处理的缺陷和血压的不平衡。Yan等[24]研究发现了ROMK存在血压性别相关的潜在钾通道。

Huang等[25]研究发现, WNK1的磷酸化位点可以被Akt1/SGK1激活, 进而通过加强ROMK的内吞作用来抑制ROMK引起血压变化。Cai等[26]研究发现, WNK1可以通过减少ERK1/2信号介导的通道溶酶体降解来增强BK通道功能。Shibuya等[27]研究揭示了WNK1可以通过磷酸化SPAK/OSR1通路来调控NKCC2, 这些研究说明, WNK1可以调控ROMK、BK和NKCC2的变化。

Yu等[28]之前的研究揭示了WNK1介导的钾离子通道在性别血压差异中的作用, 即雌雄鼠体内的WNK1激酶表达含量具有差异性, 进而影响肾脏钾离子相关通道的表达, 最终导致雌雄鼠的血压出现性别差异。

睾酮在性别血压差异方面具有着重要的作用[11-12],

睾酮是否与WNK1介导的肾脏钾离子相关通道的表达相关, 目前为止尚未有相关研究报道。

本文以C57BL/6雄鼠为研究对象, 构建去势雄鼠, 去势后注射睾酮的雄鼠。检测它们的血压、血钾、尿总钾、WNK1激酶及肾脏钾离子相关通道的变化, 探讨睾酮、WNK1激酶与肾脏钾离子相关通道之间的关系。同时构建注射WNK1抑制剂雄鼠模型, 探讨WNK1与肾脏钾离子相关通道的关系。

1 材料和方法

1.1 材料

1.1.1 实验动物 本文以C57BL/6J背景的4个月大同窝雄鼠构建实验模型, 它们来自温州医科大学实验动物中心无特定病原体(specific pathogen free, SPF)动物实验室。雄鼠体质量均在25 g左右, 给予标准规格的啮齿类食物和自由饮水。饲养环境设置为国际标准饲养条件: 人工光照模拟白天黑夜各12 h循环, 室内温度为(21±2) ℃, 湿度为(55±2)%。本研究符合温州医科大学实验动物伦理委员会所制定的伦理学标准。

1.1.2 试剂 实验用睾酮购自阿拉丁生化科技股份有限公司。睾酮试剂盒购自CUSABIO公司。橄榄油购自麦克林生化科技有限公司。WNK1抑制剂购自MCE公司。麻药avertin(三溴乙醇)购自Sigma公司。TRIzol® RNA分离试剂购自Life Technologies公司。反转录试剂盒购自TaKaRa公司。PCR反应体系和荧光定量试剂盒购自Bio-Rad公司。蛋白酶抑制剂(Cocktail tablets)购自Roche公司。聚偏二氟乙烯(polyvinylidene fluoride, PVDF)膜(0.22 μm)购自Millipore公司。BCA蛋白浓度测定试剂盒购自Thermo Fisher Scientific公司。组织OCT包埋剂购自Sakura Finetechnical公司。实验所用引物购自生工生物工程有限公司。WNK1抗体购自Alpha Diagnostic International公司。磷酸化WNK1(phospho-WNK1)购自Cell Signaling Technology公司。免疫荧光所用WNK1抗体购自生工生物工程上海有限公司。NKCC2抗体购自Stress Marq Biosciences公司。ROMK抗体购自Alomone Labs公司。免疫荧光所用ROMK抗体购自Santa Cruz公司。ENaC抗体购自Alomone Labs公司。内参β-actin购自Beyotime Biotechnology公司。

1.2 方法

1.2.1 小鼠分组 选取2个月大的同窝雄鼠, 将小鼠分成手术组和正常组。手术组包括正常雄鼠, 去势雄鼠和去势后注射睾酮雄鼠。正常组包括正常雄鼠, 正常雄鼠注射WNK1抑制剂。

1.2.2 去势雄鼠和去势后注射睾酮雄鼠模型的建立 给正常雄鼠进行假手术, 将小鼠麻醉后仰放在手术台上, 给小鼠下腹部消毒, 距小鼠外生殖器1~2 cm处用手术剪开一个2 cm左右的小口, 先剪开皮肤, 再剪开筋膜, 不切除睾丸, 先将筋膜缝合, 再将皮肤缝合。去势小鼠和去势后注射睾酮小鼠实验操作: 腹腔开口后, 暴露腹腔脂肪, 顺着脂肪将左下侧的睾丸从腹腔取出, 先在睾丸两侧用手术线结扎好, 再将睾丸剪下, 将其他的脂肪放回腹腔, 再用同样的办法将右侧睾丸剪下, 最后缝合筋膜和皮肤。

1.2.3 睾酮给药实验 小鼠手术恢复两个月后, 开始进行注射实验, 经过多次预实验, 最终选择在手术组小鼠中给已去势后注射睾酮雄鼠腹腔注射50 μg/kg橄榄油溶解的睾酮, 同时正常雄鼠和去势雄鼠注射橄榄油做对照, 连续注射15天。

1.2.4 小鼠注射WNK1抑制剂实验 经多次预实验, 最终选定给实验组小鼠连续注射6 mg/kg WNK1抑制剂3天, 其中WNK1抑制剂溶解于含有10%二甲基亚砜(dimethylsulfoxide, DMSO)的橄榄油中, 对照组小鼠注射溶有10% DMSO的橄榄油。

1.2.5 小鼠血压的测量 血压测量采用的是日本Softron公司生产的型号为BP-98A的无创小鼠血压计, 采用尾套法测量。先使小鼠适应测血压操作, 连续适应几天之后再开始测血压。每天测20~30个循环, 取小鼠状态稳定时的值做统计分析。

