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· 基础研究 ·

CYLD 在急性胰腺炎肺损伤中的表达及其与 NF- κ B 通路关系的体外研究

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摘要

目的: 探讨急性胰腺炎 (AP) 致急性肺损伤 (ALI) 时肺泡巨噬细胞 (AM) 中肿瘤抑制因子 cylindromatosis (CYLD) 的表达及其与 NF- κ B 炎症反应信号通路的关系。

方法: 60 只成年 SD 大鼠随机均分为实验组与对照组; 经支气管肺泡灌洗获取大鼠 AM 后, 实验组给予 TNF- α 刺激 (AP 致 ALI 体外模拟), 对照组给予等量生理盐水, 每组 AM 分别在处理后 0、1、3、6、12 h, 行相关炎症因子, 以及 CYLD、NF- κ Bp65、NF- κ B 必须调节蛋白 (NEMO) 及 I κ B α 蛋白表达检测。

结果: 对照组各时间点上, AM 中释放的各炎症因子、以及 CYLD、NF- κ B 通路相关蛋白的表达水平均无明显变化 (均 $P>0.05$)。与对照组比较, 实验组各项指标在 0 h 均无差异 (均 $P>0.05$), 但其后时间点均有统计学差异 (均 $P<0.05$); TNF- α 、IL-1 β 、IL-6、NO 的释放均在 1 h 明显升高, 且达到峰值, 其后缓慢下降; 从 1 h 开始, CYLD 蛋白表达明显下调、NF- κ Bp65 和 I κ B α 蛋白表达明显上调, 其后均有所恢复; NEMO 蛋白表达从 1 h 明显上调, 3 h 时表达降低, 6、12 h 表达量再次回升。实验组 AM 中 CYLD 与 NF- κ Bp65、NEMO 及 I κ B α 的表达呈明显负相关 ($r=-0.759$ 、 -0.849 、 -0.813 , 均 $P<0.05$)。

结论: AM 中 CYLD 的表达可能在 AP 致 ALI 时降低, 进而其对 NF- κ B 炎症反应信号通路的抑制减弱。上调 CYLD 的表达可能是减轻 AP 致 ALI 的有效途径。

关键词

胰腺炎; 急性肺损伤; Cylindromatosis; NF- κ B
中图分类号: R657.5

CYLD expression during acute lung injury caused by acute pancreatitis and its relation with NF- κ B pathway: an in vitro study

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Abstract

Objective: To investigate the expression of tumor suppressor factor cylindromatosis (CYLD) in alveolar macrophages (AMs) and its relation with NF- κ B-dependent inflammatory signaling pathway during acute lung injury (ALI) caused by acute pancreatitis (AP).

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Methods: Sixty adult SD rats were equally randomized into experimental group and control group. AMs were harvested by bronchoalveolar lavage, which were treated with TNF- α (for simulation of AP-induced ALI in vitro) in experimental group, and treated with the same amount of normal saline in control group, respectively. In these AMs, the levels of inflammatory factors and the expressions of CYLD, NF- κ Bp65, NF- κ B-essential modulator (NEMO) and I κ B α were determined at 0, 1, 3, 6 and 12 h after treatment.

Results: At each time point, the releasing levels of all the determined inflammatory factors and expression levels of CYLD and NF- κ B pathway-related proteins showed no significant change in control group (all $P>0.05$). In experimental group compared with control group, all parameters showed no significant difference at 0-h time point (all $P>0.05$), but had statistical difference at all the later time points (all $P<0.05$); the releasing levels of TNF- α , IL-1 β , IL-6 and NO were all significantly increased and reached their peak values at 1-h time point and then gradually decreased; from 1-h time point, the CYLD expression was significantly down-regulated, while the expressions of NF- κ Bp65 and I κ B α were significantly up-regulated, and then all were slightly recovered later; the NEMO expression was significantly up-regulated at 1-h time point, and declined at 3-h time point, and was elevated again at 6- and 12-h time point. CYLD expression had significantly negative correlation with the expressions of NF- κ Bp65, NEMO and I κ B α in the AMs of experimental group ($r=-0.759, -0.849, \text{ and } -0.813$, all $P<0.05$) respectively.

