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PAF 通过 PKCβ 途径对系膜细胞分泌细胞外基质的影响

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摘要 **目的** 探讨血小板活化因子(PAF)是否通过高糖可激活蛋白激酶 C-β(PKCβ)途径调节高糖高脂环境下细胞外基质(ECM)的分泌。**方法** 将人肾小球系膜细胞分成 6 组:正常对照组、PAF 组、PAF + LY333531 组、高糖高脂组、高糖高脂 + PAF 组、高糖高脂 + PAF + LY333531 组;ELISA 法测定各组中纤维联接蛋白(Fn)及Ⅳ型胶原(Col Ⅳ)含量,实时定量 PCR 测定 PKCβ Ⅰ mRNA 表达水平。**结果** 与正常对照组比较,其他 5 组 Fn、Col Ⅳ、PKCβ Ⅰ mRNA 表达增加($P<0.05$);与 PAF 组比较,PAF + LY333531 组 Fn、Col Ⅳ、PKCβ Ⅰ mRNA 表达减少($P<0.05$);与高糖高脂 + PAF + LY333531 组比较,高糖高脂组、高糖高脂 + PAF 组 Fn、Col Ⅳ、PKCβ Ⅰ mRNA 表达显著增加($P<0.05$)。**结论** 高糖高脂环境下 PAF 通过促进 PKCβ Ⅰ表达刺激 Fn、Col Ⅳ分泌;当加入 LY333531 时,ECM 分泌减少,PKCβ Ⅰ抑制剂对肾脏可能具有一定的保护作用。

关键词 血小板活化因子;细胞外基质;高糖高脂;PKCβ Ⅰ

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糖尿病肾病(diabetic nephropathy,DN)是糖尿

Effects of modified acidic fibroblast growth factor on the proliferation activity cultured hepatocytes *in vitro*

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Abstract ***Objective*** To explore effects of modified acidic fibroblast growth factor (MaFGF) on the growth of hepatocytes cultured *in vitro*. ***Methods*** Hepatocytes of SD rats were isolated, cultured *in vitro* and subculture for three generations. After being identified, the cells were used in the experiment: ① Hepatocytes was observed by inverted phase contrast microscope; ② The experiment were randomly divided into control and experimental group, MaFGF with different concentration were added into the culture medium of hepatocytes, after 24, 48, 72 h, the mitogenic effects were measured by the MTT test; ③ The MTT test was used to detect the effects of MaFGF on the proliferation of the hepatocytes in 24 h. The growth curve was plotted. ***Results*** ① MaFGF potentiated the proliferation of hepatocytes, which was no significant effect with dose increased. The proliferative rate of 24 h were higher than that of the other time points ($P<0.05$). there was statistical difference between the MaFGF group(4.68×10^{-3} ~ 7.80×10^{-3} mg/L) and the control group ($P<0.05$). However, the mitogeoic activities of each MaFGF group were insignificant; ② Four days after MaFGF(6.24×10^{-3} mg/L) added into the medium, the population of the hepatocytes was larger than that of the control ($P<0.05$). The number of the hepatocytes incubated with MaFGF (12.4×10^{4}) was 2.1 times as large as the control(6×10^{4}) in the 10th day($P<0.05$). ***Conclusion*** MaFGF has a certain role in promoting the proliferation of the hepatocytes at the appropriate concentration and time. However, the effects don't have concentration dependent manner.

Key words MaFGF; hepatocytes; cell culture; proliferation

病慢性并发症微血管病变之一，主要表现为肾功能损害。高糖高脂环境下血小板活化因子(platelet-activating factor，PAF)表达增加，并刺激细胞外基质(extracellular matrix，ECM)分泌增加，与DN的发生发展密切相关[1]。高糖可激活蛋白激酶C-β(protein kinase C，PKCβ)途径，进一步刺激肾小球系膜细胞和成纤维细胞分泌胶原(collagen，Col)Ⅰ、Ⅲ、Ⅳ及纤维联接蛋白(fibronectin，Fn)等ECM[2]。Fn及Col Ⅳ是ECM的主要组成成分，异常分泌及沉积于肾脏可致肾小球硬化[3]。尽管PAF及PKCβI途径均与ECM分泌有关，但在DN发生发展过程中，PAF与PKCβⅠ途径的关系目前仍未阐明。该研究探讨在高糖高脂环境下，PAF、PKCβI途径与ECM三者的关系，以进一步揭示高糖高脂环境下DN的发病机制。

