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(收稿日期:2020-03-25,修回日期:2020-05-10)

引用本文:简宇,范婷,代凌云,赵春虎.姜黄素通过上调miR-124减轻脂多糖诱导的大鼠急性肺损伤[J].安徽医药,2022,26(8):1515-1519.**DOI:10.3969/j.issn.1009-6469.2022.08.007.**

◇药学研究◇

姜黄素通过上调miR-124减轻脂多糖诱导的大鼠急性肺损伤

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摘要: 目的 探究姜黄素(Cur)对脂多糖(LPS)诱导的大鼠急性肺损伤(ALI)的保护作用及可能机制。**方法** 体外培养大鼠肺泡巨噬细胞NR8383,采用双荧光素酶报告基因实验验证微小RNA-124(miR-124)与肿瘤坏死因子受体相关因子6(TRAF6)的靶向关系。SD大鼠采用随机数字表法分为对照组(NC组)、LPS组、LPS+Cur组、LPS+Cur+inhibitor-NC组、LPS+Cur+inhibitor组,每组10只。LPS组腹腔注射LPS 10 mg/kg制备ALI大鼠模型,LPS+Cur组在ALI模型制备30 min后尾静脉注射Cur 200 mg/kg,LPS+Cur+inhibitor-NC组、LPS+Cur+inhibitor组大鼠尾静脉分别注射miR-124 inhibitor-NC、miR-124 inhibitor 50 mg/kg 1周后腹腔注射LPS 10 mg/kg,30 min后尾静脉注射Cur 200 mg/kg,NC组注射等量生理盐水30 min后尾静脉注射等量生理盐水。干预24 h后,HE染色观察肺组织病理变化,酶联免疫吸附试验(ELISA)检测血清肿瘤坏死因子-α(TNF-α)、白细胞介素-6(IL-6)水平,分别采用实时荧光定量PCR(RT-qPCR)、蛋白质印迹法(WB)检测肺组织miR-124、TRAF6 mRNA及蛋白表达。**结果** 双荧光素酶报告基因实验结果显示,miR-124可直接靶向TRAF6 3′UTR区负调控其表达。动物实验结果表明,NC组大鼠肺组织结构清晰,肺泡结构完整,无明显异常改变;LPS组、LPS+Cur+inhibitor组大鼠肺组织结构破坏严重,肺间质增厚,大量炎性细胞浸润,弥漫性充血、渗出,肺泡萎缩;LPS+Cur组、LPS+Cur+inhibitor-NC组大鼠肺组织损伤减轻。与NC组比较,LPS组大鼠血清TNF-α、IL-6、肺组织TRAF6 mRNA及蛋白表达水平显著增加,miR-124表达水平显著降低($P<0.05$);与LPS组比较,LPS+Cur组大鼠血清TNF-α、IL-6、、肺组织TRAF6 mRNA及蛋白表达水平显著降低,miR-124表达水平显著增加($P<0.05$);与LPS+ Cur+inhibitor-NC组比较,LPS+ Cur+inhibitor组大鼠血清TNF-α、IL-6、肺组织TRAF6 mRNA及蛋白表达水平显著增加,miR-124表达水平显著降低($P<0.05$)。**结论** Cur可通过上调miR-124靶向抑制TRAF6减轻LPS诱导的大鼠ALI及炎症反应,发挥治疗作用。

关键词: 急性肺损伤; 姜黄素; 微小RNA-124; 肿瘤坏死因子受体相关因子6; 大鼠

Curcumin alleviates lipopolysaccharide-induced acute lung injury in rats by up-regulating miR-124

