

# 肝素通过核转录因子- $\kappa$ B 信号通路减少脂多糖刺激人内皮细胞趋化因子的表达

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**【摘要】** 目的 观察肝素对脂多糖(LPS)刺激人内皮细胞分泌趋化因子水平的影响,探讨肝素对核转录因子- $\kappa$ B(NF- $\kappa$ B)信号通路的作用机制。方法 体外培养人肺微血管内皮细胞株(HPMEC),取传代培养至第3~5代的细胞用于实验。将细胞分成对照组、LPS刺激组、1 kU/L或10 kU/L肝素+LPS组及NF- $\kappa$ B抑制剂甲苯磺酰赖氨酸氯甲酮(TLCK)组(TLCK+LPS组)。LPS刺激组加LPS 10 mg/L;不同剂量肝素预处理组于LPS刺激前15 min分别加入1 kU/L或10 kU/L的普通肝素;TLCK+LPS组于LPS刺激前30 min加用TLCK 10  $\mu$ mol/L;对照组加入与LPS等量的磷酸盐缓冲液(PBS)。各组于LPS刺激后1 h收集细胞,采用免疫荧光法观察NF- $\kappa$ B核移位情况,以明确肝素对NF- $\kappa$ B的活化作用;LPS刺激后3 h和6 h收集细胞上清液,采用酶联免疫吸附试验(ELISA)测定白细胞介素-8(IL-8)及单核细胞趋化蛋白-1(MCP-1)的水平,以明确肝素对LPS刺激HPMEC产生趋化因子的影响以及对NF- $\kappa$ B信号通路的作用机制。结果 ① 荧光显微镜下显示,对照组NF- $\kappa$ B大部分位于胞质中;LPS刺激组NF- $\kappa$ B由胞质向胞核转移明显增多;1 kU/L和10 kU/L肝素预处理均可明显抑制LPS诱导的NF- $\kappa$ B活化,以10 kU/L肝素效果较好。② 与对照组比较,LPS刺激后3 h和6 h细胞上清液中IL-8及MCP-1水平均明显增高[IL-8 (ng/L): 3 h为387.1 $\pm$ 26.4比23.8 $\pm$ 8.1, 6 h为645.5 $\pm$ 69.6比125.7 $\pm$ 18.7; MCP-1 (ng/L): 3 h为3654.9 $\pm$ 467.9比721.6 $\pm$ 61.3, 6 h为8178.5 $\pm$ 792.6比1324.7 $\pm$ 148.7,均 $P$ <0.05];与LPS刺激组比较,1 kU/L和10 kU/L肝素预处理均可明显降低IL-8及MCP-1水平[IL-8 (ng/L): 3 h为315.3 $\pm$ 24.8、275.8 $\pm$ 31.1比387.1 $\pm$ 26.4, 6 h为557.8 $\pm$ 43.3、496.9 $\pm$ 38.7比645.5 $\pm$ 69.6; MCP-1 (ng/L): 3 h为2924.1 $\pm$ 267.9、2668.3 $\pm$ 522.6比3654.9 $\pm$ 467.9, 6 h为7121.7 $\pm$ 557.2、6563.9 $\pm$ 576.4比8178.5 $\pm$ 792.6,均 $P$ <0.05],以10 kU/L肝素作用较明显;而NF- $\kappa$ B抑制剂TLCK亦可明显抑制LPS诱导的IL-8、MCP-1水平增高[IL-8 (ng/L): 3 h为162.4 $\pm$ 21.3比387.1 $\pm$ 26.4, 6 h为274.1 $\pm$ 22.6比645.5 $\pm$ 69.6; MCP-1 (ng/L): 3 h为1478.2 $\pm$ 138.5比3654.9 $\pm$ 467.9, 6 h为3667.6 $\pm$ 259.4比8178.5 $\pm$ 792.6,均 $P$ <0.05]。结论 LPS刺激HPMEC后IL-8及MCP-1的水平增加,肝素可能通过调节NF- $\kappa$ B信号通路降低趋化因子水平,从而发挥保护作用。

**【关键词】** 内皮细胞; 普通肝素; 核转录因子- $\kappa$ B; 白细胞介素-8; 单核细胞趋化蛋白-1

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## Unfractionated heparin inhibits lipopolysaccharide-induced expression of chemokines in human endothelial cells through nuclear factor- $\kappa$ B signaling pathway