Conclusion: CYLD expression may be decreased in the AMs during ALI secondary to AP, thus its inhibition to NF- κ B-dependent inflammatory signaling pathway is lessened. Up-regulating CYLD expression may probably be an effective approach for alleviating AP-induced ALI.

Key words Pancreatitis; Acute Lung Injury; Cylindromatosis; NF- κ B

CLC number: R657.5

急性肺损伤 (ALI) 是急性胰腺炎 (AP) 早期出现的胰腺外并发症^[1]。其发病机制可能与 NF- κ B 信号通路过度活化导致大量炎症介质释放有关^[2]。如能抑制 NF- κ B 过度活化将有助于减轻 ALI。研究^[3-5]表明, 肿瘤抑制因子 cylindromatosis (CYLD) 是 NF- κ B 活化的负性调节因子。笔者前期研究初步证实 CYLD 在 AP 导致 ALI 过程中可能起负性调控作用^[6], 但其具体机制不清。研究^[7-8]提示, NF- κ B 必须调节蛋白 (NF- κ B-essential modulator, NEMO) 作为 NF- κ B 上游调控因子在其活化中起重要作用。本实验拟通过体外模拟 AP 致 ALI 发生, 观察肺泡巨噬细胞 (AM) 中 CYLD、NF- κ B、NEMO、I κ B α 的表达情况, 探索 CYLD 调控 NF- κ B 信号通路的可能机制, 为临床治疗 ALI 提供新思路。

1 材料与方法

1.1 实验动物及分组

成年健康 SD 大鼠 60 只 (中国医学科学院实验动物研究所提供), 雌雄不拘, 体质量 250~300 g。动

物实验前喂养 1 周, 普通饲料, 随意饮水, 规律昼夜时间 (各 12 h), 室温 20~25 $^{\circ}\text{C}$, 术前禁食 12 h, 不禁饮水。随机分成实验组与对照组, 每组 30 只。

1.2 支气管肺泡灌洗及细胞处理

采用腹腔放血的方法分批处死各组动物, 经肺泡灌洗获取 AM, 然后将灌洗液 2 000 r/min 离心 10 min 获取细胞, 用 1640 培养液悬浮细胞后于 37 $^{\circ}\text{C}$ 、5% CO_2 细胞培养箱中温育 1 h。获得 AM 以 0.4% 胎盘蓝染色, 判定活性大于 90%, Wright 染色判定纯度大于 90%, 且无污染。实验组给予 10 $\mu\text{g}/\text{L}$ 的 TNF- α 刺激, 模拟 AP 致 ALI^[9-10], 对照组各时相给予等量生理盐水, 分别在处理后的 0、1、3、6、12 h, 以 2 000 r/min 离心 10 min 收集上清液和 AM。

1.3 上清液中 AM 分泌的 TNF- α 、IL-1 β 、IL-6 及 NO 水平测定

应用购自 Biosource 公司的酶联免疫吸附法 (ELISA) 试剂盒测定 TNF- α 、IL-1 β 、IL-6 含量, 应用化学比色法检测上清液中 NO 含量, 试剂盒购自南京建成生物公司, 所有步骤严格按照产品说明书操作。

1.4 Western blot 法测定各组 AM 中 CYLD、NF- κ Bp65、NEMO 及 I κ B α 活性水平

应用购自北京普利莱基因技术有限公司的胞核/胞质蛋白制备试剂盒提取AM胞质蛋白和核蛋白并测定浓度后,行聚丙烯酰胺凝胶电泳。结果用计算机扫描,用图像分析系统(GelPro 3.0)测定目的条带与内参的光密度值,并取两者的比值作半定量分析。分别检测AM中CYLD、NF- κ Bp65、NEMO及I κ B α 的活性水平。

1.5 统计学处理

数据以平均数 \pm 标准差($\bar{x}\pm s$)表示。组间差异显著性的比较采用单因素方差分析。 $P<0.05$ 为差异有统计学意义。相关性采用线性分析,数据处理均用SPSS 22.0软件完成。

