

补体旁路激活产物刺激内皮细胞NF- κ B、p38MAPK、JAK2通路活化及抑制剂的干预研究

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摘要 研究补体旁路激活产物刺激人微血管内皮细胞NF- κ B、p38MAPK、JAK2通路活化的作用及相关抑制剂对其活化的干预作用。采用眼镜蛇毒因子(cobra venom factor, CVF)特异激活血清补体旁路。将旁路活化的血清作用于人微血管内皮细胞, 通过ELISA测定NF- κ B、p38MAPK及JAK2的活化情况, 分别采用上述3个通路的抑制剂PDTC、SB203580、AG490干预其活化及黏附分子ICAM-1的表达。结果显示, 补体旁路激活产物能导致人微血管内皮细胞JAK2、p38MAPK、NF- κ B通路活化, 分别在刺激2, 15, 30 min后, 分别检测到3个通路的活化高峰; PDTC、SB203580、AG490可分别抑制NF- κ B、p38MAPK、JAK2的活化。AG490对内皮细胞活化后的ICAM-1蛋白表达有明显的抑制作用, PDTC、SB203580对ICAM-1表达没有明显影响。表明AG490对JAK2通路的抑制可有效下调内皮细胞ICAM-1的表达, 提示JAK2通路有可能是补体相关炎症的潜在干预靶点。

关键词 补体; 补体旁路途径; 内皮细胞; 核转录因子; 丝裂原活化蛋白激酶; Janus激酶; 细胞间黏附分子; 眼镜蛇毒因子

Activation of NF- κ B, p38MAPK, and JAK2 in Endothelial Cells Induced by Activated Complement Alternative Pathway and Intervention by Inhibitors

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Abstract The activation of NF- κ B, p38MAPK, and JAK2 in human microvascular endothelial cells induced by activated complement alternative pathway and intervention by inhibitors were investigated in this paper. Cobra venom factor (CVF) was used to activate normal human serum. The activation of NF- κ B, p38MAPK, and JAK2 were measured by ELISA method after exposure of human microvascular endothelial cells to activated complement of the alternative pathway. The effect of PDTC, SB203580, and AG490 on inhibiting activation of NF- κ B, p38MAPK and JAK2, and the expression of ICAM-1 were measured after pretreated with endothelial cells. The results showed that JAK2, p38MAPK, and NF- κ B were activated after exposure of human microvascular endothelial

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cells to activated complement. The maximum activation of JAK2, p38MAPK, and NF- κ B was arrived after cells exposed to activated serum complement for 2, 15, 30 min, respectively. The activation of JAK2, p38MAPK, and NF- κ B was inhibited by AG490, SB203580, and PDTC, respectively. AG490 significantly inhibited the expression of ICAM-1 in endothelial cells induced by activated complement alternative pathway, whereas PDTC and SB203580 had no effect. Inhibition of JAK2 by AG490 effectively down-regulated the expression of ICAM-1, indicates that JAK2 may be a potential target for intervention in inflammation induced by complement.

Key words complement; complement alternatial pathway; endothelial cell; NF- κ B; p38MAPK; JAK2; ICAM-1; cobra venom factor

血管内皮细胞不仅是血管平滑肌与血液的物理屏障, 还具有重要的生理调节功能, 与诸多疾病的发生、发展有密切的关系^[1]。一系列病理生理刺激会导致内皮细胞的活化和损伤, 其中, 补体是重要的损伤因素^[2], 尤其是补体旁路途径的过度激活在某些病症和病理损伤中起着重要的作用^[3]。补体旁路激活会导致内皮细胞活化, 从而导致可能的炎症和损伤^[4-5]。我们之前的研究表明, 补体旁路激活产物会导致人微血管内皮细胞的活化和损伤^[6]。但对于补体旁路激活产物对人微血管内皮细胞中炎症相关通路的作用目前仍不清楚。为进一步认识补体旁路激活对内皮细胞的作用及其与病变及病理损伤的相关性, 为临床药物干预及相关新药研究提供参考, 本文进行了补体旁路激活产物刺激人微血管内皮细胞NF- κ B、p38MAPK、JAK2通路活化的作用及相关抑制剂的干预研究。