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Abstract: Objective To investigate the protective effect and its possible mechanism of Curcumin (Cur) on lipopolysaccharide (LPS)-induced acute lung injury (ALI) in rats.**Methods** Alveolar macrophages NR8383 of rats was cultured *in vitro*, double luciferase reporter gene assay was used to verify the targeting relationship between microRNA-124 (miR-124) and tumor necrosis factor receptor associated factor 6 (TRAF6). SD rats were randomly divided into control group (NC group), LPS group, LPS+Cur group, LPS+Cur+inhib-

itor NC group, LPS+Cur+inhibitor group, with 10 in each group. In LPS group, 10 mg/kg LPS was injected intraperitoneally to prepare ALI rat model. In LPS+Cur group, after 30 minutes of ALI model preparation, Cur 200 mg/kg was injected into tail vein, while the rats in LPS+Cur+inhibitor NC group and LPS+Cur+inhibitor NC group were injected with miR-124 inhibitor NC and miR-124 inhibitor 50 mg/kg via caudal vein, respectively, and LPS 10 mg/kg was injected intraperitoneally 1 week later, and then Cur 200 mg/kg was injected into tail vein 30 minutes later. The rats in NC group were injected with equal amount of normal saline into tail vein 30 minutes after injection of the same amount of normal saline. After 24 hours of intervention, HE staining was used to observe the pathological changes of lung tissue, the levels of serum tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were detected by enzyme-linked immunosorbent assay (ELISA), and the mRNA and protein expressions of miR-124 and TRAF6 were detected by real-time fluorescence quantitative PCR (RT-qPCR) and Western blot (WB) respectively.**Results** Double luciferase reporter gene showed that miR-124 could directly target TRAF63 ′UTR region and negatively regulate its expression. The results of animal experiments showed that the lung tissue structure of rats in NC group was clear, the alveolar structure was complete, and there was no obvious abnormal change; the lung tissue structure of rats in LPS group and LPS+Cur+inhibitor group was damaged seriously, the lung interstitium was thickened, a large number of inflammatory cells were infiltrated, diffuse hyperemia, exudation and alveolar atrophy were found; in addition, the lung injury in LPS+Cur group and LPS+Cur+inhibitor NC group was reduced. Compared with those in NC group, the expression levels of TNF-α, IL-6 in serum, TRAF6 mRNA and protein in lung tissue in LPS group were significantly higher, and the expression level of miR-124 was significantly lower (P<0.05); compared with those in LPS group, the expression levels of TNF-α, IL-6 in serum, TRAF6 mRNA and protein in lung tissue in LPS+Cur group were significantly lower, and the expression level of miR-124 was significantly higher (P<0.05); in addition, compared with those in LPS+Cur+inhibitor NC group, the expression levels of TNF-α, IL-6 in serum, TRAF6 mRNA and protein in lung tissue in LPS+Cur+inhibitor group were significantly higher, and the expression level of miR-124 was significantly lower (P<0.05).**Conclusion** Cur can reduce the ALI and inflammatory response induced by LPS by target inhibition of TRAF6 by up-regulating miR-124, and play a therapeutic role.

Key words: Acute lung injury; Curcumin; MicroRNA-124; Tumor necrosis factor receptor associated factor 6; Rats

急性肺损伤(acute lung injury,ALI)是常见危重症呼吸系统疾病,主要以低氧血症和进行性呼吸窘迫为临床特征,病死率高达30%~45%[1-2]。其病因多样、发病机制复杂,目前尚未完全阐明,但研究表明,炎症反应在ALI发生发展中发挥重要作用[3]。姜黄素(curcumin,Cur)是广泛存在于姜黄、莪术、郁金等中药根茎中的一种酚性色素,具有抗炎、抗氧化、抗肿瘤等多种药理活性,已有研究证实,Cur可有效减轻脂多糖(lipopolysaccharide,LPS)诱导及脓毒症相关ALI,在肺部疾病中具有良好的应用前景,但其具体作用机制尚不清楚[4-5]。微小RNA(microRNA,miRNA)是一类内源性非编码小RNA,可通过对其靶基因转录后调控发挥生物学功能,其中miR-124是一个重要炎症相关miRNA[6],有研究报道,miR-124可靶向抑制肿瘤坏死因子受体相关因子6(tumor necrosis factor receptor associated factor 6,TRAF6)抑制下游核因子-κB(nuclear factor-κB,NF-κB)发挥抗炎作用[7-8],但Cur是否也通过该通路对ALI大鼠发挥保护作用,尚未可知。因此本研究自2019年9月至2020年5月探究Cur对LPS诱导ALI大鼠的保护作用及对miR-124及其下游靶基因的表达影响,以期揭示其作用机制,为优化Cur临床应用提供进一步参考。

