



氧化三甲胺通过PI3K/AKT/SREBP1通路诱导肾纤维化*

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【摘要】目的 探讨尿毒症毒素氧化三甲胺(trimethylamine N-oxide, TMAO)在肾脏纤维化中的作用及其机制。**方法** 将20只雄性BALB/c小鼠随机平均分为对照组和TMAO组,每组10只。对照组腹腔注射生理盐水, TMAO组腹腔注射含TMAO的生理盐水[20 mg/(kg·d)],每日1次,持续8周;使用HE染色与Masson染色法观察分析小鼠肾脏切片病理及纤维化水平;采用免疫组化法检测肾脏组织中 α -肌动蛋白(alpha smooth muscle actin, α -SMA)、重组人纤维连接蛋白片段(recombinant human fibronectin fragment, Fibronectin)、胆固醇调节元件结合蛋白-1(sterol-regulatory element binding protein 1, SREBP1)水平;采用Western blot检测肾脏组织中 α -SMA、SREBP1、磷脂酰肌醇3激酶(phosphatidylinositol 3 kinase, PI3K)、磷酸化PI3K(phospho-PI3K, p-PI3K)、蛋白激酶B(protein kinase B, PKB, 又称AKT)和磷酸化AKT(phospho-AKT, p-AKT)蛋白水平。分别用SREBP1 siRNA和PI3K/AKT抑制剂组处理HK2细胞,检测对TMAO效应的逆转作用。**结果** 动物实验显示,与对照组相比,腹腔注射TMAO后小鼠出现肾脏组织病理损伤和纤维化,纤维化标志物 α -SMA、Fibronectin表达升高(P 均 <0.05)。相对于对照组, TMAO处理组小鼠肾脏中SREBP1表达升高(P <0.05), PI3K磷酸化比值、AKT磷酸化比值也发生了上调(P 均 <0.05)。细胞实验验证了上述结果。利用siRNA干扰肾小管上皮细胞中SREBP1表达后,纤维化指标蛋白表达下降(P <0.05);利用PI3K-AKT通路抑制剂LY294002孵育HK2细胞后, TMAO导致的SREBP1高表达被抑制(P <0.05)。**结论** TMAO可能通过促进PI3K/AKT/SREBP1通路而诱导肾脏纤维化。

【关键词】 氧化三甲胺 肾脏纤维化 PI3K/AKT通路 SREBP1

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【Abstract】 Objective To investigate the role and mechanism of trimethylamine N-oxide (TMAO), a uremic toxin, in renal fibrosis. **Methods** A total of 20 male BALB/c mice were randomly and evenly assigned to a Control group and a TMAO group. Mice in the Control group received intraperitoneal injection of normal saline, while mice in the TMAO group received intraperitoneal injection of TMAO (20 mg/[kg·d]). The injection was given once a day for 8 weeks. Histopathology and fibrosis of kidney were observed by H&E staining and Masson staining. Immunohistochemistry was performed to determine the levels of alpha smooth muscle actin (α -SMA), recombinant human fibronectin fragment (Fibronectin), and sterol-regulatory element binding protein 1 (SREBP1). Western blot was performed to determine α -SMA, SREBP1, phosphatidylinositol 3 kinase (PI3K), phospho-phosphatidylinositol 3 kinase (p-PI3K), protein kinase B (PKB, also known as AKT), and phospho-AKT (p-AKT) protein levels. HK2 cells were treated with SREBP1 small interfering RNA (siRNA) and PI3K/AKT inhibitor, respectively, and the reversal of the effects of TMAO was examined. **Results** Animal experiments showed that, compared with the Control group, the mice treated with TMAO experienced pathological damage and fibrosis of the kidney tissue and the expression levels of fibrosis markers, α -SMA and Fibronectin, in the kidney were increased (all P <0.05). According to the findings from further investigation, the TMAO-treatment group showed increased expression of SREBP1 and an up-regulation of PI3K phosphorylation ratio and AKT phosphorylation ratio compared with those of the Control group (all P <0.05). Cell experiments produced results similar to those of the animal experiment. After siRNA interference with SREBP1 expression, the expression levels of fibrosis marker proteins decreased (P <0.05). Besides, the high expression of SREBP1 caused by TMAO was inhibited after HK2 cells were incubated with LY294002, a PI3K-AKT pathway inhibitor (P <0.05). **Conclusion** TMAO may induce renal fibrosis by promoting the PI3K/AKT/SREBP1 pathway.

