论著·基础医学

支链氨基酸代谢障碍激活mTOR-ATF4-Chop/Trib3信号通路诱导肾小球足细胞凋亡促进糖尿病肾病进展

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[摘要] **目的** 探讨支链氨基酸(branched chain amino acids,BCAAs)分解代谢障碍诱导肾小球足细胞损伤的分子机制,并促进高脂饮食诱导的肥胖小鼠糖尿病肾病发生发展的作用和机制研究。**方法** (1)细胞实验:①提取6~8周龄C57BL/6J雄性小鼠(体质量20~25 g)原代肾小球足细胞进行体外培养,采用完全随机化法分为3组(n=4):低糖组(low glucose,LG,5.5 mmol/L)、高糖组(high glucose,HG,25 mmol/L)、高糖+3 mmol/L BCAA(HG+BCAA)组,Western blotting检测其BCAAs代谢相关分子(PPM1K、BCKD、BCKD pSer293)、足细胞功能标志物(Nephrin、Synaptopodin和WT1)和凋亡分子Cleaved Caspase-3的表达水平;免疫组化和Western blotting检测其mTOR信号通路(p70s6K pThr389、p70s6k、ATF4 pSer166和ATF4)及凋亡通路分子(Chop和Trib3)的表达水平。②体外培养ATF4敲除成纤维细胞系,采用完全随机化法分为2组(n=4):对照组和BCAA组,Western blotting检测其凋亡通路分子的表达水平。③提取6~8周龄C57BL/6J小鼠原代肾小球足细胞进行体外培养,采用完全随机化法分为3组(n=4):HG(25 mmol/L)组、HG+3 mmol/L BCAA(HG+BCAA)组、HG+3 mmol/L BCAA+雷帕霉素(HG+BCAA+Rap)组,Western blotting检测其mTOR信号通路及凋亡通路分子的表达水平。(2)动物实验:构建高脂饮食(high fat diet,HF)诱导肥胖的小鼠模型,将6~8周龄C57BL/6J雄性小鼠采用随机数字表法随机分为4组:HF组、HF/配对组(HF/Paired,食物摄入量与HF/BCAA组匹配)、HF/BCAA组(饮水添加4%BCAAs)和HF/BCAA+Rap干预组。PAS染色、油红O染色和肾甘油三酯水平检测肾脏病理形态及脂质沉积;生化检测分析尿液微量白蛋白、肌酐含量及尿白蛋白/肌酐比值;免疫荧光/RT-PCR检测肾小球足细胞功能分子(Nephrin和Synaptopodin)和纤维化相关分子(Collagen Ⅳ)表达水平;免疫组化及Western blotting检测肾脂代谢分子(FABP4、CD36、PGC1α和PPARα)及mTOR信号通路及凋亡通路分子的表达水平。**结果** (1)细胞实验:①高糖处理足细胞PPM1K表达降低,BCKD pSer293表达升高(P<0.000 1)。BCAAs下调高糖处理的足细胞Nephrin表达(P<0.000 1)和Synaptopodin表达(P<0.01),上调Cleaved Caspase-3表达(P<0.000 1)。BCAAs显著上调足细胞p70s6K pThr389、p70s6k、ATF4 pSer166、ATF4、Chop和Trib3的蛋白表达(P<0.000 1)。②敲除ATF4显著阻断BCAAs激活mTOR诱导的Chop和Trib3表达(P<0.000 1)。③mTOR抑制剂Rap显著抑制ATF4 pSer166、ATF4、Chop和Trib3的表达(P<0.000 1)。(2)动物实验:①外源性补充BCAAs升高高脂饮食小鼠尿微量白蛋白/肌酐比值(P<0.000 1),加重肾小球滤过膜损伤,同时下调肾小球WT1、Nephrin和Synaptopodin的表达(P<0.000 1),上调肾小球p70s6k pThr389的表达(P<0.01)和Chop的表达(P<0.000 1);并促进肾小管管型形成和肾脏脂质蓄积(P<0.000 1),上调肾小管FABP4和CD36的表达。②mTOR抑制剂Rap阻断mTOR-ATF4-Chop/Trib3

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通路,下调肾小球ATF4 pSer166、ATF4、Chop和Trib3的表达(P<0.000 1),改善肾脏脂质沉积(P<0.000 1)、肾小管管型形成及蛋白尿(P<0.000 1)。**结论** BCAAs代谢障碍通过激活mTOR-ATF4-Chop/Trib3信号通路诱发肾小球足细胞功能障碍和凋亡,导致肾脏脂质沉积、肾小管管型形成和肾纤维化进展,促进糖尿病肾病发生发展。

[**关键词**] 糖尿病肾病;支链氨基酸;激活转录因子4;哺乳动物雷帕霉素靶蛋白
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Branched-chain amino acid metabolism disorder promotes the progression of diabetic kidney disease by inducing podocyte apoptosis in glomeruli *via* activating the mTOR-ATF4-Chop/Trib3 signaling pathway

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[**Abstract**] **Objective** To investigate the molecular mechanisms by which branched-chain amino acids (BCAAs) catabolic disorder induces glomerular podocyte injury, and to elucidate its role and mechanism in promoting the pathogenesis of diabetic nephropathy (DN) in a high-fat diet (HFD)-induced mouse model of obesity. **Methods** (1) Cellular Experiments: ① Primary glomerular podocytes were isolated from 6- to 8-week-old male C57BL/6J mice (weight 20 to 25 g) and cultured, and then were randomly divided into 3 groups (n=4): low-glucose (LG, 5.5 mmol/L) group, high-glucose (HG, 25 mmol/L) group, and HG+3 mmol/L BCAA group. Western blotting was used to detect the expression of BCAA metabolism-related molecules (PPM1K, BCKD, and BCKD pSer293), podocyte functional markers (Nephrin, Synaptopodin, and WT1), and the apoptotic molecule Cleaved Caspase-3. Immunohistochemistry staining and Western blotting were performed to detect the expression levels of mTOR signaling pathway molecules (p70s6K pThr389, p70s6K, ATF4 pSer166, and ATF4) and apoptotic pathway molecules (Chop and Trib3). ② ATF4-knockout fibroblast cells were randomly divided into control (n=4) and BCAA (n=4) groups. Western blotting was used to detect the expression levels of apoptosis pathway molecules. ③ Primary glomerular podocytes isolated from 6- to 8-week-old C57BL/6J mice were randomly divided into 3 groups (n=4): HG (25 mmol/L) group, HG + 3 mmol/L BCAA group, and HG + 3 mmol/L BCAA + rapamycin (HG + BCAA + Rap) group. Western blotting was performed to assess the expression levels of mTOR signaling pathway and apoptotic pathway molecules. (2) Animal Experiments: An HFD-induced mouse model of obesity was established. Male C57BL/6J mice aged 6 to 8 weeks were randomly divided into an HFD group, an HFD/Paired group (food intake matched to the HFD/BCAA group), an HFD/BCAA group (4% BCAAs supplemented in drinking water), and an HFD/BCAA + Rap intervention group. Periodic acid-Schiff (PAS) staining, Oil Red O staining, and detection of renal triglyceride content were performed to assess renal pathological morphology and lipid deposition. Biochemical assays were applied to detect the urinary microalbumin and creatinine levels, as well as to calculate the urinary albumin-to-creatinine ratio. Glomerular podocyte functional markers (Nephrin and Synaptopodin) and fibrosis-related molecules (Collagen IV) were detected by immunofluorescence assay and RT-PCR. The expression levels of renal lipid metabolism-related molecules (FABP4, CD36, PGC1α, and PPARα), as well as the mTOR signaling pathway and apoptotic pathway molecules were detected by immunohistochemistry and Western blotting. **Results** (1) Cellular Experiments: ① High-glucose treatment decreased PPM1K expression and increased BCKD pSer293 expression in the podocytes (P<0.000 1). BCAAs downregulated the expression of Nephrin (P<0.000 1) and Synaptopodin (P<0.01) and upregulated Cleaved Caspase-3 expression (P<0.000 1) in the podocytes under high glucose condition. BCAAs significantly upregulated the protein levels of p70s6K

