PPAR-γ调控的自噬通路在染料木黄酮抑制肝星状细胞激活过程中的作用

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摘要:目的 探讨染料木黄酮在体外抑制肝星状细胞(HSC)活化的效应以及PPAR-γ调控的自噬通路在其中发挥的作用。**方法** 将体外培养的HSC细胞分别给予不同浓度的染料木黄酮作用48 h后,用Western blot法测定HSC激活标志蛋白α-SMA、α1(I) collagen的表达以及自噬相关蛋白LC3 Ⅱ\p62和PPAR-γ的表达;利用自噬抑制剂和PPAR-γ抑制剂验证自噬在染料木黄酮抑制HSC激活过程中的作用以及PPAR-γ对自噬的调控作用。**结果** 染料木黄酮作用于HSC细胞后,HSC活化标志蛋白α-SMA、α1(I)collagen的水平明显降低(P<0.05),而自噬相关蛋白LC3 Ⅱ的表达明显升高、泛素结合蛋白p62水平明显降低,同时PPAR-γ表达明显增加(P<0.05);与单纯染料木黄酮刺激组相比,用3-MA处理后HSC活化标志蛋白α-SMA、α1(I)collagen的表达明显升高;并且加入PPAR-γ抑制剂后,自噬相关蛋白LC3 Ⅱ的表达明显降低、泛素结合蛋白p62水平明显增加(P<0.05)。**结论** PPAR-γ调控的自噬通路在染料木黄酮抑制HSC细胞激活的过程中发挥着重要作用。

关键词:染料木黄酮;肝星状细胞;自噬;PPAR-γ

Role of PPAR-γ-regulated autophagy in genistein-induced inhibition of hepatic stellate cell activation

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Abstract: Objective To investigate the inhibitory effect of genistein on activation of hepatic stellate cells (HSCs) *in vitro* and the role of the autophagy pathway regulated by PPAR-γ in mediating this effect. **Methods** Cultured HSC-T6 cells were exposed to different concentrations of genistein for 48 h, and HSC activation was verified by detecting the expressions of α-SMA and α1(I) collagen; autophagy activation in the cells was determined by detecting the expressions of LC3-II and p62 using Western blotting. The autophagy inhibitor 3-MA was used to confirm the role of autophagy in genistein-induced inhibition of HSC activation. A PPAR-γ inhibitor was used to explore the role of PPAR-γ in activating autophagy in the HSCs. **Results** Genistein at concentrations of 5 and 50 μmol/L significantly inhibited the expressions of α-SMA and α1(I) collagen (P<0.05), markedly up-regulated the expressions of PPAR-γ and the autophagy-related protein LC3-II (P<0.05) and significantly down-regulated the expression of the ubiqutin-binding protein p62 (P<0.05) in HSC-T6 cells. The cells pretreated with 3-MA prior to genistein treatment showed significantly increased protein expressions of α-SMA and α1(I) collagen compared with the cells treated with genistein only (P<0.05). Treatment with the PPAR-γ inhibitor obviously lowered the expression of LC3-II and enhanced the expression p62 in genistein-treated HSC-T6 cells, suggesting the activation of the autophagy pathway. **Conclusion** PPAR-γ-regulated autophagy plays an important role in mediating genistein-induced inhibition of HSC activation *in vitro*.

Keywords: genistein; hepatic stellate cells; autophagy; PPAR-γ

肝纤维化是多种慢性肝病发展为肝硬化的中间过程,早期有效的抗纤维化治疗能控制疾病发展,降低病死率。目前已有一些药物用于肝纤维化的治疗[1-2],但各种药物的疗效有限并具有不同程度的不良反应。因此,寻找安全有效的天然化合物是治疗肝纤维化的重点。

染料木黄酮是一种功能活性很强的植物化学物,其具有广泛的药理活性[3],其中就包括抗炎、抗氧化和抑制纤维化的作用[4-7]。由于其天然无毒的特性,染料木黄酮是一种具有广泛应用前景的抗纤维化药物。已有研究证实染料木黄酮可以明显减轻肝脏的纤维化程度[8-9]。并且在细胞水平发现染料木黄酮可以抑制HSC细胞激活[10-11]。但是,染料木黄酮抑制HSC细胞活化的分子机制尚不清楚。因此,本研究主要通过PPAR-γ调控的细胞自噬探索染料木黄酮对HSC细胞的影响,为其临床应用提供实验依据。

1 材料和方法

1.1 材料

HSC-T6细胞由Dr. Scott Friedman (Mt. Sinai School of Medicine, New York, NY)惠赠,表型为活化的HSC,具有纤维化特性。染料木黄酮(Sigma);

