•研究论文•

金雀异黄素调控miR-21表达促进LPS活化的RAW264.7细胞凋亡

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摘要: 本文旨在研究金雀异黄素 (genistein, GEN) 对脂多糖 (lipopolysaccharide, LPS) 活化的RAW264.7细胞凋亡的影响, 探讨GEN抗动脉粥样硬化的药理学作用机制。应用LPS活化RAW264.7细胞, qRT-PCR检测肿瘤坏死因子-α (tumor necrosis factor-α, TNF-α) 和白介素-6 (interleukin 6, IL-6) mRNA表达, Western blot检测环氧合酶-2 (cyclooxygenase-2, COX-2) 和诱导型一氧化氮合酶 (inducible nitric oxide synthases, iNOS) 蛋白表达。使用GEN预处理细胞2 h, 再与LPS共孵育24 h后, CCK8检测细胞活力, Annexin V-FITC/PI法检测细胞凋亡, qRT-PCR检测CHOP、caspase-3和miR-21基因表达, Western blot检测CHOP和caspase-3蛋白表达。结果显示, 1 000 ng·mL^{-1} LPS上调RAW264.7细胞TNF-α、IL-6、COX-2和iNOS基因或蛋白表达; GEN呈浓度依赖性下调LPS活化的RAW264.7细胞活力, 增加凋亡率, 上调CHOP和caspase-3表达, 并下调miR-21表达; 慢病毒介导的miR-21 up抑制LPS活化的RAW264.7细胞CHOP和caspase-3表达, 与GEN作用相反; 慢病毒介导的miR-21 down促进LPS活化的RAW264.7细胞CHOP和caspase-3表达, 与GEN具有协同作用。这些结果提示, 金雀异黄素能够促进LPS活化的RAW264.7细胞凋亡, 其作用机制可能与下调miR-21表达, 激活内质网应激反应性凋亡途径有关。

关键词: 金雀异黄素; RAW264.7细胞; 脂多糖; miR-21; 凋亡

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Genistein regulates miR-21 to promote the apoptosis in LPS-activated RAW264.7 cells

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Abstract: The research is aimed to investigate the effect of genistein (GEN) on the apoptosis in lipopolysaccharide (LPS)-activated RAW264.7 cells and explore the pharmacological mechanism of GEN anti-atherosclerosis (AS). RAW264.7 cells were activated by LPS, the level of TNF-α and IL-6 mRNA were detected by qRT-PCR, the expression of COX-2 and iNOS were detected by Western blot. RAW264.7 cells were pretreated with GEN for 2 h, and then incubated with LPS for 24 h. After that, CCK8 kit was used for the cell viability, Annexin V-FITC/PI kit for the apoptosis of cell. qRT-PCR was used to detect the level of CHOP, caspase-3 and miR-21. Western blot was used to detect the expression of CHOP and caspase-3. Results showed that LPS (1 000 ng · mL^{-1}) increased the expression of TNF-α, IL-6, COX-2 and iNOS in RAW264.7 cells compared with that in control group. GEN inhibited the cell activity and the level of miR-21, promoted the expression of CHOP and caspase-3 in LPS-activated RAW264.7 cells in a dose-dependent manner. miR-21 up inhibited the expression of CHOP and caspase-3 in LPS-activated RAW264.7 cells and this process was reversed by GEN treatment. miR-21 down promoted the expression of CHOP and caspase-3, which were further enhanced by GEN. These results indicate that GEN

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promotes the apoptosis of RAW264.7 cells activated by LPS through down regulating miR-21 and activating endoplasmic reticulum (ER) stress pathway.