pThr389, p70s6K, ATF4 pSer166, ATF4, Chop, and Trib3 in the podocytes (P<0.000 1). ② Knockout of ATF4 significantly blocked BCAA-induced Chop and Trib3 expression activated by mTOR (P<0.000 1). ③ The mTOR inhibitor Rap significantly suppressed the expression of ATF4 pSer166, ATF4, Chop, and Trib3 (P<0.000 1). (2) Animal Experiments: ① Exogenous BCAA supplementation increased the urinary albumin-to-creatinine ratio (P<0.000 1), aggravated the damage to glomerular filtration barrier, downregulated the expression of WT1, Nephrin, and Synaptopodin (P<0.000 1), and upregulated glomerular p70s6K pThr389 (P<0.01) and Chop (P<0.000 1) expression. Additionally, BCAA supplementation promoted renal tubular cast formation and renal lipid accumulation (P<0.000 1), and upregulated the expression of FABP4 and CD36 in renal tubules of HFD-fed mice. ② The mTOR inhibitor Rap blocked the mTOR-ATF4-Chop/Trib3 pathway, downregulated the expression of ATF4 pSer166, ATF4, Chop, and Trib3 (P<0.000 1), and ameliorated renal lipid deposition (P<0.000 1), renal tubular cast formation, and proteinuria (P<0.000 1). **Conclusion** BCAA metabolism disorder induces glomerular podocyte dysfunction and apoptosis by activating the mTOR-ATF4-Chop/Trib3 signaling pathway, leading to renal lipid deposition, renal tubular cast formation, and progression of renal fibrosis, thereby promoting the development and progression of DN.

[**Key words**] diabetic nephropathy; branched-chain amino acids; activating transcription factor 4; mammalian target of rapamycin

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糖尿病肾脏病(diabetic kidney disease, DKD)是糖尿病常见且严重的微血管并发症之一,已成为我国终末期肾病(end stage renal disease, ESRD)的首要病因,患病率持续攀升,疾病负担沉重[1-4]。然而,DKD进展存在显著个体异质性,部分患者即使在严格控制血糖、血脂等传统危险因素后,仍不可避免进展为ESRD,提示存在尚未明确的内源性代谢危险因素驱动DKD发生发展[5]。

支链氨基酸(branched chain amino acids,BCAAs)(包括亮氨酸、异亮氨酸和缬氨酸)是必需氨基酸[6],其分解代谢受一系列体内限速酶的精细调控,包括支链α-酮酸脱氢酶(branched chain keto acid dehydrogenase E1 subunit alpha, BCKD)及支链α-酮酸脱氢酶磷酸酶(mitochondrial phosphatase 2C, PPM1K)和支链α-酮酸脱氢酶激酶(branched chain keto acid dehydrogenase kinase, BCKDK)[7]。前期研究显示,BCAAs与糖尿病并发症密切相关[8-11]。DKD模型小鼠血浆及肾脏中存在BCAAs及其代谢中间产物的显著蓄积[12],且尿液BCAAs水平可预测1型糖尿病患者DKD的进展风险[13],提示BCAAs代谢紊乱在DKD中发挥关键作用。

足细胞是肾小球滤过屏障的核心组成部分,其损伤与丢失是DKD早期的标志性病理改变,且损伤程度与蛋白尿产生和肾功能恶化密切相关[14-19]。因此,阐明DKD早期足细胞丢失及功能障碍的分子机制,对解析DKD发病机制、开发早期干预策略具有重要意义。足细胞功能高度依赖代谢稳态[20]。但BCAAs代谢障碍是否以及如何参与足细胞损伤,进而促进DKD进展,目前尚不明确。

在DKD病理微环境中,mTOR(主要为mTORC1)异常激活可直接诱导足细胞损伤与死亡[21-22];而ATF4作为关键应激响应转录因子,可通过调控下游凋亡通路介导足细胞功能障碍与凋亡,参与DKD进展[23];且mTOR可磷酸化ATF4使其活化[24],这提示mTOR可能通过调控ATF4影响DKD的发生发展。BCAAs作为已知的mTORC1激活剂[25-26],可能通过调控mTOR-ATF4信号轴介导足细胞损伤,但这一机制尚未在DKD中得到证实。

因此,本研究通过构建高脂饮食联合外源性BCAAs补充的小鼠模型,并结合原代肾小球足细胞培养,旨在探讨BCAAs代谢障碍诱导肾小球足细胞损伤的分子机制,为DKD的防治提供新靶点。

1 材料与方法

1.1 实验材料

1.1.1 实验细胞 参照先前已报道的标准化实验方法进行原代足细胞培养[27]:选取6~8周龄C57BL/6J雄性小鼠(体质量20~25 g),无菌条件下分离肾脏肾小球,并将小鼠的肾小球纯度纯化至95%以上[28]。将纯化后的肾小球接种于Ⅰ型胶原蛋白包被的6孔培养板;采用添加10%胎牛血清、100 U/mL青霉素及100 μg/mL链霉素的RPMI 1640完全培养基,置于37 ℃、5%CO_2的恒温培养箱中培养。培养3 d后,足细胞从肾小球组织块中迁移出,用PBS缓冲液轻轻洗涤2次,去除未附着的肾小球及杂质细胞;继续培养至第6天,待足细

胞充分从肾小球迁出,免疫荧光染色检测Nephrin和WT1检测足细胞纯度,用于后续实验。

1.1.2 实验动物 成年6~8周龄雄性C57BL/6J小鼠(体质量20~25 g)购自第四军医大学动物中心。ATF4敲除成纤维细胞获赠于上海复旦大学Terytty Yang Li教授。所有动物实验按照美国国立卫生研究院实验室动物指南进行,并获得第四军医大学动物管理和使用委员会的许可。本实验所有动物实验按照中国人民解放军第四军医大学实验动物福利与伦理委员会的要求进行,动物伦理批准号为IACUC-20230250。所有小鼠饲养于第四军医大学实验动物中心SPF级别动物房内,饲养温度保持在(26±2)℃和55%±15%相对湿度,光照/黑暗周期为12 h。所有小鼠喂养16周。由于本实验是在高脂喂养的小鼠模型进行的,C57BL/6J雌性小鼠具有抵抗高脂饮食喂养诱导的肥胖表型[29-30],很难造成肥胖模型。因此本实验所有高脂喂养小鼠为雄性C57BL/6J小鼠。

麻醉与安乐死操作:涉及解剖或侵入性操作时,采用1%戊巴比妥钠(50 mg/kg)腹腔注射麻醉,麻醉起效后再进行操作;安乐死采用过量麻醉法,避免小鼠因操作不当产生痛苦。

当小鼠出现以下任意一种预期或意外的不良事件时,立即实施安乐死,终止实验,避免其承受不必要的痛苦:①体质量与体态:单次体质量下降>15%(连续2次监测确认);出现明显恶病质(身体消瘦、脊柱突出、行动迟缓)。②生理状态:持续腹泻>3 d,伴采食量下降>50%;呼吸急促、发绀(口鼻呈青紫色);严重脱水(眼窝凹陷、皮肤弹性丧失)。③损伤与感染:皮肤大面积咬伤、溃疡,无法愈合;严重真菌感染或全身感染(体温升高>39.5 ℃、精神高度萎靡)。

1.1.3 实验试剂 RIPA裂解液和PMSF购自赛文创新(北京)科技有限公司;TRIzol试剂和Pierce™ BCA蛋白检测试剂盒购自Thermofisher(美国公司);磷酸酶抑制剂和SDS-PAGE蛋白上样缓冲液(5×)购自上海碧云天生物科技有限公司;Immobilon Western化学发光HRP底物和氧化铁(Ⅲ)购自Sigma(美国)贸易有限公司;辣根过氧化物酶偶联抗兔IgG抗体以及辣根过氧化物酶偶联抗小鼠IgG抗体购自北京中杉金桥生物技术有限公司;Hieff UNICON® Universal Blue qPCR SYBR Green Master Mix、Hifair® Ⅲ 1st Strand cDNA Synthesis SuperMix for qPCR (gDNA digester plus)和RPMI 1640培养基购自翌圣生物科技(上海)股份有限公司;无菌PBS缓冲液、通用型组织固定液(中性)、DAPI染色试剂(即用型)和抗荧光淬灭封片剂购自武汉赛维尔生物科技有限公司;柠檬酸三钠购自天津市天力化学试剂有限公司;改良油红O染色试剂盒和糖原PAS染色试剂盒(含苏木素)购自北京索莱宝科技有限公司;胶原酶A和DNase I购自北京云肽生物科技有限公司;肌酐测定试剂盒购自南京建成生物工程研究所;Albumin ELISA检测试剂盒和Triglyceride Assay检测试剂盒购自艾博抗(上海)贸易有限公司。