收稿日期:2019-02-12
基金项目:上海市卫生和计划生育委员会科研课题青年项目(20174Y0201)
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DMEM(Gibco)；胎牛血清(杭州四季青公司)；TRIzol(Invitrogen)；血小板衍生生长因子-BB(PDGF-BB)、PPAR-γ inhibitor T0070907(Selleck Chemicals)；3-MA(Sigma)；α1(I)collagen、α-SMA一抗、p62一抗、LC3一抗、PPAR-γ一抗(Cell Signaling Technology)；CO_2培养箱(Thermo)；高速冷冻离心机(Thermo)；倒置相差显微镜(Zeiss)；凝胶成像分析系统、低压电泳仪(Bio-Rad)。

1.2 方法

1.2.1 细胞培养　将冷冻保存于超低温冰箱中的HSC-T6复苏后接种于含100 mL/L胎牛血清的高糖DMEM培养液中，37 ℃、50 mL/L CO_2条件下培养。当细胞呈单层致密状时，采用2.5 g/L胰蛋白酶消化后传代。每次试验均在呈指数生长的细胞中进行。

1.2.2 蛋白质免疫印记法分析蛋白表达　HSC-T6细胞分别加入0、0.5、5、50 μmol/L的染料木黄酮，培养48 h后，提取细胞总蛋白，做SDS-PAGE电泳，将蛋白转移至硝酸纤维滤膜上。膜用体积分数为5%的脱脂奶粉封闭1 h，加入各分子的抗体，4 ℃过夜。TBST漂洗3次，加入过氧化酶标记山羊抗兔IgG(1∶5000)，置于水平脱色摇床上孵育2 h，TBS漂洗3次，ECL光化学法显色。凝胶成像分析系统分析扫描，测定各条带积分光密度值，计算目的条带与内参照的光密度比值。

1.2.3 统计学方法　采用SPSS20.0软件进行统计学分析，单因素方差分析，各组数据以均数±标准差表示，以P<0.05为差异有统计学意义。

2 结果

2.1 染料木黄酮在体外抑制PDGF-BB诱导的HSC活化

HSC活化标志物，α平滑肌肌动蛋白(α-SMA)，α1Ⅰ型胶原蛋白[α1(I) collagen]的表达均显着上调(图1A、B)。Western印迹分析显示用染料木黄酮处理后，显著抑制了HSC活化标记物α-SMA，α1(I)collagen的表达。

2.2 染料木黄酮对PPAR-γ调控的自噬通路的影响

染料木黄酮处理以浓度依赖性地促进了HSCs自噬标记LC3Ⅱ蛋白的表达，还以浓度依赖的方式显著降低了泛素结合蛋白p62的表达(图2A、B)。同时，PPAR-γ蛋白的表达水平随着染料木黄酮刺激浓度的增大而明显增加(图2C、D)。

2.3 在体外抑制自噬对染料木黄酮拮抗HSC细胞活化作用的影响

蛋白质免疫印迹分析表明，与对照组相比，Genistein处理显着增加LC3Ⅱ蛋白的表达，但自噬特异性抑制剂3-MA处理后削弱了Genistein对自噬的激活强度(图3A、B)。用染料木黄酮处理显著降低了HSC活化标记蛋白α-SMA和α1(I)collagen的表达，而3-MA处理后显著损害了染料木黄酮抑制HSC活化的能力(图3C、D)。

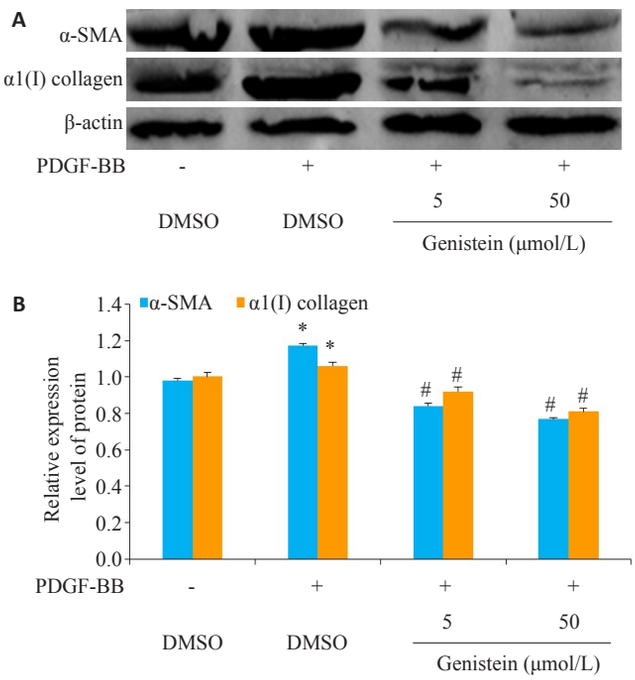

图1 染料木黄酮对HSC细胞增殖的影响

Fig.1 Effect of genistein on the proliferation of cultured hepatic stellate cells. **A**: Expression of α-SMA and α1(I) collagen in HSC cells detected by Western blotting; **B**: Analysis of relative expression of the proteins. *P<0.05 *vs* control group, #P<0.05 *vs* PDGF-BB group.

