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低度增高的棕榈酸与脂多糖联合对胰岛 β 细胞活力的影响 *

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摘要 目的:探讨低度增高的棕榈酸联合脂多糖对胰岛 β 细胞活力的影响及可能机制。**方法:**采用 0.15 mmol/L 棕榈酸和 50 ng/mL 脂多糖单独、联合刺激大鼠胰岛 β 细胞株 INS-1 细胞 24h 后,通过 CCK8 法检测细胞活力,Western blot 方法检测细胞内神经鞘脂代谢的关键酶 - 中性神经酰胺酶(NCDase)的蛋白表达水平。进一步建立过表达 NCDase 基因重组质粒 pEGFP-C3-NCDase 并转染 INS-1 细胞后,用棕榈酸、脂多糖联合刺激 24h,再通过 CCK8 法检测细胞活力。**结果:**与对照组相比,0.15 mmol/L 棕榈酸或 50 ng/mL 脂多糖分别刺激 INS-1 细胞 24h 后对其细胞活力的影响无统计学意义($P>0.05$),但二者联合刺激可明显降低 INS-1 细胞的活力($P<0.05$)。与对照组相比,单独棕榈酸或脂多糖刺激 INS-1 细胞后并不影响细胞内 NCDase 的蛋白表达,但联合刺激可显著下调 NCDase 的表达 ($P<0.05$);与 pEGFP-C3+ 棕榈酸 + 脂多糖组相比,pEGFP-C3-NCDase 明显削弱了棕榈酸联合脂多糖对 INS-1 细胞活力的抑制作用($P<0.05$)。**结论:**低度增高的棕榈酸与脂多糖协同刺激可产生 β 细胞毒性作用,其机制可能与下调胰岛 β 细胞内 NCDase 表达有关。

关键词:棕榈酸;脂多糖;中性神经酰胺酶;胰岛 β 细胞

中图分类号:R-33;R587.1 **文献标识码:**A **文章编号:**1673-6273(2019)09-1634-04

Effect of Low-grade Increase of Palmitate and Lipopolysaccharide on the Viability of Pancreatic β -cells*

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ABSTRACT Objective: To explore the effect of low-grade increase of palmitate and lipopolysaccharide on the viability of pancreatic β -cells and its possible mechanism. **Methods:** The cell viability of rat pancreatic β -cell line INS-1 was detected by CCK8 assay after being incubated with 0.15 mmol/L palmitate and 50 ng/mL lipopolysaccharide for 24 h respectively or together. The expression of NCDase protein in INS-1 cells was detected by Western blot assay. Stable clones of INS-1 cell line transfected with recombinant plasmids pEGFP-C3-NCDase and pEGFP-C3 vector were established. Then they were incubated with palmitate and lipopolysaccharide for 24 h. The cell viability was detected by CCK8 assay. **Results:** Compared with the control INS-1 cells, the viability of INS-1 cells was hardly affected by the stimulation of palmitate or lipopolysaccharide alone. However, the cell viability was significantly decreased after treatment with palmitate and lipopolysaccharide together($P<0.05$). Compared with palmitate or lipopolysaccharide alone, the expression of NCDase protein in INS-1 cells was significantly decreased after incubated with palmitate and lipopolysaccharide ($P<0.05$). Compared with pEGFP-C3-palmitate plus lipopolysaccharide treatment group, NCDase overexpression alleviated the cytotoxicity which was induced by palmitate and lipopolysaccharide in INS-1 cells ($P<0.05$). **Conclusions:** The synergistic effect of low-grade increase of palmitate and lipopolysaccharide on the cytotoxicity of β -cells is related to the down-regulation of NCDase protein in pancreatic β -cells.