1 材料与方法

1.1 材料 人肾小球系膜细胞(human mesangial cells，HMCs)由东南大学附属中大医院内分泌科孙子林教授惠赠[1]；D-葡萄糖(货号：MB2510，大连美伦生物技术有限公司)，溶血卵磷脂(货号：L4129，美国Sigma公司)，人Col IV ELISA试剂盒(货号：YG05870B，上海雅吉生物科技有限公司)；人Fn ELISA试剂盒(货号：SBJ-H1058，南京森贝伽生物科技有限公司)；PKCβI抑制剂LY333531(货号：1401，美国ApexBio公司；PAF C-16(货号：360906，美国Cayman公司)。

1.2 方法

1.2.1 细胞培养及分组 HMCs以含10%胎牛血清的DMEM培养液行传代培养，待细胞成融合状态后更换无血清的培养基，同步化12 h，进入静止期后分组，放置于37 ℃且含5% CO_2的无菌潮湿培养箱中培养24 h。离心后细胞上清液用于测定Fn、Col Ⅳ含量。实验分为6组：正常对照组(5.5 mmol/L D-葡萄糖)、PAF组(2×10^{-8} mol/L PAF C-16)、PAF+LY333531组(2×10^{-8} mol/L PAF C-16+2×10^{-7} mol/L LY333531)、高糖高脂组(30 mmol/L D-葡萄糖+20 mg/L溶血软磷脂)、高糖高脂+PAF组(30 mmol/L D-葡萄糖+20 mg/L溶血软磷脂+2×10^{-8} mol/L PAF C-16)、高糖高脂+PAF+LY333531组(30 mmol/L D-葡萄糖+20 mg/L溶血软磷脂+2×10^{-8} mol/L PAF C-16+2×10^{-7} mol/L LY333531)。

1.2.2 ELISA法测定上清液中Fn、Col Ⅳ、PAF水平 按照ELISA说明书步骤提示分别测定各组中细胞上清液中Fn、Col Ⅳ含量。各组均设置3个复孔，实验均重复3次。

1.2.3 实时荧光定量PCR测定PKCβⅠmRNA表达水平 提取细胞中总RNA，逆转入为cDNA，行实时荧光定量PCR检测。PKCβⅠmRNA上游引物为：5′-GGGGGCGACCTCATGTAT-3′；下游引物为5′-GCAATTTCTGCAGCGTAAAA-3′。人GAPDH引物序列如下：5′-ACACCCACTCCTCCACCTTT-3′；下游引物为：5′-TTACTCCTTGGAGGCCATGT-3′。PCR反应体系为：预变性95 ℃、10 min，变性95 ℃、10 s，随后退火56 ℃、20 s，延伸72 ℃、15 s重复40个循环，所有步骤均重复3次，采用$2^{-\Delta\Delta CT}$法分析目的基因的相对表达量。

1.3 统计学处理 采用SPSS 18.0统计软件进行分析，计量资料以$\bar{x}\pm s$表示，组间两两比较采用t检验或单因素方差分析(one way ANOVA)，$P<0.05$为差异有统计学意义。

2 结果

2.1 高糖高脂对ECM分泌的影响 与正常对照组相比，高糖高脂组Fn、Col Ⅳ显著增加($P<0.05$)，见表1；与正常对照组相比，高糖高脂组PKCβⅠmRNA表达增加($P<0.05$)，见表2。

表1 PAF及LY333531对系膜细胞Fn、Col IV的影响($n=3$，$\bar{x}\pm s$)

分组	Fn(mg/L)	Col Ⅳ(μg/L)
正常对照	3.90±0.43	4.54±0.74
PAF	7.05±0.05$^{*\#\triangle\nabla}$	13.71±0.88$^{*\#\triangle\nabla}$
PAF+LY333531	3.81±0.13*	5.31±0.81*
高糖高脂	7.89±0.34$^{*\triangle\nabla}$	16.32±1.55$^{*\triangle\nabla}$
高糖高脂+PAF	9.11±0.10$^{*\nabla}$	22.89±0.34$^{*\nabla}$
高糖高脂+PAF+LY333531	5.23±0.24*	11.40±0.72*
F值	214.00	172.44
P值	0.00	0.00