1 材料与方法

1.1 实验动物及细胞 8周龄健康雄性清洁级SD大鼠,体质量范围为290~310 g,购自上海斯莱克实验动物有限责任公司,动物生产许可证号SCXK(沪)2017-0005,使用许可证号SYXK(沪)2017-0008。本研究符合动物伦理学标准。大鼠肺泡巨噬细胞NR8383来自ATCC细胞库。

1.2 主要试剂及仪器 Cur(货号HY-N0005,纯度96.31%)购自MCE公司;LPS(货号L2630)购自Sigma-Aldrich公司;胎牛血清(fetal bovine serum,FBS,货号10099141)购自美国Gibco公司;RNA提取试剂盒(货号R0011)购自上海碧云天有限公司;PrimeScript™ RT reagent Kit (Perfect Real Time)(货号RR037A)、microRNA定量试剂盒(货号638315)、TB Green® Premix Ex Taq™ Ⅱ (Tli RNaseH Plus)(货号RR820A)购自TaKaRa生物公司;脂质体转染试剂盒Lipofectamine^{2000} Reagent(货号11668)购自美国Invitrogen公司;miR-124模拟物及其阴性对照(miR-124 mimic/NC)、miR-124抑制剂及其阴性对照(miR-124 inhibitor/inhibitor-NC)及miR-124、U6、TRAF6、GAPDH引物均由广州锐博生物科技有限公司提供;大鼠TNF-α ELISA试剂盒(货号ab236712)、IL-6 ELISA试剂盒(货号ab234570)、兔源一抗anti-TRAF6(货号ab40675)、anti-β-actin(ab8227)、二抗羊抗兔IgG(货号ab6721)均购自英国Abcam公司;MODEL550型酶标仪购自美国Bio-Rad公司;Forma Steri-Cycle i160二氧化碳培养箱购自美国Thermo

Fisher公司；ix75荧光显微镜购自日本Olympus公司等。

1.3 方法

1.3.1 细胞培养 NR8383细胞常规复苏后采用含20%FBS、1% P/S Ham′s F-12K培养基置于37 ℃、5%二氧化碳培养箱中培养，待细胞汇合至80%~90%时，用0.05%胰蛋白酶消化处理以1∶3比例进行传代。

1.3.2 双荧光素酶报告基因实验 构建与miR-124种子区结合的TRAF6基因3’UTR野生型（Pmir-GLO-TRAF6-Wt）和突变型（Pmir-GLO-TRAF6-Mut）双荧光报告载体。构建PmiR-GLO-TRAF6野生型和突变型重组载体后，采用脂质体转染法将miRNA（miR-124 mimic或NC）和重组载体（野生型或突变型）共转染入NR8383细胞中，于二氧化碳培养箱继续培养。每组设立5个复孔，48 h后用PLB裂解细胞，收集细胞裂解液，离心后取上清检测萤火虫荧光素信号和海肾荧光素信号，以海肾荧光素酶活性为对照，计算其相对活性。

1.3.3 ALI大鼠模型建立及分组 SD大鼠采用随机数字表法分为对照组（NC组）、LPS组、LPS+Cur组、LPS+Cur+inhibitor-NC组、LPS+Cur+inhibitor组，每组10只。LPS组腹腔注射LPS 10 mg/kg制备ALI大鼠模型[9]，LPS+Cur组在ALI模型制备30 min后尾静脉注射Cur 200 mg/kg，LPS+Cur+inhibitor-NC组、LPS+Cur+inhibitor组大鼠尾静脉分别注射miR-124 inhibitor-NC、miR-124 inhibitor 50 mg/kg 1周后腹腔注射LPS 10 mg/kg，30 min后尾静脉注射Cur 200 mg/kg，NC组注射等量生理盐水30 min后尾静脉注射等量生理盐水。24 h后腹腔麻醉处死大鼠，分别采集心脏血及肺组织标本待检。