1.2.6 血睾酮及血和尿电解质分析 血睾酮检测如下: 采用Elisa试剂盒检测睾酮的含量。将酶标板取出, 设1个空白对照孔, 不加任何液体, 每个标准点依次各设2孔, 每孔加入相应标准品50 μL, 其余每个标准品加入待测标本50 μL。每孔加入酶结合物50 μL, 再按同样的顺序加入抗体50 μL, 充分混匀, 贴上不干胶片, 置于37 ℃下温育1 h。手工洗板, 弃去孔内液体, 洗涤液洗板3次, 拍板拍干。每孔加显色液A液50 μL, 显色液B液50 μL, 振荡混匀后, 37 ℃避光显色15 min, 每孔加终止液50 μL。用酶标仪在450 nm波长处依序测量各孔的光密度。

血钾检测如下: 小鼠麻醉后眼眶静脉取血, 低速离心取上清, 用火焰分光光度计检测血清钾水平。

尿钾检测如下: 小鼠置于代谢笼, 期间供给常

规饲料和自由饮水, 待其稳定后收集尿样, 用火焰分光光度计检测尿钾离子浓度。

1.2.7 WNK1激酶及肾脏钾离子相关通道的mRNA含量的检测 将3组小鼠同时杀死取肾脏, 按照TRIzol试剂盒操作说明裂解分离提取总RNA。反转录得到cDNA, 用实时荧光定量方法分析肾脏相关基因的转录水平。其中所用的引物序列如表1所示。

1.2.8 WNK1激酶及肾脏钾离子相关通道的蛋白含量检测 将小鼠杀死后, 取肾脏, 用蛋白裂解液充分裂解提取总蛋白, 测定蛋白浓度, 变性处理, 经聚丙烯酰胺凝胶电泳分离, 转移至PVDF膜。电转完毕后, 取条带于5%脱脂牛奶内封闭2 h。一抗过夜, 抗体稀释度为1:2 000。第2天回收一抗后TBST洗3次, 每次10 min。用含有1%脱脂牛奶的TBST稀释二抗, 室温孵育2 h, 稀释度为1:4 000。再用TBST洗3次后用发光液ECL法显色。为避免免疫印迹不同标本蛋白量不同造成的影响, 免疫印迹结果用β-actin进行矫正。

1.2.9 组织免疫荧光法检测小鼠WNK1及肾脏钾离子相关通道的表达 小鼠肾脏的固定及切片如下: 将小鼠用剪刀迅速打开腹腔, 取下左侧肾脏后, 将小鼠左肾动脉端用动脉夹固定。打开下腔静脉, 经左心室插入头皮针连接的注射器, 用磷酸缓冲盐(phosphate buffer saline, PBS)溶液冲洗另一侧肾脏, 肾脏颜色变白后用4%多聚甲醛固定肾脏。纵向切成等量2份, 放入多聚甲醛中固定3 h, 然后放入到30%蔗糖溶液(PBS溶液配制) 4 ℃过夜脱水。用OCT包埋剂包埋肾脏, 放入液氮中冷冻。用冰冻切片机4 μm切片, 于光学显微镜下观察切片样品的结构是否完整, 选择组织平整的样品用于以下实验。

免疫荧光方法如下: 活化, 将切片用PBS水化5 min; 通透细胞, 将样品放入用PBS配制的0.2% TritonX 100中10 min; 然后将样品放入PBS溶液中, 摇床上摇晃洗涤3次, 每次10 min; 消除本底荧光, 将样品转入到PBS配制的0.1% NH_4Cl溶液中40 min; 然后用PBS洗涤3次, 每次10 min; 封闭, 甩去PBS, 擦干样品周围的水分; 用免疫组化笔在组织周围画一大小适当的圈; 取PBS配制的10%驴血清, 室温孵育1 h。一抗孵育如下: 去除封闭液, 将载玻片放入暗盒中, 用合适浓度的抗体覆盖样品4 ℃孵育过夜。二抗孵育如下: 回收一抗, 将含样品的玻片放入免疫荧光专用盒中, 加入PBS置于摇床上洗涤, 10 min×3次。加上相应二抗, 室温孵育1 h。封片步骤如下: 去除二抗, 加入PBS置于摇床上洗涤, 10 min×3次, 注意避光。加上适量抗荧光淬灭剂, 盖上盖玻片, 以待镜检。

1.2.10 统计分析 每个实验重复3~4次, 运用GraphPad Prism 5.0软件进行统计分析。所有数据采用均数±标准误差($\bar{x}$±SEM)表示, 对照组与实验组之间的数据采用两独立样本t检验, P<0.05表示差异具有统计学意义。