Key words: Palmitate; Lipopolysaccharide; NCDase; Pancreatic β -cells

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前言

临床上 2 型糖尿病(type 2 diabetes, T2DM)患者常伴有脂代谢紊乱^[1],主要表现为血浆游离脂肪酸(free fatty acid, FFA)低

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度增高,并以棕榈酸(palmitic acid,PA)含量最为丰富,FFA增高及菌群失调等导致肠道黏膜通透性受损使革兰氏阴性菌细胞壁的主要成分-脂多糖(lipopolysaccharide,LPS)呈低水平增高,LPS可作为启动子介导慢性炎症反应而参与T2DM等代谢性疾病的发生与进展^[2]。新近的研究表明低度增高的PA与LPS能产生显著的协同作用诱导免疫细胞的促炎症反应和神经细胞损伤^[3,4]。

长期FFA增高产生的脂毒性是T2DM患者 β 细胞损伤的关键因素之一^[5]。较高浓度的PA或LPS能产生明显的 β 细胞毒性作用。然而,在T2DM人群中更广泛存在的低度增高浓度的LPS或PA对 β 细胞的影响尚未见研究报道。鞘脂信号通路被认为在LPS与PA的协同作用中起着关键作用^[6],我们前期发现鞘脂信号通路亦与 β 细胞损伤关系密切,是调控 β 细胞功能的关键物质之一^[7,8]。本实验选择大鼠胰岛 β 细胞株INS-1为实验对象,主要观察低度增高的PA及LPS对INS-1细胞活力和鞘脂通路的影响,以进一步阐明细菌、脂毒性致

1 材料与方法

1.1 材料

大鼠胰岛 β 细胞株INS-1由南京中医药大学刘超教授惠赠。RPMI-1640培养基、胎牛血清购自美国Gibco公司,PA和LPS(O55:B5,L6529)购自美国Sigma公司,CCK8购自日本Dojindo公司,BCA蛋白定量试剂盒购自碧云天,ECL显色试剂盒购自德国Millipore,兔抗大鼠中性神经酰胺酶(neutral ceramidase,NCDase)购自美国Abcam公司,Rabbit anti- β actin、Anti-rabbit IgG购自美国Cell Signaling Technology公司。

1.2 细胞培养

INS-1细胞培养采用含10%胎牛血清、2 mmol/L谷氨酰胺、1 mmol/L丙酮酸钠、10 mmol/L HEPES、50 μ mol/L β -巯基乙醇、100 U/mL青霉素、100 μ g/mL链霉素的RPMI-1640培养液,在条件为37℃、5%CO₂的培养箱中培养。取对数生长期细胞用于实验。

1.3 棕榈酸配制

在70℃水浴中用0.1 mol/L的NaOH溶液溶解一定量的PA,振荡混匀10min后过滤,配成100 mmol/L的PA储存液。在55℃水浴中用去离子水配制50 g/L的BSA溶液过滤,将上述PA溶液和BSA溶液按1:19体积比混合配成5 mmol/L的PA/50 g/L BSA复合液,复合液在水浴中振荡10s后继续水浴10 min,室温冷却过滤后置于-20℃保存^[9]。

1.4 CCK8检测细胞活力

将INS-1细胞以 1×10^4 个/孔接种于96孔细胞培养板,培养24 h待细胞贴壁后予以药物干预。实验1:①正常对照组;②PA不同浓度处理组:PA 0.1、0.15、0.2、0.25、0.3 mmol/L。实验2:①正常对照组;②LPS不同浓度处理组:LPS 1、5、10、50 ng/mL。实验3:①正常对照组;②PA 0.15 mmol/L组;③LPS 50 ng/mL组;④PA 0.15 mmol/L联合LPS 50 ng/mL组。实验4:①pEGFP-C3组;②pEGFP-C3-NCDase组;③pEGFP-C3+PA+LPS组;④pEGFP-C3-NCDase+PA+LPS组。上述四组实验均于药物干预24 h后检测,每孔加入占孔内培养液体积10%的CCK8,继续37℃、5%CO₂培养1-4 h后,用酶标仪检测450 nm下各