1 材料与方法

1.1 材料

人微血管内皮细胞株HMEC由本实验室传代培养; RPMI-1640培养基购自GIBCO; 胎牛血清(fetal bovine serum, FBS)为天津灏洋生物产品; 眼镜蛇毒因子(cobra venom factor, CVF)的制备和激活补体活性测定同文献^[7]; 正常人血清(normal human serum, NHS)由本实验室健康志愿者献血制备, 分装后于-80 °C冻存备用; 灭活人血清(inactivated normal human serum, INHS)由NHS 56 °C孵育30 min而得; 人P-selectin、ICAM-1 ELISA试剂盒购自武汉博士德生物工程有限公司; 核因子- κ B(NF- κ B)抑制剂吡咯烷二硫氨基甲酸(pyrrolidinedithiocarbamate, PDTC)、脂多糖(lipopolysaccharide, LPS; *Escherichia coli* O55:B5)购自美国Sigma公司; 丝裂原活化蛋白激酶(mitogen-activated protein kinase, MAPK)抑制剂

SB203580、Janus激酶2(JAK2)抑制剂AG490购自江苏碧云天生物研究所; NF- κ B p65、p-JAK2、p-P38 MAPK ELISA试剂盒为上海达为科生物有限公司产品; 其余试剂均为符合实验要求的进口或国产试剂。

1.2 仪器

Forma 3111型CO₂培养箱(美国Thermo公司); Nikon TS100倒置相差显微镜(日本Nikon公司); Milli Q超纯水系统和Elix纯水系统(美国Millipore公司); Molecular Devices Spectra MAX-190连续波长酶标仪(美国MD公司); 5810R冷冻离心机(德国Eppendorf公司); Revco超低温冰箱(美国Thermo公司)。

1.3 方法

1.3.1 内皮细胞培养 人微血管内皮细胞株HMEC由本实验室传代培养, 用含20%胎牛血清的RPMI-1640培养基在37 °C, 5% CO₂饱和湿度培养箱内培养, 收集对数生长期细胞进行实验。

1.3.2 血清活化 参照前期已发表的文献^[6]的方法, 将NHS与CVF(6.5×10^4 U/L)等体积混合, 在37 °C水浴孵育30 min, 将孵育产物(CVF-activated complement, 以下简称CAC)分装, -80 °C冻存备用。实验以INHS与CVF的孵育混合物作为对照。

1.3.3 补体旁路激活产物诱导内皮细胞的活化 将HMEC以 1×10^4 /孔, 接种于96孔细胞培养板, 培养24 h后弃上清, 加入60 μ L CAC, 用无血清RPMI-1640培养基补足至200 μ L, 分别在不同时间取培养上清按试剂盒说明书测定P-selectin、ICAM-1。具体检测方法如下: 取100 μ L稀释的样品于已包被的酶标板中, 37 °C反应90 min后加入100 μ L抗体工作液, 37 °C反应60 min后用PBS洗板3次, 加入亲和素-过氧化物酶复合物工作液100 μ L, 37 °C反应30 min后用PBS洗板5次, 加入90 μ L TMB显色液, 37 °C避光反应25~30 min后加入终止液, 用酶标仪在450 nm处测定吸光度(D)值。根据D值及标准曲线计算所测样品浓度, 再乘

以稀释倍数,即为所取样品的实际浓度。

1.3.4 补体旁路激活产物刺激内皮细胞NF- κ B、p38MAPK、JAK2通路活化的检测 将HMEC以 1×10^4 /孔接种于96孔细胞培养板,培养24 h后弃上清,加入60 μ L CAC,用无血清RPMI-1640培养基补足至200 μ L,各组分别刺激不同时间后弃上清,加入胰酶在20 $^{\circ}$ C条件下快速消化2~3 min,在4 $^{\circ}$ C离心5 min,收集细胞,弃上清,加入50 μ L 4 $^{\circ}$ C的PBS悬浮细胞,将细胞悬液置于-80 $^{\circ}$ C快速冻存,将冻结的细胞置于12~15 $^{\circ}$ C水浴中快速化冻,反复冻融6次后在4 $^{\circ}$ C条件下3 000 r/min离心20 min收集上清,检测NF- κ B、p38MAPK、JAK2的活化情况。具体检测方法如下:取50 μ L稀释的样品于已包被的酶标板中,37 $^{\circ}$ C反应30 min后加入50 μ L酶标抗体,37 $^{\circ}$ C反应30 min后用PBS洗板3次,加入50 μ L显色液A和50 μ L显色液B,37 $^{\circ}$ C避光反应15 min后加入终止液,用酶标仪在450 nm处测定吸光度(D)值。根据D值及标准曲线计算所测样品浓度,再乘以稀释倍数,即为所取样品的实际含量。实验同时设正常细胞组、阳性对照组,其中阳性对照LPS终浓度为100 μ g/L。