与对照组比较：$^{*}P<0.05$；与PAF+LY333531组比较：$^{\#}P<0.05$；与高糖高脂+PAF组比较：$^{\triangle}P<0.05$；与高糖高脂+PAF+LY333531组比较：$^{\nabla}P<0.05$

2.2 PAF对系膜细胞分泌ECM影响 与正常对照组相比，PAF组Fn、Col Ⅳ明显增多($P<0.05$)，见表1；与正常对照组相比，PAF组PKCβⅠmRNA表达增加($P<0.05$)，见表2。

2.3 高糖高脂环境下PAF对ECM分泌的影响 高糖高脂+PAF组比正常对照组、高糖高脂组及PAF组Fn、Col Ⅳ增加更为显著($P<0.05$)；与正常对照组相比，其余5组Fn、Col Ⅳ表达均增加($P<$

0.05),见表1;高糖高脂+PAF组比正常对照组、高糖高脂组及PAF组PKCβ Ⅰ mRNA增加更为显著($P<0.05$);与正常对照组相比,其余5组PKCβ Ⅰ mRNA表达均增加($P<0.05$),见表2。

2.4 LY333531对PAF及高糖高脂环境下ECM分泌的影响 与PAF组相比,PAF+LY333531组Fn、Col Ⅳ下降明显($P<0.05$);与高糖高脂+PAF+LY333531组相比,高糖高脂组及高糖高脂+PAF组Fn、Col Ⅳ表达显著增加($P<0.05$,表1);与PAF组相比,PAF+LY333531组PKCβ Ⅰ mRNA下降明显($P<0.05$);与高糖高脂+PAF+LY333531组相比,高糖高脂组及高糖高脂+PAF组PKCβ Ⅰ mRNA表达显著增加($P<0.05$),见表2。

表2 PKCβ Ⅰ mRNA在各组中的表达差异倍数($n=3$,$\bar{x}\pm s$)

分组	表达差异倍数
正常对照	16.00±0.00
PAF	2.68±0.17*#△▽
PAF+LY333531	1.85±0.39*
高糖高脂	2.12±0.31*△▽
高糖高脂+PAF	3.59±0.41*▽
高糖高脂+PAF+LY333531	2.76±0.57*
F值	18.13
P值	0.00

与对照组比较:* $P<0.05$;与PAF+LY333531组比较:# $P<0.05$;与高糖高脂+PAF组比较:△ $P<0.05$;与高糖高脂+PAF+LY333531组比较:▽ $P<0.05$

3 讨论

DN是糖尿病主要的慢性并发症之一,是导致终末期肾衰竭的主要原因。DN发病机制除肾脏局部胰岛素抵抗、炎症反应[4]外,还与ECM异常沉积肾小球硬化密切相关[1]。Col Ⅳ和Fn是ECM的重要成分,其异常分泌、沉积于肾脏将导致肾小球硬化及肾功能恶化[5]。糖尿病被认为是一种慢性炎症反应[6],既往研究[7]提示慢性炎症反应促进ECM异常分泌,参与DN发展的病理生理过程。PAF作为炎症因子刺激ECM分泌[7],是否与PKCβ Ⅰ途径有关仍尚未明确。该实验通过研究高糖高脂及PAF环境中应用PKCβ Ⅰ抑制剂LY333531观察ECM表达变化,以进一步探讨肾小球硬化的病理机制。

高糖高脂环境下,炎症因子可持续性表达,表达增多的炎症因子能进一步刺激肾小球系膜细胞及内皮细胞分泌ECM,参与肾小球硬化的发生发展过程[8]。高糖高脂上调PAF表达,该研究提示表达增加的PAF进一步促进Col Ⅳ和Fn的分泌,这与Reznichenko et al[9]的研究结果一致,表明PAF促进ECM过度沉积于肾小球系膜细胞是加速肾小球硬化的主要原因。

