研究论文

PI3K/Akt/mTOR信号通路在脂多糖诱导的大鼠肝星状细胞自噬中的作用

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摘要 该文探讨了磷脂酰肌醇3-激酶(PI3K)/蛋白激酶B(Akt)/哺乳动物雷帕霉素靶蛋白(mTOR)信号通路在脂多糖(LPS)诱导的大鼠肝星状细胞-T6(HSC-T6)自噬中的作用。体外培养HSC-T6细胞, 随机分为对照组、LPS组、雷帕霉素(Rapamycin, Rapa)组、LPS+Rapa组、LY294002组、LPS+LY294002组, SC79组、LPS+SC79组, 各组经相应处理后, 单丹磺酰尸胺(MDC)染色法观察自噬溶酶体变化; 细胞免疫荧光法检测各组微管相关蛋白轻链II(LC3 II)表达; Western blot检测各组通路蛋白p-Akt、p-mTOR、Akt、mTOR及自噬相关蛋白LC3 II、Beclin1的表达; qRT-PCR检测各组*LC3 II*和*Beclin1* mRNA的表达。结果显示, LPS+Rapa组、LPS+LY294002组较LPS组的自噬溶酶体、*LC3 II*荧光亮点含量无明显差异($P>0.05$), LPS+SC79组较LPS组的自噬溶酶体、*LC3 II*荧光亮点含量明显减少($P<0.05$); Western blot显示, LPS+Rapa组、LPS+LY294002组较LPS组LC3 II、Beclin1、p-Akt、p-mTOR蛋白表达水平无明显差异($P>0.05$), LPS+SC79组较LPS组LC3 II、Beclin1含量明显减少, p-Akt、p-mTOR蛋白表达水平明显增加($P<0.05$); qRT-PCR显示LPS+Rapa组、LPS+LY294002组较LPS组*LC3 II*、*Beclin1* mRNA含量无明显差异($P>0.05$), LPS+SC79组较LPS组*LC3 II*、*Beclin1* mRNA含量明显减少($P<0.05$)。该项研究结果表明, LPS可能通过抑制PI3K/Akt/mTOR信号通路促进HSC-T6细胞自噬。

关键词 信号通路; 肝星状细胞; 脂多糖; 自噬

Role of PI3K/Akt/mTOR Signaling Pathway in Autophagy of Rat Hepatic Stellate Cells Induced by Lipopolysaccharide

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Abstract This work was to investigate the role of phosphatidylinositol 3-kinase (PI3K)/protein kinase B(Akt)/mammalian target of rapamycin (mTOR) signaling pathway in the lipopolysaccharide (LPS)-induced autophagy of rat hepatic stellate cells. HSC-T6 cells were cultured *in vitro* and randomly divided into control group, LPS group, Rapa group, LPS+Rapa group, LY294002 group, LPS+LY294002 group, SC79 group and LPS+SC79

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group. After treatment, the changes of autophagic lysosome were observed by mono-dansylcadaverine (MDC) staining; the expression of microtubule-associated protein light chain II (LC3 II) was detected by immunofluorescence assay; the expressions of p-Akt, p-mTOR, Akt, mTOR, LC3 II and Beclin1 in each group were detected by Western blot; the expressions of *LC3 II* and *Beclin1* mRNA in each group were detected by qRT-PCR. The results showed that there were no significant differences in the levels of autophagic lysosome and LC3 II fluorescent spots in comparison between LPS group and LPS+Rapa group, or LPS+LY294002 group (P>0.05), but the levels of autophagic lysosome and LC3 II fluorescent spots particles in LPS+SC79 group were significantly lower than those in LPS group (P<0.05). Western blot showed that there were no significant differences in the levels of LC3 II, Beclin1, p-Akt and p-mTOR in comparison between LPS group and LPS+Rapa group, or LPS+LY294002 group (P>0.05), but the levels of LC3 II and *Beclin1* in LPS+SC79 group were significantly lower than those in LPS group, and the levels of p-Akt and p-mTOR protein were significantly increased (P<0.05). qRT-PCR showed that there were no significant differences in the expressions of LC3 II and *Beclin1* mRNA in comparison between LPS group and LPS+Rapa group, or LPS+LY294002 group (P>0.05), but the expressions of *LC3 II* and *Beclin1* mRNA were significantly decreased in LPS+SC79 group compared with LPS group (P<0.05). It is possibly that LPS promote autophagy in HSC-T6 cells by inhibiting PI3K/Akt/mTOR signaling pathway.