2.4 在体外抑制PPAR-γ活性后对染料木黄酮激活HSC细胞自噬的影响

蛋白质免疫印迹分析表明，与对照组相比，染料木黄酮处理显着增加LC3Ⅱ蛋白的表达，但10 nmol/L T0070907处理后LC3Ⅱ蛋白的表达明显降低(图4A、B)。同样，染料木黄酮处理显着降低了泛素结合蛋白p62的表达，但10 nmol/L T0070907处理后p62蛋白的表达明显增加(图4A、B)。

3 讨论

肝纤维化是由许多慢性肝损伤引起的肝脏组织代偿性反应，若得不到有效治疗，常常会发展为肝硬化，肝功能衰竭，门静脉高压和肝细胞癌[1]。肝移植是晚期肝纤维化患者唯一可用的治疗方法[12]，而肝纤维化在一定程度上是可逆的。因此，探讨抗纤维化治疗的方案对于肝脏疾病的防治具有重大意义[13-16]。有研究发现，染料木黄酮可以抑制酒精、高脂饮食等各种原因引起的肝纤维化的进程。而染料木黄酮作为大豆异黄酮中的一种主要活性因子，广泛存在于豆科植物中，并具有多种重要的生物活性，包括抗炎、抗氧化、防治疗骨质疏松和抗肿瘤等作用[17-20]。染料木黄酮除了来源广泛外，还具有