组实验对象的吸光度。

1.5 Western blot检测NCDase的蛋白表达

INS-1细胞按 3×10^5 个/孔接种于6孔培养板,培养至70%融合后弃去原培养液,加入含药培养液:空白组(只含培养液,无PA、LPS),PA 0.15 mmol/L组,LPS 50 ng/mL组,PA 0.15 mmol/L+LPS 50 ng/mL组。药物作用24 h后,用预冷PBS洗涤细胞,加入含1%PMSF的RIPA蛋白裂解液后置于冰上30 min,继以高速14000 r/min离心30 min,取上清用BCA法测定蛋白浓度,加入上样缓冲液,100℃变性5-10 min,取适量蛋白上样于12%的SDS-PAGE胶。电泳后转印至PVDF膜上,5%脱脂奶粉封闭1.5 h,加入一抗于4℃孵育过夜,PBST洗涤3次(10 min/次)后,加入HRP标记的二抗于室温孵育2 h,再用PBST洗涤3次(10 min/次),ECL试剂处理曝光。扫描胶片,用Photoshop 6.0和Image J软件进行图片灰度分析。

1.6 pEGFP-C3-NCDase重组质粒转染及筛选

pEGFP-C3空载质粒购自Clontech;pEGFP-C3-NCDase重组质粒由Prof.Hannun(Medical University of South Carolina, USA)馈赠。按Invitrogen公司操作说明,应用脂质体Lipofectamine™ 2000介导pEGFP-C3空质粒和pEGFP-C3-NCDase重组质粒转染INS-1细胞,转染24 h后,用加有G418(终浓度为400 μ g/mL)的1640全培养基筛选,每3-4天更换一次含有G418的培养液,筛选两周后,更换G418浓度为200 μ g/mL,继续维持筛选,约两周后显微镜下可见转染组均有分裂相细胞并形成集落,根据质粒带GFP的特点,挑取GFP阳性单克隆株转入培养瓶中继续培养,获得稳定表达的细胞系。

1.7 统计学方法

数据采用GraphPad Prism 5软件进行统计学分析。数据用 $\bar{x} \pm s$ 表示,组间比较用方差分析和t检验。以P<0.05为差异具有统计学意义。

2 结果

2.1 PA、LPS对INS-1细胞活力的影响

与对照组相比,PA在0.2~0.3 mmol/L浓度范围内,对INS-1细胞活力有明显的抑制作用(P<0.05),而PA 0.1~0.15 mmol/L干预24 h后对于INS-1细胞活力的影响无明显统计学意义(图1A)。与对照组相比,LPS在浓度1、5、10、50 ng/mL时刺激INS-1细胞24 h,对细胞活力的影响无统计学意义(图1B)。与空白对照组相比,0.15 mmol/L PA或50 ng/mL LPS单独对于INS-1细胞活力的抑制作用均无统计学意义,但两者联合作用后的INS-1细胞活力较正常对照组及单独PA、LPS组均明显下降,且差异具有统计学意义(P<0.05)(图1C)。

2.2 PA联合LPS对INS-1细胞内NCDase蛋白表达的影响

PA 0.15 mmol/L或LPS 50 ng/mL单独刺激INS-1细胞24 h后,细胞内NCDase的蛋白表达水平与对照组相比无明显差异,但两者联合作用于INS-1细胞后可明显下调细胞内NCDase的蛋白表达(P<0.05)(图2)。

2.3 过表达NCDase对PA联合LPS抑制INS-1细胞活力的影响

通过转染pEGFP-C3-NCDase重组质粒,瞬时高表达NCDase(图3A),在此基础上建立转有NCDase重组质粒的INS-1稳定细胞系,稳定高表达NCDase(图3B)。PA 0.15 mmol/L联合