Keywords signaling pathway; hepatic stellate cells; lipopolysaccharide; autophagy

肝纤维化(hepatic fibrosis, HF)作为肝硬化的必经病理过程, 是对反复发生的慢性肝损伤的适应性反应, 其基本病理特点为细胞外基质(extracellular matrix, ECM)合成与降解失衡, 导致ECM过度沉积于肝脏, 而肝星状细胞(hepatic stellate cells, HSCs)的活化是肝纤维化形成的重要条件[1]。自噬(autophagy)是一种将部分蛋白质和受损细胞器包裹在双膜的自噬体中, 并向溶酶体进行消化的过程, 从而实现蛋白质和细胞器更新, 为细胞提供能量[2]。近年来的研究表明, 自噬在肝纤维化的发生发展中发挥重要作用, 它诱导HSCs活化, 促进肝纤维化[3]。因此, 干预HSCs自噬可能是治疗肝纤维化的一个潜在的治疗手段[4]。本实验室先前的研究显示, LPS可诱导HSC-T6细胞发生自噬, 进一步研究发现, 其促进细胞自噬的作用可能并非通过NF-kB通路[5]。PI3K/Akt/mTOR通路在巨噬细胞、牙龈成纤维细胞等细胞中发现是调控细胞自噬至关重要的信号通路之一[6], 但其在LPS诱导的HSCs自噬中的作用尚未见报道。本研究使用LPS及mTOR抑制剂Rapa、PI3K抑制剂LY294002、Akt激动剂SC79预处理HSC-T6细胞后观察其对自噬的影响, 探讨LPS诱导HSCs自噬的机制。

1 材料与方法

1.1 材料与试剂

1.1.1 实验细胞 实验细胞为大鼠肝星状细胞系HSC-T6(齐氏生物科技有限公司)。

1.1.2 主要试剂 胎牛血清、DMEM高糖培养基、FITC-羊抗兔IgG抗体购自武汉博士德生物工程有限公司; β-actin抗体、Akt抗体、mTOR抗体购自Bio-Red公司; p-Akt(Ser473)抗体、p-mTOR(Ser2448)抗体购自Cell Signaling Technology公司; LPS、LC3抗体购自Sigma公司; Beclin1抗体购自爱必信(上海)生物科技有限公司; MDC试剂盒购自南京建成生物工程研究所; LY294002、雷帕霉素、SC79购自MCE公司; qRT-PCR试剂盒购自北京聚合美生物科技有限公司。

1.2 主要方法

1.2.1 细胞培养和分组 HSC-T6细胞生长于含10%胎牛血清的高糖DMEM培养基中, 常规培养, 取处于指数生长期的细胞接种于6孔板, 并随机分为对照组、LPS组(0.1 mg/L LPS处理12 h)、Rapa组(200 μmol/L Rapa处理12 h)、LPS+Rapa组(200 μmol/L Rapa预处理12 h后给予0.1 mg/L LPS处理12 h)、LY294002组(20 μmol/L LY294002处理12 h)、LPS+LY294002组(20 μmol/L LY294002预处理12 h后给予0.1 mg/L LPS处理12 h)、SC79组(5 mg/L SC79处理12 h)、LPS+SC79组(5 mg/L SC79预处理12 h后给予0.1 mg/L LPS处理12 h)。