1.2 实验方法

1.2.1 动物实验 将小鼠采用随机数表法随机分为3组:高脂饮食(high fat diet,HF)组、HF/配对(HF/Paired,食物摄入量与HF/BCAA组匹配)组、HF加BCAA喂养(HF/BCAA,60%高脂饮食+含4% BCAAs的饮用水)组。所有小鼠可自由获取水,并喂养60%高脂饮食(D12492,Research Diets)16周。

1.2.2 HF、HF/BCAA和HF/Paired饮食喂养方案 如先前所述[9,31]进行。本研究首先评估了HF/BCAA组的总体质量(A/g),并计算了每天消耗的总热量(B/cal)=(提供的总热量-未食用食物中的热量)/d。然后,本研究确定了每克体质量消耗的热量[C/(cal/g)]=B/A。接下来,本研究测量了HF/Paired组的总体质量(D/g),并计算了第二天应给予HF/Paired组的高脂饮食食物量(E/cal)=C×D。此外,给HF组和HF/Paired组提供溶解在饮用水中的4%氨基酸混合物(除Gln和Asn外的所有20种天然存在的氨基酸,18种氨基酸混合物中BCAA占据19%)。18种氨基酸的混合物的比例是参照动物饲料中酪蛋白的18种氨基酸的天然比例,并参照文献NEWGARD[11]中高BCAA饮食组对照小鼠混合氨基酸比例。HF/Paired组饮用水中的BCAA浓度为4%×19%=0.76%。BCAA(4%)以亮氨酸:异亮氨酸:缬氨酸比例为2:1:1溶解在饮用水中。每日分两批次投喂,分别于8时与18时进行,避免动物饥饿。HF/BCAA+Rap组在HF/BCAA组的条件下以0.5 mg/kg体质量的剂量腹腔注射雷帕霉素。

1.2.3 小鼠肾脏过碘酸-希夫(periodic acid-Schiff stain,PAS)染色 将小鼠肾脏组织石蜡切片65 ℃烤片2 h后用二甲苯脱蜡,并用梯度乙醇进行水化。向切片滴加过碘酸溶液,室温孵育10 min,蒸馏水漂洗3次;随后滴加Schiff试剂,室温避光孵育15 min,用亚硫酸氢钠溶液漂洗2次,蒸馏水冲洗终止反应。切片经Mayer苏木素染液复染细胞核5 min,1%盐酸水溶液分化30 s,稀碳酸锂溶液返蓝,蒸馏水冲洗干净后,通过梯度乙醇脱水、二甲苯透明,中性树胶封片。最后在光学显微镜下观察肾小球系膜区、肾小管基底膜的PAS阳性染色情况,并采集图像,采用Image J软件定量分析阳性面积/肾小球面积。

1.2.4 小鼠肾脏油红O染色 实验制备4 μm厚的肾脏冰冻切片,经蒸馏水冲洗后,浸入60%异丙醇中漂洗30 s。将油红O储备液与蒸馏水按体积3:2比例

混合配制工作液,对切片染色10 min;随后用60%异丙醇轻柔漂洗以去除背景染色,再经蒸馏水冲洗干净。切片浸入Mayer苏木素染液中染核1 min,用1%盐酸水溶液进行分化处理,之后经蒸馏水漂洗或稀碳酸锂溶液返蓝,擦干切片边缘多余水分。最后进行封片操作,在显微镜下观察肾脏组织脂质沉积情况并采集图像。

1.2.5 尿液样本的生化分析 收集小鼠24 h尿液,采用小鼠Albumin ELISA检测试剂盒(Abcam,ab207620)检测小鼠尿液中白蛋白含量。小鼠24 h尿液肌酐采用肌酐测定试剂盒(南京建成,C011-2-1)检测。小鼠肾脏甘油三酯采用Triglyceride Assay检测试剂盒检测(Abcam,ab178780)。以上均在酶标仪对应波长下读取吸光度比值后计算浓度。尿白蛋白肌酐比值(urinary albumin-creatinine ratio,UACR)=尿微量白蛋白/(mg/L)÷尿肌/(g/L),单位为mg/g。

1.2.6 小鼠肾小球分离 将实验小鼠麻醉后,依次暴露小鼠的胸腔和腹腔。经左心室灌注4~5 mL含1%氧化铁的PBS直至双侧肾脏外观明显变黑。随后取肾脏剪碎,置于含1 mg/mL胶原酶A和100 U/mL DNase I的消化液中37 ℃消化30 min。终止消化后,将混合液通过100 μm细胞筛过滤,收集滤液置于磁体上,利用氧化铁的磁性吸附效应分离并纯化肾小球。通过相差显微镜观察,确认每个样本中肾小球的纯度达到95%以上。

1.2.7 免疫荧光染色 将小鼠肾脏组织石蜡切片在65 ℃烤片2 h后用二甲苯脱蜡,并用梯度乙醇进行水化。用蒸馏水冲洗2次后将切片置于0.1 mol/L柠檬酸钠缓冲液(pH=6.0)中,采用沸水浴进行抗原修复。自然冷却至室温后滴加3%过氧化氢封闭内源性过氧化物酶活性,然后将切片用5%正常山羊血清封闭1 h,随后4 ℃孵育一抗过夜。次日用PBS洗涤后,切片滴加荧光标记二抗室温孵育1 h,滴加DAPI染核剂室温避光孵育5 min,最后加入抗荧光淬灭剂封片,并通过FV4000共聚焦显微镜采集图像,用Image J软件进行定量分析。

使用的一抗如下:Col4a5(1∶100,Abcam # ab6586);FABP4(1∶1 000,Proteintech # 67167-1-Ig);CD36(1∶500,Proteintech # 66395-1-Ig);PGC1α(1∶1 000,Proteintech # 66369-1-Ig);PPARα(1∶250,Abcam # ab61182);NPHS2(1∶300,Proteintech # 20384-1-AP);SYNPO(1∶500,Proteintech # 21064-1-AP);DDIT3(1∶500,Abcam # ab11419);P-P70s6K(1∶1 500,Servicebio # GB153805)。

1.2.8 Western blotting实验 收取细胞样本后,加入现配的含有磷酸酶抑制剂和蛋白酶抑制剂的RIPA裂解液,使用超声破碎细胞样本后,冰上裂解30 min。获取组织样本后,按照60 mg组织样本/200 μL RIPA裂解液加入裂解液,使用Servicebio三维冷冻研磨仪(KZ-5F-3D)研磨,冰上裂解30 min。裂解完成后15 000×*g* 4 ℃离心15 min,取上清液使用BCA试剂盒检测蛋白浓度,加入(5×)上样缓冲液制备样本,95 ℃煮10 min使蛋白变性。制备好的蛋白样品进行SDS-PAGE电泳并转至PVDF膜。用5%脱脂奶粉室温封闭1.5 h,通用型抗体稀释液稀释的一抗4 ℃孵育过夜,TBST清洗后二抗室温孵育1 h,取等体积的A液和B液新鲜配制发光工作液,使用化学发光仪对蛋白条带进行可视化。

