

急性呼吸窘迫综合征大鼠血小板 c-Jun 氨基末端蛋白激酶磷酸化水平的变化

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【摘要】 目的 探讨急性呼吸窘迫综合征(ARDS)时血小板活化的信号通路。方法 将30只健康SD大鼠按随机数字表法分为对照组($n=6$)和模型组($n=24$)。采用经尾静脉注射油酸0.25 mL/kg制备ARDS模型;对照组给予等量生理盐水。模型组于制模后2、6、24、72 h取腹主动脉血,分离血小板,采用蛋白质免疫印迹试验(Western Blot)检测血小板丝裂素活化蛋白激酶(MAPKs)通路中的主要蛋白激酶c-Jun氨基末端蛋白激酶(JNK)的磷酸化(pJNK)水平;处死动物取肺组织,计算肺系数(肺质量/体质量 $\times 100\%$)及肺湿/干质量(W/D)比值;苏木素-伊红(HE)染色后,光镜下观察肺组织病理学变化。结果 与对照组相比,ARDS模型组大鼠制模后2 h血小板pJNK水平即明显增高(灰度值: 0.72 ± 0.09 比 0.22 ± 0.01),6 h达峰值(灰度值: 0.91 ± 0.03 比 0.22 ± 0.01),之后逐渐降低,至72 h仍明显高于对照组(灰度值: 0.39 ± 0.06 比 0.22 ± 0.01 ,均 $P < 0.05$)。ARDS模型组大鼠制模后2 h大鼠肺系数和肺W/D比值即较对照组明显升高[分别为(1.30 ± 0.20)%比(0.60 ± 0.10)%、 6.00 ± 0.60 比 3.30 ± 0.30],之后随时间延长逐渐降低,但直至72 h肺系数和肺W/D比值仍明显高于对照组[分别为(0.90 ± 0.10)%比(0.60 ± 0.10)%、 4.80 ± 0.70 比 3.30 ± 0.30 ,均 $P < 0.05$]。光镜下显示,对照组大鼠肺组织无明显病理学改变。模型组制模后2 h即可见明显的肺泡水肿和间质水肿,炎性细胞浸润,小血管扩张、充血,肺泡内有大量蛋白渗出物;24 h病变达极期;72 h肺泡腔液体渗出大部分被吸收,肺泡腔缩小,肺泡间隔增厚,炎性细胞浸润减轻,纤维组织增生,有微血栓形成。结论 ARDS时肺组织除发生病理学改变外,血小板也发生了活化,且其活化过程与JNK信号转导通路启动有关。

【关键词】 急性呼吸窘迫综合征; 血小板活化; 丝裂素活化蛋白激酶; 信号转导; c-Jun氨基末端蛋白激酶

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Study on the changes of platelet c-Jun N-terminal kinase phosphorylation in rats with acute respiratory distress syndrome Liu Hong, Fan Xiaozhi, Tian Xinqiang, Li Bing

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【Abstract】 Objective To investigate the signal pathway of platelet activation in acute respiratory distress syndrome (ARDS). **Methods** Thirty healthy Sprague-Dawley (SD) rats were randomly divided into control group ($n=6$) and model group ($n=24$). The model of ARDS was reproduced by intravenous injection of oleic acid (0.25 mL/kg), and the rats in control group were injected with the same amount of normal saline. The blood of abdominal aorta was collected at 2, 6, 24, and 72 hours after model reproduction, the platelets were separated, and c-Jun N-terminal kinase phosphorylation (pJNK) levels which was one of major protein kinases in the mitogen-activated protein kinases (MAPKs) signal pathway was determined by Western Blot. The rats were sacrificed, the lung tissues were harvested, and lung coefficient (lung weight/body weight $\times 100\%$) and lung wet/dry (W/D) ratio were calculated. Pathological changes in the lung tissue were observed with hematoxylin-eosin (HE) staining in light microscope. **Results** Compared with the control group, platelet pJNK level in ARDS model group was significantly increased at 2 hours after model reproduction (gray value: 0.72 ± 0.09 vs. 0.22 ± 0.01), and peaked at 6 hours (gray value: 0.91 ± 0.03 vs. 0.22 ± 0.01), then it was decreased gradually. It was