【Key words】 Trimethylamine N-oxide Renal fibrosis PI3K/AKT pathway SREBP1

慢性肾脏病(chronic kidney disease, CKD)是一种肾脏结构和功能慢性损害的疾病,以肾小球滤过率持续降低引起肾功能减退为主要表现;而肾纤维化是CKD向终

末期肾脏病进展的关键和共同病理途径^[1]。因此,肾纤维化常被用作控制CKD发生发展、预防及治疗并发症的首要靶点^[2-5]。目前已有大量证据表明:硫酸吡啶酚(indoxyl sulfate, IS)^[6]、同型半胱氨酸(homocysteine, Hcy)^[7]、马尿酸(hippuric acid, HA)^[8]及晚期糖基化终末产物(advanced glycation end products, AGEs)^[9]等多种尿毒症毒素可通过介导炎症、氧化应激及细胞凋亡而促进肾纤维化^[1, 10]。

* 国家自然科学基金(No. U22A20279, No. 82322012, No. 82170705)和重庆市英才计划(No. cstc2021jcyj-bgzxm0145)资助

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出版日期: 2023-11-20

其中,氧化三甲胺(trimethylamine N-oxide, TMAO)是肠道菌群通过代谢高胆碱食物而产生的尿毒症毒素^[11],常在CKD患者体内蓄积^[12]。临床研究提示,高TMAO水平与CKD及心血管并发症的发生发展密切相关^[13]。但TMAO在肾纤维化过程中的分子机制仍未完全阐明。

甾体调节元件结合蛋白(sterol-regulatory element binding protein, SREBPs)家族是一组转录因子,可以通过控制胆固醇、磷脂、三酰甘油和脂肪酸的合成来调节脂质稳态^[14]。研究发现磷脂酰肌醇3激酶/蛋白激酶B通路(phosphatidylinositol 3 kinase/protein kinase B signaling pathway, PI3K/AKT)激活可以调节SREBP1表达水平进而影响气道重塑^[15]。同时,既往文献报道TMAO可通过调节PI3K/AKT通路影响动脉粥样硬化等疾病进程^[16]。然而,TMAO是否能通过PI3K/AKT通路调控SREBP1表达而影响肾纤维化仍不清楚。本研究采用体内外实验方法,探讨TMAO对PI3K/AKT/SREBP1通路的影响及其介导的肾纤维化的调控作用,以期为进一步开发肾纤维化新的靶向药物提供理论依据。

1 材料和方法

1.1 实验材料及主要试剂

20只SPF级6~8周龄雄性BALB/c小鼠(体质量23~25 g)购自陆军军医大学实验动物中心,样本数量根据自由度(E)与既往TMAO相关动物研究^[11, 17]综合决定($E = \frac{\text{总样本量}}{\text{分组数目}}$,当数值 $E < 10$,表示样本量过小;当 $10 \leq E \leq 20$,表示样本量合适;当 $E > 20$,表示样本量过大)。动物饲养条件为恒温(20~26 ℃),湿度40%~70%,明暗周期为12 h/12 h。所涉及的动物实验按照陆军军医大学动物保护委员会的规定进行,所有实验方案均经陆军军医大学实验动物福利伦理审查委员会批准(编号:AMUWEC20224500)。

兔源SREBP1抗体、兔源 α -肌动蛋白(alpha smooth muscle actin, α -SMA)抗体、兔源重组人纤维连接蛋白片段(recombinant human fibronectin fragment, Fibronectin)抗体购于Abcam公司(美国)、兔源p-AKT购自CST公司(美国)。兔源AKT抗体、鼠源PI3K抗体、兔源p-PI3K抗体购自Zen BioScience公司(成都)。抗鼠或兔抗体购于Beyotime Biotechnology公司(上海)。DAB显色液和免疫组织化学试剂盒购自ZSGB-BIO公司(北京)。改良Masson三色染色液购自solarbio公司(北京)。人肾小管上皮细胞HK2购自ATCC细胞库(美国)。青霉素-链霉素混合液、DMEM F12细胞培养基和胰酶(含0.02% EDTA)购自meilunbio公司(大连),胎牛血清和Lipofectamine™3000购自Thermo Fisher Scientific公司(美国), si-SREBP1购自Biomec Biotechnology公司(江苏), Tris-Glycine SDS-