2 结果

2.1 AM 分泌 TNF- α 、IL-1 β 、IL-6 及 NO 活性变化

对照组可检测到低水平的TNF- α 活性,且各时间点无明显变化($P>0.05$);实验组AM分泌的TNF- α 活性0 h与对照组无明显差异($P>0.05$),自1 h即明显升高($P<0.05$),且达到峰值,至3、6、12 h仍维持高水平,但呈逐渐降低的趋势,12 h时TNF- α 活性仍高于0 h($P<0.05$);AM分泌IL-1 β 、IL-6及NO活性各组变化情况与TNF- α 的变化趋势相似(表1-4)。

表 1 两组 AM 中 TNF- α 释放水平比较 ($n=6$, pg/mL)

Table 1 Comparison of the TNF- α releasing levels in the AMs of the two groups ($n=6$, pg/mL)

组别	0 h	1 h	3 h	6 h	12 h
对照组	83.0 \pm 7.8	89.2 \pm 4.5	85.3 \pm 7.2	79.7 \pm 8.1	91.6 \pm 11.3
实验组	87.7 \pm 3.8	1 008.3 \pm 6.7 ¹⁾	410.9 \pm 10.6 ¹⁾	270.3 \pm 5.6 ¹⁾	204.7 \pm 14.8 ¹⁾

注:1)与对照组相同时间点比较, $P<0.05$

Note: 1) $P<0.05$ vs. control group at the same time point

表 2 两组 AM 中 IL-1 β 释放水平比较 ($n=6$, pg/mL)

Table 2 Comparison of the IL-1 β releasing levels in the AMs of the two groups ($n=6$, pg/mL)

组别	0 h	1 h	3 h	6 h	12 h
对照组	9.0 \pm 1.3	11.4 \pm 2.6	13.5 \pm 4.1	10.2 \pm 1.7	9.7 \pm 1.8
实验组	8.6 \pm 2.3	218.5 \pm 16.4 ¹⁾	152.0 \pm 3.9 ¹⁾	101.6 \pm 2.9 ¹⁾	68.2 \pm 4.1 ¹⁾

注:1)与对照组相同时间点比较, $P<0.05$

Note: 1) $P<0.05$ vs. control group at the same time point

表 3 两组 AM 中 IL-6 释放水平比较 ($n=6$, pg/mL)

Table 3 Comparison of the IL-6 releasing levels in the AMs of the two groups ($n=6$, pg/mL)

组别	0 h	1 h	3 h	6 h	12 h
对照组	68.3 \pm 2.3	75.4 \pm 5.3	72.7 \pm 6.8	66.7 \pm 6.0	70.8 \pm 4.9
实验组	72.8 \pm 2.6	512.6 \pm 6.1 ¹⁾	358.6 \pm 8.0 ¹⁾	241.2 \pm 9.4 ¹⁾	196.1 \pm 4.0 ¹⁾

注:1)与对照组相同时间点比较, $P<0.05$

Note: 1) $P<0.05$ vs. control group at the same time point

表 4 两组 AM 中 NO 释放水平比较 ($n=6$, nmol/mL)

Table 4 Comparison of the NO releasing levels in the AMs of the two groups ($n=6$, nmol/mL)

组别	0 h	1 h	3 h	6 h	12 h
对照组	0.414 \pm 0.040	0.472 \pm 0.055	0.483 \pm 0.047	0.428 \pm 0.013	0.446 \pm 0.028
实验组	0.445 \pm 0.033	1.206 \pm 0.113 ¹⁾	1.045 \pm 0.077 ¹⁾	0.740 \pm 0.072 ¹⁾	0.610 \pm 0.047 ¹⁾