also significantly higher than that of control group till 72 hours after model reproduction (gray value: 0.39 ± 0.06 vs. 0.22 ± 0.01 , all $P < 0.05$). Lung coefficient and lung W/D ratio in ARDS model group were significantly increased at 2 hours after model reproduction as compared with those of control group [$(1.30 \pm 0.20)\%$ vs. $(0.60 \pm 0.10)\%$, 6.00 ± 0.60 vs. 3.30 ± 0.30], then they were decreased gradually. They were also significantly higher than those of control group till 72 hours after model reproduction [$(0.90 \pm 0.10)\%$ vs. $(0.60 \pm 0.10)\%$, 4.80 ± 0.70 vs. 3.30 ± 0.30 , all $P < 0.05$]. It was showed by light microscopy that lung tissue of rats in the control group had no significant pathological changes. At 2 hours after model reproduction in model group, clearly visible alveolar edema and interstitial edema, interstitial lung infiltration of inflammatory cells, small blood vessels dilation and congestion were found, and there were a lot of protein exudates. The lesions of lung peaked at 24 hours. At 72 hours, absorption of most of fluid leaking in alveolar, alveolar space narrow, alveolar septum thickening, the reduction of inflammatory cells infiltration, fibrous tissue proliferation, and micro thrombosis formation were found. **Conclusion** In ARDS, in addition to pathological changes in the lung tissue, platelet activation occurs, and its activation process is related to the priming of JNK signal transduction pathways.

【Key words】 Acute respiratory distress syndrome; Platelet activation; Mitogen-activated protein kinase; Signal transduction; c-Jun N-terminal kinase

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急性呼吸窘迫综合征(ARDS)是由多种病因引起的一种特殊类型的急性呼吸衰竭(呼衰),是临床常见危重病,病死率较高^[1-2]。虽然近50年来进行了大量研究,但ARDS的发病机制仍不清楚。本课题组前期研究显示,ARDS时血液循环中存在高凝状态,血小板活化可能在ARDS发病中起到重要作用^[3]。本研究在既往研究基础上,探讨ARDS时血小板活化的信号转导通路,进而探讨ARDS发病的分子机制。

1 材料与方法

1.1 实验动物:健康SD大鼠30只,体质量250~400 g,由北京维通利华实验动物技术有限公司提供,动物合格证号:11400700064520。

1.2 动物分组及模型制备:将30只大鼠按随机数字表法分为对照组($n=6$)和模型组($n=24$)。采用经尾静脉注射油酸(分析纯,天津市风船化学试剂公司产品)0.25 mL/kg制备ARDS模型;对照组注射等量生理盐水。模型组于制模后2、6、24、72 h分别经腹主动脉取血并处死动物,取肺组织备检。

本实验动物处置方法符合动物伦理学标准。

1.3 检测指标及方法

1.3.1 蛋白质免疫印迹试验(Western Blot)测定血小板磷酸化c-Jun氨基末端蛋白激酶(pJNK)水平:于制模后各时间点取大鼠腹主动脉血约10 mL,分离血小板并提取蛋白,加入 $5 \times$ 加样缓冲液并混匀,94℃加热5 min,进行十二烷基硫酸钠-聚丙烯酰胺凝胶电泳(SDS-PAGE),转至聚偏二氟乙烯(PVDF)膜,10%脱脂奶粉封闭2 h;按1:1000用缓冲液稀释一抗(兔抗鼠),室温孵育0.5 h后4℃冰箱孵

育过夜;室温下再孵育0.5 h;用含吐温20的Tris-HCl缓冲液(TBST)洗膜10 min \times 3次;按1:2000用缓冲液稀释二抗(羊抗兔),室温孵育2 h,洗膜10 min \times 3次。用电化学发光法(ECL)显色成像;应用ImageLab系统软件测定JNK的磷酸化水平,其表达量以pJNK与JNK总蛋白的灰度值比值表示。