使用的抗体如下:PPM1K(1∶1 000,Abcam # ab224424);BCKDHA(phosphor Ser293)(1∶1 000,Abcam # ab302504);BCKDHA(1∶1 000,Abclonal # A9806);Beta Actin(1∶20 000,Proteintech # 66009-1-Ig);FABP4(1∶5 000,Proteintech # 67167-1-Ig);CD36(1∶1 000,Proteintech # 66395-1-Ig);PGC1α(1∶5 000,Proteintech # 66369-1-Ig);PPARα(1∶500,Abcam # ab61182);WT1(1∶1 000,Abcam # ab267377);Nephrin(1∶5 000,Abclonal # A28348);SYNPO(1∶1 000,Proteintech # 21064-1-AP);Cleaved Caspase-3(1∶1 000,Cell Signaling Technology # 9661);ATF4(phosphor Ser166)(1∶1 000,Cell Signaling Technology # 2325);ATF4(1∶1 000,Proteintech # 60035-1-Ig);DDIT3(1∶3 000,Abcam # ab11419);Trib3(1∶500,Abclonal # A3523);P70s6K(phosphor Thr389)(1∶500,Servicebio # GB113805-100);P70s6K(1∶500,Servicebio # GB111133-100)。

1.2.9 RT-PCR实验 使用TRIzol试剂从肾脏组织中提取总RNA。使用逆转录试剂盒合成cDNA。使用Applied Biosystems 7300仪器进行基于SYBR Green Master Mix的RT-PCR。选择*β-actin*作为内参基因,相对mRNA表达水平通过$2^{-\Delta\Delta Ct}$方法进行计算。每个实验独立重复3次。

使用的引物对序列如下:*β-actin*(上游引物:CCACCATGTACCCAGGCATT;下游引物:CGGACTCATCGTACTCCTGC);*Collagen* Ⅳ(上游引物:GGACAAAGGACAAGCTGGGATAA;下游引物:CCAACTCTCCTTTGTCACCTTTC)。

1.3 统计学分析

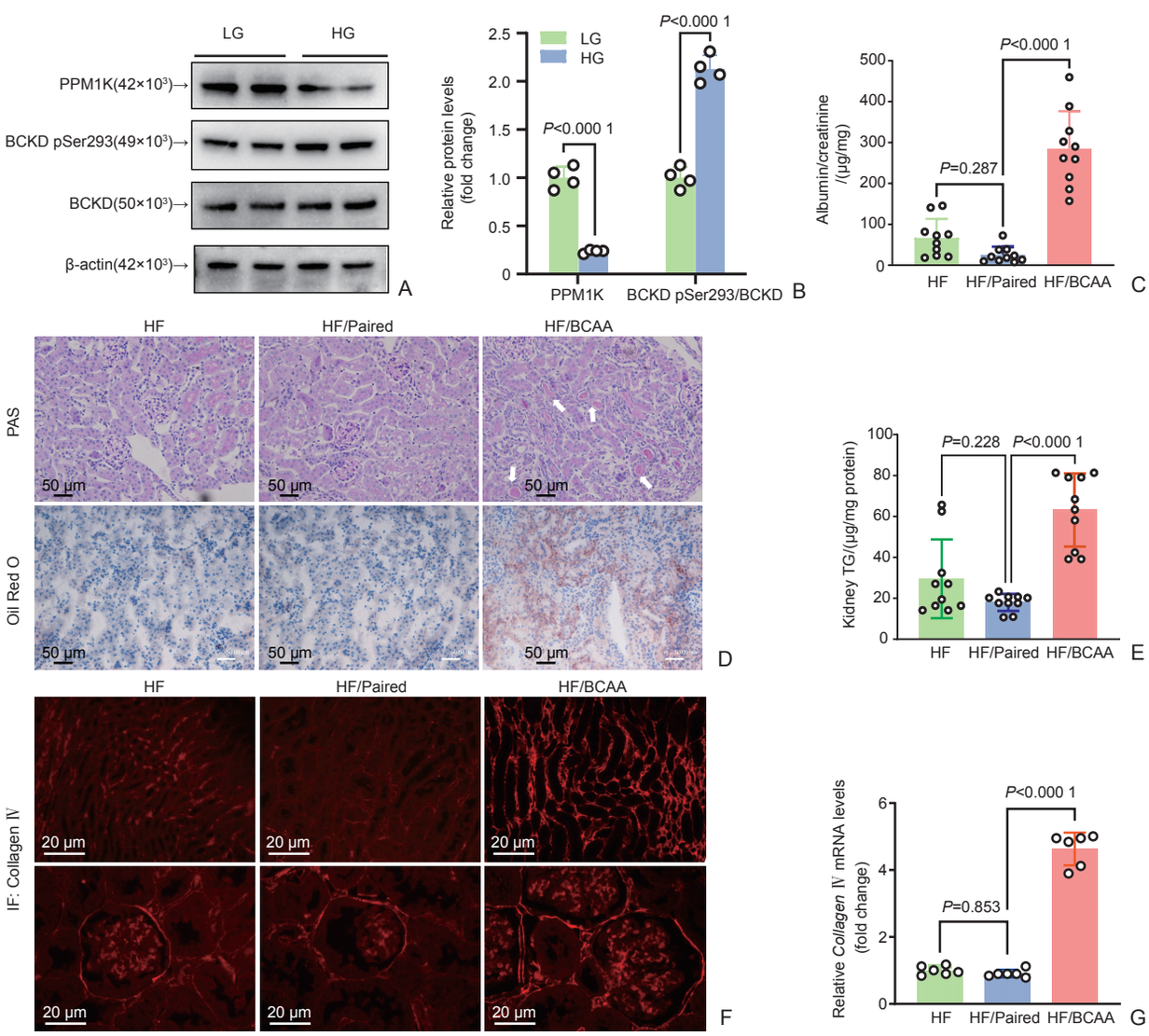
采用GraphPad Prism 8软件进行统计分析。数据均以$\bar{x}\pm s$表示,两组比较采用非配对双尾t检验进行评估,多组比较采用单因素方差分析进行分析,$P<0.05$被认为差异具有统计学意义。

2　结果

2.1　BCAAs代谢障碍加剧高脂饮食诱导糖尿病患者肾损伤

首先，为明确高糖是否引起足细胞BCAAs代谢障碍，本研究采用高糖处理原代足细胞，检测BCAAs分解代谢关键分子的表达。Western blotting结果显示，与LG组相比，HG组足细胞中BCAAs代谢关键酶PPM1K蛋白表达显著降低，而磷酸化的BCKD（BCKD pSer293）蛋白表达明显升高（$P<0.000\ 1$，图1A、B），提示高糖可诱导足细胞出现BCAAs分解代谢障碍。

为了探究BCAAs代谢障碍对糖尿病肾损伤的影响，本研究向高脂饮食喂养的小鼠外源性补充BCAAs，构建BCAAs蓄积小鼠模型。为排除外源性补充BCAAs所致的饮食摄入差异的干扰，设置HF组、HF/BCAA组和HF/Paired组。结果显示，HF组和HF/Paired组肾损伤很轻，这与既往研究中单纯高脂饮食并不能诱导C57BL/6J小鼠DKD的结论一致。相较于HF/Paired组，HF/BCAA组小鼠的尿白蛋白/肌酐比值显著升高（$P<0.000\ 1$，图1C）。PAS染色结果显示HF/



A：representative Western blotting images of BCAA catabolism-related proteins in cultured primary podocytes in indicated groups（n=4）；B：quantitative analysis of relative BCAA catabolism-related protein levels in cultured primary podocytes in indicated groups（n=4）；C：urinary albumin-to-creatinine ratio in indicated groups（n=10）；D：PAS staining（top）and Oil Red O red staining（bottom）of mouse kidney sections in indicated groups；E：quantitative analysis of renal triglyceride levels in indicated groups（n=10）；F：representative immunostaining images of Collagen Ⅳ of mouse kidney sections；G：quantitative analysis of relative *Collagen* Ⅳ mRNA expression in mouse kidneys（n=6）. IF：immunofluorescence

图1　BCAAs代谢障碍促进高脂饮食诱导的小鼠糖尿病肾损伤

Figure 1　BCAAs exacerbate high-fat diet induced diabetic nephropathy（DN）

BCAA组小鼠肾小管管型形成明显增加(图1D);油红O染色及甘油三酯测量结果均证实,HF/BCAA组肾脏脂质滴蓄积明显增加(P<0.000 1,图1D、E)。此外,*Collagen* Ⅳ的免疫荧光染色及RT-PCR检测结果显示,HF/BCAA组肾脏*Collagen* Ⅳ表达水平均显著高于HF/Paired组(P<0.000 1,图1F、G),提示BCAAs蓄积可促进肾脏纤维化进程。综上,BCAAs代谢障碍促进高脂饮食诱导小鼠肾脏脂质沉积、肾小管管型形成及纤维化进展,加剧高脂饮食诱导的DKD发生发展。