1.2.2 MDC染色 将处理好的接种于载玻片的各组细胞吸弃培养基, 按照试剂盒说明处理各组爬片, 紫外激发光源下荧光显微镜观察并拍照, Image Pro

Plus分析图片。

1.2.3 细胞免疫荧光法检测LC3 II 将处理好的接种于载玻片的各组细胞吸弃培养基, PBS洗涤后, 4%多聚甲醛固定。洗涤后加入0.5% Triton X-100孵育10 min。洗涤后用5% BSA封闭1 h, 加入LC3抗体4 ℃孵育过夜。洗涤后加入荧光二抗, 室温避光孵育1 h。洗涤后加入DAPI染色, 洗涤后封片, 荧光显微镜下观察并拍照, Image Pro Plus分析图片。

1.2.4 Western blot 检测通路蛋白和自噬相关蛋白含量。各组细胞PBS洗涤后RIPA裂解提取蛋白, 通过BCA法测定并调整蛋白浓度。8% SDS和12% SDS聚丙烯酰胺凝胶进行蛋白电泳, 并将蛋白质电转移至NC膜上, 5%牛奶室温封闭2 h, 一抗4 ℃孵育过夜。TBST洗涤3次后, 加入HRP标记的羊抗兔IgG于37 ℃恒温气浴70 min, TBST洗涤3次后, 伯乐化学发光成像仪显影, 使用Image Lab分析条带灰度值。

1.2.5 实时荧光定量qRT-PCR检测*LC3 II*和*Beclin1*的mRNA表达 提取各组细胞总RNA, 反向转录成cDNA, 以cDNA为模板, 采用SYBR Green进行实时荧光定量PCR反应; 反应参数: 95 ℃ 60 s, 1个循环; 95 ℃ 15 s, 58 ℃ 45 s, 72 ℃ 45 s, 37个循环, 样本中目的基因的计算方法为$2^{-\Delta\Delta Ct}$。大鼠*LC3 II*上游引物5′-GAG TGG AAG ATG TCC GGC TC-3′, 下游引物5′-CCA GGA GGA AGA AGG CTT GG-3′; 大鼠*Beclin1*上游引物5′-CGT GGA GAA AGG CAA GAT TGA AGA-3′, 下游引物5′-GTG AGG ACA CCC AAG CAA GAC C-3′; 大鼠*GAPDH*上游引物5′-CAA CGG GAA ACC CAT CAC CA-3′, 下游引物5′-ACG CCA GTA GAC TCC ACG ACA T-3′。