1.3.2 肺系数测定:处死动物取全肺,称肺质量,计算肺系数(肺质量/体质量 $\times 100\%$)。

1.3.3 肺湿/干质量比值(W/D)测定:取大鼠右肺,称湿质量,置80℃烤箱内烘干至恒重,称干质量,并计算肺W/D比值。

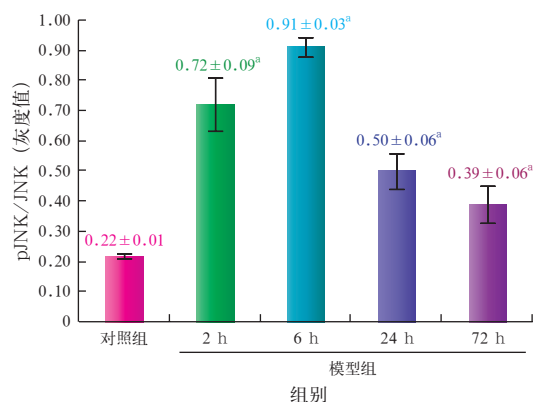
1.3.4 肺组织病理学观察:取大鼠左肺,进行大体观察后,置于10%甲醛溶液中固定1周。在矢状面取0.3~0.5 cm组织,常规切片,进行苏木素-伊红(HE)染色,光镜下观察肺组织病理学改变。

1.4 统计学方法:采用Graphpad Prism 5软件进行数据分析,计量资料以均数 \pm 标准差($\bar{x} \pm s$)表示,多组间比较采用单因素方差分析,两两比较采用Newman-Kuels q 检验,组内各时间点比较采用配对 t 检验,以 $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 动物一般情况:模型组大鼠于制模后2~3 min即开始出现呼吸困难,瘫软无力,活动减少,爬行式行走,进食减少;24 h后症状开始好转;72 h接近正常。对照组大鼠无异常变化。

2.2 血小板内JNK磷酸化水平变化(图1):与对照组相比,模型组制模后2 h血小板pJNK水平即明显增高,6 h达峰值,之后逐渐降低,至72 h仍明显高于对照组(均 $P < 0.05$)。



注: pJNK 为磷酸化 c-Jun 氨基末端蛋白激酶; 与对照组比较, ^a*P*<0.05

图1 各组大鼠血小板 pJNK 水平的变化

2.3 肺系数和 W/D 比值变化(表 1): 模型组大鼠制模后 2 h 肺系数及肺 W/D 比值即较对照组明显升高(均 *P*<0.05), 之后随时间延长逐渐降低, 但直至 72 h 仍明显高于对照组(均 *P*<0.05)。

表 1 各组大鼠肺系数和肺湿 / 干质量比值的比较 ($\bar{x} \pm s$)

组别	动物数(只)	肺系数(%)	肺湿 / 干质量比值
对照组	6	0.60 ± 0.10	3.30 ± 0.30
模型 2 h 组	6	1.30 ± 0.20 ^a	6.00 ± 0.60 ^a
模型 6 h 组	6	1.10 ± 0.10 ^a	5.60 ± 0.20 ^a
模型 24 h 组	6	0.90 ± 0.10 ^a	5.30 ± 1.40 ^a
模型 72 h 组	6	0.90 ± 0.10 ^a	4.80 ± 0.70 ^a

注: 与对照组比较, ^a*P*<0.05

2.4 肺组织病理学观察

2.4.1 大体观察显示: 对照组大鼠肺脏呈粉红色, 表面光滑, 纹理清晰, 大小正常。模型组制模后 2 h 双肺明显肿胀, 体积增大, 重量增加, 外观呈鲜红或深红色花斑状, 随时间延长逐渐变为暗红色; 72 h 肺脏肿胀减轻, 外观呈灰白色, 弹性差。

2.4.2 光镜下显示: 对照组大鼠肺组织结构清晰,

肺泡腔干净, 肺泡壁无增厚(图 2A)。模型组制模后 2 h 即可见明显的肺泡水肿和间质水肿, 肺间质有炎性细胞浸润, 小血管扩张、充血, 肺泡内可见大量染色深红均一的蛋白渗出物(图 2B); 6 h 透明膜大量形成, 肺间质和肺泡出血, 肺间质炎性细胞浸润加重(图 2C); 24 h 肺泡、肺间质有大量炎性细胞浸润及明显的肺间质和肺泡出血, 肺组织实变, 病变达到极期(图 2D); 72 h 肺泡腔液体渗出物大部分吸收, 肺泡腔缩小, 肺泡间隔增厚, 炎性细胞浸润减轻, 纤维组织增生, 有微血栓形成(图 2E)。