2.2 BCAAs代谢障碍促进高脂饮食诱导的小鼠肾小球滤过膜损伤

为阐明BCAAs蓄积加剧肾脏损伤的潜在机制,本研究检测小鼠肾脏脂质β氧化和脂质重吸收关键分子表达水平。免疫组化染色显示,HF/BCAA组小鼠肾脏肾小管高表达脂质转运相关蛋白FABP4和CD36。Western blotting结果也证实HF/BCAA组小鼠肾脏FABP4和CD36表达显著升高(图2A、B),提示BCAAs代谢障碍可促进肾小管对脂质的摄取与转运。同时,脂质β氧化关键调控分子PGC1α和PPARα的免疫组化染色及Western blotting结果显示,高脂饮食补充BCAAs并不影响其表达水平(图2C、D)。由于正常情况下,脂肪酸很难通过肾小球滤过膜进入肾小管;只有在肾小球滤过膜破损的情况下,脂肪酸进入肾小管才会被肾小管重吸收。上述结果表明,BCAAs代谢障碍并不抑制肾小管上皮细胞脂质β氧化,而是促进肾小管脂质摄取促进脂质蓄积于肾脏,提示BCAAs代谢障碍诱导肾小球滤过膜损伤。

因此,本研究通过透射电镜检测各组小鼠的肾小球滤过膜情况。结果显示,高脂饮食补充BCAAs造成肾小球足细胞足突大量融合(图2E,黄色箭头所指),显著升高肾小球滤过膜厚度(P<0.000 1,图2E、F)、上调肾小球足细胞足突的宽度(P<0.000 1,图2E、G)。综上,本研究发现高脂饮食补充BCAAs显著损伤小鼠肾小球滤过膜,并诱导肾小球足细胞损伤。

2.3 BCAAs代谢障碍加重高脂饮食诱导的小鼠足细胞功能障碍和凋亡

为明确BCAAs代谢障碍对足细胞的影响,本研究首先检测各组小鼠肾脏足细胞特异性标志物的表达情况。免疫荧光染色结果显示,相较于HF/Paired组,HF/BCAA组小鼠肾脏中足细胞特异性标志物Nephrin和Synaptopodin的表达明显降低(图3A、B);Western blotting结果证实,HF/BCAA组肾脏中WT1、Nephrin及Synaptopodin的蛋白表达水平均明显降低(P<0.000 1,图3C、D)。在原代培养的肾小球足细胞,相较于低糖培养条件下的足细胞,高糖培养条件下的足细胞Nephrin及Synaptopodin的表达显著降低,BCAAs更显著降低高糖培养条件下足细胞Nephrin(P<0.000 1)及Synaptopodin(P<0.01)的表达(图3E、F),这些数据证实BCAAs代谢障碍诱导足细胞功能障碍。

由于WT1的表达水平和足细胞个数成正比,在体水平,BCAAs显著降低高脂饮食诱导的小鼠肾脏足细胞WT1水平。因此本研究推测,BCAAs代谢障碍诱导高脂喂养小鼠肾小球足细胞丢失。因此,本研究采用高糖或高脂联合BCAAs处理原代培养的足细胞,Western blotting检测足细胞凋亡标志物Cleaved Caspase-3的蛋白水平,结果显示HG+BCAA组Cleaved Caspase-3的表达显著升高(P<0.000 1,图3G、H),证实BCAAs促进高糖诱导的足细胞凋亡。综上,BCAAs代谢障碍促进糖尿病小鼠足细胞功能障碍和凋亡,促进高脂喂养小鼠DKD进展。

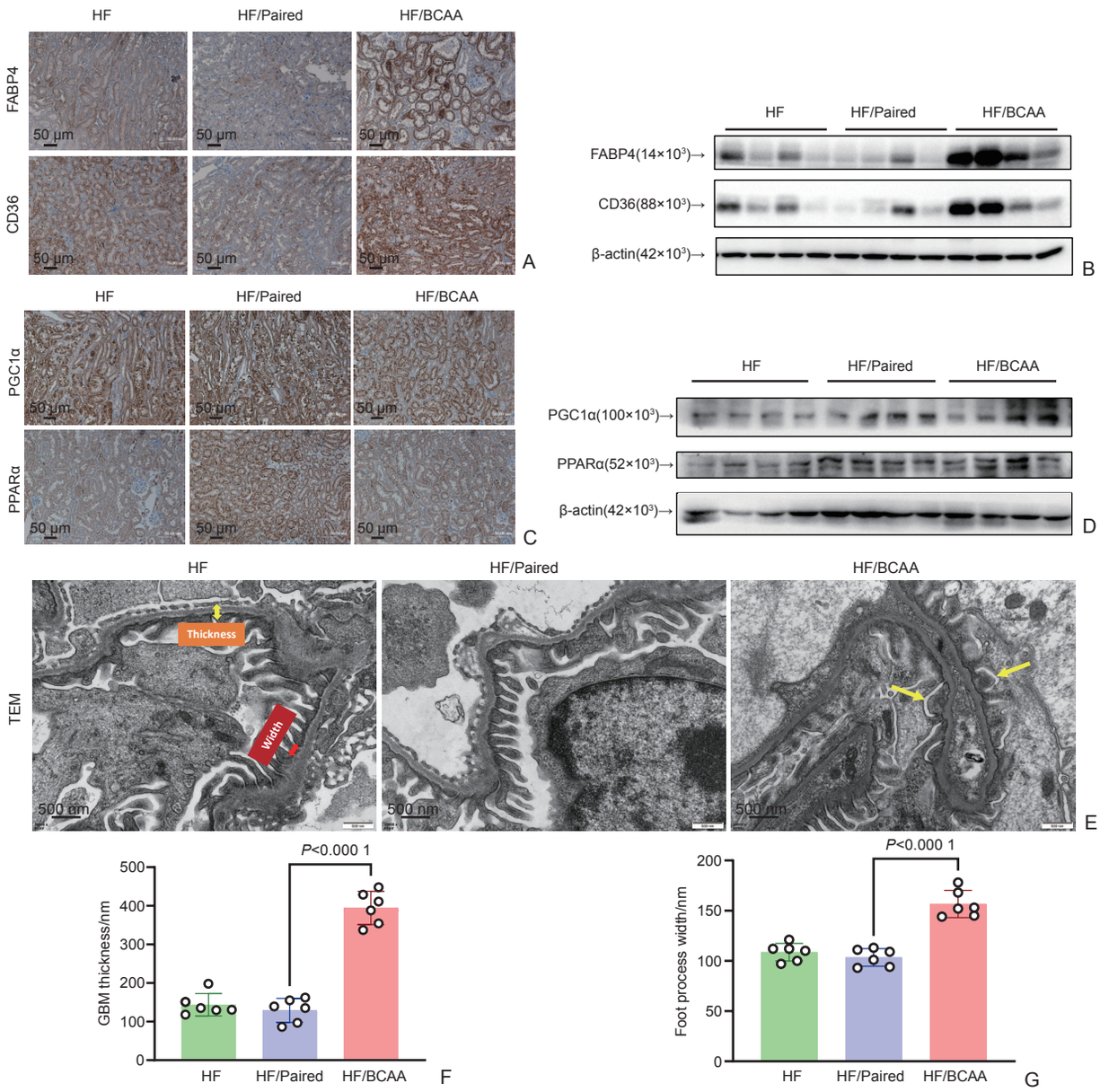
2.4 BCAAs代谢障碍显著激活足细胞mTORC1-ATF4-Chop/Trib3信号通路