PAGE电泳缓冲液、TBST封闭-洗涤缓冲液和Tris-Gly转膜缓冲液购自Sango Biotech公司(上海), LY294002购自美国MedChemExpress(MCE)公司。

1.2 方法

1.2.1 动物分组及模型建立

取20只BALB/c小鼠平均分为两组($n = 10$): ①对照组:腹腔注射生理盐水,每日1次,持续8周; ②TMAO组:腹腔注射含TMAO的生理盐水(20 mg/kg),每日1次,持续8周。8周后,异氟烷麻醉小鼠,处死,心脏灌注后,取肾脏,沿肾组织冠状面纵轴分为两半,分别放于体积分数为4%多聚甲醛固定和-80 ℃冰箱中备用。

1.2.2 小鼠肾组织病理学检查与免疫组织化学(IHC)染色分析

小鼠肾组织经固定脱水包埋后进行病理切片,厚度约为2 μm 。经过烤片、脱蜡后,一部分进行HE染色与Masson染色;另一部分进行IHC染色:使用柠檬酸钠缓冲液进行抗原修复,经过15 min 3%过氧化氢酶阻断液阻断后,孵育Fibronectin(1:150)、 α -SMA(1:100)、SREBP1(1:150)一抗稀释液4 ℃过夜, PBS洗涤3次(5 min/次), 37 ℃孵育二抗1 h, PBS洗涤后,采用现配DAB显色液显色,经苏木精核染,1%盐酸乙醇分化2~3 s,中性树脂封片后扫描分析。各组切片随机选取3个视野,使用ImageJ软件分析 α -SMA、Fibronectin、SREBP1阳性区域比例并进行量化,具体方法参考文献^[15]。

1.2.3 细胞培养及分组

HK2细胞采用DMEM F12完全培养基(含1%双抗,10%胎牛血清)培养。细胞放置在含体积分数为5%CO₂、空气湿度为80%的37 ℃细胞恒温培养箱中进行细胞培养,按1:3比例传代,每隔2~3 d传代1次。实验按如下顺序进行: ①将细胞分为Control组和TMAO组,每组3个复孔,分别在正常培养基和含100 $\mu\text{mol/L}$ TMAO的培养基中处理24 h后,观察TMAO对HK2细胞 α -SMA、Fibronectin和SREBP1表达的影响; ②将细胞分为Control组、siSREBP1组、TMAO组和TMAO+siSREBP1组,每组3个复孔,分别在正常培养基(Control组和siSREBP1组)和含100 $\mu\text{mol/L}$ TMAO的培养基(TMAO组和TMAO+siSREBP1组)中处理24 h后,观察siRNA抑制SREBP1表达后TMAO对HK2细胞 α -SMA和Fibronectin的诱导效应有无变化; ③将细胞分为Control组和TMAO组,每组3个复孔,分别在正常培养基和含100 $\mu\text{mol/L}$ TMAO的培养基中处理24 h后,观察TMAO对HK2细胞PI3K、p-PI3K、AKT和p-AKT表达的影响; ④将细胞分为Control组、TMAO组和TMAO+LY294002组,每组3个复孔,分别在正常培养基、含100 $\mu\text{mol/L}$ TMAO或100 $\mu\text{mol/L}$ TMAO+20 $\mu\text{mol/L}$ PI3K/AKT抑制剂LY294002的培养基中处理24 h后,观察

TMAO对SREBP1的诱导效应有无变化。

1.2.4 细胞转染

将HK2细胞在6孔板中培养至细胞融合度为60%~80%时进行转染。首先混合Lipofectamine™3000和Opti MEM得到混合液1,然后将siRNA与Opti MEM混合得到混合液2,静置15 min后,再将前两份混合液混合。静置30 min后加入无血清培养基混合,用于处理各孔细胞。转染6 h后,用完全培养基培养细胞。