Key words: genistein; RAW264.7 cell; lipopolysaccharide; miR-21; apoptosis

动脉粥样硬化 (atherosclerosis, AS) 是一种以动脉壁脂质和炎症细胞蓄积为特征的、复杂的血管炎症性疾病[1,2]。巨噬细胞在AS发生发展中扮演重要角色, 参与慢性炎症过程, 促进活化巨噬细胞凋亡, 从而有效抑制AS的早期发展[3]。由此可见, 药物干预活化巨噬细胞凋亡对防护AS具有积极的临床意义。金雀异黄素 (genistein, GEN) 又称为染料木素或染料木黄酮, 是异黄酮类化合物中活性最强的一种, 大豆是其最丰富的来源。WHO-CARDIAC等临床研究显示, GEN可以抑制AS进程, 对心脑血管调节功能有益, 具有开发成心血管保护药物的性质[4], 但其作用机制尚未完全明确。鉴于此, 本课题组前期一直围绕GEN及其衍生物抗AS的药理学作用机制进行有关研究, 通过体内 (新西兰兔、*Apoe*$^{-/-}$小鼠)、体外实验证实GEN可以明显抑制AS[5-7]。本研究以小鼠巨噬细胞RAW264.7细胞为研究对象, 以脂多糖 (lipopolysaccharides, LPS) 为刺激因素, 探究GEN对活化巨噬细胞内质网应激凋亡相关靶点的影响及作用机制, 为GEN的临床应用提供实验依据。

材料与方法

试剂和抗体 GEN, 纯度≥98%, Sigma公司; LPS, 纯度≥99%, Solarbio公司; 胎牛血清、DMEM培养基、青霉素/链霉素、磷酸盐缓冲液 (phosphate buffered saline, PBS), BI公司; CCK8试剂盒、Annexin V-FITC/PI细胞凋亡检测试剂盒、Trizol、HiScript Ⅱ Q RT SuperMix for qPCR (+ gDNA wiper)、miRNA 1st Strand cDNA Synthesis Kit (by stem-loop)、AceQ qPCR SYBR Green Master Mix、GAPDH抗体, Vazyme公司; RIPA细胞裂解液、BCA蛋白浓度测定试剂盒, Beyotime公司; 环氧合酶-2 (cyclooxygenase-2, COX-2) 抗体和诱导型一氧化氮合酶 (inducible nitric oxide synthases, iNOS) 抗体, Affinity公司; CHOP抗体, Cell Signaling Technology公司; caspase-3抗体, ImmunoWay公司; β-actin抗体, CMCTAG公司; 鼠IgG和兔IgG, ComWin Biotech公司; 慢病毒颗粒, Genechem公司。

细胞 RAW264.7细胞购于中国科学院细胞库, 常规培养于含10%胎牛血清和100 U·mL^{-1}青霉素/链霉素的DMEM完全培养基, 置于37 ℃、5% CO_2细胞培养箱中培养, 所有实验均在细胞处于对数生长期时进行。将RAW264.7细胞接种于12孔板中 (每毫升5×10^{4}个), 培养24 h, 分别按照慢病毒感染说明书加入miR-21 up-NC (阴性对照)、miR-21 up、miR-21 down-NC (阴性对照)、miR-21 down的慢病毒颗粒。

qRT-PCR 使用Trizol法提取总RNA, 分光光度计检测RNA纯度和浓度。根据mRNA和miRNA逆转录试剂说明书进行逆转录, 采用SYBR Green荧光染料法进行相对定量检测。基因表达水平以$2^{-\triangle\triangle CT}$法进行分析。引物序列见表1。

Table 1 Primer sequence. TNF-α: Tumor necrosis factor-α; IL-6: Interleukin 6

Gene		Primer sequence (5'-3')
TNF-α	Forward	CAAGGGACAAGGCTGCCCCG
	Reverse	GCAGGGGCTCTTGACGGCAG
IL-6	Forward	AACGATGATGCACTTGCAGA
	Reverse	CTCTGAAGGACTCTGGCTTTG
CHOP	Forward	AATAACAGCCGGAACCTGAGGA
	Reverse	ACTCAGCTGCCATGACTGCAC
Caspase-3	Forward	GAGCCAGAGCAGAGACTTGG
	Reverse	CATCATCCACACAAACCAGAA
GAPDH	Forward	TGATGACATCAAGAAGGTGGTGAA
	Reverse	TGGGATG-GAAATTGTGAGG GAGAT
miR-21	Forward	GTGCAGGGTCCGAGGT
	Reverse	GCCGCTAGCTTATAAGACTGATGT
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT
miR-21	RT primer	GTCGTATCCAGTGCAGGGTCCGAGGT ATTCGCACTGGATACGACTCAACA
U6	RT primer	AAAATATGGAACGCTTCACGAATTTG