LPS 50 ng/mL 刺激稳定转染 pEGFP-C3 空质粒和 pEGFP-C3-NCDase 重组质粒的 INS-1 细胞 24 h 后, CCK8 检测细胞活力结果显示: 与 pEGFP-C3 空质粒组比较,

pEGFP-C3+PA+LPS 组细胞活力明显下降 ($P<0.05$), 而与 pEGFP-C3+PA+LPS 组相比, pEGFP-C3-NCDase+PA+LPS 组细胞活力明显上升 ($P<0.05$) (图 3C)。

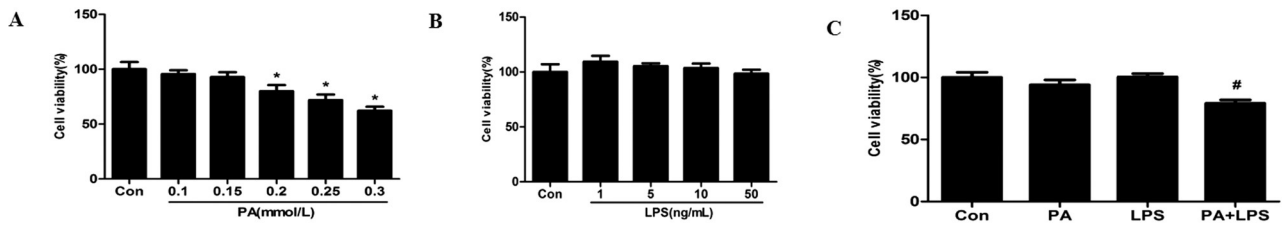


图 1 不同浓度 PA、LPS、PA 联合 LPS 对 INS-1 细胞活力的影响

Fig.1 Effect of different concentrations of PA, LPS, or the combination of PA and LPS on the viability of INS-1 cells

Note: * $P<0.05$ vs. Con; # $P<0.05$ vs. Con, PA, LPS

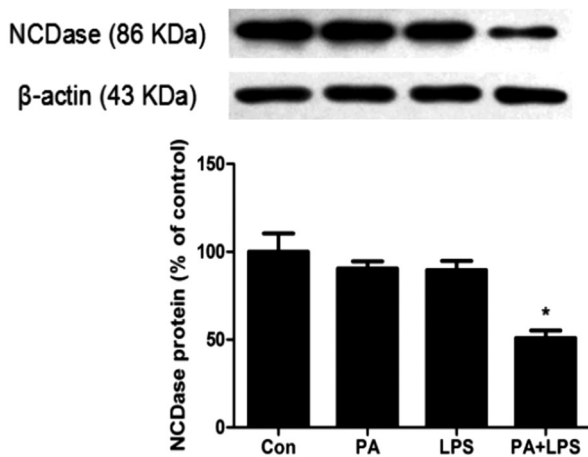


图 2 PA 联合 LPS 对 INS-1 细胞内 NCDase 蛋白表达的影响

Fig.2 Effects of PA and LPS on the NCDase protein expression in INS-1 cells

Note: * $P<0.05$ vs. Con, PA, LPS.

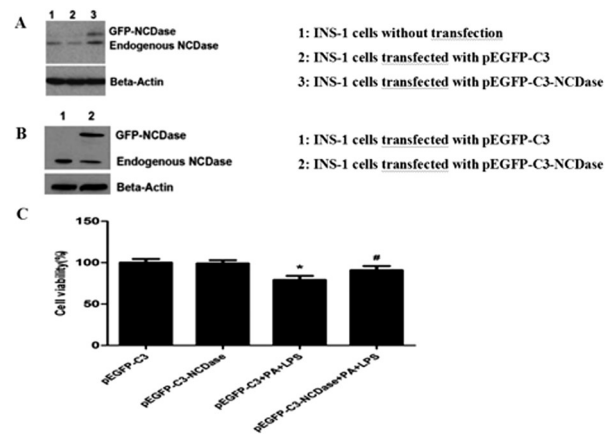


图 3 NCDase 过表达对 PA 联合 LPS 抑制 INS-1 细胞活力的影响

Fig.3 Effect of NCDase overexpression on the viability of INS-1 cells after incubated with PA and LPS

Note: * $P<0.05$ vs. pEGFP-C3; # $P<0.05$ vs. pEGFP-C3+PA+LPS.