2 结果

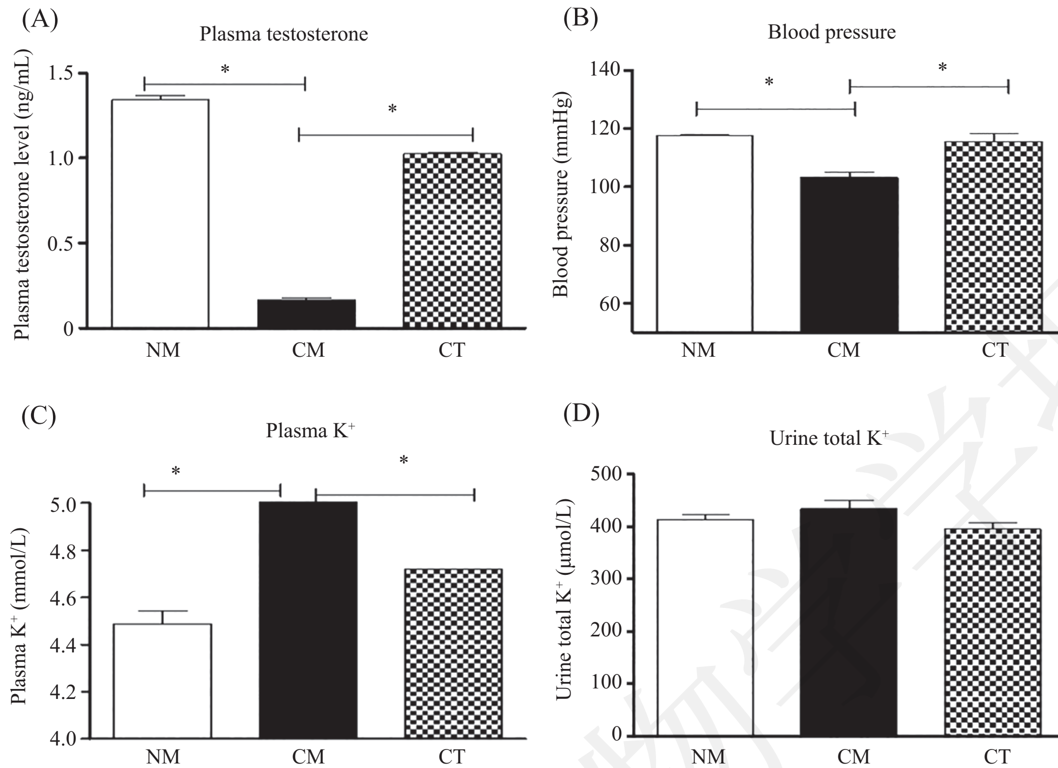
2.1 小鼠血睾酮、血压及钾代谢结果

如图1A所示, 正常雄鼠的血睾酮含量在1.34 ng/mL左右, 去势后雄鼠的血睾酮含量仅为正常雄鼠的八分之一左右, 给去势雄鼠注射睾酮之后, 血睾酮的含量

表1 主要引物序列

Table 1 Main primer sequences

目的基因 Objective gene	引物序列 Primer sequences
WNK1	Forward 5′-GTC TGG ACA CCG AAA CCA CT-3′
	Reverse 5′-CGA ACA ATG TTG GGA TGT TG-3′
ROMK	Forward 5′-ACA GAC AAA ACT GAA CAG CAC CAC-3′
	Reverse 5′-GGA GAC CAA CCT TGC TCG TTG-3′
BK-α	Forward 5′-TCT CAG CAT TGG TGC CCT CGT AAT-3′
	Reverse 5′-GTA GAG GAG GAA GAA CAC GTT GAA-3′
BK-β4	Forward 5′-TGC CTT TGG GTC AAT GTA TCA GCT-3′
	Reverse 5′-GCA ATA GAA TTC ATG GTG CTT ATA-3′
NKCC2	Forward 5′-CCA GAG CGT TGT CTA AAG CA-3′
	Reverse 5′-TGG GCA GCT GTC ATC ACT TA-3′
Cyclophilin	Forward 5′-CGT GGC TCC GTT GTC TT-3′
	Reverse 5′-TGA CTT TAG GTC CCT TCT TCT TAT CG-3′



A: 去势雄鼠血睾酮降低, 注射睾酮之后血睾酮升高; B: 去势雄鼠和正常雄鼠相比, 血压降低, 注射睾酮之后, 血压升高; C: 去势雄鼠比正常雄鼠相比血钾升高, 注射睾酮之后血钾降低; D: 正常雄鼠、去势雄鼠、去势后注射睾酮之后3种小鼠24 h后尿总钾没有区别。所有实验都是重复3次获得相似结果, *P<0.05。NM: 注射橄榄油的正常雄鼠; CM: 注射橄榄油的去势雄鼠; CT: 注射睾酮的去势雄鼠。

A: the decrease of serum testosterone in castrated male mice and the increase of serum testosterone after testosterone injection. B: the blood pressure of castrated male mice was decreased and the blood pressure was increased after testosterone injection. C: the serum potassium of castrated male mice is higher than that of normal male mice, and decreased after testosterone injection. D: no difference in total urine potassium after 24 h between the three kinds of male mice. All the experiments were repeated three times with similar results, *P<0.05. NM: normal male mice injected with olive oil; CM: castrated male mice injected with olive oil; CT: castrated male mice injected with testosterone.

图1 正常雄鼠、去势雄鼠和去势后注射睾酮雄鼠的血睾酮、血压、血钾和尿总钾的分析

Fig.1 Analysis of serum testosterone, blood pressure, blood potassium and total potassium of urine in normal male, castrated male mice and castrated male mice injected with testosterone

有升高, 是去睾丸雄鼠的七倍左右。去势雄鼠血压是103 mmHg, 而正常雄鼠血压是117 mmHg左右, 血压显著降低(P<0.05)。再给去势雄鼠注射15天50 μg/kg睾酮之后, 发现血压又显著回升至115 mmHg(图1B), 说明睾酮可以使血压升高。比较去势雄鼠和正常雄鼠的血液和尿液电解质, 发现去势雄鼠的血钾升高。去势雄鼠注射睾酮之后, 血钾降低(图1C), 三种雄鼠尿总钾无明显差别(图1D), 说明睾酮可以使血钾降低。