Western blot 使用蛋白裂解液裂解细胞并离心 (12 000 ×*g*, 4 ℃) 提取蛋白, 应用BCA蛋白定量试剂盒测定蛋白浓度后将其变性。制备12% SDS-PAGE凝胶, 取40 μg样品, 电泳后转膜, 将蛋白转移至PVDF膜 (polyvinylidene difluoride membranes, PVDF) 上, 用5%脱脂牛奶-TBST (TBS包含0.05% Tween-20) 室温封闭1 h, TBST洗涤3次。然后加入一抗4 ℃孵育过夜, 一抗稀释倍数按照说明书进行 [iNOS (1∶2 000)、COX-2 (1∶2 000)、CHOP (1∶1 000)、caspase-3 (1∶1 000)、GAPDH (1∶10 000) 和β-actin (1∶10 000)], GAPDH和β-actin为内参, TBST洗涤3次。之后加入二抗, 室温孵育1 h, TBST洗涤3次, 使用ECL发光剂和化学发光成像系统显影。

CCK8检测细胞活力 使用DMEM完全培养基轻柔吹打RAW264.7细胞, 制备每毫升5×10^{4}个单细胞

悬液, 将其接种于96孔板, 每孔100 μL。置于37 ℃、5% CO_2细胞培养箱中培养24 h后, 加不同浓度GEN (10、20、40 μmol·L^{-1}) 预处理2 h, 再与1 000 ng·mL^{-1} LPS共孵育24 h。然后小心吸弃上清, PBS洗涤2次, 加入90 μL新鲜完全培养基和10 μL CCK8溶液, 轻轻摇晃培养板使试剂充分混匀, 继续放入培养箱内孵育2 h。使用多功能酶标仪 (BioTek Instruments) 检测各孔在450 nm处的吸光度, 并设置空白孔、对照孔, 每组3个复孔。细胞活力%= (处理孔−空白孔)/ (对照孔−空白孔)×100%。

AnnexinV-FITC/PI双染法检测细胞凋亡 制备每毫升5×10^{4}个RAW264.7单细胞悬液, 将其接种于12孔板, 每孔1 mL。在37 ℃、5% CO_2细胞培养箱中培养24 h后, 加不同浓度GEN (10、20、40 μmol·L^{-1}) 预处理2 h, 再与1 000 ng·mL^{-1} LPS共孵育24 h。使用无EDTA的胰蛋白酶消化细胞, 并收集于离心管中离心 (800 r·min^{-1}, 7 min), PBS洗涤2次; 用200 μL结合缓冲液重悬细胞至浓度为每毫升4×10^{5}个, 取195 μL细胞 (约8×10^{4}个细胞) 悬液加入5 μL AnnexinV-FITC, 轻轻混匀后室温避光孵育10 min; 用200 μL结合缓冲液洗涤细胞 (800 r·min^{-1}, 7 min); 再用190 μL结合缓冲液重悬细胞, 加入5 μL碘化丙啶溶液, 轻轻混匀后上流式细胞仪检测各组细胞凋亡率。

统计学分析 数据均采用均数±标准差表示, 采用SPSS20.0统计软件和GraphPad Prism 5进行*t*检验或单因素方差分析。*P*<0.05表示差异具有统计学意义。

结果

1 GEN对LPS活化的RAW264.7细胞活力与凋亡的影响

1.1 构建LPS活化的RAW264.7细胞模型 LPS (1 000 ng·mL^{-1}, 24 h) 组细胞肿瘤坏死因子 (tumor necrosis factor-*α*, TNF-*α*)、白细胞介素-6 (interleukin 6, IL-6) mRNA表达及COX-2、iNOS蛋白表达均高于对照组 (*P*<0.05), 见图1。由此, 本研究选取1 000 ng·mL^{-1} LPS孵育24 h作为活化RAW264.7细胞的最佳浓度和时间。

1.2 GEN对LPS活化的RAW264.7细胞活力与凋亡的影响 分别使用10、20、40 μmol·L^{-1} GEN预处理细胞2 h, 再与LPS共孵育24 h。与对照组相比, LPS明显增加细胞活力 (*P*<0.05); GEN则呈浓度依赖性下调LPS活化的RAW264.7细胞活力, 并促进凋亡, 其中LPS组凋亡率为5.3%, GEN组 (10、20、40 μmol·L^{-1}) 凋亡率依次为5.8%、14%、36.1% (图2A、B)。此外, LPS