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**【Abstract】 Objective** To determine the effect of unfractionated heparin (UFH) on lipopolysaccharide (LPS)-induced expression of chemokines and nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway. **Methods** Human pulmonary microvascular endothelial cells (HPMECs) were cultured in vitro, and the cells between passages 3 and 5 were used in the experiments. The cells were divided into control group, LPS challenge group, 1 kU/L or 10 kU/L UFH+LPS group, and NF- $\kappa$ B inhibitor N-tosyl-L-lysyl chloromethyl-ketone (TLCK) group (TLCK+LPS group). HPMECs in LPS challenge group were treated with 10 mg/L LPS. UFH pretreatment with different dosages groups were treated with 1 kU/L or 10 kU/L UFH 15 minutes before LPS challenge. Cells in the TLCK+LPS group were treated with 10  $\mu$ mol/L of TLCK 30 minutes before the addition of LPS, and HPMECs in control group were treated with an equal volume

of phosphate-buffered saline (PBS) instead. The cells were harvested 1 hour after LPS challenge, and the nuclear translocation of NF- $\kappa$ B was determined by immunofluorescence assay to detect the effect of UFH on NF- $\kappa$ B activation. The levels of interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1) in cell culture supernatants were determined by enzyme linked immunosorbent assay (ELISA) 3 hours and 6 hours after LPS challenge to detect the effect of UFH on LPS induced expression of chemokines and its mechanism of effect on NF- $\kappa$ B signaling pathway in HPMECs. **Results** ① In the control group, NF- $\kappa$ B was mostly located in the cytosol as shown by immunofluorescence. Treatment of HPMECs with LPS significantly increased the translocation of NF- $\kappa$ B from the cytosol to nucleus. UFH suppressed LPS-induced NF- $\kappa$ B activation both in 1 kU/L and 10 kU/L dosages, and 10 kU/L UFH gave even better results. ② Compared with control group, the levels of IL-8 and MCP-1 in the supernatants in LPS challenge group were significantly increased at 3 hours and 6 hours after LPS challenge [IL-8 (ng/L):  $387.1 \pm 26.4$  vs.  $23.8 \pm 8.1$  at 3 hours,  $645.5 \pm 69.6$  vs.  $125.7 \pm 18.7$  at 6 hours; MCP-1 (ng/L):  $3654.9 \pm 467.9$  vs.  $721.6 \pm 61.3$  at 3 hours,  $8178.5 \pm 792.6$  vs.  $1324.7 \pm 148.7$  at 6 hours, all  $P < 0.05$ ]. Compared with that of LPS challenge group, in 1 kU/L and 10 kU/L UFH pretreatment groups, the levels of IL-8 and MCP-1 were significantly decreased [IL-8 (ng/L):  $315.3 \pm 24.8$ ,  $275.8 \pm 31.1$  vs.  $387.1 \pm 26.4$  at 3 hours,  $557.8 \pm 43.3$ ,  $496.9 \pm 38.7$  vs.  $645.5 \pm 69.6$  at 6 hours; MCP-1 (ng/L):  $2924.1 \pm 267.9$ ,  $2668.3 \pm 522.6$  vs.  $3654.9 \pm 467.9$  at 3 hours,  $7121.7 \pm 557.2$ ,  $6563.9 \pm 576.4$  vs.  $8178.5 \pm 792.6$  at 6 hours, all  $P < 0.05$ ]. The results indicated that 10 kU/L UFH yielded better results. However, inhibition study using the known NF- $\kappa$ B inhibitor TLCK could decrease LPS-induced increase in IL-8 and MCP-1 levels [IL-8 (ng/L):  $162.4 \pm 21.3$  vs.  $387.1 \pm 26.4$  at 3 hours,  $274.1 \pm 22.6$  vs.  $645.5 \pm 69.6$  at 6 hours; MCP-1 (ng/L):  $1478.2 \pm 138.5$  vs.  $3654.9 \pm 467.9$  at 3 hours;  $3667.6 \pm 259.4$  vs.  $8178.5 \pm 792.6$  at 6 hours, all  $P < 0.05$ ]. **Conclusions** The levels of IL-8 and MCP-1 were increased obviously in LPS treated HPMECs. UFH might suppress LPS-activated NF- $\kappa$ B signaling pathway, contributing to the inhibitory effects of chemokines in HPMECs.