1.3.5 抑制剂对补体旁路激活产物刺激内皮细胞NF- κ B、p38MAPK、JAK2活化的抑制 将HMEC以 1×10^4 /孔接种于96孔细胞培养板,培养24 h后弃上清,分别加入含不同浓度抑制剂的无血清RPMI-1640培养基140 μ L预处理细胞1 h,再加入60 μ L CAC,培养不同时间收集细胞,按试剂盒说明书裂解细胞后检测NF- κ B、p38MAPK、JAK2。取样及测定方法同上。

1.3.6 抑制剂对ICAM-1表达的影响 将对数生长

期HMEC以 1×10^4 /孔,接种于96孔细胞培养板,培养24 h后弃上清,加入含单一或不同抑制剂组合的无血清RPMI-1640培养基140 μ L预处理细胞1 h,再加入60 μ L CAC,培养4 h后取培养上清测定ICAM-1。测定方法同上。

1.4 统计学分析

实验数据用 $\bar{x} \pm s$ 表示,采用SPSS16.0软件进行单因素方差分析, $P < 0.05$ 有统计学意义。

2 结果

2.1 补体旁路激活产物对内皮细胞黏附分子表达的影响

补体旁路活化能诱导内皮细胞表达P-selectin和ICAM-1明显增加(图1)。

2.2 补体旁路激活产物对NF- κ B、p38MAPK、JAK2通路的活化

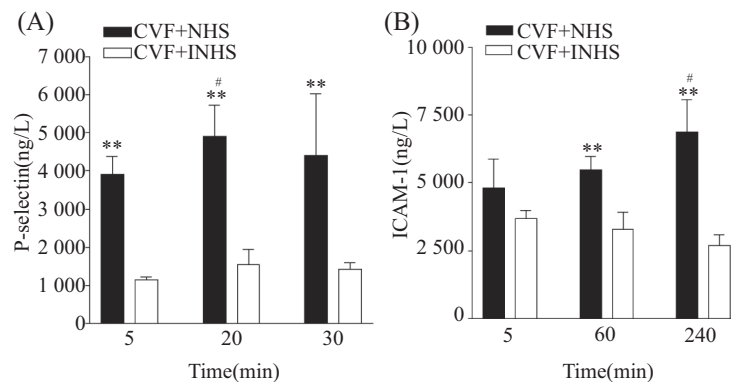
补体旁路激活产物能诱导内皮细胞JAK2、p38MAPK、NF- κ B通路活化。分别在刺激2, 15, 30 min后,分别检测到JAK2、p38MAPK、NF- κ B通路的活化高峰(图2)。

2.3 抑制剂对内皮细胞NF- κ B、p38MAPK、JAK2通路活化的抑制

抑制剂对NF- κ B、p38MAPK、JAK2通路的活化有一定的抑制作用(图3)。

2.4 抑制剂对内皮细胞黏附分子表达的影响

PDTC和SB203580对ICAM-1的表达无明显影响(图4A和4B)。AG490对ICAM-1有一定的抑制作用(图4C)。三种抑制剂合用时对ICAM-1蛋白表达的抑制作用最强(图4D)。

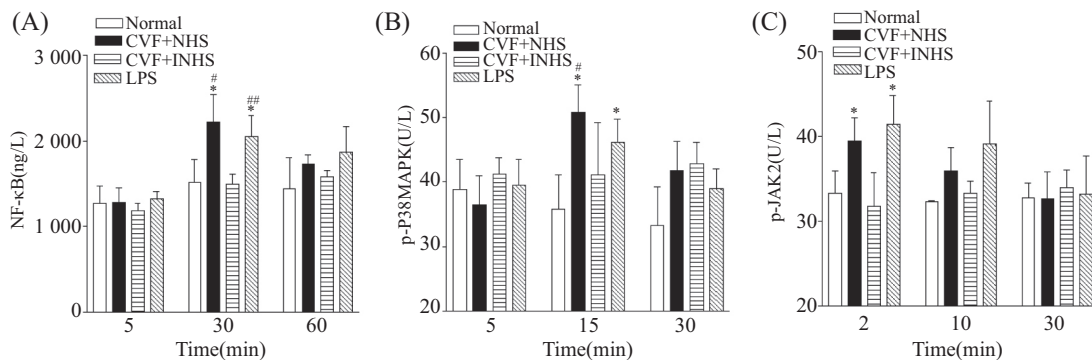


A: P-selectin; B: ICAM-1. # $P < 0.05$, 与5 min组相比; ** $P < 0.01$, 与对照组相比。n=4。

A: P-selectin; B: ICAM-1. # $P < 0.05$ vs 5 min group, ** $P < 0.01$ vs control group. n=4。