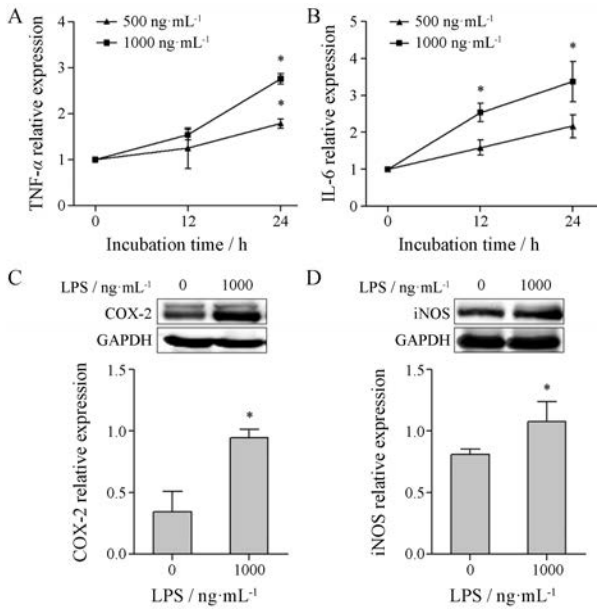

Figure 1 The effect of lipopolysaccharide (LPS) on RAW264.7 cells. RAW264.7 cells were activated by LPS (500 or 1 000 ng·mL^{-1}) for 12 h or 24 h. qRT-PCR detected the level of TNF-*α* (A) and IL-6 (B) mRNA. Western blot detected the protein expression of COX-2 (C) and iNOS (D). n = 3, $\bar{x} \pm s$. $^*P$<0.05 *vs* control group. COX: Cyclooxygenase-2; iNOS: Inducible nitric oxide synthases

下调CHOP、caspase-3 mRNA和蛋白表达, 而GEN则上调其表达 (*P*<0.05, 图2C～E)。由此, 本研究选择10 μmol·L^{-1} GEN进行后续实验。

2 GEN对LPS活化的RAW264.7细胞miR-21表达的影响

与对照组相比, LPS上调RAW264.7细胞miR-21表达; 与LPS组相比, GEN下调LPS活化的RAW264.7细胞miR-21表达 (*P*<0.05, 图3)。

3 慢病毒介导的miR-21对LPS活化的RAW264.7细胞凋亡的影响

3.1 慢病毒介导的miR-21 up对LPS活化的RAW264.7细胞凋亡的影响 将miR-21 up-NC和miR-21 up慢病毒颗粒分别感染至RAW264.7细胞, 培养72 h后, 荧光显微镜观察GFP表达的细胞比例达85%以上; 与miR-21 up-NC组相比, miR-21 up组miR-21表达上调约2.5倍, 可用于后续实验 (图4A、B)。与miR-21 up-NC组相比, miR-21 up抑制LPS活化的RAW264.7细胞CHOP、caspase-3 mRNA (*P*<0.05) 和蛋白表达 (图4C～E)。

3.2 慢病毒介导的miR-21 down对LPS活化的RAW264.7细胞凋亡的影响 将miR-21 down-NC和miR-21 down慢病毒颗粒分别感染至RAW264.7细胞, 培养72 h后, 荧光显微镜观察GFP表达的细胞比例达