1.3.4 HE染色观察肺组织病理学变化 取部分肺组织于4%多聚甲醛中固定，进行石蜡包埋，切片（4 μm），苏木精-伊红（HE）染色，于光镜下观察肺组织病理学变化。

1.3.5 ELISA检测血清TNF-α、IL-6水平 常规分离血清，采用酶联免疫吸附试验（enzyme-linked immunosorbent assay，ELISA）检测各组大鼠血清TNF-α、IL-6水平，具体步骤严格按照试剂盒说明书进行。

1.3.6 RT-qPCR检测肺组织miR-124、TRAF6 mRNA表达水平 采用RNA提取试剂盒提取肺组织总RNA，浓度及纯度检测合格后，反转录得到cDNA，置于-20 ℃保存备用。采用实时荧光定量PCR（real-time quantitative PCR，RT-qPCR）扩增miR-124、TRAF6 mRNA目的片段。RT-qPCR采用20 μL反应体系：TB Green Premix Ex Taq Ⅱ（2×）10 μL，ROX Reference Dye Ⅱ（50×）0.4 μL，cDNA（50 μg/L）2 μL，上下游引物（10 μmol/L）各0.8 μL，ddH_2O 6.0 μL。反应条件为：95 ℃ 30 s；95 ℃ 5 s，60 ℃ 34 s，40个循环。采用$2^{-\Delta\Delta Ct}$法对肺组织miR-124、TRAF6 mRNA相对表达水平进行定量分析。

1.3.7 蛋白质印迹（Western blotting）检测肺组织TRAF6蛋白表达 采用蛋白抽提试剂盒提取肺组织总蛋白，BCA试剂盒检测蛋白浓度，置于-80 ℃保存备用。取40 μg蛋白样品，进行6%SDS-PAGE电泳后PVDF膜转膜，室温封闭，添加一抗（anti-TRAF6，稀释比1∶5 000；anti-β-actin，稀释比1∶1 000）4℃孵育过夜，洗膜后，添加二抗IgG（稀释比1∶5 000）室温孵育1.5 h，洗膜，显色，曝片，拍照，观察并分析蛋白条带灰度值。

1.4 统计学方法

采用SPSS 25.0软件进行统计学分析。计量资料以$\bar{x} \pm s$表示，两组比较采用t检验，多组数据比较采用单因素方差分析，进一步任意两两比较采用SNK-q检验，以P<0.05为差异有统计学意义。

2 结果

2.1 miR-124可靶向TRAF6基因表达

通过TargetScan、miRanda、miRTarBase在线生物信息学软件预测TRAF6基因3′UTR区可能包含miR-124的互补序列（图1），提示TRAF6可能是miR-124的作用靶点。双荧光素酶报告实验结果显示，共转染miR-124 mimic和Pmir-GLO-TRAF6野生型重组质粒NR8383细胞的荧光素酶活性显著降低（P<0.05），而共转染miR-124 mimic和Pmir-GLO-TRAF6突变型重组质粒、转染miR-124 NC和Pmir-GLO-TRAF6野生及突变型重组质粒的NR8383细胞的荧光素酶活性差异无统计学意义（P>0.05）（图2），提示miR-124可通过靶向TRAF6 3′UTR区负调控其表达，二者存在直接靶向关系。

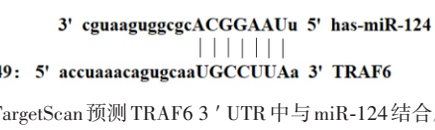


图1 TargetScan预测TRAF6 3′UTR中与miR-124结合序列

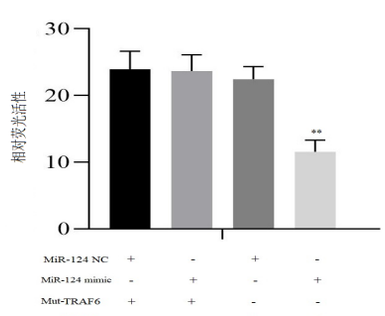


图2 NR8383细胞的相对荧光素酶活性

2.2 各组大鼠肺组织病理学变化 NC组大鼠肺组织结构清晰,肺泡结构完整,无明显异常改变;LPS组、LPS+Cur+inhibitor组大鼠肺组织结构破坏严重,肺间质增厚,大量炎性细胞浸润,弥漫性充血、渗出,肺泡萎缩;LPS+Cur组、LPS+Cur+inhibitor-NC组大鼠肺组织损伤减轻,见图3。