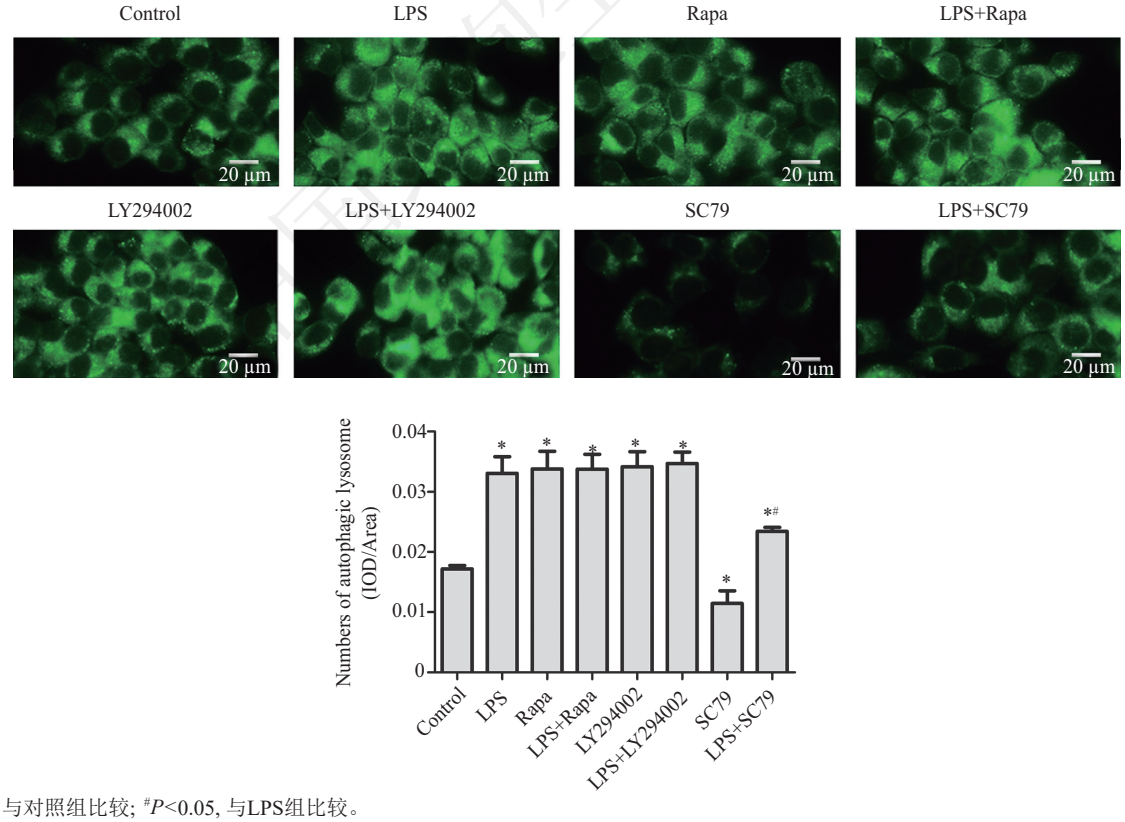
1.3 统计学分析

采用SPSS 25.0软件进行统计分析。计量资料以均数±标准差($\bar{x}\pm s$)表示, 组间差异采用单因素方差分析(One-Way ANOVA), 两两比较用*LSD*检验, 以$P<0.05$为差异有统计学意义。

2 结果

2.1 自噬溶酶体的变化

自噬溶酶体变化结果(图1)显示: LPS+Rapa组、LPS+LY294002组较LPS组的自噬溶酶体含量无明显差异($P>0.05$); LPS+SC79组较LPS组的自噬溶酶体含量