PKC是AGC(cAMP依赖性蛋白激酶/PKG/PKC)家族的成员,是在许多细胞功能中起关键作用并影响多条信号转导途径的丝氨酸/苏氨酸相关蛋白激酶[10];其与ECM合成、细胞生长和凋亡、白细胞黏附和细胞因子活化密切相关[10]。高糖诱导线粒体超氧化可激活肾脏PKCβ[11];链脲佐菌素诱导大鼠DN模型的研究[12]显示,肾小球硬化和肾小管纤维化形成的同时伴随着PKCβ及转化生长因子-β(transforming growth factor-β,TGF-β)的表达增加,而进一步接受特定的PKCβ抑制剂LY333531治疗后大鼠TGF-β表达下降,且尿白蛋白量降低。LY333531抑制PKCβ活性、降低纤维化生长因子TGF-β的过表达可减少肾小管间质纤维化及肾小球损伤,具有一定的肾脏保护作用[12];体外实验报道[13]糖尿病患者的系膜组织中Fn、Col Ⅳ表达增加,研究[13]还表明糖尿病肾小球系膜扩张和基底膜增厚的发展与TGF-β/PKCβ有关。另一些研究[14]同样提示,LY333531可抑制高糖刺激肾小球系膜细胞ECM分泌和TGF-β表达,表明PKCβ基因缺失或使用LY333531可使肥大的肾脏细胞及肾小球过滤得到一定程度改善;LY333531可抑制高糖刺激肾小球系膜细胞ECM分泌,减少DN大鼠肾小球硬化和肾小管间质纤维化的程度。肾小球基底膜的增厚和ECM在肾小球系膜组织和肾小管间质中异常沉积是DN的主要病理变化。炎症因子通过激活PKC依赖途径刺激Fn合成,而PAF作为强烈的炎症因子激活PKCβ通路并促进ECM分泌[15]参与DN发展。本研究结果提示高糖高脂联合PAF环境中使用PKCβ Ⅰ抑制剂可降低Fn、Col Ⅳ分泌,进一步揭示PAF可通过介导PKCβ Ⅰ途径促进ECM分泌,其具体是否与TGF-β/PKCβ Ⅰ途径有关,仍需下一步研究验证。

综上所述,高糖高脂血症环境中,PAF可通过介导PKCβ Ⅰ表达刺激肾小球系膜细胞分泌ECM,参与肾小球硬化发生发展,而PKCβ Ⅰ特异性抑制剂LY333531可降低高糖高脂及PAF刺激ECM分泌的水平,因此通过研究PKCβ Ⅰ抑制剂可能为DN的治疗提供新的思路及发现。

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Effects of PAF on extracellular matrix secreted by mesangial cells by PKCβ Ⅰ pathway

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Abstract ***Objective*** To investigate whether platelet activating factor(PAF) regulates extracellular matrix(ECM) secretion in high glucose(HG) and lysophosphatidylcholine(LPC) environment by PKCβ Ⅰ pathway. ***Methods*** Human glomerular mesangial cells were divided into six groups: normal control group, PAF group, PAF + LY333531 group, HG + LPC group, HG + LPC + PAF group, HG + LPC + PAF + LY333531 group. ELISA assay was used to detect Fn, Col Ⅳ content in supernatant of each group. Real-time quantitative PCR was used to determine the expression level of PKCβ Ⅰ mRNA. ***Results*** Compared with normal control group, the expressions of Fn and Col Ⅳ and PKCβ Ⅰ RNA in other five groups were significantly increased($P<0.05$). Compared with PAF group, the expressions of Fn and Col Ⅳ and PKCβ Ⅰ RNA in group PAF + LY333531 were decreased($P<0.05$), and the expressions of Fn and Col Ⅳ and PKCβ Ⅰ RNA in groups HG + LPC and HG + LPC + PAF were significantly higher than those in group HG + LPC + PAF + LY333531($P<0.05$). ***Conclusion*** PAF can stimulate the secretion of Fn and Col Ⅳ by promoting the expression of PKCβ Ⅰ in the high glucose and high fat environment. When the LY333531 is added, the secretion of ECM is reduced and the PKCβ Ⅰ inhibitor may have protective effect on the kidney.

Key words platelet activating factor; extracellular matrix; high glucose and high lysophosphatidylcholine; PKCβ Ⅰ