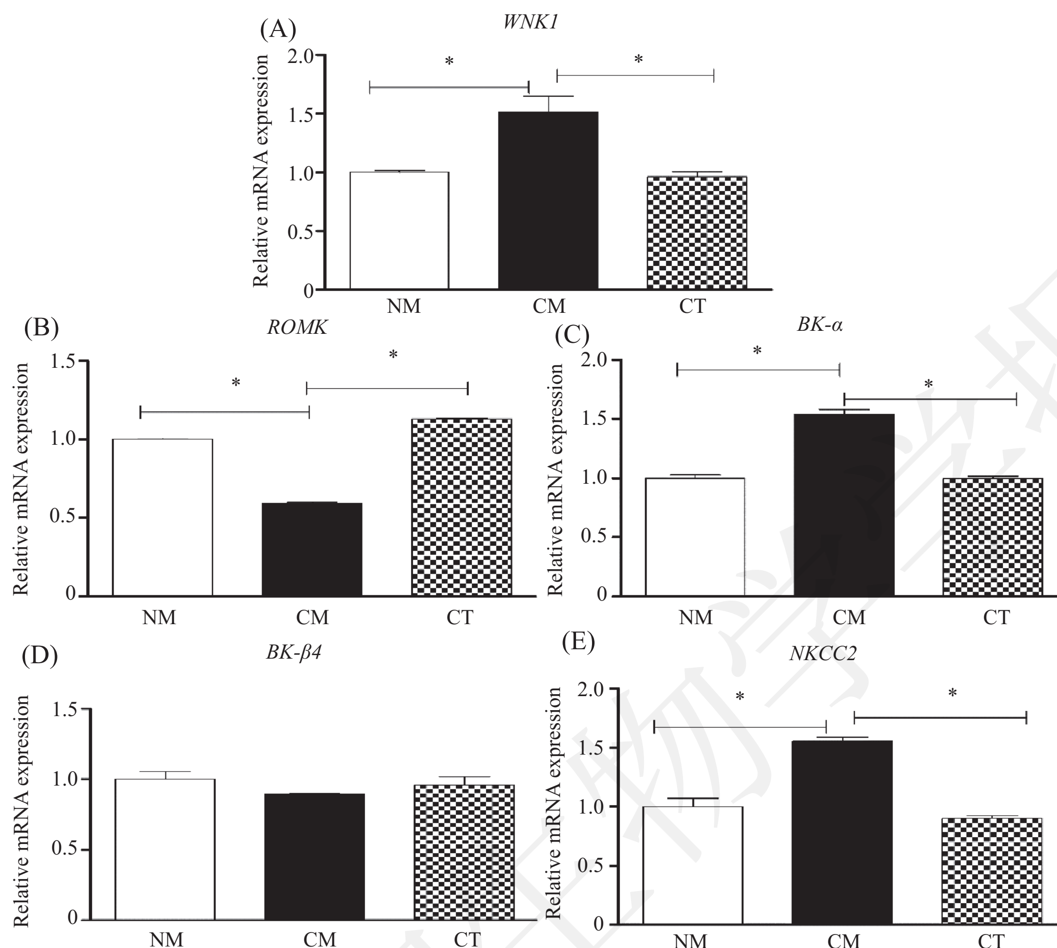
以上结果证实了睾酮可以通过调节钾代谢来影响血压, 进而表明睾酮是血压升高的一个重要调节因子。

2.2 小鼠*WNK1*及肾脏钾离子相关通道蛋白的mRNA表达情况

为了研究*WNK1*及肾脏钾离子相关通道蛋白的基因表达水平, 本文运用QPCR技术检测三种小鼠的*WNK1*、*ROMK*、*BK*及*NKCC2*的mRNA表达情况, 结果如图2所示。从图2中可以看出, 和正常雄鼠相比, 去势雄鼠的*WNK1*、*BK-α*和*NKCC2*明显升高(图2A、图2C和图2E), 且*ROMK*的表达明显下降(图2B), 给去势雄鼠注射睾酮之后, *WNK1*、*BK-α*和*NKCC2*又下降(图2A、图2C和图2E), 而*ROMK*表达回升(图2B), 三种雄鼠*BK-β4*没有明显区别(图2D)。QPCR结果说明, 睾酮在转录水平上引起了钾离子通道上游激酶WNK1及肾脏钾离子相关通道ROMK、BK和NKCC2的变化。

2.3 小鼠WNK1及肾脏钾离子相关通道蛋白表达情况

为了进一步探究睾酮具体影响小鼠血压及血钾的机制。本文运用WB技术检测正常雄鼠、去势雄鼠和去势后注射睾酮雄鼠的WNK1、磷酸化



A: 三种雄鼠*WNK1*的mRNA表达情况; B: 三种雄鼠*ROMK*的mRNA表达情况; C: 三种雄鼠*BK-α*的mRNA表达情况; D: 三种雄鼠*BK-β4*的mRNA表达情况; E: 三种雄鼠*NKCC2*的mRNA表达情况。内参基因是*cyclophilin*。所有实验都是重复3次获得相似结果, $^*P<0.05$。NM: 注射橄榄油的正常雄鼠; CM: 注射橄榄油的去势雄鼠; CT: 注射睾酮的正常雄鼠。

A: the mRNA expression of *WNK1* in three male mice. B: the mRNA expression of *ROMK* in three male mice. C: the mRNA expression of *BK-α* in three male mice. D: the mRNA expression of *BK-β4* in three male mice. E: the mRNA expression of *NKCC2* in three male mice. The housekeeping gene is cyclophilin. All the experiments were repeated three times with similar results, $^*P<0.05$. NM: normal male mice injected with olive oil; CM: castrated male mice injected with olive oil; CT: castrated male mice injected with testosterone.