注:1)与对照组相同时间点比较, $P<0.05$

Note: 1) $P<0.05$ vs. control group at the same time point

2.2 Western blot 检测 CYLD 和 NF- κ Bp65 表达

对照组各时间点CYLD蛋白表达呈高水

平,且表达量无明显变化($P>0.05$);实验组1 h时CYLD蛋白表达与对照组相比已有明显下

降 ($P < 0.05$)，随着时间进行，其后各时相较对照组均呈现低表达 ($P < 0.05$)。对照组各时相NF- κ Bp65蛋白表达呈低水平，实验组1 h

时NF- κ Bp65蛋白表达与对照组相比明显上升 ($P < 0.05$)，且达到峰值，其后各时相较对照组相比亦呈高表达，但趋势逐渐降低 (图1-2)。

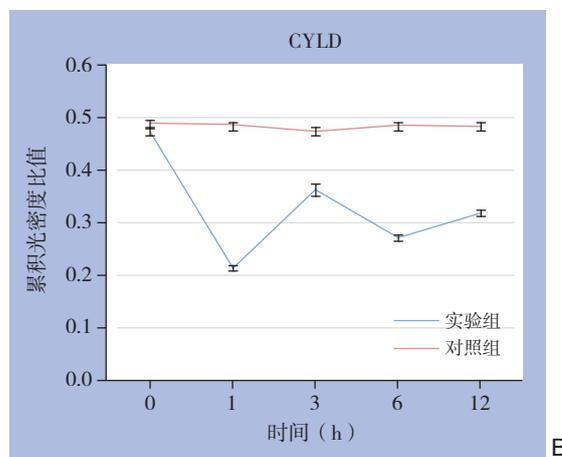
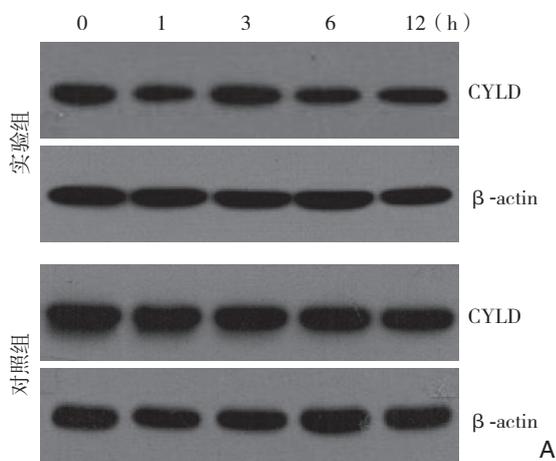


图1 Western blot 检测 CYLD 蛋白表达

A: 电泳图; B: 定量比较

Figure 1 CYLD protein expression determination by Western blot analysis

A: Electrophoretic diagram; B: Quantitative comparison

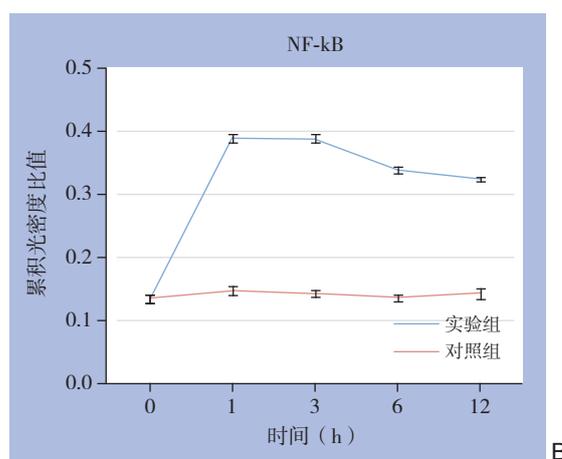
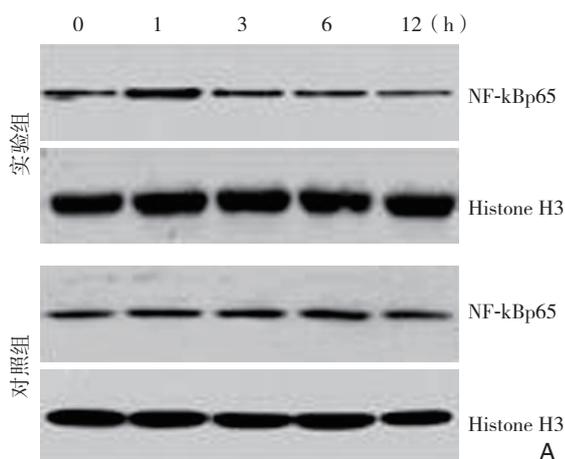