**【Key words】** Endothelial cell; Unfractionated heparin; Nuclear factor- $\kappa$ B; Interleukin-8; Monocyte chemoattractant protein-1

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脓毒症是宿主防御系统对入侵的病原微生物作出的过度全身反应<sup>[1]</sup>,在炎症因子作用下,一方面可激活中性粒细胞等炎性细胞,促进其在内皮上滚动、黏附,并进一步促进其释放炎症因子,同时内皮细胞得到活化,由正常抗凝表面变成促凝表面,促进微血栓形成;另一方面单核细胞活化释放组织因子增多,激活体内凝血系统,导致凝血活化。炎症与凝血相互作用,形成恶性循环,最终导致多器官功能障碍的发生<sup>[2-3]</sup>。大量实验及临床研究表明,微血栓形成和内皮细胞损伤是脓毒症发生发展成多器官功能障碍综合征(MODS)的两个关键环节<sup>[4-5]</sup>。在内皮细胞功能障碍的发生中,活化的炎性细胞起到了重要作用,其中白细胞在内皮细胞表面滚动、黏附、游走并趋化到炎症组织是感染过程和机体免疫反应的关键,趋化因子可调控此过程<sup>[6]</sup>。白细胞介素-8(IL-8)和单核细胞趋化蛋白-1(MCP-1)是两种具有代表性的趋化因子,分别趋化中性粒细胞和单核细胞<sup>[7]</sup>。核转录因子- $\kappa$ B(NF- $\kappa$ B)调控400多种基因的转录,在炎症、凋亡、增殖、免疫调节等过程中发挥核心作用<sup>[8-10]</sup>。肝素作为传统的抗凝剂,具有抗凝、抗炎、抗凋亡等多种生物学作用,可能成为

治疗脓毒症的新亮点<sup>[11-18]</sup>。本研究通过观察肝素对脂多糖(LPS)刺激人内皮细胞趋化因子水平的影响,探讨肝素对NF- $\kappa$ B信号通路的可能作用,以寻求肝素在临床脓毒症患者中应用的理论依据。

## 1 材料与方 法

**1.1 主要实验材料及试剂:**人肺微血管内皮细胞株(HPMEC)购自上海拜力生物技术有限公司;LPS(O55:B5,美国Sigma公司);胎牛血清、DMEM培养基(美国HyClone公司);人IL-8和MCP-1酶联免疫吸附试验(ELISA)试剂盒购于美国R&D公司;抗NF- $\kappa$ B多克隆抗体购于美国Cell Signaling公司;其余均为国产分析纯试剂。

**1.2 细胞培养:**HPMEC细胞培养于含10%胎牛血清的DMEM培养基中,置于37℃、5%CO<sub>2</sub>饱和湿度培养箱中培养,1~2d换液1次,直至90%融合,取第3~5代细胞用于实验。

**1.3 细胞分组及处理方法:**按照 $1 \times 10^9$ /L密度将HPMEC接种于6孔板,每孔2mL,过夜达70%融合后将细胞分为对照组、LPS刺激组、1kU/L或10kU/L肝素+LPS组、NF- $\kappa$ B抑制剂甲苯磺酰赖氨酸氯甲酮(TLCK)组(TLCK+LPS组)。LPS刺激组

加入 10 mg/L LPS;不同剂量肝素预处理组于 LPS 刺激前 15 min 应用既定剂量普通肝素预处理;TLCK+LPS 组于 LPS 刺激前 30 min 加入 TLCK 10 μmol/L;对照组加入与 LPS 等量的磷酸盐缓冲液(PBS)。

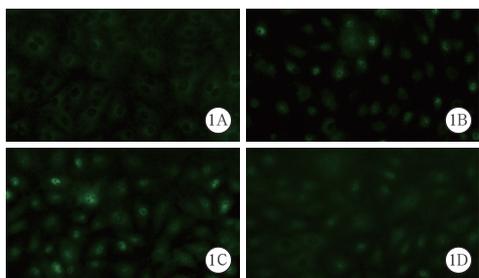
**1.4 免疫荧光观察:**将生长良好的细胞传代后种植于玻片上,待细胞融合成单层时用于实验。各组于 LPS 刺激 1 h 后应用 2% 多聚甲醛固定细胞,0.1% 曲通 100(Triton X-100)打孔,1% 牛血清蛋白(BSA)封闭后,依次加入 NF-κB p65 一抗(1:500 稀释)和异硫氰酸荧光素(FITC)标记的二抗(1:1000 稀释)。荧光显微镜下观察 NF-κB 活化情况,并采集图片,荧光标记的 NF-κB 呈亮绿色。