1.2.5 Western blot实验

取各组小鼠肾组织和细胞用RAPI裂解液提取总蛋白,采用10%SDS-PAGE胶上样,电泳、转膜,然后用Western blot快速封闭液封闭30 min后,4℃孵育Fibronectin、 α -SMA、SREBP1、PI3K、p-PI3K、AKT和p-AKT一抗稀释液(稀释比例均为1:1000)过夜。TBST洗涤3次,每次15 min。再在37℃孵育稀释好的二抗(稀释比例为1:2000)1 h。TBST洗涤3次,每次15 min,加ECL曝光显色。用Image J软件得出灰度值,以目的蛋白与 β -肌动蛋白(β -

actin)灰度值之比作为目的蛋白的相对表达量。

1.2.6 统计学方法

数据用 $\bar{x} \pm s$ 表示。采用SPSS 19.0软件进行统计分析,文中数据均服从正态分布,两组间比较采用两独立样本 t 检验,多组间比较采用单因素方差分析,组间两两比较使用LSD法, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 TMAO诱导小鼠肾脏纤维化

HE染色显示(图1A),对照组小鼠肾脏中肾小球结构正常且肾小管上皮细胞排列整齐;与对照组相比,TMAO组小鼠肾脏中肾小管明显扩张变形,出现空泡样改变,肾小球萎缩,肾间质纤维化增加。Masson染色提示,相较于对照组,TMAO组小鼠肾组织肾纤维化面积较大($P < 0.05$),且主要分布于肾间质。免疫组化结果提示(图1B):TMAO组小鼠肾组织中Fibronectin、 α -SMA蛋白黄染面积更大、颜色更深,分析结果提示Fibronectin表达水平、

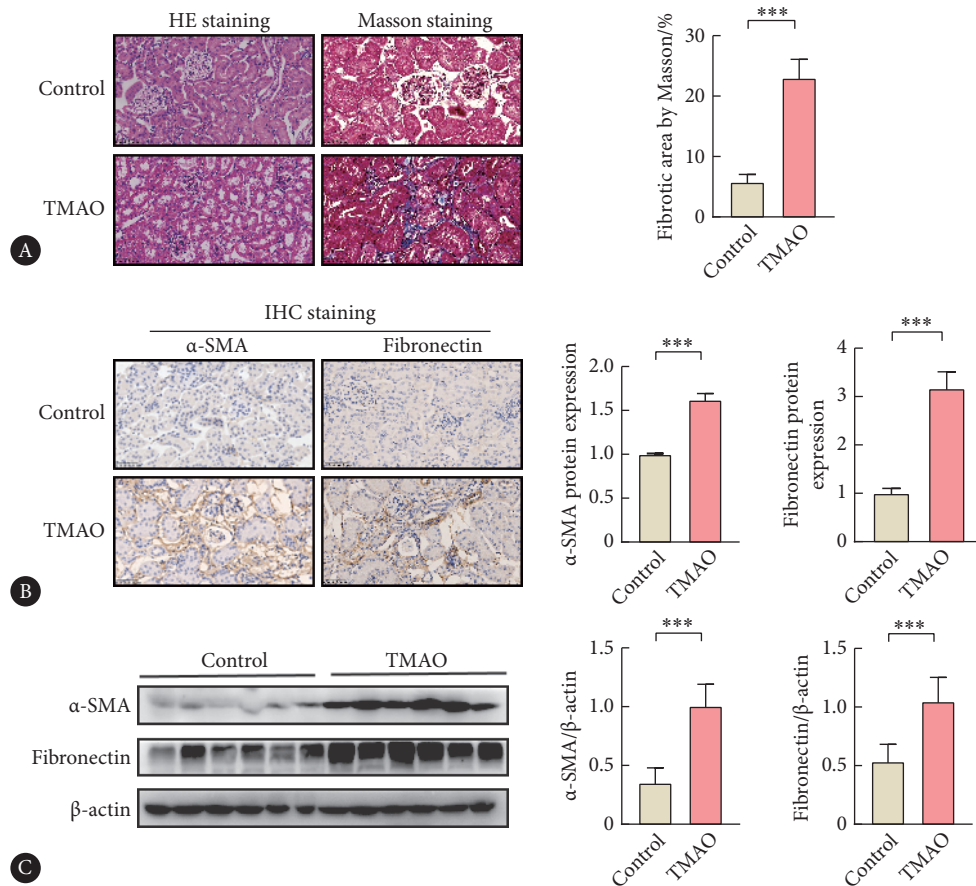


图1 腹腔注射TMAO诱导小鼠肾脏纤维化

Fig 1 Intrapertoneal injection of TMAO to induce renal fibrosis in mice

A, Pathological changes of renal tissues after intraperitoneal injection of TMAO (H&E staining and Masson staining, $\times 400$). *** $P < 0.001$, $n = 3$. B, Immunohistochemistry examination of α -SMA and Fibronectin in kidneys, $\times 400$. *** $P < 0.001$, $n = 3$. C, Western blot was used to analyze the expression of α -SMA and Fibronectin in kidneys of the Control and the TMAO groups. *** $P < 0.001$, $n = 6$.