3 讨论

ARDS 是临床上比较常见的疾病, 在美国每年发病约 20 万例, 病死率接近 50%, 早期或轻度阶段曾被称为 ALI^[4]。ARDS 可由多种病因引起, 但其发病机制尚不十分清楚。近年来, 对血小板的功能和特性有了很多新发现, 使人们对血小板如何在呼吸系统分子和细胞生物学中起作用有了新的看法^[5]。血小板除参与止血和血栓形成过程外, 还参与免疫、炎症、动脉硬化、肿瘤生长和转移、血管生成和淋巴管的发育等多种病理过程^[6-11]。血小板可表达 Toll 样受体 (TLRs)、CD14、CD40、Fc 受体 (FcR) 等多种免疫受体, 以及释放大量接近活化的细胞因子, 如白细胞介素-1β (IL-1β)、转化生长因子-β (TGF-β)、调节活化正常 T 细胞表达与分泌的趋化因子 (RANTES)、单核细胞趋化因子-1 (MCP-1)、CD40L 等。这些受体和分子共同促使血小板招募白细胞到血管损伤点, 释放炎性因子, 产生微粒物质, 诱发凝血酶生成。这些过程已被证实对动脉粥样硬化、脓毒症、肝炎、血管再狭窄、ALI 和移植排斥反应等疾病的发病过程至关重要^[8]。

已知在生理状态和 ARDS 时, 血小板、血小板前体与肺有多层次的交互作用, 血小板发生活化并可能在 ARDS 发病中起重要作用^[5, 12-13], 但活化的

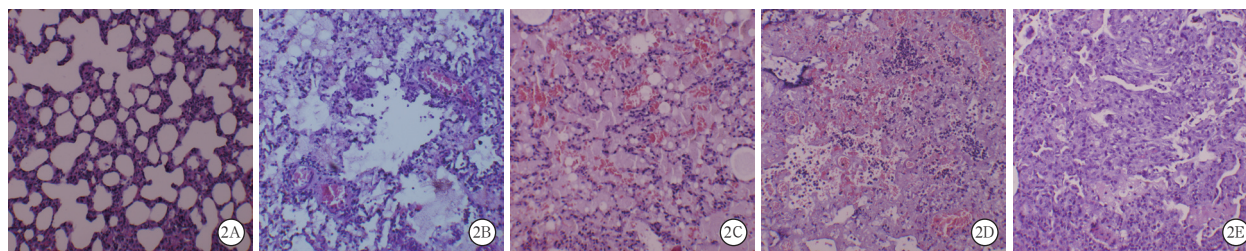


图 2 光镜下观察对照组和急性呼吸窘迫综合征 (ARDS) 模型组大鼠制模后各时间点肺组织病理学改变 对照组 (A) 肺组织结构清晰, 肺泡腔干净; 模型组制模后 2 h (B) 肺泡腔内有大量液体渗出, 肺泡间隔有炎性细胞浸润, 小血管扩张、充血; 6 h (C) 肺泡腔内有大量液体渗出, 肺透明膜形成, 肺泡腔、肺间质出血; 24 h (D) 肺泡腔内有液体渗出, 肺泡腔、肺间质出血, 炎性细胞浸润; 72 h (E) 肺泡腔缩小, 肺泡间隔增宽, 炎性细胞浸润, 有灶性分布的肺间质纤维化 HE 染色 中倍放大