3 讨论

正常人体血浆中 LPS 浓度很低,而在肥胖人群、T2DM 患者体内呈低度增高,可能与高脂饮食促进 LPS 的转运吸收、肠道菌群失调及通透性增加等有关^[10,11]。与 LPS 类似,T2DM 患者体内 PA 亦呈低水平增高^[12]。研究表明较高浓度 LPS ($\geq 1 \mu\text{g/mL}$)^[13]或 PA ($\geq 0.3 \text{ mmol/L}$)^[14]可明显抑制 INS-1 细胞活力,而本研结果显示低度增高浓度的 LPS (50 ng/mL) 或 PA (0.15 mmol/L) 单独作用对 INS-1 细胞无明显毒性作用,而二者联合可明显降低 INS-1 细胞活力,说明低度增高的 PA 联合 LPS 对于 INS-1 细胞产生了协同毒性效应。

已有文献报道,低度增高的 PA 联合 LPS 协同作用 RAW 264.7 巨噬细胞时通过调节鞘脂代谢以促进炎症因子的表达^[15],提示低度增高的 PA 与 LPS 的协同效应与调控鞘脂信号分子的代谢有关。近年研究显示神经鞘脂除了作为细胞的膜性结构成分外,更是一关键的决定细胞命运的信号途径^[16],并在 FFA 诱导的 β 细胞损伤中扮演重要角色^[17]。在神经鞘脂信号通路中,神经酰胺 (ceramide, Cer) 是中心分子,其在细胞内有两条生成途径: (1) 利用丝氨酸和软脂酰-CoA 从头合成; (2) 由神经鞘磷脂经神经鞘磷脂酶水解生成^[18]。Cer 通过激活外源性死亡受体、

内质网应激、线粒体氧化应激、活化 c-Jun 氨基末端激酶及 Caspase-3 等途径促进 β 细胞凋亡、参与 T2DM 的发生和进展^[19]。现已证实 PA 可以为 Cer 的从头合成途径提供原料从而诱导 Cer 生成增多损害胰岛 β 细胞^[20]。

NCDase 是细胞内 Cer 分解代谢的关键限速酶,本课题组前期研究发现 β 细胞表达 NCDase^[21],并且高浓度 PA (0.5 mmol/L) 可通过抑制 INS-1 细胞内 NCDase 表达和活性、提高 Cer 水平^[22],从而加剧 Cer 堆积、促进细胞凋亡。此外,我们还发现,过表达 NCDase 通过增加 Cer 降解改善 FFA 诱导的 INS-1 细胞凋亡^[23]。在本研究中,我们进一步发现低度增高的 LPS 与 PA 协同导致 INS-1 细胞活性显著降低,同时 NCDase 表达明显降低,而高表达 NCDase 能显著改善低度增高的 PA 与 LPS 协同诱导的 INS-1 细胞毒性,提示 NCDase 表达降低导致 Cer 降解减少是 PA 协同 LPS 抑制 INS-1 细胞活力的重要机制。此外,未来的研究尚需进一步阐明低度增高的 PA 协同 LPS 抑制 NCDase 表达的具体机制。

综上所述,虽然低度增高的 PA 或 LPS 对于 INS-1 细胞活力及 NCDase 表达无明显影响,但二者协同通过降低 NCDase 表达调控鞘脂信号分子进而抑制 INS-1 细胞活力。由于临床上 T2DM 患者广泛存在低度增高的 PA 或 LPS,本研究的结果提

示对于这部分患者需及时采取干预措施以保护胰岛 β 细胞,从而为 T2DM 的防治提供新的干预靶点。

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