为了探索BCAAs激活造成足细胞功能障碍和死亡的分子机制,本研究检测了足细胞整合应激反应信号通路。ATF4作为应激与代谢稳态核心转录因子可通过特异性结合下游靶基因Chop与Trib3并驱动其转录激活启动凋亡信号。结果显示,BCAAs可以显著激活足细胞ATF4磷酸化,上调Trib3和Chop的蛋白水平(P<0.000 1,图4A、B)。ATF4可以被mTORC1直接磷酸化,mTORC1通过促进ATF4磷酸化促进细胞整合应激反应,而亮氨酸是激活mTORC1最强的氨基酸。本研究进一步检测mTORC1的直接下游分子p70s6k的磷酸化水平。结果显示,BCAAs可以显著上调足细胞中p70s6k pThr389的表达水平(P<0.001,图4C、D),表明BCAAs代谢障碍显著激活足细胞mTORC1信号通路。

为了证明BCAAs通过激活mTORC1-ATF4信号通路诱导细胞凋亡,本研究在ATF4 KO的成纤维细胞上给予BCAAs,观察BCAAs对其Trib3和Chop表达水平的影响。结果显示,ATF4 KO并不影响p70s6k pThr389表达,但是显著阻断了BCAAs激活mTORC1诱导Trib3和Chop的表达(P<0.000 1,图4E、F)。此外,免疫组化染色显示,高脂饮食补充BCAAs显著升高肾小球p70s6k pThr389和Chop的蛋白水平(图4G、H)。这些结果证实,BCAAs显著激活mTORC1-p70s6k-ATF4-Chop/Trib3信号通路,这可能是BCAAs诱导足细胞损伤和凋亡机制。

2.5 抑制mTORC1显著改善BCAAs代谢障碍促进的DKD进展

为了证明BCAAs代谢障碍通过激活mTORC1促进DKD发生发展,本研究采用mTORC1特异性抑制剂Rap。结果显示,Rap显著抑制HG+BCAA诱导的足细胞ATF4磷酸化、Chop和Trib3蛋白的表达(P<0.000 1,

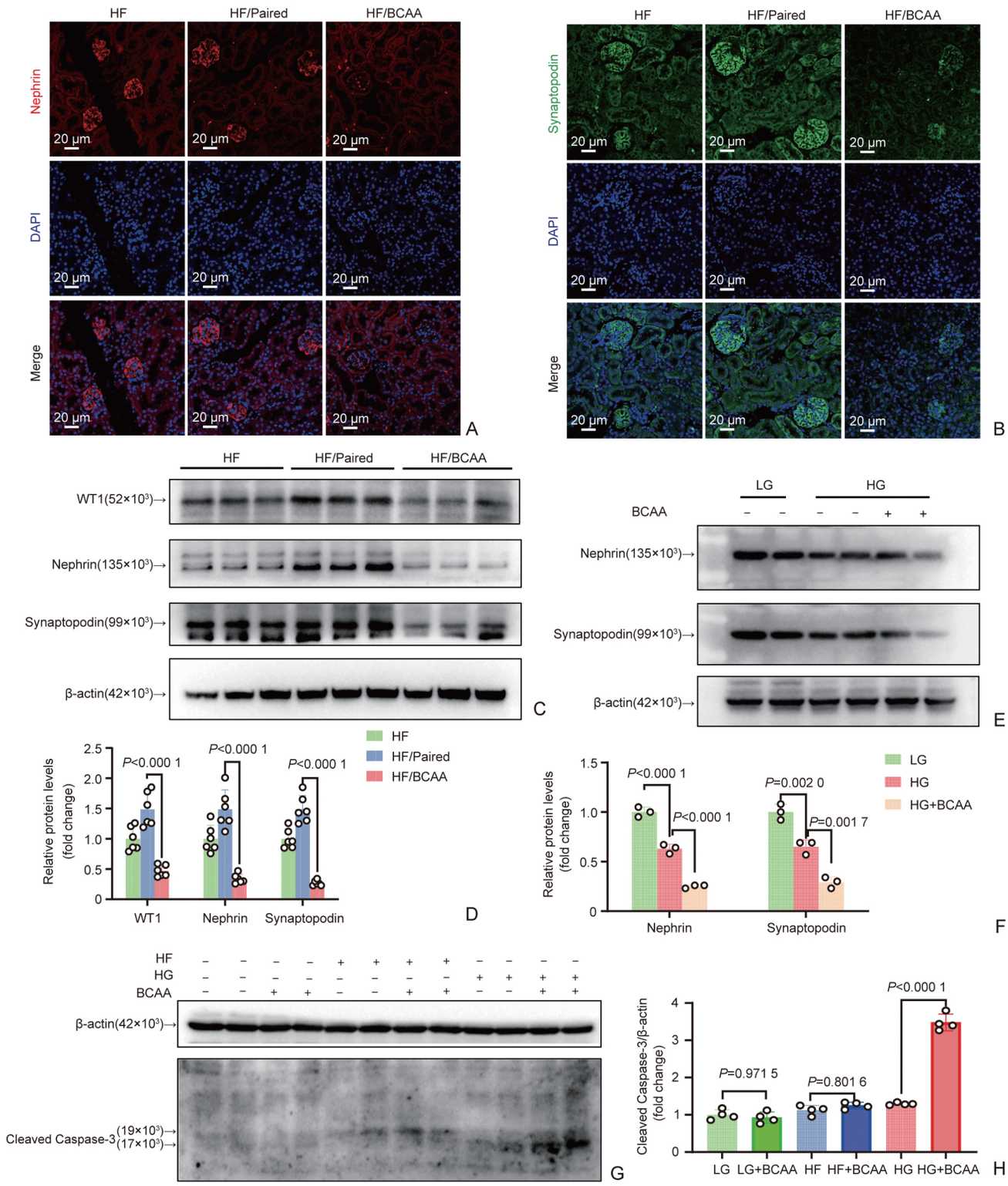


A: representative immunohistochemistry images of FABP4 and CD36 in mouse renal sections; B: representative Western blotting images of FABP4 and CD36 in mouse kidneys (n=4); C: representative immunohistochemistry images of PGC1α and PPARα in mouse kidney sections; D: representative Western blotting images of PGC1α and PPARα in mouse kidneys (n=4); E to G: representative TEM images of glomerular podocytes (E) and measurements of GBM thickness (F) and foot process width (G). TEM: transmission electron microscopy; GBM: glomerular filtration barrier. Yellow arrow: massive fusion of podocyte foot processes in the glomerulus

图 2 BCAAs对高脂饮食小鼠肾脏脂质代谢相关分子表达的影响

Figure 2 Effects of BCAAs on the expression of renal lipid metabolism-related molecules in mice fed with high-fat diet