图1 补体旁路激活产物对内皮细胞黏附分子表达的影响

Fig.1 Expression of P-selectin and ICAM-1 after exposure of endothelial cells to activated complement for various times

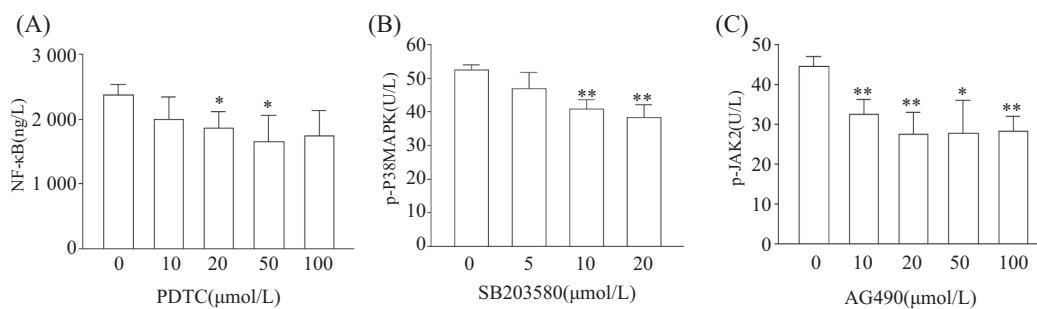


A: NF-κB; B: p38MAPK; C: JAK2. #*P*<0.05, ##*P*<0.01, 与5 min组相比; **P*<0.05, 与正常细胞组相比。n=3。

A: NF-κB; B: p38MAPK; C: JAK2. #*P*<0.05, ##*P*<0.01 vs 5 min group, **P*<0.05 vs normal group. n=3。

图2 补体旁路激活产物对内皮细胞NF-κB、p38MAPK、JAK2通路的活化

Fig.2 Activation of NF-κB, p38MAPK, and JAK2 in endothelial cells after exposed to activated complement for various times

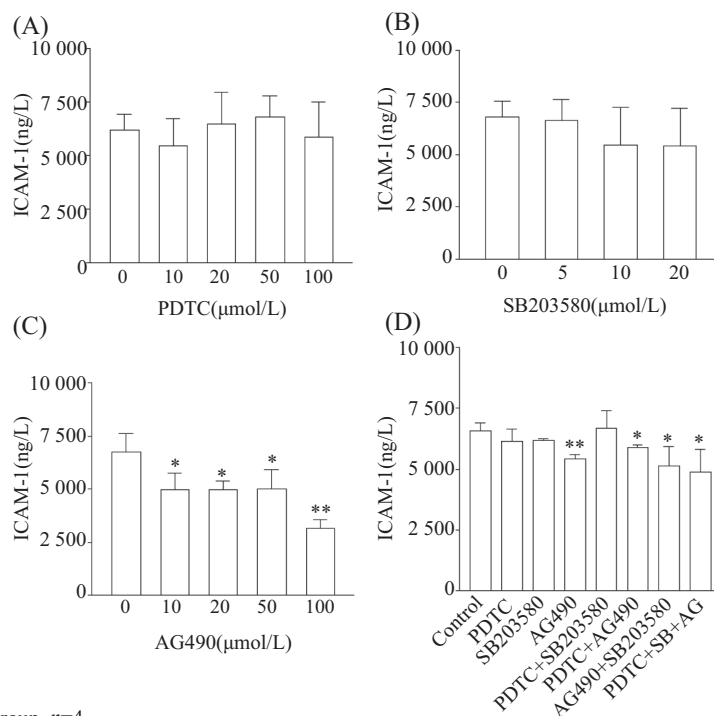


A: NF-κB; B: p38MAPK; C: JAK2. **P*<0.05, ***P*<0.01, 与0 μmol/L组相比。n=3。

A: NF-κB; B: p38MAPK; C: JAK2. **P*<0.05, ***P*<0.01 vs 0 μmol/L group. n=3。

图3 抑制剂对补体旁路激活产物导致的内皮细胞NF-κB、p38MAPK、JAK2通路活化的抑制

Fig.3 Effects of inhibitors on activation of NF-κB, p38MAPK, and JAK2 in endothelial cells after exposed to activated complement, respectively