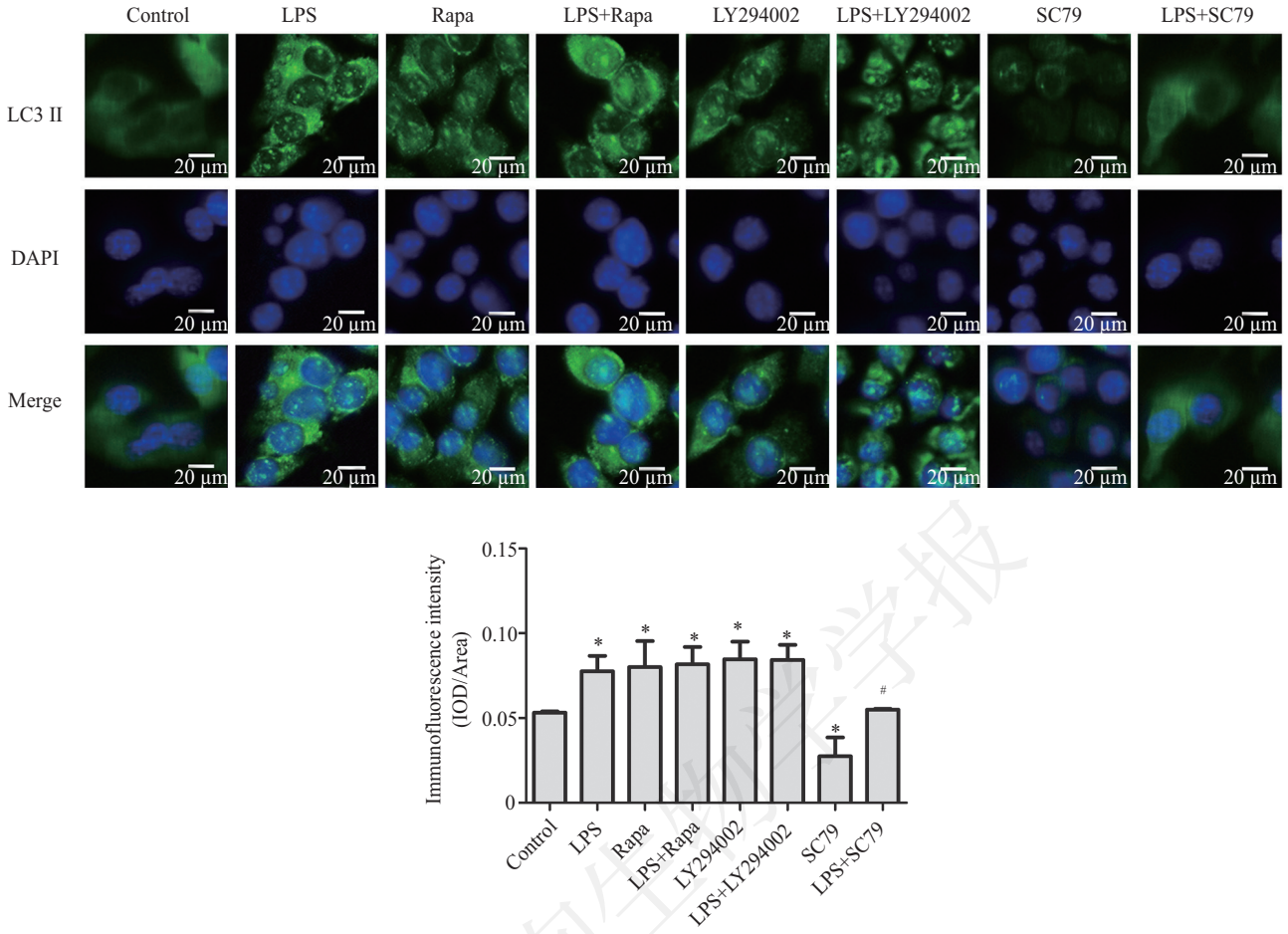


*$P<0.05$, 与对照组比较; #$P<0.05$, 与LPS组比较。

*$P<0.05$ compared with control group; #$P<0.05$ compared with LPS group.

图1 MDC染色显示不同处理因素对HSC-T6细胞自噬溶酶体的影响

Fig.1 MDC staining of autophagic lysosomes among different treatments in HSC-T6 cells



#*P*<0.05, 与对照组比较; ##*P*<0.05, 与LPS组比较。

**P*<0.05 compared with control group; ##*P*<0.05 compared with LPS group。

图2 免疫荧光法观察不同处理因素对HSC-T6细胞LC3 II细胞内分布的影响

Fig.2 The expression of LC3 II among different groups detected by immunofluorescence

明显减少(*P*<0.05)。

2.2 LC3 II细胞免疫荧光标记情况

结果(图2)显示: LPS+Rapa组、LPS+LY294002组较LPS组的荧光亮点表达无明显差异(*P*>0.05), 且胞质、胞核中均出现荧光亮点; LPS+SC79组较LPS组的荧光亮点表达明显减少(*P*<0.05)。

2.3 Western blot检测结果

结果(图3)显示: LPS+Rapa组、LPS+LY294002组较LPS组LC3 II、Beclin1蛋白表达无显著差异(*P*>0.05); LPS+SC79组较LPS组LC3 II、Beclin1蛋白表达显著减少(*P*<0.05)。结果(图4)显示: LPS+LY294002组、LPS+Rapa组较LPS组p-Akt、p-mTOR蛋白表达无显著差异(*P*>0.05); LPS+SC79组较LPS组p-Akt、p-mTOR蛋白表达显著增加(*P*<0.05)。