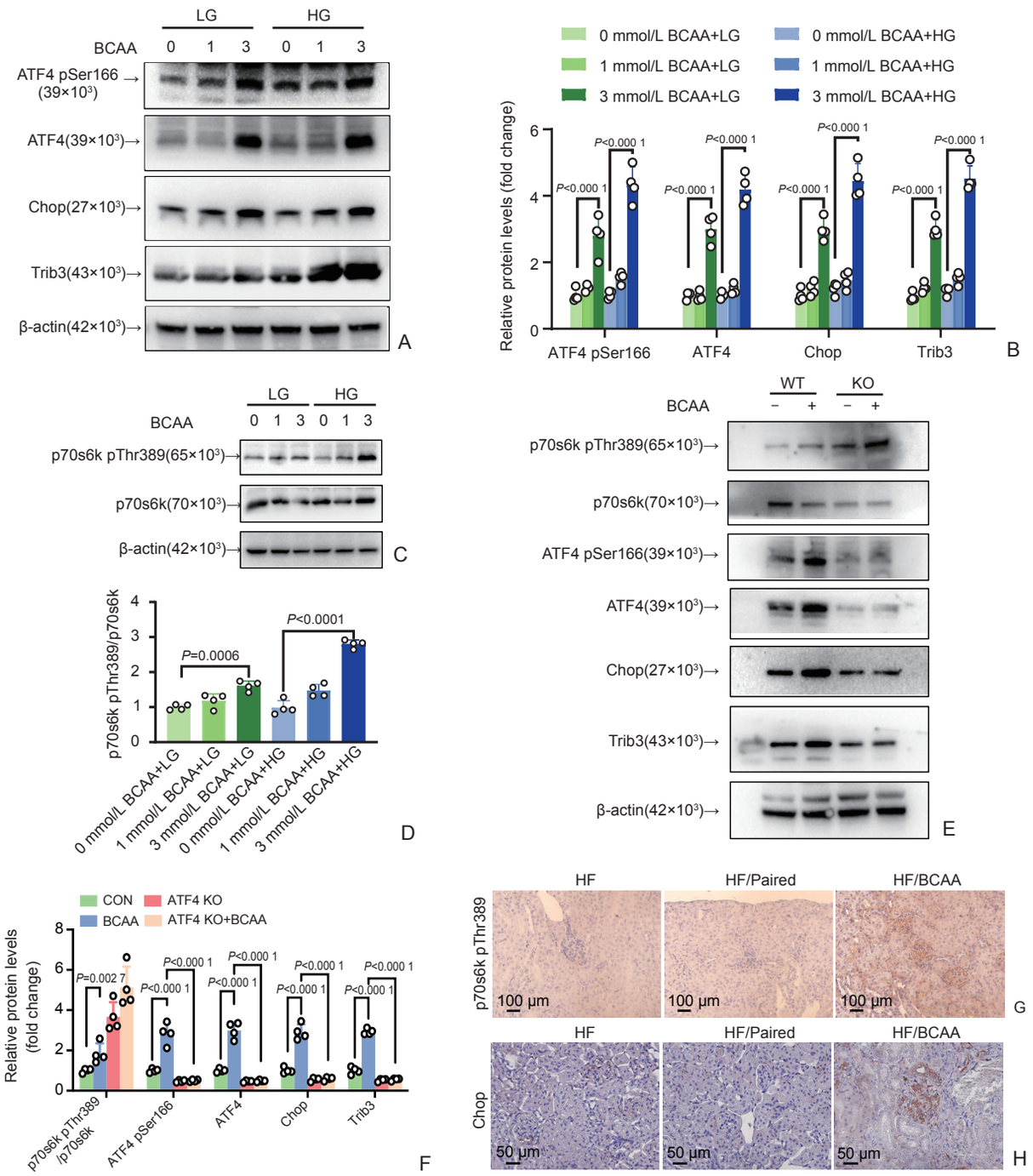
图5A、B),表明Rap可有效阻断BCAAs代谢障碍激活的p70s6k-ATF4-Chop/Trib3凋亡通路。进一步,本研究在HF/BCAA组小鼠中给予Rap灌胃。油红O染色及PAS染色结果显示,相较于HF/BCAA组,HF/BCAA+Rap组小鼠肾脏的脂质沉积明显减少,肾小管管型数量显著减少(图5C)。本研究提取和纯化小鼠肾小球(图5D)。Western blotting检测发现,Rap显著降低HF/BCAA小鼠肾小球p70s6k pSer389、ATF4 pSer166、Chop及Trib3的蛋白表达水平(P<0.000 1,图5E)。接着,本研究检测了各组小鼠尿微量白蛋白浓度,结果显示相较于HF/BCAA组,HF/BCAA+Rap组尿微量白蛋白显著降低(P<0.000 1,图5F),小鼠肾脏甘油三酯含量检测显示,结果显示Rap显著降低HF/BCAA小鼠肾脏甘油三酯含量(P<0.000 1,图5G)。以上结果表明,BCAAs通过激活mTORC1促进足细胞功能障碍和死亡,促进DKD发生发展。



A: representative immunofluorescence images of Nephrin (red) and DAPI (blue) in mouse renal sections; B: representative immunofluorescence images of Synaptopodin (green) and DAPI (blue) in mouse renal sections; C: representative Western blotting images of WT1, Nephrin and Synaptopodin in mouse kidneys; D: quantitative analysis of relative protein expression in panel C (n=6); E: representative Western blotting images of Nephrin and Synaptopodin proteins in cultured podocytes in indicated groups; F: quantitative analysis of relative protein levels in panel E (n=3); G: representative Western blotting images of Cleaved Caspase-3 in podocytes in indicated groups; H: quantitative analysis of relative protein expression in panel G (n=4)

图 3 BCAAs对糖尿病条件下足细胞功能及死亡的影响

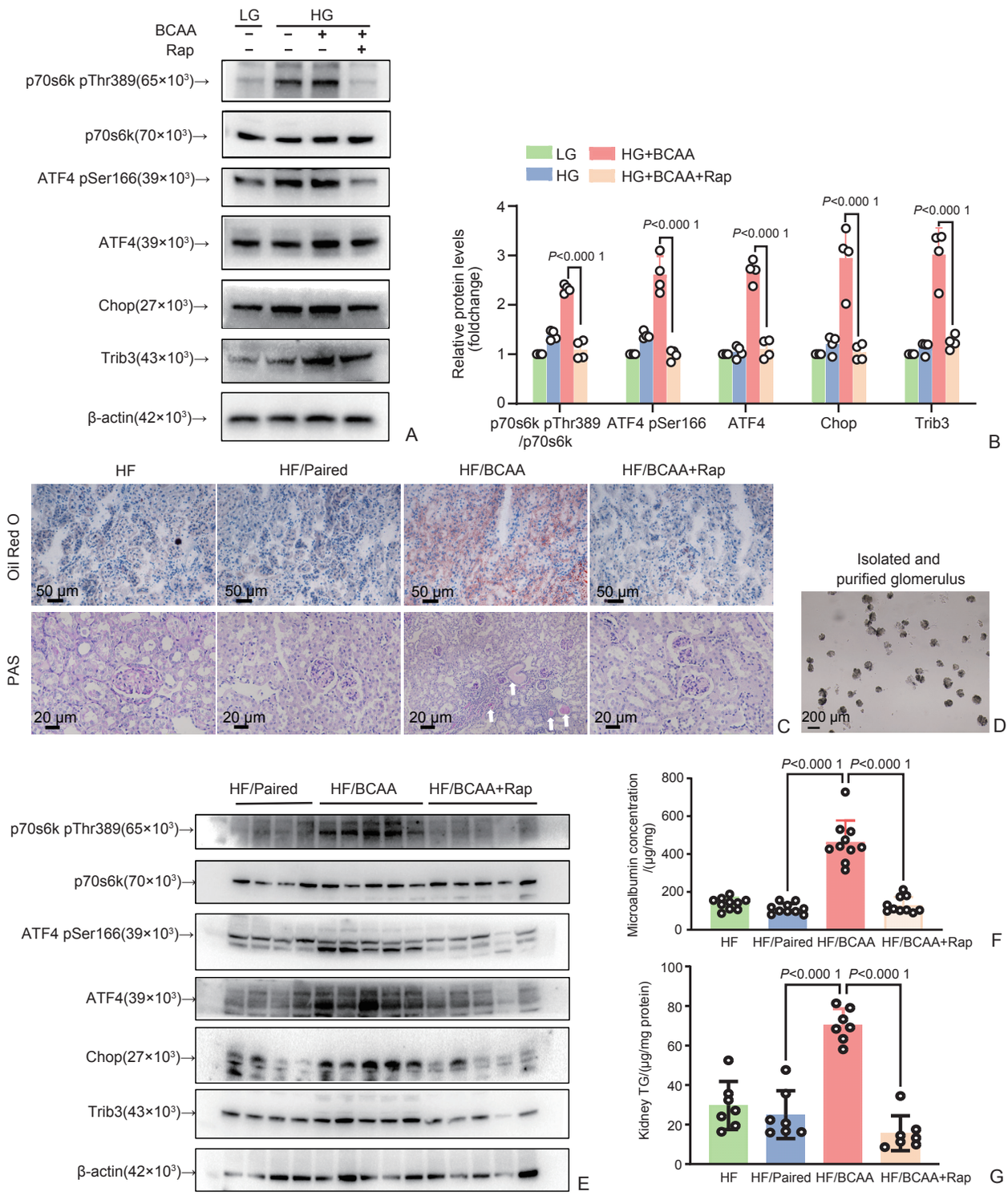
Figure 3 Effects of BCAAs on podocyte function and death in diabetic conditions