图2 Western blot 检测 NF- κ Bp65 蛋白表达

A: 电泳图; B: 定量比较

Figure 2 NF- κ Bp65 protein expression determination by Western blot analysis

A: Electrophoretic diagram; B: Quantitative comparison

2.3 Western blot 检测 NEMO 和 I κ B α 表达

对照组各时相NEMO蛋白表达呈低水平，实验组1 h时NEMO蛋白表达与对照组相比明显升高 ($P < 0.05$)，3 h时表达降低，但仍高于对照组 ($P < 0.05$)，6、12 h时又回升，呈现高表达。对照组各时相I κ B α 蛋白表达呈低水平，实验组1 h时I κ B α 蛋白表达与对照组相比明显升高

($P < 0.05$)，随着时间进行，其后各时相较对照组均呈现高表达 (图3-4)。

2.4 相关性分析

实验组AM中CYLD与NF- κ Bp65、NEMO及I κ B α 的表达呈高度负相关 ($r = -0.759$ 、 $r = -0.849$ 、 $r = -0.813$ ，均 $P < 0.05$)。

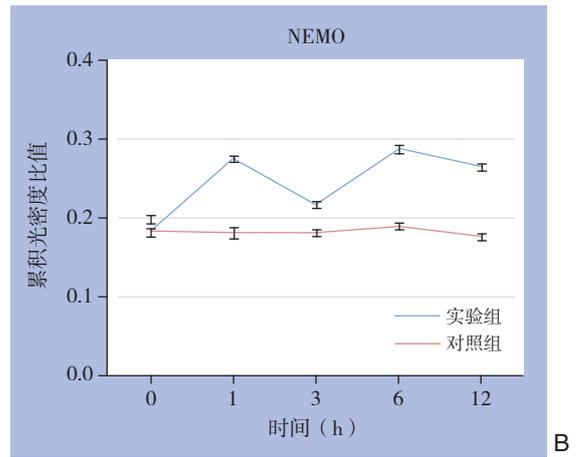
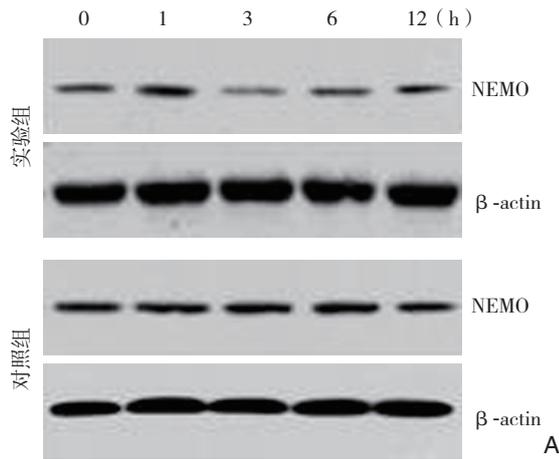