**1.5 趋化因子测定:**各组分别于 LPS 刺激后 3 h 和 6 h 收集细胞上清液,1000 r/min(离心半径 3 cm)离心 20 min 去除细胞碎屑,应用 ELISA 法测定上清液中 IL-8 和 MCP-1 的表达。操作严格按照试剂盒说明书进行,每组样本设 3 个复孔。

**1.6 统计学处理:**采用 SPSS 13.0 统计软件,计量资料以均数 ± 标准差( $\bar{x} \pm s$ )表示,各组间比较采用单因素方差分析,两两比较方差齐时采用 LSD 法,方差不齐时采用非参数秩和检验。 $P < 0.05$  为差异有统计学意义。

**2 结果**

**2.1 肝素对 NF-κB 活化的影响(图 1):**荧光显微镜下显示,对照组 NF-κB 大部分位于胞质中;LPS 刺激 1 h 后 NF-κB 由胞质向胞核内转移,发生活化;而肝素预处理可抑制 NF-κB 活化,以 10 kU/L 肝素作用较好。



**图 1** 荧光显微镜下观察肝素预处理对脂多糖(LPS)刺激人肺微血管内皮细胞(HPMEC)1 h 后核转录因子-κB(NF-κB)活化的影响 磷酸盐缓冲液(PBS)对照组(A)可见 NF-κB(呈亮绿色)绝大部分位于胞质中;LPS 刺激组(B)NF-κB 由胞质向胞核内转移,大部分分布于胞核内;1 kU/L 肝素 + LPS 组(C)可见 NF-κB 部分分布于胞质,部分分布于胞核;10 kU/L 肝素 + LPS 组(D)NF-κB 核移位的抑制作用更明显 免疫荧光 低倍放大

**2.2 肝素对 IL-8、MCP-1 蛋白水平的影响以及对 NF-κB 信号通路的作用(表 1):**LPS 刺激 HPMEC 后,IL-8 及 MCP-1 的蛋白水平均较对照组明显增高(均  $P < 0.05$ ),且随刺激时间的延长逐渐上调,6 h 达到高峰。肝素预处理可降低 IL-8、MCP-1 的水平,以 10 kU/L 肝素作用较好(均  $P < 0.05$ )。加入 NF-κB 抑制剂 TLCK 10 μmol/L 预处理后,IL-8 及 MCP-1 蛋白水平较 LPS 刺激组明显降低(均  $P < 0.05$ )。表明 LPS 可能通过调节 NF-κB 信号通路诱导内皮细胞损伤及趋化因子释放。

**3 讨论**

脓毒症时除炎性因子参与外,趋化因子在调控中性粒细胞活化和游走过程中也起关键作用。趋化因子是一个大家族,根据氨基酸序列前 2 个半胱氨酸残基位置的不同,主要分成 α 趋化因子(CXC)家族和 β 趋化因子(CC)家族两大类<sup>[19]</sup>。CXC 家族的代表是 IL-8,主要作用于中性粒细胞;CC 家族的代表是 MCP-1,主要作用于单核细胞。早期研究表明,内皮细胞是释放 IL-8 的主要细胞,在炎症、感染及应激等情况下其水平明显增高<sup>[19-21]</sup>。MCP-1 是最重要的趋化因子,可调节单核/巨噬细胞的迁移和浸润,主要参与脓毒症过程中一系列细胞事件,从而严重损害血管内皮及其周围组织<sup>[22-23]</sup>;有研究发现其水平可以准确预测脓症患者 28 d 病死率,与患者预后密切相关<sup>[24]</sup>。MCP-1、IL-6、IL-8 及肿瘤坏死因子-α(TNF-α)是参与脓毒症过程的主要炎性因子,是反映血管炎症的标志物<sup>[25]</sup>。本课题组前期研究证实,体外 LPS 刺激内皮细胞损伤后,