α -SMA表达水平显著上升($P < 0.05$)。Western blot结果显示(图1C): 对照组小鼠肾脏中纤维化指标蛋白Fibronectin蛋白、 α -SMA蛋白含量均低于TMAO组($P < 0.05$)。

2.2 TMAO诱导肾小管上皮细胞纤维化

如图2所示, 与Control组相比, TMAO组肾小管上皮细胞Fibronectin、 α -SMA蛋白表达水平上调($P < 0.05$), 提示TMAO能够诱导肾小管上皮细胞纤维化指标上调, 促进肾脏纤维化。

2.3 TMAO诱导小鼠肾脏组织与肾小管上皮细胞SREBP1表达增加

Western blot结果显示, 在小鼠肾脏样本中, TMAO腹腔注射诱导SREBP1蛋白水平上调($P < 0.05$) (图3A, 图3C); 免疫组化结果显示, SREBP1主要分布在肾小管中; 且相对于对照组, TMAO组小鼠肾脏切片SREBP1表达水平增加($P < 0.05$) (图3B, 图3D)。Western blot结果显示, TMAO处理后肾小管上皮细胞中SREBP1蛋白表达量较Control组增加($P < 0.05$) (图3E)。

2.4 抑制SREBP1缓解TMAO诱导的肾小管上皮细胞纤维化

如图4所示, Control组、siSREBP1组、TMAO组和TMAO+siSREBP1组肾小管上皮细胞中SREBP1蛋白表达差异有统计学意义($P < 0.05$), 纤维化指标Fibronectin蛋白表达和 α -SMA蛋白表达差异亦有统计学意义($P < 0.05$)。TMAO处理后, SREBP1显著上调($P < 0.05$), 肾小管上皮细胞纤维化指标蛋白Fibronectin和 α -SMA表达上升($P < 0.05$)。使用siSREBP1抑制SREBP1的表达后, 相对于TMAO组, TMAO+siSREBP1组Fibronectin、 α -SMA表达下降($P < 0.05$), 提示siSREBP1可以显著抑制TMAO诱

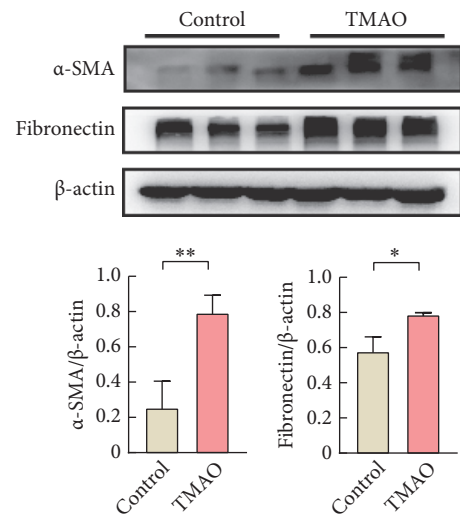


图2 TMAO对肾小管上皮细胞肾纤维化相关蛋白的影响

Fig 2 Effects of TMAO on renal fibrosis-related proteins in renal tubular epithelial cells

* $P < 0.05$, ** $P < 0.01$. $n = 3$.

导的肾小管上皮纤维化。

2.5 TMAO通过PI3K/AKT信号通路上调SREBP1表达

如图5A所示, 相对于对照组小鼠, PI3K、AKT蛋白水平在TMAO组小鼠肾脏中无明显变化, p-PI3K/PI3K与p-AKT/AKT比值上升($P < 0.05$), 提示PI3K/AKT通路在TMAO处理的小鼠肾脏中被激活。类似变化发生在TMAO处理的肾小管上皮细胞中。如图5B所示, TMAO处理组的PI3K、AKT蛋白表达水平相较于对照组未见明显变化($P > 0.05$), p-PI3K/PI3K与p-AKT/AKT比值上升($P < 0.05$)。如图5C示, Control组、TMAO组和TMAO+