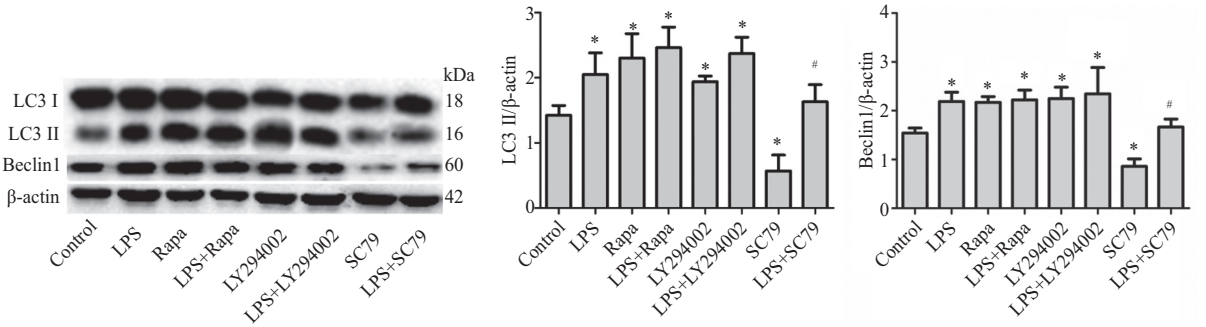
2.4 实时荧光定量qRT-PCR检测结果

结果(图5)显示: LPS+Rapa组、LPS+LY294002组较LPS组*LC3 II*、*Beclin1* mRNA含量无明显差异(*P*>0.05); LPS+SC79组较LPS组*LC3 II*、*Beclin1* mRNA含量明显增加(*P*<0.05)。

3 讨论

细菌脂多糖(LPS)是革兰氏阴性细菌的细胞壁成分, 是已知最强烈的炎症诱导因子之一。研究表明, 各种急、慢性肝损伤时, 由于肠黏膜通透性的改变、细菌易位的增加及肝脏清除能力的降低, 血浆LPS浓度甚至在早期就明显升高, 谓之"肠源性内毒素血症"[7-8]。目前已经公认, 肠源性内毒素血症可加重各种肝脏疾病的发生与发展, 包括肝纤维化。LPS通过活化HSCs而促进肝纤维化[7], 但其机制及信号通路尚不完全清楚。

近年来发现, HSCs自噬可促进HSCs活化, 干预自噬可能对治疗肝纤维化有重大意义[3,9-10]。本实验

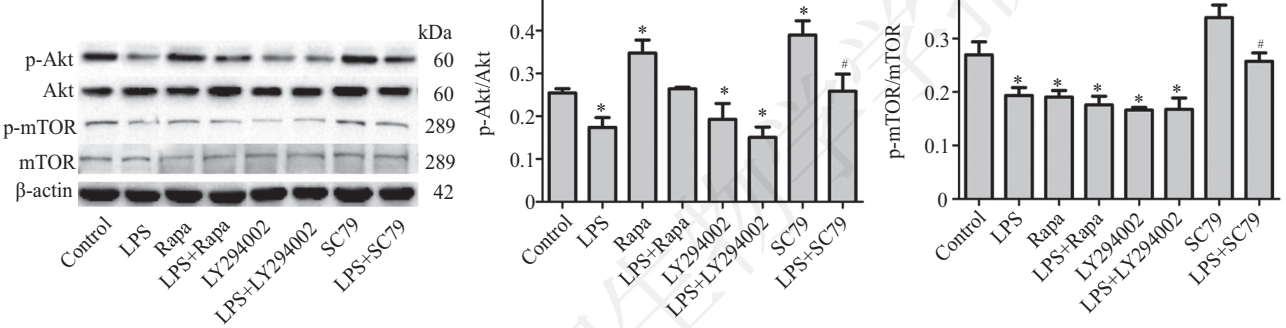


*P<0.05, 与对照组比较; #P<0.05, 与LPS组比较。

*P<0.05 compared with control group; #P<0.05 compared with LPS group.

图3 Western blot检测不同处理因素对HSC-T6细胞中LC3 II及Beclin1含量的影响

Fig.3 Levels of LC3 II and Beclin1 in HSC-T6 cells administrated with different conditions were detected by Western blot