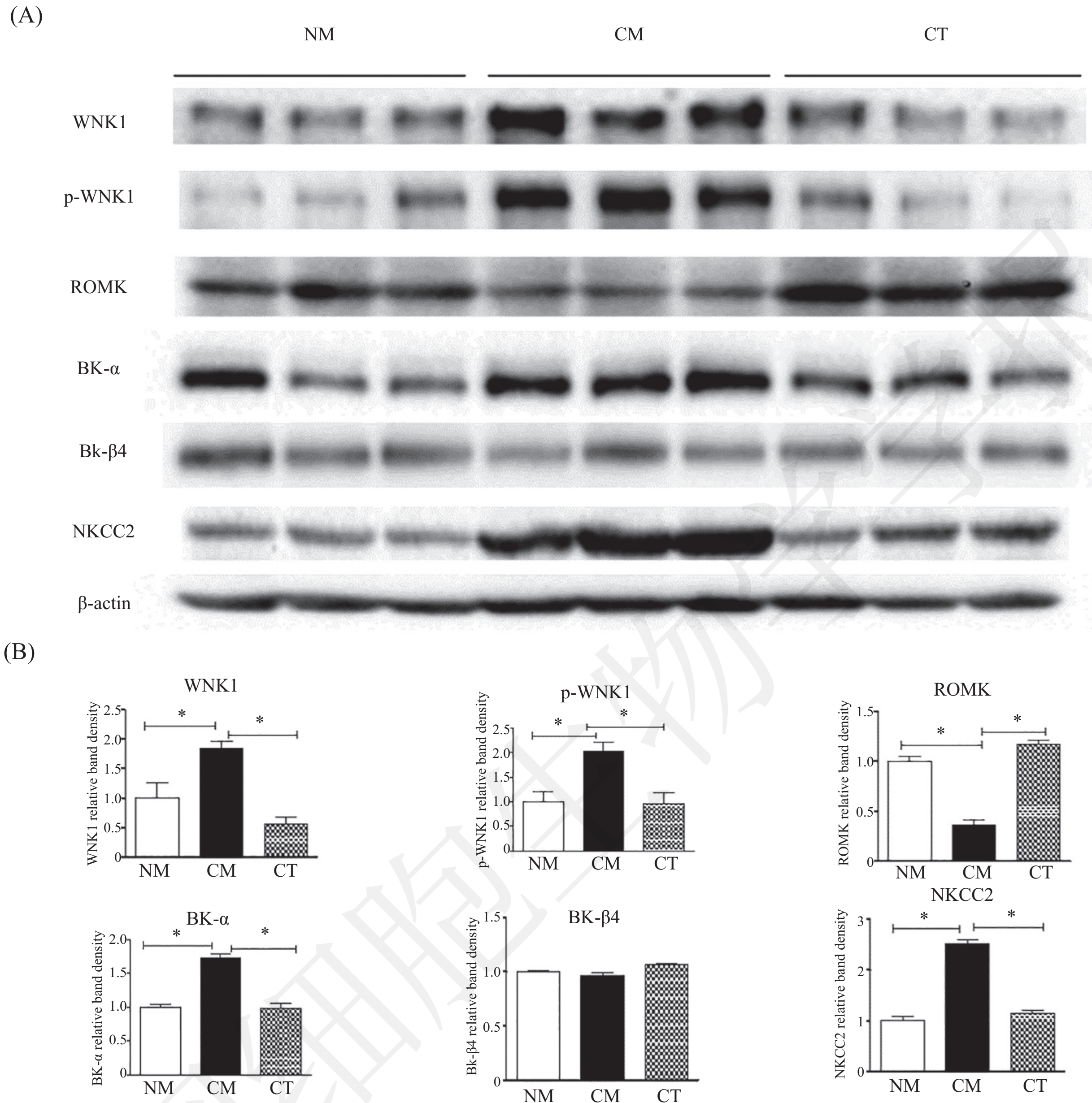
图2 正常雄鼠、去势雄鼠和去势后注射睾酮雄鼠的*WNK1*、肾脏钾离子相关通道*ROMK*、*BK-α*、*BK-β4*和*NKCC2*的mRNA表达情况

Fig.2 mRNA expression of *WNK1*, renal potassium ion channels *ROMK, BK-α*, *BK-β4* and *NKCC2* in normal male mice, castrated male mice and castrated male mice injected with testosterone after castration

WNK1、ROMK、BK和NKCC2的翻译水平的表达结果, 结果如图3所示。从图3A和图3B中可以看出, 跟正常雄鼠相比, 去势雄鼠的WNK1、磷酸化WNK1、BK-α和NKCC2蛋白表达量升高, 去势雄鼠接受睾酮注射后WNK1、磷酸化WNK1、BK-α和NKCC2蛋白表达又降低。BK-β4在三种小鼠中蛋白表达量没有区别。去势雄鼠的ROMK蛋白表达量比正常雄鼠降低, 且在接受睾酮注射后, 表达量升高。

2.4 小鼠WNK1及肾脏钾离子相关通道免疫荧光表达情况

本文采用免疫荧光实验来观察WNK1、ROMK、BK和NKCC2在肾小管的荧光强弱, 从侧面验证mRNA和蛋白的表达结果, 结果呈现在图4中。图4A和图4B表明, 去势雄鼠和正常雄鼠相比, WNK1在肾小管荧光表达增强, 注射睾酮的去势雄鼠WNK1的荧光变弱。和正常雄鼠相比, 去势雄鼠的ROMK在肾小管处免疫荧光强度较弱, 且在接受睾酮注射后, 荧光强度增强。去势雄鼠和正常雄鼠相比, BK-α的荧光强度增强, 当去势雄鼠注射睾酮之后, BK-α的荧光强度减弱。BK-β4在三种小鼠中荧光强度没有区别。去势雄鼠和正常雄鼠相比, NKCC2的免疫荧光增强, 注射睾酮后荧光变弱。



A: WNK1、p-WNK1、ROMK、BK-α、BK-β4和NKCC2的蛋白印迹分析; B: WNK1、p-WNK1、ROMK、BK-α、BK-β4和NKCC2的灰度值结果。所有实验都是重复3次获得相似结果, **P*<0.05。NM: 注射橄榄油的正常雄鼠; CM: 注射橄榄油的去势雄鼠; CT表示注射睾酮的去势雄鼠。

A: the protein imprinting analysis of WNK1, p-WNK1, ROMK, BK-α, BK-β4 and NKCC2. B: the gray value results of WNK1, p-WNK1, ROMK, BK-α, BK-β4 and NKCC2 respectively. All the experiments were repeated three times with similar results, **P*<0.05. NM: normal male mice injected with olive oil; CM: castrated male mice injected with olive oil; CT: castrated male mice injected with testosterone.