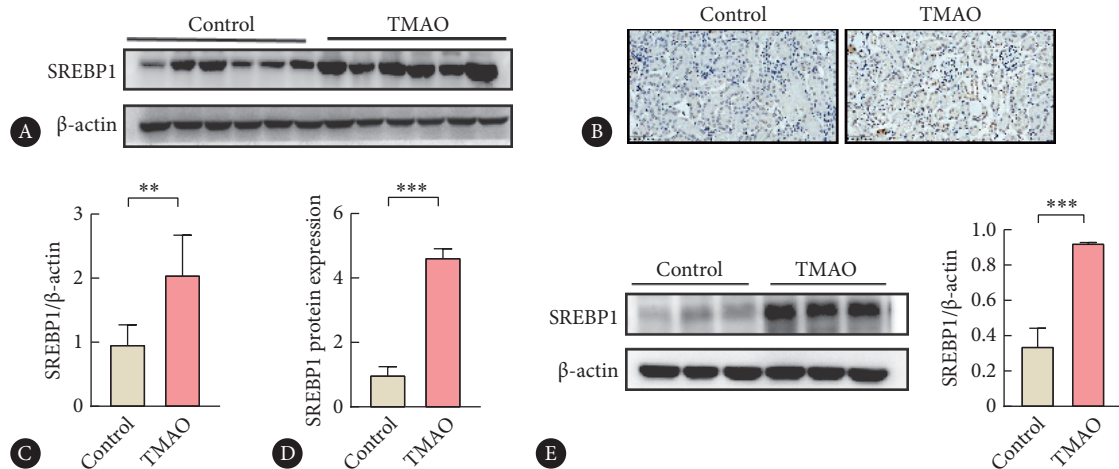


图3 TMAO诱导小鼠肾脏与肾小管上皮细胞SREBP1表达增加

Fig 3 TMAO induces increased SREBP1 expression in mouse kidney and tubular epithelial cells

A, Representative Western blot results for SREBP1 in renal cortex (mice); B, immunohistochemical staining of kidney sections with SREBP1 ($\times 400$) (mice); C, densitometric quantification of SREBP1 from the Western blot data (** $P < 0.01$, $n = 6$) (mice); D, densitometric quantification of SREBP1 from the immunohistochemical staining result (** $P < 0.001$, $n = 3$) (mice); E, Western blot analysis of SREBP1 expression in renal tubule epithelial cells in the Control and the TMAO groups (** $P < 0.001$, $n = 3$).

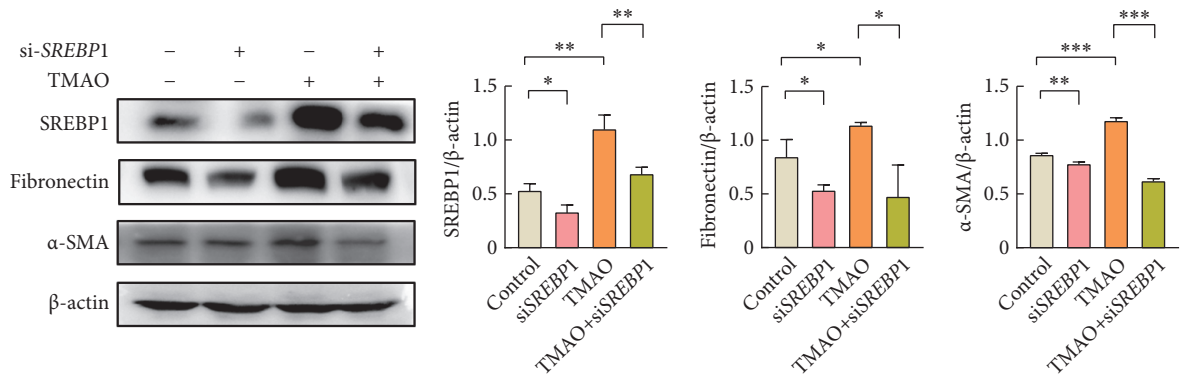


图 4 抑制SREBP1缓解TMAO诱导的肾小管上皮细胞纤维化

Fig 4 Inhibition of SREBP1 alleviates the fibrosis of TMAO-induced tubular epithelial cells

Western blot was used to analyze the expression levels of SREBP1, Fibronectin, α-SMA and β-actin in renal tubular epithelial cells in the Control group, the TMAO group, the siSREBP1 group and the TMAO+siSREBP1 group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. $n = 3$.