2.3 各组大鼠血清TNF-α、IL-6表达水平变化 与NC组比较,LPS组大鼠血清TNF-α、IL-6表达水平显著增加(P<0.05);与LPS组比较,LPS+Cur组大鼠血清TNF-α、IL-6表达水平显著降低(P<0.05);与LPS+ Cur+inhibitor-NC组比较,LPS+ Cur+inhibitor组大鼠血清TNF-α、IL-6表达水平显著增加(P<0.05),见表1。

表1 各组大鼠血清肿瘤坏死因子-α(TNF-α)、白细胞介素-6(IL-6)表达水平变化/(ng/L,$\bar{x}\pm s$)

组别	鼠数	TNF-α	IL-6
NC组	10	6.14±1.21	5.49±1.06
LPS组	10	45.28±4.85①	32.74±2.27①
LPS+Cur组	10	29.26±2.72①②	21.44±2.12①②
LPS+ Cur+inhibitor-NC组	10	29.87±2.59①②	21.65±2.18①②
LPS+ Cur+inhibitor组	10	34.59±3.02①②③	28.97±2.29①②③
F值		212.52	262.99
P值		<0.001	<0.001

注:①与NC组比较,P<0.05。②与LPS组比较,P<0.05。③与LPS+ Cur+inhibitor-NC组比较,P<0.05。

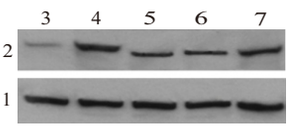
2.4 各组大鼠肺组织miR-124、TRAF6 mRNA相对表达水平比较 与NC组比较,LPS组大鼠肺组织miR-124表达水平显著降低、TRAF6 mRNA表达水平显著增加(P<0.05);与LPS组比较,LPS+Cur组大鼠肺组织miR-124表达水平显著增加、TRAF6 mRNA表达水平显著降低(P<0.05);与LPS+ Cur+inhibitor-NC组比较,LPS+ Cur+inhibitor组大鼠肺组织miR-124表达水平显著降低、TRAF6 mRNA表达水平显著增加(P<0.05),见表2。

2.5 各组大鼠肺组织TRAF6蛋白表达量比较 与NC组比较,LPS组大鼠肺组织TRAF6蛋白表达量显著增加(P<0.05);与LPS组比较,LPS+Cur组大鼠肺组织TRAF6蛋白表达量显著降低(P<0.05);与LPS+ Cur+inhibitor-NC组比较,LPS Cur+inhibitor组大鼠肺组织TRAF6蛋白表达量显著增加(P<0.05),见图4,表2。

表2 各组大鼠肺组织微小RNA-124(miR-124)与肿瘤坏死因子受体相关因子6(TRAF6)相对表达水平比较/$\bar{x}\pm s$

组别	鼠数	miR-124	TRAF6 mRNA	TRAF6蛋白
NC组	10	1.01±0.09	0.99±0.07	0.11±0.01
LPS组	10	0.37±0.04①	5.15±0.53①	1.07±0.10①
LPS+Cur组	10	0.74±0.06①②	3.51±0.32①②	0.38±0.04①②
LPS+ Cur+inhibitor-NC组	10	0.73±0.07①②	3.56±0.34①②	0.37±0.03①②
LPS+ Cur+inhibitor组	10	0.48±0.04①②③	4.67±0.41①②③	0.45±0.04①②③
F值		157.90	192.86	447.11
P值		<0.001	<0.001	<0.001

注:①与NC组比较,P<0.05。②与LPS组比较,P<0.05。③与LPS+ Cur+inhibitor-NC组比较,P<0.05。



注:1—β-actin;2—肿瘤坏死因子受体相关因子6(TRAF6);3—NC组;4—LPS组;5—LPS+Cur组;6—LPS+Cur+inhibitor-NC组;7—LPS+Cur+inhibitor组。