图3 正常雄鼠、去势雄鼠和去势后注射睾酮雄鼠的WNK1、p-WNK1、肾脏钾离子相关通道ROMK、BK-α、BK-β4和NKCC2的蛋白表达情况

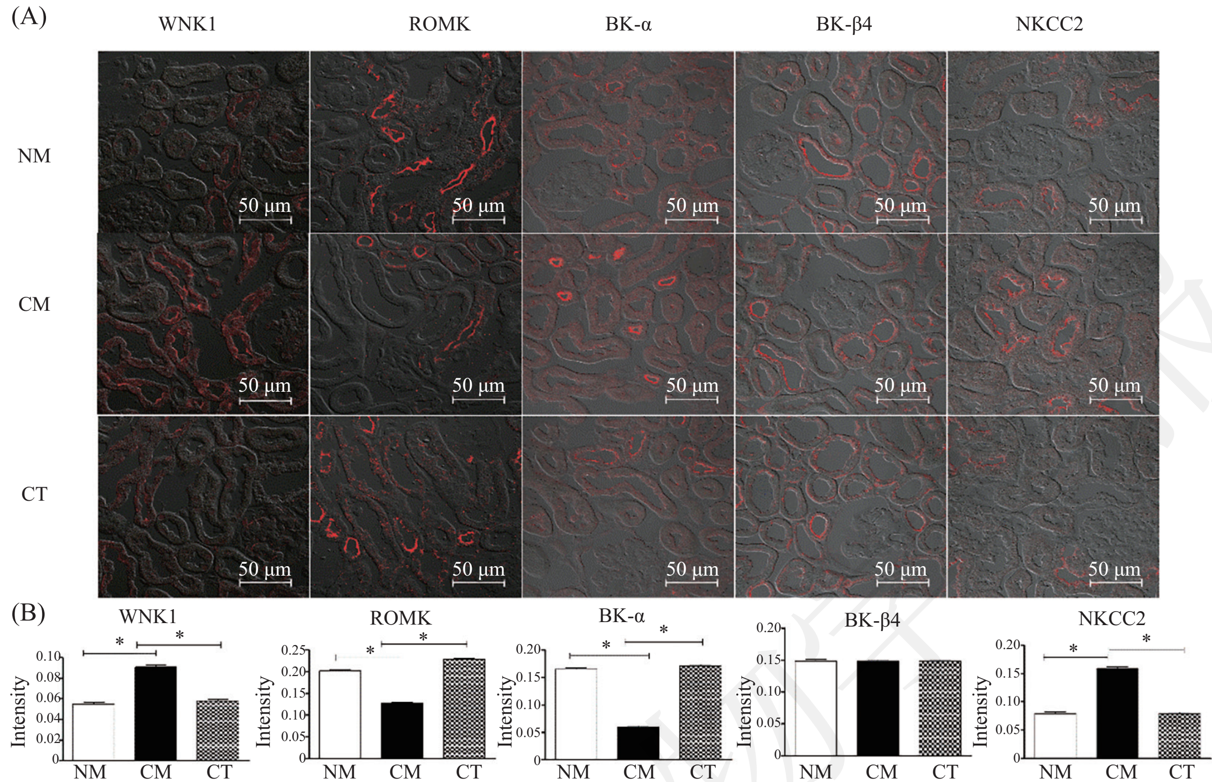
Fig.3 The protein expression of WNK1, p-WNK1, renal potassium ion channels ROMK, BK-α, BK-β4 and NKCC2 in normal male mice, castrated male mice and male mice injected with testosterone after castration

2.5 WNK1抑制剂注射小鼠钾代谢结果

为了进一步研究WNK1是否可以调控肾脏钾离子相关通道, 本文开展了小鼠注射WNK1抑制剂实验, 发现小鼠的钾代谢发生了变化, 结果如图5所示。从图5中可以看出, 相比于未注射WNK1抑制剂的正常小鼠, 注射WNK1抑制剂的小鼠血钾和尿总钾出现降低现象, 这表明WNK1抑制剂引起了小鼠钾代谢的变化。

2.6 WNK1抑制剂注射小鼠WNK1及钾离子相关通道的蛋白表达情况

为了进一步研究WNK1是否调控了肾脏钾离子

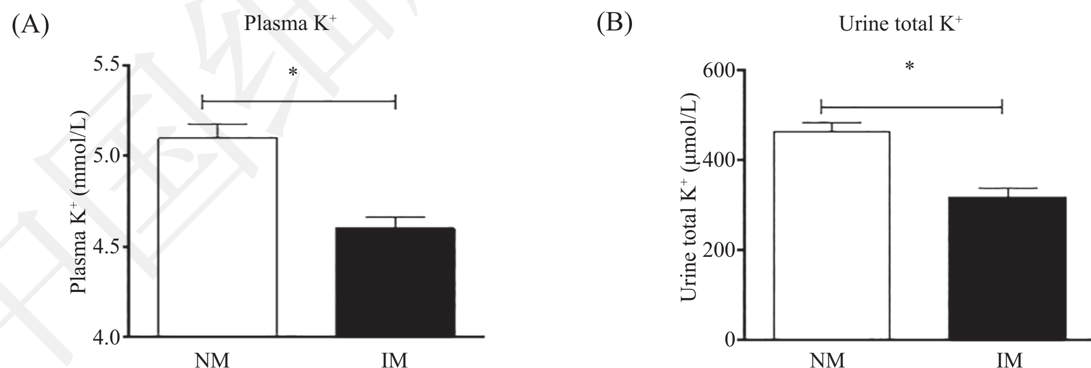


A: 三种小鼠的WNK1、ROMK、BK-α、BK-β4和NKCC2的免疫荧光表达结果; B: WNK1、ROMK、BK-α、BK-β和NKCC2在正常雄鼠、去势雄鼠和去势后注射睾酮雄鼠免疫荧光亮度结果。所有实验都是重复3次获得相似结果, $^*P<0.05$。

A: the results of WNK1, ROMK, BK-α, BK-β and NKCC2 immunofluorescence expression in three kinds of mice. B: the results of immunofluorescence intensity of WNK1, ROMK, BK-α, BK-β and NKCC2 in normal male mice, castrated male mice and testosterone male mice after castration. All the experiments were repeated three times with similar results, $^*P<0.05$.