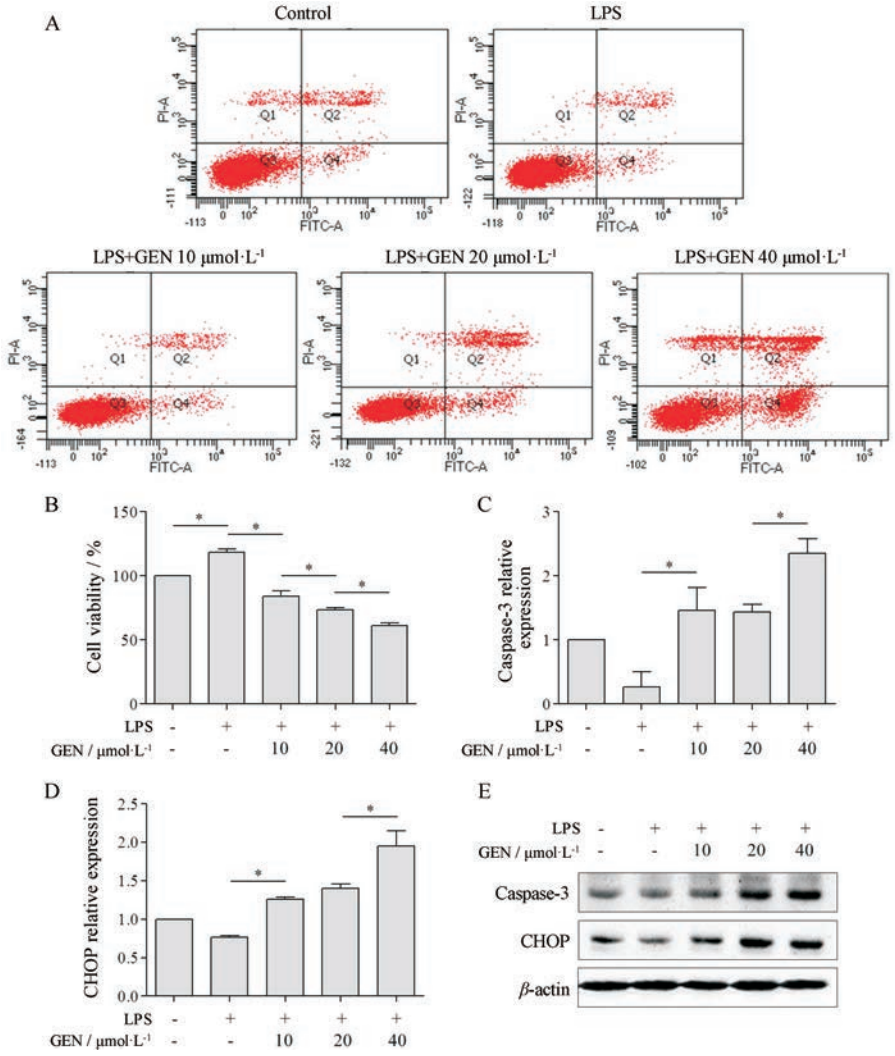

Figure 2 The effect of genistein (GEN) on the cell viability and apoptosis of LPS-activated RAW264.7 cells. RAW264.7 cells were pretreated with GEN (10、20、40 μmol·L^{-1}) for 2 h, then incubated with LPS (1 000 ng·mL^{-1}) for 24 h. Annexin V-FITC/PI kits and CCK8 kits detected cell apoptosis rate (A) and viability (B), respectively. qRT-PCR detected the level of caspase-3 (C) and CHOP (D) mRNA. Western blot detected the protein expression of caspase-3 and CHOP (E). n = 3, $\bar{x} \pm s$. $^{*}P$<0.05

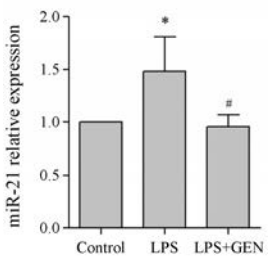

Figure 3 The effect of GEN on the level of miR-21 in LPS-activated RAW264.7 cells. RAW264.7 cells were pretreated with GEN (10 μmol·L^{-1}) for 2 h, then incubated with LPS (1 000 ng·mL^{-1}) for 24 h. qRT-PCR was used for the level of miR-21. n = 3, $\bar{x} \pm s$. $^{*}P$<0.05 *vs* control group; $^{\#}P$<0.05 *vs* LPS group

85% 以上; 与 miR-21 down-NC 组相比, miR-21 down 组 miR-21 表达明显下调约 70% (P<0.05), 可用于后续实验 (图 5A、B)。与 miR-21 down-NC 组相比, miR-21 down 促进 LPS 活化的 RAW264.7 细胞 CHOP、caspase-3 mRNA (P<0.05) 和蛋白表达 (图 5C～E)。

4 慢病毒介导的 miR-21 对 GEN 促进 LPS 活化的 RAW264.7 细胞凋亡的影响

4.1 慢病毒介导的 miR-21 up 对 GEN 促进 LPS 活化的 RAW264.7 细胞凋亡的影响 与 miR-21 up-NC+LPS+GEN 组相比, miR-21 up+LPS+GEN 组 RAW264.7 细胞 CHOP、caspase-3 mRNA (P<0.05) 和蛋白表达均下调 (图 6)。