具体机制和信号转导通路仍不清楚。丝裂素活化蛋白激酶(MAPKs)是细胞跨膜信号转导过程中位于下游的一组丝氨酸/苏氨酸蛋白激酶,包括细胞外信号调节激酶(ERKs)、JNKs和p38MAPK 3个亚家族。该信号通路存在于许多细胞内,在细胞外刺激信号向细胞及其核内转导并引起细胞发生生物学反应的过程中起重要作用。哺乳类动物细胞内有多条并行的MAPKs信号转导通路,不同的细胞外刺激通过不同的通路相互调控,从而介导不同的细胞生物学反应。国外学者发现,血小板内主要存在ERK2、JNK1、p38MAPK 3种MAPKs,血小板活化时,JNK1参与了其聚集、黏附和分泌活动^[14-15]。毕辉等^[16]研究发现,在体外使用凝血酶诱导的大鼠和人血小板活化过程中,大鼠血小板胞质中游离钙离子和JNK1以及人血小板中P-选择素均参与了这一过程。王灵聪等^[17]研究表明,人支气管上皮细胞存在脂多糖(LPS)-ERK信号通路。马明明等^[18]研究提示,芹菜素可拮抗LPS引起的小鼠ALI,其保护作用可能与抑制p38MAPK信号通路活化有关。但有关ARDS时血循环中血小板内的MAPKs信号转导通路的实验研究报道较少。本研究结果显示,模型组制模后2~24 h大鼠血循环中血小板pJNK水平显著增高,6 h达峰值,之后逐渐降低,其变化趋势与肺系数、肺W/D比值和肺组织病理学基本一致。

综上,本实验结果表明,ARDS时血小板确实发生了活化,并存在JNK信号转导通路的启动;JNK信号通路的启动有可能参与了血小板的活化过程。但本研究仅对ARDS时血小板活化信号通路进行了初步探索,今后尚需大量实验研究进行深入探讨。