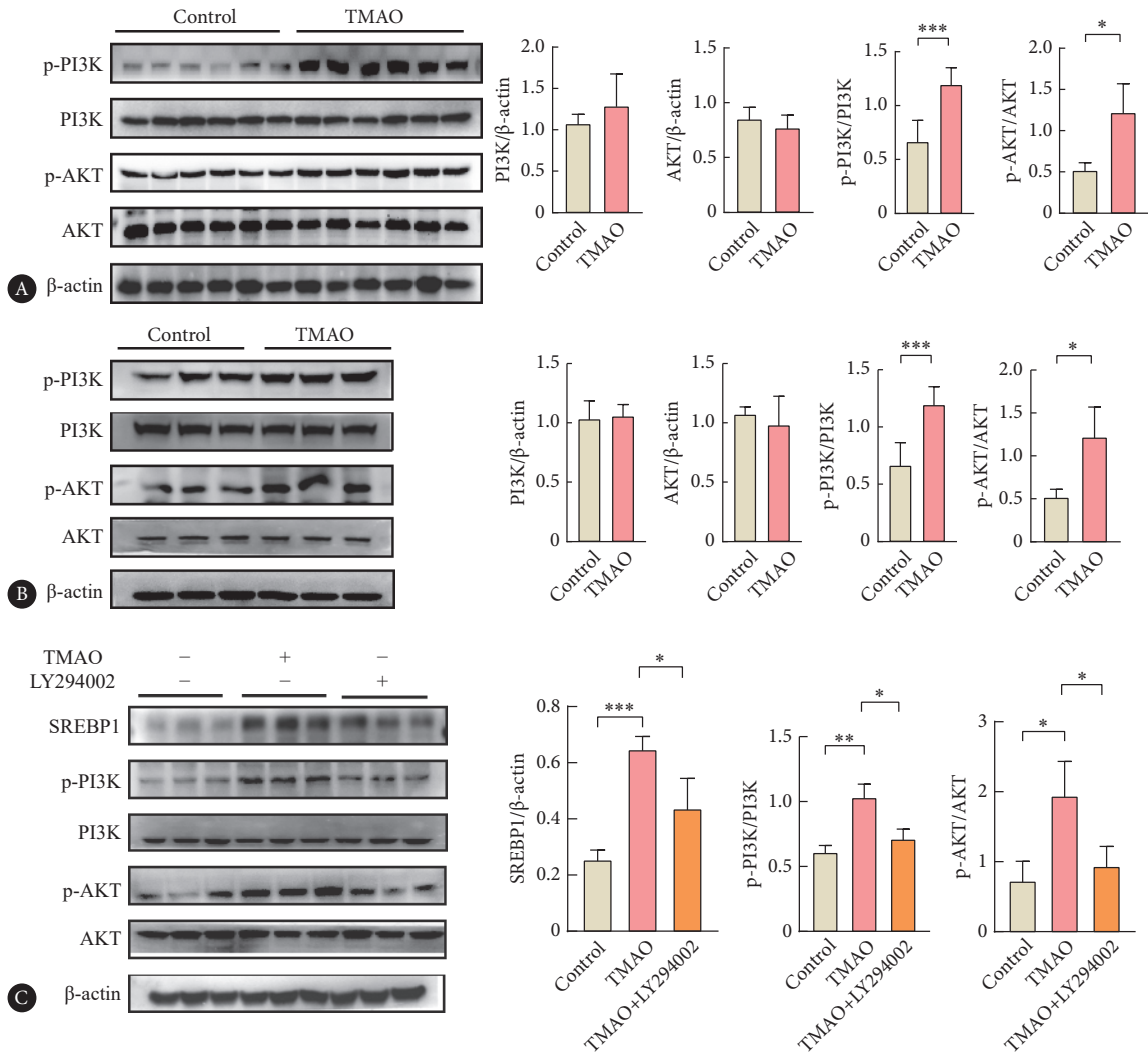


图 5 TMAO通过PI3K/AKT通路上调SREBP1表达

Fig 5 TMAO upregulates SREBP1 expression through the PI3K/AKT pathway

A, Western blot was used to determine the expression of PI3K, AKT, p-PI3K, and p-AKT in the kidneys of mice in the control group and the TMAO group ($n = 10$); B, Western blot was used to determine the expression of PI3K, AKT, p-PI3K, and p-AKT in renal tubular epithelial cells of the control group and the TMAO group ($n = 3$); C, Western blot was performed to determine the expression of SREBP1, AKT, and p-AKT expression in renal tubular epithelial cells in the Control group, the TMAO group, and the TMAO+PI3K/AKT inhibitor group ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