4.2 慢病毒介导的 miR-21 down 对 GEN 促进 LPS 活化的 RAW264.7 细胞凋亡的影响 与 miR-21 down-NC+LPS+GEN 组相比, miR-21 down+LPS+GEN 组 RAW264.7 细胞 CHOP、caspase-3 mRNA (P<0.05) 和蛋

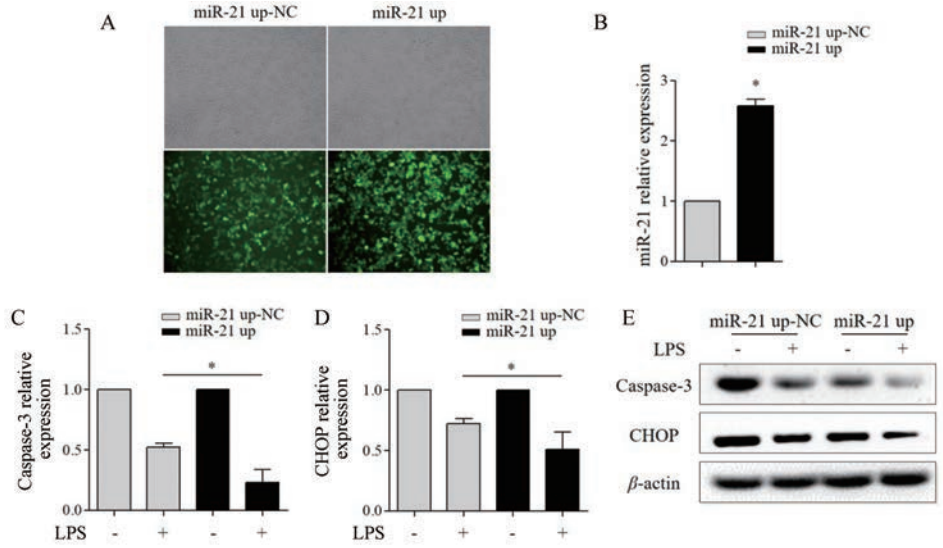

Figure 4 The effect of miR-21 up on the apoptosis of LPS (1 000 ng·mL^{-1})-activated RAW264.7 cells. A: RAW264.7 cells infected with miR-21 up-NC (multiplicity of infection, MOI = 50) and miR-21 up (MOI = 50) lentivirus with GFP were captured under phase contrast and fluorescence microscopy (×200); B: qRT-PCR detected the level of miR-21; qRT-PCR detected the level of caspase-3 (C) and CHOP (D) mRNA; E: Western blot detected the protein expression of caspase-3 and CHOP. n = 3, $\bar{x} \pm s$. $^*P$<0.05