图4 正常雄鼠、去势雄鼠和去势后注射睾酮雄鼠的WNK1、肾脏钾离子相关通道ROMK、BK-α、BK-β4和NKCC2的免疫荧光结果

Fig.4 Immunofluorescence of WNK1, renal potassium ion channels ROMK, BK-α, BK-β4 and NKCC2 in normal male mice, castrated male mice and castrated male mice injected with testosterone

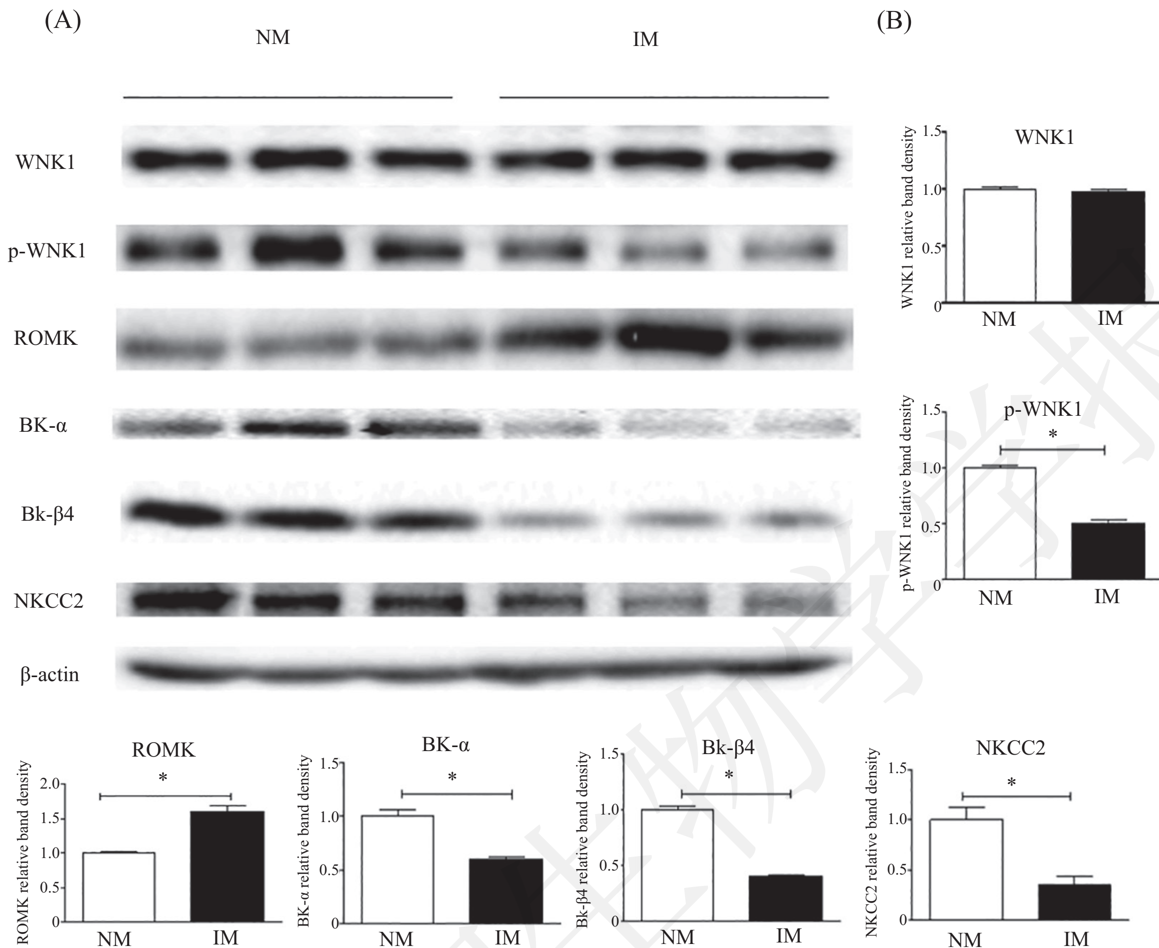


A: 和未注射WNK1抑制剂的雄鼠相比, 注射WNK1抑制剂后雄鼠血钾降低。B: 和未注射WNK1抑制剂的雄鼠相比, 注射WNK1抑制剂24 h后雄鼠的尿总钾降低。所有实验都是重复3次获得相似结果, $^*P<0.05$。NM: 注射10% DMSO溶于橄榄油的小鼠; IM: 注射WNK1抑制剂溶于含有10% DMSO的橄榄油的小鼠。

A: the serum potassium of male mice injected with WNK1 inhibitor was lower than that of male mice not injected with WNK1 inhibitor. B: the total urine potassium of male mice injected with WNK1 inhibitor is lower than that of male mice not injected with WNK1 inhibitor after 24 h. All the experiments were repeated three times with similar results. $^*P<0.05$. NM: mice injected with 10% DMSO in olive oil; IM: mice injected with WNK1 inhibitor which was dissolved in olive oil containing 10% DMSO.

图5 正常雄鼠和注射WNK1抑制剂的雄鼠血钾和尿总钾的分析

Fig.5 Analysis of blood potassium and total potassium of urine in normal male mice and male mice injected with WNK1 inhibitor



A: WNK1、p-WNK1、ROMK、BK-α、BK-β4和NKCC2的蛋白印迹分析。B: WNK1、p-WNK1、ROMK、BK-α、BK-β4和NKCC2的灰度值结果。所有实验都是重复3次获得相似结果, *P<0.05。NM: 注射溶有10% DMSO的橄榄油小鼠; IM: 注射WNK1抑制剂溶于含有10% DMSO的橄榄油的小鼠。

A: the protein imprinting analysis of WNK1, p-WNK1, ROMK, BK-α, BK-β4 and NKCC2. B: the gray value results of WNK1, ROMK, BK-α, BK-β4 and NKCC2 respectively. All the experiments were repeated three times with similar results, *P<0.05. NM: mice injected with 10% DMSO in olive oil; IM: mice injected with WNK1 inhibitor which was dissolved in olive oil containing 10% DMSO.