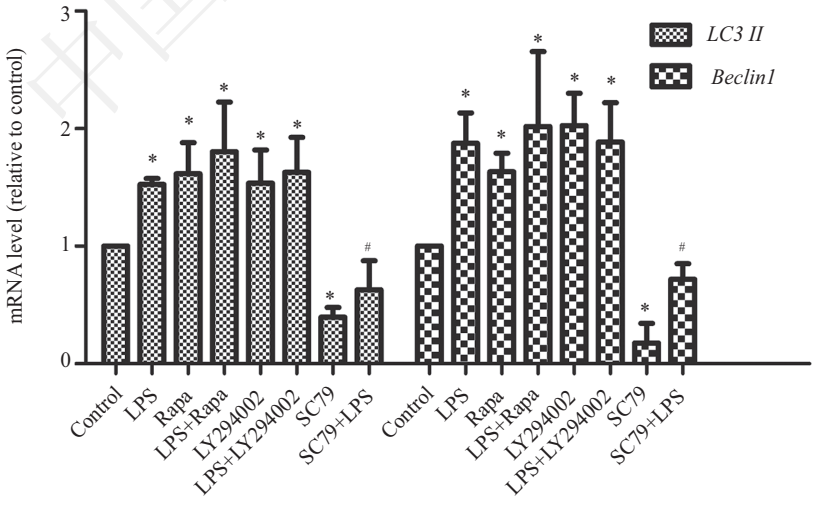


*P<0.05, 与对照组比较; #P<0.05, 与LPS组比较。

*P<0.05 compared with control group; #P<0.05 compared with LPS group.

图4 Western blot检测不同处理因素对HSC-T6细胞中p-Akt及p-mTOR含量的影响

Fig.4 Levels of p-Akt and p-mTOR in HSC-T6 cells administrated with different conditions were detected by Western blot



*P<0.05, 与对照组比较; #P<0.05, 与LPS组比较。

*P<0.05 compared with control group; #P<0.05 compared with LPS group.

图5 实时荧光定量qRT-PCR检测不同处理因素对HSC-T6细胞中*LC3 II*及*Beclin1* mRNA含量的影响

Fig.5 Levels of *LC3 II* and *Beclin1* mRNA expressions in HSC-T6 cells administrated with different conditions were detected by qRT-PCR

室先前的研究发现, LPS可诱导HSC-T6发生自噬, 但并非通过NF-κB通路[5]。最近的研究发现, LPS诱导巨噬细胞自噬, LPS可通过TLR4将细胞外信号转导到细胞内, 进而通过PI3K/Akt轴激活下游mTOR实现自噬的调控[11]。mTOR是PI3K和Akt下游的一个重要作用靶点, 是自噬的一个关键负性调节因子, mTOR的两种复合物形式包括: mTOR复合物1(mTOR complex 1, mTORC1)负责自噬调控; mTOR复合物2(mTOR complex 2, mTORC2)与细胞周期有关[12]。当mTORC1被抑制时, ULK1(unc-51 like kinase 1)复合物被激活后进一步激活PI3K复合物III发生囊泡成核, 随后起源于内质网的膜结构在两种泛素共轭体系Atg5-Atg12和LC3 II介导下延伸和闭合形成自噬体, 自噬体与溶酶体融合形成自噬溶酶体[13]。此外Liu等[2]发现, LPS通过抑制PI3K/Akt/mTOR信号传导途径诱导人牙龈成纤维细胞的自噬; Chen[8]等发现, LPS干预人肝星状细胞(human hepatic stellate cell line, LX-2)后, p-mTOR、p-Akt水平下调, 提示Akt/mTOR信号通路介导LPS诱导LX-2自噬, 促进肝纤维化。基于以上, 我们推测, LPS或许通过PI3K/Akt/mTOR信号通路调控HSC-T6自噬。

在本实验中, 我们发现, LPS、Rapa/ly294002均能促进自噬溶酶体的形成, SC79则抑制该过程。其中LPS能抑制Akt、mTOR磷酸化水平, 并能有效提高LC3 II、Beclin1 mRNA和蛋白水平, 而使用SC79预处理后, Akt、mTOR磷酸化水平提高, LC3 II、Beclin1 mRNA和蛋白水平降低, 这说明, LPS可能通过抑制PI3K/Akt/mTOR信号通路促进HSC-T6细胞自噬。 然而使用Rapa/ly294002预处理后再加LPS刺激, Akt、mTOR磷酸化水平及LC3 II、Beclin1 mRNA和蛋白水平均无进一步的明显变化, 表明PI3K/Akt/mTOR信号通路可能是LPS诱导HSC-T6发生自噬的重要信号通路, 具体机制有待于进一步研究阐明。

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