图4 蛋白质印迹法检测各组大鼠肺组织TRAF6蛋白表达

3 讨论

ALI是外伤、感染、脓毒症等一系列刺激因素引起的复杂呼吸系统疾病,发病机制复杂,主要以肺内炎症反应失控导致的严重低氧血症、弥漫性肺损伤、肺水肿等为主要病理特征,伴随一系列基因表达失调等,但尚未完全明确[10]。目前临床尚缺乏ALI特效疗法,因此积极探究新型治疗方案,并深入研究其作用机制具有重要意义。肺泡巨噬细胞介导的固有免疫在ALI发生发展中发挥重要作用,LPS是革兰阴性菌细胞壁主要成分,是内毒素和重要群特异性抗原,LPS感染机体刺激肺组织可活化肺泡巨噬细胞膜上Toll样受体(TLRs),继而通过激活NF-κB诱导炎症级联反应,导致ALI发生[11-12]。本研究显示,LPS组大鼠肺组织出现严重损伤,肺间质增厚,大量炎性细胞浸润,弥漫性充血、渗出,肺泡萎缩,且血清促炎因子TNF-α、IL-6表达水平显著增加,提示LPS诱导ALI模型成功。而Cur可减轻ALI大鼠肺组织病理变化及TNF-α、IL-6炎性因子分泌。Cur是一种具有抗炎、抗氧化、抗血脂、抗肿瘤等多种功能的膳食多酚,研究表明,Cur可通过上调线粒体融合蛋白2减轻脓毒症小鼠ALI[13],还可通过抑制NF-κB信号通路减轻流感病毒感染引起的巨噬细胞活化和肺部炎症[14],且对LPS诱导的ALI有显著治疗作用[15],提示Cur可减轻LPS诱导的大鼠ALI,减轻肺组织炎症反应。

MiRNAs在进化过程中高度保守,主要通过识别靶基因3′UTR区抑制蛋白翻译或促进靶基因降解,在炎症调控反应中发挥重要作用。研究显示,MiR-124在多种细胞中特异表达,是一种影响炎症通路的重要miRNA,与多种炎症性疾病密切相关[16-17],miR-124靶基因众多,本研究通过在线软件

预测及文献检索发现，miR-124可靶向抑制TRAF6表达发挥抗狼疮性肾炎[18]、ALI肺泡巨噬细胞炎症[19]等作用，本研究经双荧光素酶报告基因实验结果显示，与其他对照组比较，共转染miR-124 mimic和Pmir-GLO-TRAF6野生型重组质粒NR8383细胞的荧光素酶活性显著降低，提示TRAF6是miR-124的直接作用靶点。TRAF6是TLR4信号通路中关键蛋白，TRAF6激活可进而引起TNF-α、IL-1β、IL-6等促炎因子免疫应答，引发炎症级联反应，与肺损伤成正相关[20]。本研究结果显示，与NC组比较，LPS组大鼠肺组织miR-124表达水平显著降低，TRAF6 mRNA及蛋白表达水平显著增加，而Cur处理可显著增加miR-124表达，抑制TRAF6 mRNA及蛋白表达，提示Cur可能通过促进miR-124表达抑制TRAF6表达，减轻LPS诱导的ALI大鼠肺组织炎症反应，发挥治疗作用。为进一步验证上述结果，本研究在LPS+Cur基础上转染miR-124 inhibitor，结果显示，与转染miR-124 inhibitor-NC对照比较，转染miR-124 inhibitor可显著降低Cur治疗效果，进一步验证Cur可通过miR-124/TRAF6轴发挥作用，但可能不是唯一途径，具体相关通路有待进一步深入研究。

综上所述，Cur可通过上调miR-124靶向抑制TRAF6减轻LPS诱导的大鼠ALI及炎症反应，发挥治疗作用。但是否存在其他信号通路参与，及具体作用机制有待进一步深入探究。

（本文图3见插图8-2）

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（收稿日期:2020-07-16,修回日期:2020-09-05）