P*<0.05, *P*<0.01 vs control group. n=4。

图4 抑制剂对补体旁路激活产物诱导的内皮细胞黏附分子表达的影响

Fig.4 Effects of inhibitors on expression of ICAM-1 in endothelial cells after exposed to activated complement

3 讨论

补体系统作为机体天然的防御屏障,参与特异和非特异性的免疫调节^[8]。补体系统的激活通过经典、旁路、凝集素途径。补体的过度激活,尤其旁路途径的过度激活能够导致内皮细胞的结构和功能发生变化,继而引发后续的炎症和损伤^[4-6]。本研究中,采用眼镜蛇毒因子CVF特异激活血清补体旁路。CVF是从眼镜蛇毒中分离纯化出来的一种高度特异的补体旁路激活蛋白,与补体C3b有高度的同源性。CVF在血清环境中与补体B因子特异结合,形成CVF·B复合物,CVF·B能被补体D因子特异识别和酶切,进而形成CVF·Bb。CVF·Bb具有旁路途径C3/C5转化酶的特异活性,可持续激活补体旁路^[9-10],产生C3a、C3b、C5a以及攻膜复合物等多种活化产物。这种旁路激活的方式和产物与体内病理生理条件下补体旁路的过度激活高度一致。

在本研究中,补体旁路激活产物刺激内皮细胞后,导致JAK2、p38MAPK、NF-κB通路活化。分别在刺激2, 15, 30 min后,分别检测到JAK2、p38MAPK、NF-κB通路的活化高峰。从结果可以看到,补体旁路激活产物对HMECJAK2、p38MAPK、NF-κB通路的活化与阳性对照LPS的作用是类似的。与文献报道的LPS激活人脐静脉内皮细胞(HUVEC)JAK2、p38MAPK、NF-κB通路的活化效果也是类似的^[11-12]。上述情况提示,虽然补体旁路激活产物与LPS刺激内皮细胞的机制不同,但效应却可能是相似的,且不同来源的内皮细胞HMEC和HUVEC在受到相同刺激后JAK2、p38MAPK、NF-κB通路活化的作用也是类似的。

之前研究表明,补体旁路激活产物刺激内皮细胞后可导致内皮细胞出现典型的I型活化和II型活化,表现为P-selectin、E-selectin、ICAM-1等表达上调^[6]。本实验用补体旁路激活产物刺激HMEC后,观察到NF-κB、p38MAPK及JAK2通路活化。NF-κB、p38MAPK及JAK2是细胞内重要的信号通路,在炎症的发生发展中起着重要的作用^[13-15],与炎症相关因子如IL-8、MCP-1等的转录表达密切相关^[16-17]。本研究提示,补体旁路激活产物刺激内皮细胞活化和相关炎症因子转录表达与NF-κB、p38MAPK及JAK2通路有关。因此,在进一步的实验中我们研究了NF-κB抑制剂PDTC、p38MAPK抑制剂SB203580、JAK2抑制剂AG490对上述通路活化及黏附分子ICAM-1蛋白表达的干预

作用。结果表明,AG490、PDTC和SB203580分别对JAK2、NF-κB、p38MAPK通路活化有抑制作用,但只有AG490能抑制ICAM-1蛋白的表达,提示JAK2通路与ICAM-1表达密切相关。PDTC、SB203580对补体旁路激活诱导的内皮细胞ICAM-1表达无明显影响。目前,NF-κB通路抑制剂PDTC对炎症相关黏附分子ICAM-1的表达的抑制作用尚存在争议。有文献报道,PDTC能抑制高半胱氨酸诱导的HUVEC的NF-κB的活化,并能抑制ICAM-1的表达^[18]。也有文献报道,射线刺激内皮细胞ICAM-1蛋白表达增加,PDTC并不能抑制此条件下的ICAM-1的增加^[19]。在本实验条件下PDTC虽能抑制NF-κB的活化,但不能阻断ICAM-1蛋白的表达。提示NF-κB虽然是炎症反应的中心环节,但由于炎症诱发因素的多样性和应答调控的复杂性,对信号通路本身活化的有效抑制并不一定能抑制或阻断下游靶标基因的转录调控。这也说明炎症相关的细胞信号通路在炎症反应中的调控是错综复杂的,进一步揭示这种网络调控的机制将有助于加深对炎症的认识和理解。

本研究工作表明,补体旁路激活产物会导致内皮细胞JAK2、p38MAPK、NF-κB通路发生活化。其中,JAK2通路及ICAM-1的转录表达密切相关,对其活化的有效调控和抑制可能是补体相关炎症的潜在有效的干预策略。

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