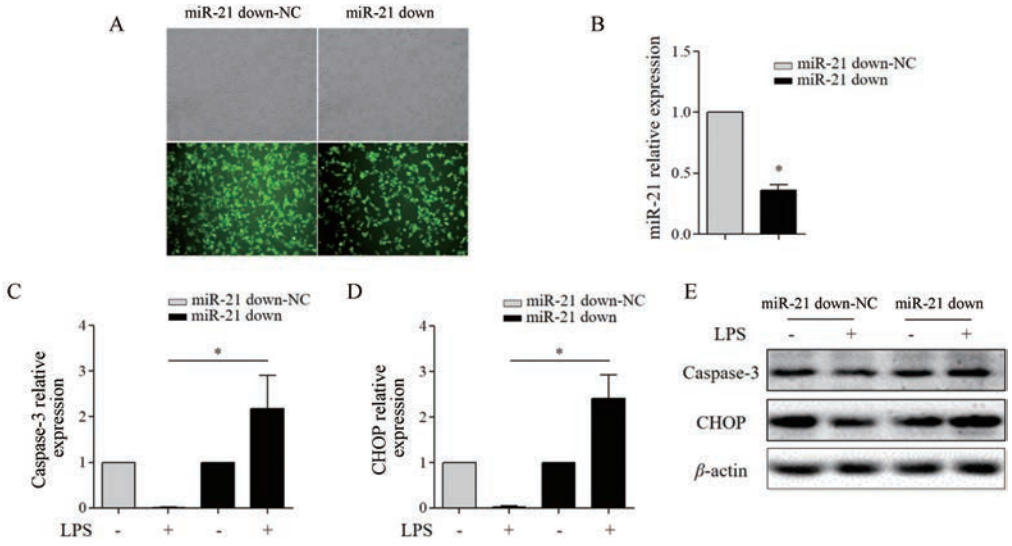

Figure 5 The effect of miR-21 down on the apoptosis of LPS (1 000 ng·mL^{-1})-activated RAW264.7 cells. A: RAW264.7 cells infected with miR-21 down-NC (MOI = 50) and miR-21 down (MOI = 50) lentivirus with GFP were captured under phase contrast and fluorescence microscopy (×200); B: qRT-PCR detected the level of miR-21; qRT-PCR detected the level of caspase-3 (C) and CHOP (D) mRNA; E: Western blot detected the protein expression of caspase-3 and CHOP. n = 3, $\bar{x} \pm s$. $^*P$<0.05

白表达均上调 (图7)。

讨论

血管慢性炎症是促进AS发展的因素之一, 巨噬细胞作为主要的免疫细胞, 全程参与此过程[8]。LPS是革兰阴性细菌细胞壁的主要成分之一, 能够活化巨噬细胞, 引发损伤性炎症反应, 激活先天性免疫[9]。本研究结果显示, LPS能够刺激小鼠巨噬细胞RAW264.7产生大量炎症细胞因子和诱导型酶, 如TNF-α、IL-6、COX-2和iNOS, 同时也能够促进巨噬细胞增殖, 抑制凋亡相关蛋白CHOP和caspase-3的表达, 与Cheng和De Santis等[10,11]的研究结果一致。以上结果表明, LPS可能通过抑制巨噬细胞凋亡, 促进慢性炎症反应。另有文献[12-14]报道, 促进活化巨噬细胞凋亡不仅可以减少炎症细胞因子, 如IL-6、单核细胞趋化蛋白-1 (monocyte chemotactic protein-1, MCP-1) 等的释放, 避免炎症反

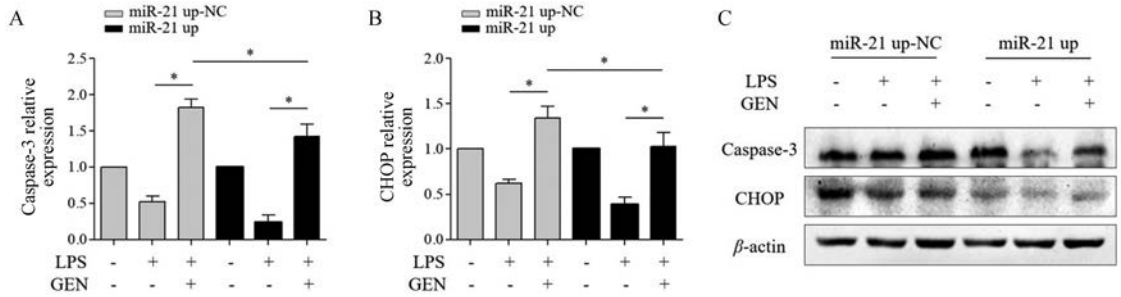

Figure 6 The effect of GEN (10 μmol·L^{-1}) on the apoptosis of LPS (1 000 ng·mL^{-1})-activated RAW264.7 cells infected with miR-21 up (MOI = 50). qRT-PCR detected the level of caspase-3 (A) and CHOP (B) mRNA; C: Western blot detected the protein expression of caspase-3 and CHOP. n = 3, $\bar{x} \pm s$. *P<0.05