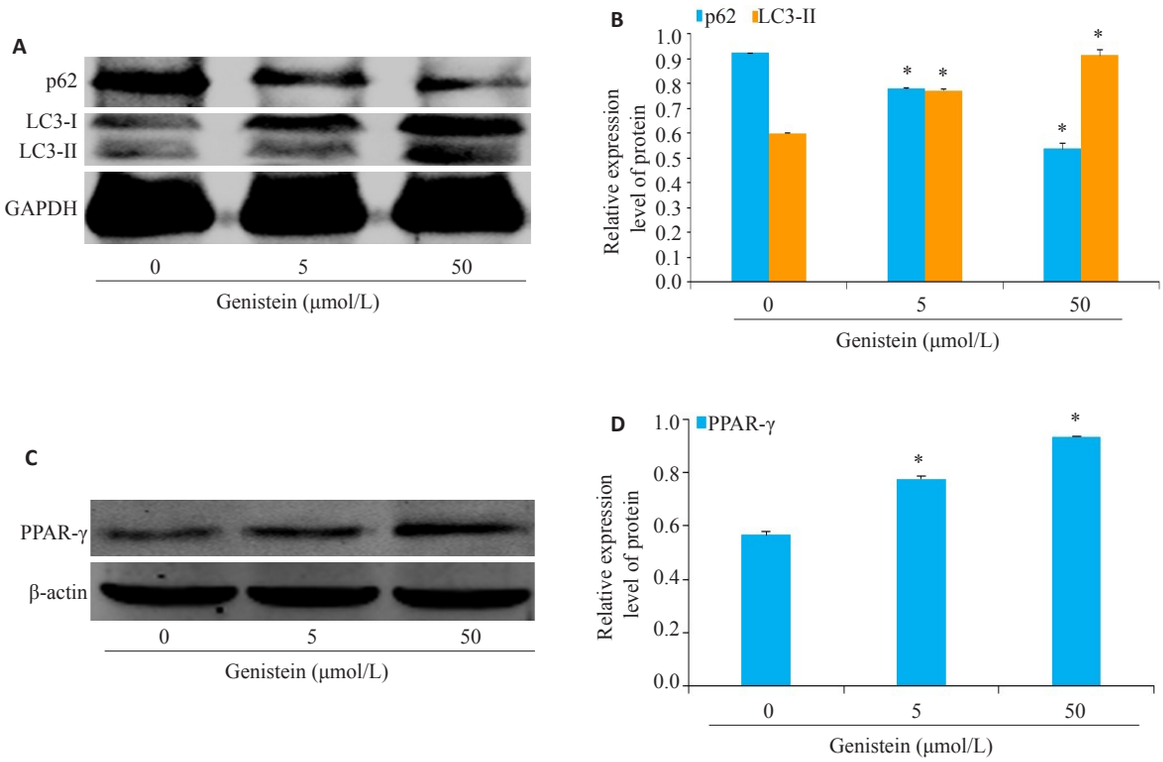

图2 染料木黄酮对PPAR-γ调控的自噬通路的影响

Fig.2 Effect of genistein on the activation of autophagy in HSC-T6 cells. **A**: Expression of p62 and LC3-II in HSC-T6 cells detected by Western blotting; **B**: Analysis of relative expression of the proteins; **C**: Expression of PPAR-γ in HSC-T6 cells detected by Western blotting; **D**: Analysis of relative expression of the protein. *P<0.05 *vs* control group.

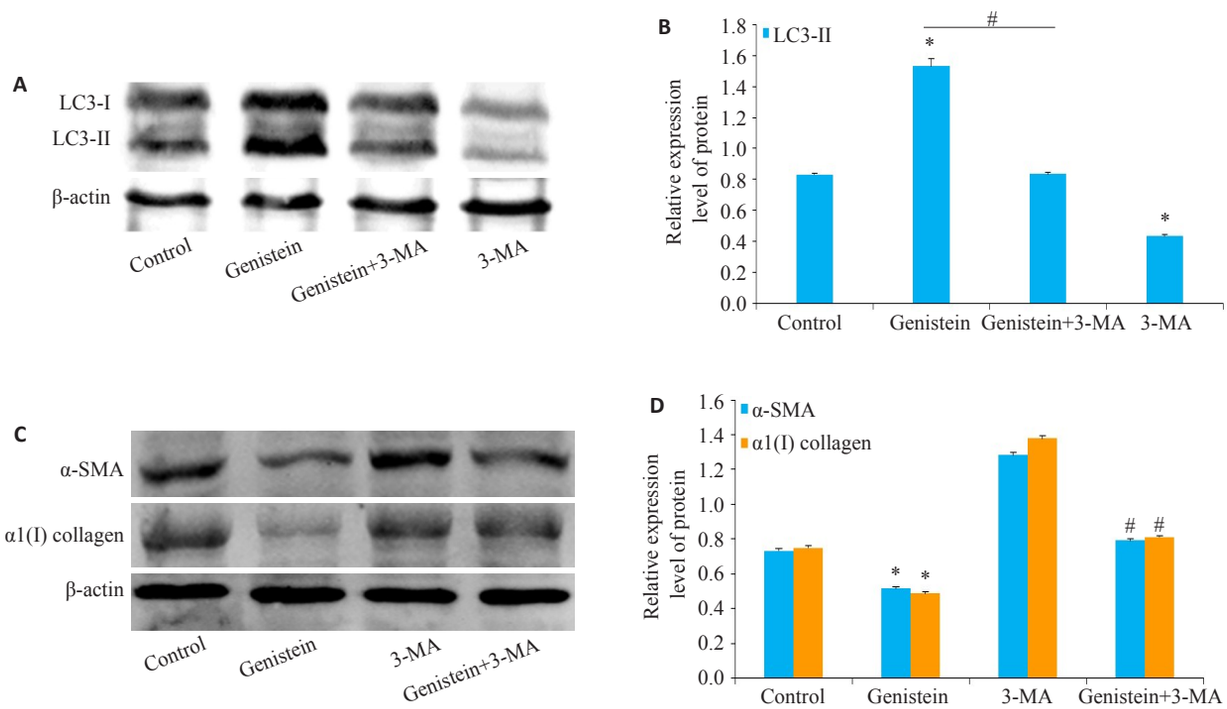

图3 自噬在染料木黄酮抑制HSC激活过程中的作用

Fig.3 Role of autophagy in mediating genistein-induced inhibition of HSC activation. **A**: Expression of LC3-II in HSC-T6 cells detected by Western blotting; **B**: Analysis of the relative expression of LC3-II protein; **C**: Expression of α-SMA and α1(I) collagen in HSC-T6 cells detected by Western blotting; **D**: Analysis of relative expression of the proteins. *P<0.05 *vs* control group, #P<0.05 *vs* genistein group.