**表 1** 肝素预处理对 LPS 刺激 HPMEC 后各时间点 IL-8 和 MCP-1 蛋白水平的影响以及对 NF-κB 信号通路的作用( $\bar{x} \pm s$ )

组别	样本数(孔)	IL-8 (ng/L)		MCP-1 (ng/L)	
		3 h	6 h	3 h	6 h
PBS 对照组	3	23.8 ± 8.1	125.7 ± 18.7	721.6 ± 61.3	1324.7 ± 148.7
LPS 刺激组	3	387.1 ± 26.4 <sup>a</sup>	645.5 ± 69.6 <sup>a</sup>	3654.9 ± 467.9 <sup>a</sup>	8178.5 ± 792.6 <sup>a</sup>
1 kU/L 肝素 + LPS 组	3	315.3 ± 24.8 <sup>b</sup>	557.8 ± 43.3 <sup>b</sup>	2924.1 ± 267.9 <sup>b</sup>	7121.7 ± 557.2 <sup>b</sup>
10 kU/L 肝素 + LPS 组	3	275.8 ± 31.1 <sup>b</sup>	496.9 ± 38.7 <sup>bc</sup>	2668.3 ± 522.6 <sup>b</sup>	6563.9 ± 576.4 <sup>bc</sup>
TLCK+LPS 组	3	162.4 ± 21.3 <sup>b</sup>	274.1 ± 22.6 <sup>b</sup>	1478.2 ± 138.5 <sup>b</sup>	3667.6 ± 259.4 <sup>b</sup>
F 值		61.281	106.442	378.364	411.817
P 值		0.000	0.005	0.001	0.000

注: LPS 为脂多糖,HPMEC 为人肺微血管内皮细胞,IL-8 为白细胞介素-8,MCP-1 为单核细胞趋化蛋白-1,NF-κB 为核转录因子-κB,PBS 为磷酸盐缓冲液,TLCK 为 NF-κB 抑制剂 N-苄基磺酰赖氨酸甲酯;与 PBS 对照组比较,<sup>a</sup> $P < 0.05$ ;与 LPS 刺激组比较,<sup>b</sup> $P < 0.05$ ;与 1 kU/L 肝素 + LPS 组比较,<sup>c</sup> $P < 0.05$

IL-6 等细胞因子表达增加,而肝素可减少炎症因子释放<sup>[12]</sup>。本研究通过观察 LPS 刺激下内皮细胞分泌两种具有代表性的趋化因子 IL-8 和 MCP-1 水平,并探讨肝素的保护作用,证实了趋化因子与细胞因子变化趋势一致,而肝素可抑制趋化因子的表达。

NF- $\kappa$ B 是重要的转录因子,在介导细胞内信号转导过程中发挥重要作用<sup>[26]</sup>。多数细胞中 NF- $\kappa$ B 以 p65/p50 二聚体形式存在,在生理状态下, NF- $\kappa$ B 与其抑制剂 I $\kappa$ B 结合形成不具有活性的三聚体存在于胞质中;而在 LPS 的刺激下, I $\kappa$ B 发生磷酸化,与 NF- $\kappa$ B 解离,进而 NF- $\kappa$ B 以活性形式进入细胞核内,与特定靶基因启动子区的相应位点结合,发挥转录活性,调控下游基因的表达,从而参与一系列炎症过程<sup>[27-28]</sup>。为了进一步研究肝素发挥内皮细胞保护作用的机制,本研究观察了 LPS 刺激下内皮细胞 NF- $\kappa$ B 的活化情况。结果表明, LPS 刺激下 NF- $\kappa$ B 从胞质向胞核移位;而肝素预处理可明显抑制 NF- $\kappa$ B 核移位,其中以 10 kU/L 肝素效果较好。

本研究结果还显示,应用 NF- $\kappa$ B 抑制剂 TLCK 后可明显抑制 LPS 刺激内皮细胞 IL-8 和 MCP-1 表达,结合肝素对 NF- $\kappa$ B 活化的影响,表明 LPS 刺激 HPMEC 表达 IL-8 和 MCP-1 受到 NF- $\kappa$ B 调控。

综上所述,研究表明,肝素可能通过 NF- $\kappa$ B 信号通路抑制趋化因子 IL-8 和 MCP-1 的释放,从而为应用肝素治疗脓毒症提供了理论依据。

## 参考文献

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