图3 Western blot 检测 NEMO 蛋白表达

A: 电泳图; B: 定量比较

Figure 3 NEMO protein expression determination by Western blot analysis

A: Electrophoretic diagram; B: Quantitative comparison

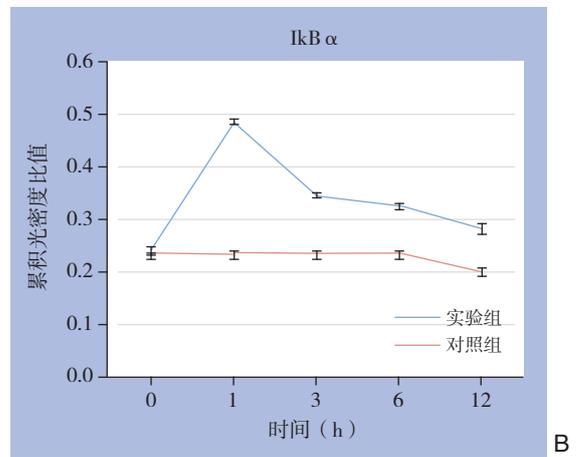
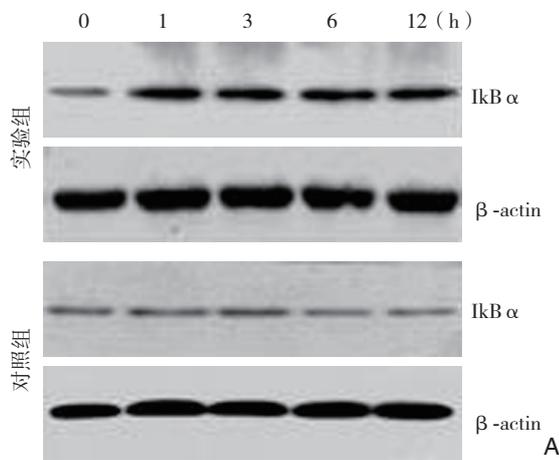


图4 Western blotting 检测 IκBα 蛋白表达

A: 电泳图; B: 定量比较

Figure 4 IκBα protein expression determination by Western blot analysis

A: Electrophoretic diagram; B: Quantitative comparison

3 讨论

AP引起ALI的实质就是多种炎症细胞参与的肺部局部炎症反应和炎症反应失控所致的肺毛细血管膜损伤^[11],是导致早期死亡的主因。研究^[2, 12]认为NF-κB信号通路活化在ALI中起关键作用,抑制其活化可能是治疗ALI的新靶点。

研究^[13-14]表明NF-κB活化可引起多种炎症介质的过量释放,导致炎症反应。本实验对正常大鼠AM行体外刺激以模拟AP致ALI模型。结果显示:实验组AM分泌的炎症介质TNF-α、IL-1β、IL-6和NO较对照组相比均在1h即明显升高,且达到峰值,其后各时相仍维持较高水平,但呈逐渐下降的趋势。证实AP时由于NF-κB的活化导致炎症介质的释放,引起炎症反应级联扩大,进而加重肺损伤。该结果与我们前期研究^[15]相符,但炎症介质分泌的峰值有所提前,可能由于体外直接刺激AM,反应比较迅速且充分,且由于培养基消

耗等因素,导致分泌峰值提前。

研究^[16-17]证实,CYLD是NF-κB的负性调控因子。同时也在炎症性疾病中起关键性作用^[18-19]。笔者前期大鼠体内研究^[20-21]也表明,CYLD表达可能与AP致ALI相关,但其具体机制尚不清。

本研究结果显示,对照组各时间点CYLD表达水平均较高,而实验组从1h即明显下降,且表达量最低,此后各时相较对照组比较均维持低水平,表明CYLD在正常的AM中有一定的表达,而AP时其表达降低。推测可能与CYLD能抑制NF-κB活化的上游调控因子有关。

NEMO作为NF-κB信号通路的上游调控因子,是IKK复合物激活唯一绝对必需的亚单位^[7]。CYLD能抑制IKK复合物的形成,进而抑制NF-κB活化^[22]。故本研究进一步检测各组AM中NF-κB p65及其上游调控因子NEMO和IκBα的表达,结果显示,实验组上述指标均于1h即明显升高,提示AM经过TNF-α刺激后,NEMO和IκBα

表达增强, 进而导致NF- κ B信号通路被迅速激活。相关性分析也显示实验组上述指标与CYLD表达呈负相关, 表明CYLD可能通过抑制NF- κ B活化和上游调控因子的表达而大量消耗, 从而导致实验组CYLD表达较对照组降低。

综上, 本研究结果提示, AM中CYLD在AP所致ALI时呈低表达, 使其对NF- κ B信号通路活性的抑制减弱, 从而导致大量炎症因子的释放。增强CYLD表达可能是减轻AP致ALI的有效途径。

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