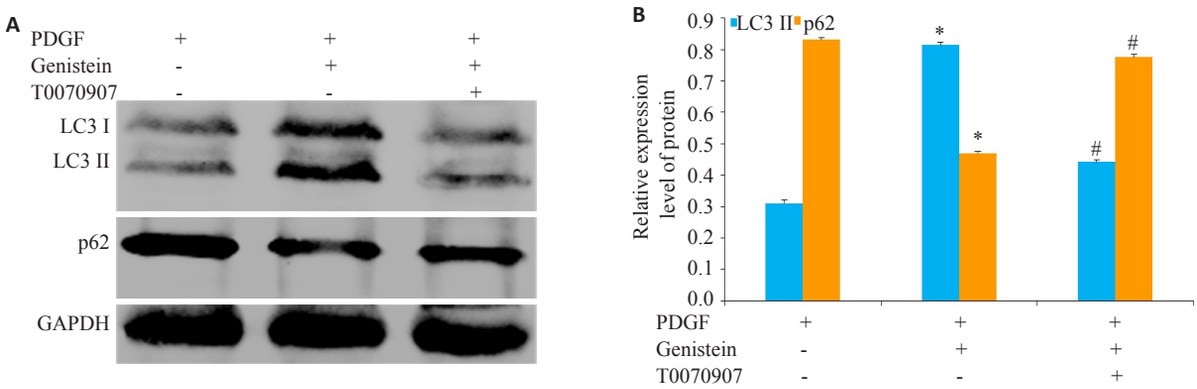

图4 PPAR-γ在染料木黄酮激活HSC自噬过程中的作用

Fig.4 Role of PPAR-γ in activating autophagy in genistein-treated HSCs. **A**: Expressions of p62 and LC3-II in HSC cells detected by Western blotting; **B**: Analysis of relative expression of the proteins. **P*<0.05 *vs* control group, #*P*<0.05 *vs* genistein group.

低毒性的优点，因此研究其肝脏保护作用具有重要的实际意义。

为了研究染料木黄酮抑制HSC细胞激活的活性，我们利用血小板衍生生长因子-BB (PDGF-BB) 在体外构建HSC活化的细胞模型[10-11]，观察染料木黄酮对其抑制效应。结果发现染料木黄酮作用后显著抑制了HSC细胞活化标志蛋白α-SMA、α1(I)collagen的表达水平。这就表明，染料木黄酮可能通过抑制HSC细胞活化，发挥抑制肝纤维化的作用，然而具体是通过怎样的分子机制，课题组进行了进一步的实验研究。

PPAR-γ在HSC细胞病理学过程中发挥着重要作用。Miyahara等[6]发现在活化的HSC中PPAR-γ的表达明显降低，当加入PPAR-γ激活剂后则显著抑制HSC的激活并促进了HSC的凋亡。同时，染料木黄酮已被证实是PPAR-γ的一种天然配体[8]，通过诱导PPAR-γ依赖的基因表达、调控PPAR-γ相关信号通路发挥重要的生理作用[11]。而我们的研究发现，染料木黄酮可以显著提高PPAR-γ的蛋白表达水平。这就表明，在HSC细胞内，染料木黄酮确实可以激活PPAR-γ，并通过其发挥作用。自噬作为抗肝纤维化的防御机制已经积累了大量的证据[21-24]。激活自噬可以改善肝纤维化的炎症微环境，并导致活化的HSC细胞衰老，促进HSC细胞凋亡，从而减轻肝脏的纤维化损伤[24-26]。PPAR-γ作为一个转录因子，可以调控细胞内自噬通路的激活[27-29]。有研究报道，激活PPAR-γ可以调控下游靶基因PTEN的表达，抑制Akt-mTOR信号通路进而激活细胞自噬，同时，PPAR-γ还可以促进Bcl-2磷酸化，促进其从Beclin1解离，从而导致自噬发生[27,30]。在目前的研究中，我们发现染料木黄酮处理后，HSC细胞内自噬相关蛋白LC3Ⅱ的表达表现出与PPAR-γ同样的增长趋势，并且利用T0070907抑制PPAR-γ的活性后，细胞自噬水平也受到了明显抑制。同时，为了验证自噬在染料木黄酮拮抗肝纤维化过程中的作用，我们利用自噬抑制剂3MA抑制染料木黄酮增加的HSC细胞自噬水平后，发现染料木黄酮抑制HSC活化的活性也受到了明显抑制。所有这些结果证明，染料木黄酮可以通过激活PPAR-γ调控的自噬通路抑制HSC增殖从而发挥抗纤维化的作用。尽管我们的数据表明染料木黄酮诱导的抗纤维化作用与自噬激活之间存在直接联系，但不能消除可能导致染料木黄酮保护作用的其他信号分子。

总之，这些结果提供了一个机制证据，即在肝纤维化中，染料木黄酮诱导的保护作用与PPAR-γ调控的自噬的激活有关。

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