A: representative Western blotting images of ATF4 pSer166, ATF4, Chop and Trib3 in cultured podocytes in indicated groups; B: quantitative analysis of relative protein expression in panel A (n=4); C: representative Western blotting images of p70s6k pSer389 and p70s6k in cultured podocytes in indicated groups; D: quantitative analysis of relative protein expression in panel C (n=4); E: representative Western blotting images of p70s6k pSer389, ATF4 pSer166, ATF4, Chop and Trib3 in podocytes in indicated groups; F: quantitative analysis of relative protein expression in panel E (n=4); G: representative immunohistochemistry images of p70s6k pSer389 in mouse renal sections; H: representative immunohistochemistry images of Chop in mouse renal sections

图 4 BCAAs通过激活p70s6k-ATF4-Chop/Trib3通路诱导足细胞凋亡的作用

Figure 4 BCAAs induce to podocyte apoptosis *via* activating the p70s6k-ATF4-Chop/Trib3 pathway



A: representative Western blotting images of p70s6k pSer389, p70s6k, ATF4 pSer166, ATF4, Chop and Trib3 in cultured podocytes in indicated groups; B: quantitative analysis of relative protein expression in panel A (n=4); C: representative Oil Red O red staining (top) and PAS staining (bottom) images of mouse kidney sections in indicated groups; D: representative image of isolated and purified mouse glomeruli; E: representative Western blotting images of p70s6k pSer389, p70s6k, ATF4 pSer166, ATF4, Chop and Trib3 in glomeruli in indicated groups; F: quantification of urinary microalbumin concentration in indicated groups (n=10); G: quantification of renal TG levels in indicated groups (n=7). TG: triglyceride

图 5 抑制 mTORC1 改善 BCAAs 代谢障碍诱导的 DN 发生发展

Figure 5 Inhibiting mTORC1 improves the development and progression of DN induced by BCAAs catabolic disorder

3 讨论

3.1 BCAAs代谢障碍是促进DKD发生发展的独立危险因素

DKD已成为我国ESRD的首要病因,疾病负担沉重[3-4]。然而,部分患者即使在严格控制血糖、血脂后仍进展为ESRD,提示存在尚未明确的内源性代谢危险因素[5]。BCAAs作为必需氨基酸,其代谢紊乱与糖尿病并发症密切相关,尿液BCAAs水平可预测1型糖尿病患者DKD进展风险,而DKD模型小鼠中存在BCAAs代谢中间产物蓄积[12-13]。本研究体外实验发现,高糖处理可诱导足细胞BCAAs分解代谢障碍,表现为PPM1K降低、BCKD磷酸化水平升高。体内实验进一步证实,外源性BCAAs蓄积显著加剧高脂饮食小鼠DKD相关肾损伤,包括尿白蛋白/肌酐比值升高、肾小管管型形成、肾脏脂质沉积及纤维化加重。本课题组前期研究也表明,高脂饮食补充BCAAs升高小鼠空腹血糖,导致小鼠糖耐量和胰岛素耐量受损[9,28]。此外,足细胞特异性敲除PPM1K促进高脂饮食诱导的DKD进展[28]。综上,无论是外源性BCAAs补充还是足细胞BCAAs分解代谢障碍,均可促进高脂饮食诱导的DKD发生发展,提示BCAAs代谢障碍是DKD进展中不可忽视的内源性危险因素。

3.2 BCAAs代谢障碍通过损伤肾小球滤过膜完整性引发肾小管脂质重吸收与管型形成

正常情况下,血浆脂肪酸几乎无法通过肾小球滤过膜进入原尿,因其多与白蛋白等载体结合形成大分子复合物,且白蛋白带负电受滤过膜负电屏障阻隔[32]。只有当滤过膜破损时,脂肪酸-白蛋白复合物才能大量进入原尿,被近端肾小管上皮细胞重吸收。本研究发现,高脂饮食补充BCAAs显著上调肾小管脂质重吸收关键分子FABP4和CD36的表达,并导致肾脏脂质蓄积,但未影响脂质β氧化关键分子PGC1α和PPARα的表达,提示BCAAs代谢障碍主要通过促进脂质摄取而非抑制氧化导致脂质蓄积。透射电镜结果显示,BCAAs蓄积造成足细胞足突融合、滤过膜增厚、足突宽度增加,提示肾小球滤过膜损伤。同时,BCAAs诱导肾小管管型形成,这是滤过膜破损后血浆蛋白大量滤过并被浓缩的典型表现。因此,BCAAs代谢障碍通过损伤肾小球滤过膜完整性,使脂质和蛋白滤过进入肾小管,进而引发肾小管脂质重吸收增加、脂质蓄积及管型形成,加剧DKD进展。

3.3 BCAAs代谢障碍通过激活mTORC1-ATF4-Chop/Trib3信号轴诱导足细胞功能障碍与凋亡

足细胞损伤与丢失是DKD早期的标志性病理事件,与蛋白尿产生及肾功能恶化密切相关[16-19]。本研究发现,BCAAs蓄积可显著降低足细胞特异性标志物Nephrin、Synaptopodin及WT1的表达,并促进高糖诱导的足细胞凋亡,表明BCAAs蓄积通过直接损伤足细胞功能紊乱并诱导其凋亡,是BCAAs促进DKD发生发展的机制。在分子机制层面,BCAAs作为mTORC1的经典激活剂[25-26],可通过结合上游感知蛋白SAR1B和Sestrin2等直接激活mTORC1[33-37]。本研究发现,BCAAs显著上调足细胞mTORC1通路的直接下游分子p70s6K磷酸化水平,提示mTORC1通路被激活。mTORC1可磷酸化ATF4并调控其活性[24],而ATF4作为整合应激反应的关键转录因子,可通过下游靶基因Chop和Trib3启动凋亡程序[38-39]。本研究显示,BCAAs显著上调足细胞ATF4磷酸化水平及Chop、Trib3表达,且ATF4敲除可显著阻断BCAAs诱导的Chop和Trib3上调,表明ATF4在该通路中发挥关键介导作用。体内实验同样观察到高脂饮食补充BCAAs小鼠肾小球中p70s6K磷酸化水平和Chop表达升高。综上,BCAAs代谢障碍通过激活mTORC1-ATF4-Chop/Trib3信号轴,诱导足细胞功能障碍与凋亡。

3.4 靶向mTORC1可有效阻断BCAAs代谢障碍诱导的DKD进展

为验证上述通路的关键作用,本研究采用mTORC1特异性抑制剂雷帕霉素[40]进行干预,结果显示,阻断BCAAs激活的p70s6k-ATF4-Chop/Trib3信号通路,下调Chop、Trib3的表达,同时改善BCAAs诱导的肾脏脂质沉积、肾小管管型形成及蛋白尿,恢复足细胞特异性标志物的表达。以上结果证实,BCAAs代谢障碍通过激活mTORC1-ATF4-Chop/Trib3通路驱动足细胞损伤与DKD进展,靶向mTORC1可有效阻断这一病理过程,为DKD防治提供了新的干预策略。

本研究也存在一定局限性:首先,动物实验中主要采用雄性小鼠构建模型,而性别差异可能影响BCAAs代谢及DKD进展,未来需纳入雌性动物进一步验证研究结论;其次,本研究聚焦于mTOR-p70s6k-ATF4-Chop/Trib3通路,BCAAs代谢紊乱是否通过其他信号通路调控足细胞功能仍需深入探索;最后,临床转化方面,本研究在前期工作中发现早期糖尿病肾病患者(eGFR≥90 mL/min/1.73 m^2,尿白蛋白/肌酐比值≥30 mg/g)尿液中BCAAs分解代谢产物4 methyl 2 oxopentanoate和methylmalonic acid明显升高。这2个BCAAs分解代谢产物可能作为DKD的诊断标志物[28]。后续研究将进一步证实其临床转化价值。

综上所述,本研究证实BCAAs代谢障碍通过激活mTORC1-ATF4-Chop/Trib3信号通路诱导足细胞功能

障碍与凋亡,进而破坏肾小球滤过膜完整性,导致肾脏脂质沉积、肾小管管型形成及纤维化进展,最终促进DKD发生发展。靶向BCAAs代谢或mTORC1-ATF4通路可能成为改善DKD患者预后的有效策略。

利益冲突 本文所有作者声明不存在利益冲突

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