图6 正常雄鼠和注射WNK1抑制剂雄鼠的WNK1、p-WNK1、肾脏钾离子相关通道ROMK、BK-α、BK-β4和NKCC2的蛋白表达情况

Fig.6 The protein expression of WNK1, p-WNK1, renal potassium ion channels ROMK, BK-α, BK-β4 and NKCC2 in normal male mice and male mice injected with WNK1 inhibitor

相关蛋白的表达, 本文检测了WNK1、ROMK、BK和NKCC2的蛋白表达情况。相比于未注射WNK1抑制剂的小鼠, 注射WNK1抑制剂小鼠的WNK1总蛋白未发生变化, 磷酸化WNK1表达明显降低, ROMK表达明显升高, BK和NKCC2的表达均降低(图6A和图6B)。这些结果表明, WNK1活性的变化可以引起ROMK、BK和NKCC2蛋白表达的变化。

3 讨论

本文的研究表明, 睾酮在ROMK、BK、NKCC2和WNK1的表达方面起着一定的调节作用, 揭示了睾酮通过WNK1调节肾脏钾离子相关通道的机制, 建立了睾酮与性别血压差异之间的联系。

WNK1在雌雄鼠血压性别差异中作用的研究此前已有报道[28]。Yu等[28]的研究发现了WNK1与雌雄鼠的血压性别差异有关, 即WNK1通过调节肾脏钾离子相关通道的变化来影响雌雄鼠的血压, 但是WNK1对肾脏离子通道起作用的上游调控因素还不清楚。本文的研究结果发现了上游调控因子睾酮在WNK1调节肾脏钾离子相关通道中起着一定的作用, 并揭示了睾酮通过WNK1调节肾脏钾离子相关通道的机制。

中国细胞生物学学报

ROMK是表达于肾脏远曲小管顶端膜上的肾脏钾离子通道, 是一个重要的钾排泄通路。WNK1对ROMK的卵母细胞的表面表达具有直接的抑制作用[29]。Hadchouel等[30]研究表明, WNK1的增加引起ROMK的表达减少,可能会导致高血钾。本文通过WNK1抑制剂实验发现, 给小鼠注射WNK1抑制剂后, WNK1的磷酸化水平降低, ROMK的表达升高, 这说明, WNK1活性的变化可以引起ROMK表达出现变化。Yu等[28]的研究首次揭示了WNK1对ROMK在钾平衡和性别血压调节方面的作用。本研究进一步发现, 去势雄鼠血钾升高, 血压降低, WNK1升高, 磷酸化的WNK1也升高, ROMK降低。当给去势雄鼠注射睾酮之后, 血钾降低, 血压升高, WNK1降低, 磷酸化的WNK1降低, ROMK升高。以上结果说明, 睾酮能够通过改变WNK1的表达量和活性来调控ROMK引起血压和血钾的变化。

BK通道至少包括α和β两种亚基, β亚基不形成孔道, 只起修饰和调节作用[31], BK-α具有重要的钾排泄功能。基于多种转基因小鼠模型的研究已经确定WNK1会正调控BK-α来诱导钾的排泄, 进而影响血压的平衡[26]。*BK-α*基因的敲除会引起钾排泄的缺失, 导致高血压[21]。本文通过给小鼠注射WNK1抑制剂, 然后检测WNK1和BK的表达, 发现注射抑制剂后, WNK1的磷酸化水平降低, BK的表达也降低, 这说明, WNK1活性的变化可以引起BK的变化。之前已有研究发现, 雌鼠和雄鼠相比BK-α的表达量增高, 雌鼠血压相对于雄鼠更低, 显示出BK-α表达量升高会引起血压降低[28]。本文的研究发现, 去势雄鼠血压降低, WNK1、磷酸化WNK1和BK-α升高, 去势雄鼠注射睾酮之后, 血压升高, WNK1、磷酸化WNK1和BK-α均降低, 这说明, 睾酮可以通过改变WNK1的表达量和活性来影响BK-α的表达, 进而影响血压。BK-β4在三种雄鼠中表达没有区别, 说明睾酮可能对BK-β4没有调控作用。但是, 增加BK-α促进了钾的排泄, 这似乎和去势雄鼠的高血钾相悖。因此, 本文认为, 升高的BK-α可能是对更高的血钾的代偿反应, 进而导致去势雄鼠的血压降低。

NKCC2作为一个重要的钾重吸收通道, 它存在于肾小管髓袢升支粗段中。WNK1对NKCC2的起着正调控作用[27]。本文通过给小鼠注射WNK1抑制剂发现WNK1磷酸化水平降低, NKCC2的表达降低, 这说明WNK1引起了NKCC2表达的变化, 这和以前的研究结果较为一致[27]。Masilamani等[32]的研究表明, 性激素会影响NKCC2的顶端等离子体膜表达量。本文的研究结果显示, NKCC2在去势雄鼠体内的表达量更高。NKCC2的缺失会显示出巴特综合征I, 巴特综合征I以严重的高血钾为特点[23], 说明NKCC2可能会促进去势雄鼠肾脏钾的潴留和更高的血钾。然而之前研究证明, NKCC2也负责血压的调节, 激活的NKCC2可以引起大鼠的高血压[33]。因此, 增加的NKCC2不符合去势雄鼠血压下降的要求, 这可能为降低去势雄鼠的血压提供了一种补偿机制。

本文的研究揭示了睾酮通过影响WNK1的表达和活性来对ROMK、BK-α和NKCC2进行调控, 进而影响钾在体内的平衡, 更细微的调控机制有待进一步的深入探究。综上所述, 本文的研究发现了睾酮可以通过WNK1来调控肾脏钾离子相关通道来调控血压, 为高血压的个体化治疗提供了新的思路。

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