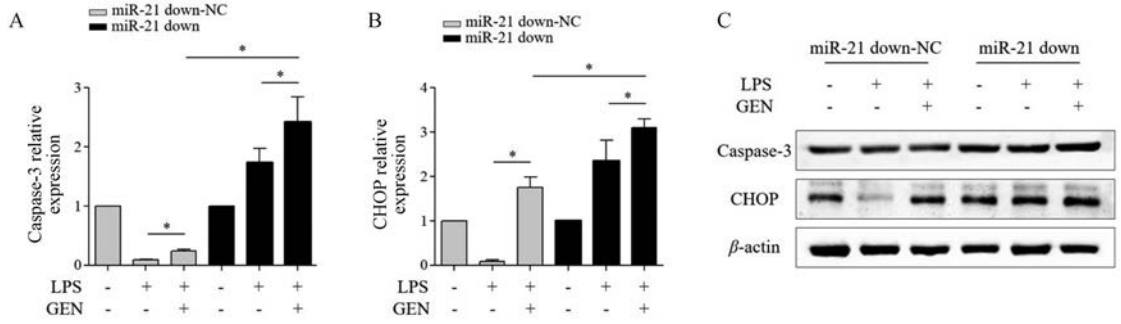

Figure 7 The effect of GEN (10 μmol·L^{-1}) on the apoptosis of LPS (1 000 ng·mL^{-1})-activated RAW264.7 cells infected with miR-21 down (MOI = 50). qRT-PCR detected the level of caspase-3 (A) and CHOP (B) mRNA; C: Western blot detected the protein expression of caspase-3 and CHOP. n = 3, $\bar{x} \pm s$. *P<0.05

应级联放大效应, 还可以减少巨噬细胞源性泡沫细胞的形成, 抑制AS早期斑块发生, 有效改善AS。

金雀异黄素富含于豆制食品中, 既能通过激活雌激素受体发挥抗氧化作用, 又能抑制酪氨酸激酶家族发挥抗细胞增殖作用, 有效抵抗低密度脂蛋白氧化和血管炎症, 发挥抗AS的作用[15]。CHOP是抗凋亡向促凋亡转换的重要信号分子, 正常情况下主要存在于细胞浆中且表达量很低, 但在应激状态下其表达明显增加, 进而导致细胞周期停滞, 细胞最终死亡[16]。Caspase是细胞凋亡的核心成分, 位于执行性凋亡蛋白的最下游, 在细胞凋亡过程中必不可少[17]。本研究结果显示, GEN能够抑制LPS活化的巨噬细胞活力, 上调CHOP和caspase-3表达, 诱导活化巨噬细胞凋亡, 同时下调miR-21表达。这些结果提示, GEN可能下调miR-21表达后, 通过内质网应激途径促进LPS活化的巨噬细胞凋亡, 发挥抗炎作用。

miR-21高表达于心血管疾病, 在心血管系统中的作用非常复杂, 其通过作用于同源性磷酸酶−张力蛋白 (phosphatase and tensin homolog, PTEN) 基因, 抑制心肌干细胞凋亡[18], 也可以通过丝裂原活化蛋白激酶激酶3抑制巨噬细胞凋亡[1]。病理状态下, miR-21作为巨噬细胞中表达量最丰富的miRNAs之一, 参与细胞增殖、迁移、凋亡及炎症[19,20]。本研究结果显示, LPS能够上调RAW264.7细胞miR-21表达, 与既往研究一致[21]。Canfránduque等[1]研究显示, 敲除miR-21能够有效促进巨噬细胞凋亡。本课题组使用慢病毒介导的miR-21构建稳定表达miR-21 up和miR-21 down的RAW264.7细胞株发现, 当miR-21表达上调时, 明显抑制LPS活化的巨噬细胞促凋亡蛋白的表达, 而miR-21 down结果与其相反, 这表明miR-21能够通过内质网应激途径抑制活化巨噬细胞的凋亡。此外, 还发现GEN与miR-21 down具有协同作用, 能够促进LPS活化的巨噬细胞凋亡。

综上推断, GEN可能下调miR-21表达, 通过激活内质网应激反应性途径, 促进LPS活化的巨噬细胞凋亡, 进而发挥抗AS的作用。但线粒体凋亡途径和死亡配体介导的外源性凋亡途径是否参与其中, 尚未得知。此外, Canfránduque和Feng等[1,21]研究显示, miR-21可以调节巨噬细胞内脂质蓄积及细胞膜上CD36的表达, 但并无文献报道GEN对miR-21这些作用的干预研究。在后续实验中, 课题组将继续研究GEN通过调控miR-21对巨噬细胞凋亡、泡沫化及炎症反应的影

响, 以进一步阐明GEN抗AS的作用机制, 为临床药物研究提供实验依据。

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