参考文献

- 乔良, 刘志. 按柏林新标准分析急诊脓毒症患者发生急性呼吸窘迫综合征的危险因素[J]. 中华危重病急救医学, 2015, 27 (7): 558-562. DOI: 10.3760/cma.j.issn.2095-4352.2015.07.004.
- Qiao L, Liu Z. Analysis of the risk factors of acute respiratory distress syndrome of Berlin new definition in patients with sepsis in emergency department [J]. Chin Crit Care Med, 2015, 27 (7): 558-562. DOI: 10.3760/cma.j.issn.2095-4352.2015.07.004.
- 葛庆岗, 姚智渊, 王铁华, 等. 急性呼吸窘迫综合征发生及预后危险因素的多中心前瞻性队列研究[J]. 中华危重病急救医学, 2014, 26 (11): 773-779. DOI: 10.3760/cma.j.issn.2095-4352.2014.11.002.
- Ge QG, Yao ZY, Wang TH, et al. Risk factors of the occurrence and death of acute respiratory distress syndrome: a prospective multicenter cohort study [J]. Chin Crit Care Med, 2014, 26 (11): 773-779. DOI: 10.3760/cma.j.issn.2095-4352.2014.11.002.
- 刘宏, 赵金垣, 刘艳云, 等. 肺循环高凝状态与成人呼吸窘迫综合征的发病机理[J]. 中华劳动卫生职业病杂志, 1996, 14 (4): 193-196.
- Liu H, Zhao JY, Liu YY, et al. Hypercoagulability of pulmonary circulation and the pathogenesis of adult respiratory distress syndrome [J]. Chin J Ind Hyg Occup Dis, 1996, 14 (4): 193-196.
- Avasarala S, Zhang F, Liu G, et al. Curcumin modulates the inflammatory response and inhibits subsequent fibrosis in a mouse model of viral-induced acute respiratory distress syndrome [J]. PLoS One, 2013, 8 (2): e57285. DOI: 10.1371/journal.pone.0057285.
- Bozza FA, Shah AM, Weyrich AS, et al. Amicus or adversary: platelets in lung biology, acute injury, and inflammation [J]. Am J Respir Cell Mol Biol, 2009, 40 (2): 123-134. DOI: 10.1165/rcmb.2008-0241TR.
- Rivera J, Lozano ML, Navarro-Núñez L, et al. Platelet receptors and signaling in the dynamics of thrombus formation [J]. Haematologica, 2009, 94 (5): 700-711. DOI: 10.3324/haematol.2008.003178.
- Rondina MT, Weyrich AS, Zimmerman GA. Platelets as cellular effectors of inflammation in vascular diseases [J]. Circ Res, 2013, 112 (11): 1506-1519. DOI: 10.1161/CIRCRESAHA.113.300512.
- Jenne CN, Wong CH, Petri B, et al. The use of spinning-disk confocal microscopy for the intravital analysis of platelet dynamics in response to systemic and local inflammation [J]. PLoS One, 2011, 6 (9): e25109. DOI: 10.1371/journal.pone.0025109.
- Li C, Li J, Li Y, et al. Crosstalk between Platelets and the Immune System: Old Systems with New Discoveries [J]. Adv Hematol, 2012, 2012: 384685. DOI: 10.1155/2012/384685.
- Ware J, Corken A, Khetpal R. Platelet function beyond hemostasis and thrombosis [J]. Curr Opin Hematol, 2013, 20 (5): 451-456. DOI: 10.1097/MOH.0b013e32836344d3.
- 吉祥, 姚芳超, 王兵, 等. 脂多糖诱导小鼠血小板减少症可能与凝血及炎症反应无关[J]. 中华危重病急救医学, 2015, 27 (9): 754-758. DOI: 10.3760/cma.j.issn.2095-4352.2015.09.011.
- Ji X, Yao FC, Wang B, et al. Thrombocytopenia induced by lipopolysaccharide may be not related to coagulation and inflammatory response [J]. Chin Crit Care Med, 2015, 27 (9): 754-758. DOI: 10.3760/cma.j.issn.2095-4352.2015.09.011.
- Weyrich AS, Zimmerman GA. Platelets in lung biology [J]. Annu Rev Physiol, 2013, 75: 569-591. DOI: 10.1146/annurev-physiol-030212-183752.
- Kroll MH, Afshar-Kharghan V. Platelets in pulmonary vascular physiology and pathology [J]. Pulm Circ, 2012, 2 (3): 291-308. DOI: 10.4103/2045-8932.101398.
- Kauskot A, Adam F, Mazharian A, et al. Involvement of the mitogen-activated protein kinase c-Jun NH₂-terminal kinase 1 in thrombus formation [J]. J Biol Chem, 2007, 282 (44): 31990-31999. DOI: 10.1074/jbc.M701596200.
- Adam F, Kauskot A, Nurden P, et al. Platelet JNK1 is involved in secretion and thrombus formation [J]. Blood, 2010, 115 (20): 4083-4092. DOI: 10.1182/blood-2009-07-233932.
- 毕辉, 王莉安, 纪红蕊, 等. 蚓激酶对活化血小板[Ca²⁺]_i、JNK1和P-选择素表达的影响[J]. 中国药理学杂志, 2011, 46 (2): 89-93.
- Bi H, Wang LA, Ji HR, et al. Effect of lumbrokinase on [Ca²⁺]_i, JNK1 and P-selection in activated platelet [J]. Chin Pharm J, 2011, 46 (2): 89-93.
- 王灵聪, 韦丽玲, 江荣林, 等. 姜黄素对脂多糖诱导人支气管上皮细胞丝裂素活化蛋白激酶信号通路的影响[J]. 中国中西医结合急救杂志, 2015, 22 (3): 239-242. DOI: 10.3969/j.issn.1008-9691.2015.03.004.
- Wang LC, Wei LL, Jiang RL, et al. Effect of curcumin on mitogen activated protein kinase signaling pathway in human bronchial epithelial cells induced by lipopolysaccharide [J]. Chin J TCM WM Crit Care, 2015, 22 (3): 239-242. DOI: 10.3969/j.issn.1008-9691.2015.03.004.
- 马明明, 李岩, 朱委委, 等. 芹菜素对脂多糖致小鼠急性肺损伤的作用机制研究[J]. 中国中西医结合急救杂志, 2014, 21 (3): 170-174. DOI: 10.3969/j.issn.1008-9691.2014.03.003.
- Ma MM, Li Y, Zhu WW, et al. The effect of apigenin on lipopolysaccharide-induced acute lung injury and its mechanism in mice [J]. Chin J TCM WM Crit Care, 2014, 21 (3): 170-174. DOI: 10.3969/j.issn.1008-9691.2014.03.003.

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