LY294002组肾小管上皮细胞中SREBP1表达水平差异有统计学意义($P < 0.05$)。相对于Control组, TMAO组中SREBP1上调($P < 0.05$)。应用PI3K/AKT通路抑制剂后, TMAO诱导的SREBP1上调被明显抑制($P < 0.05$), 提示TMAO诱导的SREBP1上调可能通过PI3K/AKT通路介导。

3 讨论

众所周知, 肾纤维化是CKD发生发展的重要病理过程, 与包括肠源性尿毒症毒素TMAO在内的多种尿毒症毒素的大量蓄积密切相关。临床研究发现血浆高水平TMAO与CKD患者肾脏病进程和死亡风险增加相关^[17]; 大鼠研究发现, 补充TMAO不仅加重了糖尿病大鼠的肾功能不全, 而且加重了肾纤维化^[18]; 小鼠研究发现, 靶向抑制肠源性TMAO的生成显著改善了慢性皮下输注异丙肾上腺素所致的肾功能障碍与纤维化的多个指标^[9]。本研究结果证实了腹腔注射TMAO能显著诱导肾间质纤维化, 为证明肠源性TMAO在体内的蓄积可能是CKD肾纤维化的关键因素提供了进一步的证据。

虽然大量研究证据提示TMAO水平与肾纤维化的关联, 但其分子机制尚未被完全阐明。SREBP1作为肾脏及其他组织中细胞脂质代谢的经典调节因子, 被报道通过多种途径介导肾纤维化的发生发展。一方面, SREBP1诱导脂质代谢紊乱间接影响肾脏纤维化^[20]。非酯化脂肪酸及其代谢物的积累进一步加重了细胞线粒体能量代谢障碍, 活性氧产生过多和内质网应激^[21]。另一方面, SREBP1作为一种弱转录因子, 被报道可以结合经典促肾纤维化细胞因子转化生长因子 β (transforming growth factor β , TGF- β)的启动子区域SRE, 直接发挥促纤维化作用^[22]。本研究体内外实验结果显示SREBP1信号被TMAO处理显著激活, 抑制SREBP1表达可以抑制TMAO诱导的肾小管上皮细胞纤维化。这些发现支持SREBP1在TMAO诱导的肾纤维化中起重要作用。PI3K/AKT信号通路是细胞内重要的信号传导途径, 参与多种生物学过程的调控^[23]。研究发现, TMAO可以激活PI3K/AKT通路促进肾成纤维细胞活化和成纤维细胞增殖^[24], 增加总胶原蛋白产量, 发挥促纤维化作用。然而很少实验探讨TMAO对小鼠肾脏和肾小管上皮细胞中PI3K/AKT通路的影响。本研究发现TMAO处理显著激活了小鼠肾脏和人肾小管上皮细胞中的PI3K/AKT通路。此外, 抑制PI3K/AKT通路可以抑制TMAO介导的SREBP1表达增强。这表明SREBP1和PI3K/AKT在肾小管上皮纤维化中存在联系。

综上所述, 本研究结果揭示了肠源性尿毒症毒素TMAO通过激活PI3K/AKT通路上调SREBP1表达, 进而促

进肾脏纤维化。这一发现不仅进一步完善了肾脏纤维化的发病机制, 而且也为下一步开发针对于PI3K/AKT/SREBP1通路的药物以治疗肾纤维化提供了理论依据, 具有一定的临床意义。

* * *

作者贡献声明 陈静负责正式分析、调查研究、验证和初稿写作, 黄英辉负责论文构思、经费获取、提供资源、监督指导和审读与编辑写作, 赵景宏负责论文构思、数据审编、经费获取、调查研究、提供资源、监督指导和审读与编辑写作。所有作者已经同意将文章提交给本刊, 且对将要发表的版本进行最终定稿, 并同意对工作的所有方面负责。

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(2023-09-27收稿, 2023-11-02修回)

编辑 余琳



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