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和厚朴酚抑制线粒体可溶蛋白诱导小胶质细胞活化的体外实验研究*

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摘要 目的:探讨线粒体可溶蛋白诱导小胶质细胞活化作用及和厚朴酚对小胶质细胞活化的影响。**方法:**培养 BV2 小胶质细胞, 分空白对照组(control)、线粒体可溶蛋白组(MDP)、和厚朴酚干预组(HNK)。ELISA 检测不同浓度线粒体可溶蛋白(MSP)刺激不同时间的细胞上清液中 IL-6 和 TNF- α 含量; RT-PCR 半定量法测定各组转录因子 Klf4 的表达情况; 倒置相差显微镜观察各组细胞形态变化。**结果:** 1. ELISA: MSP (10 μ g/ml, 100 μ g/ml) 处理 7h 时, IL-6 和 TNF- α 含量与 control 组比较显著升高 ($P < 0.05$); 100 μ g/ml MSP 处理 1、3、5、7h 时, IL-6 和 TNF- α 表达与 control 组比较明显升高 ($P < 0.05$), 且 7h 时 HNK 组含量明显低于 MDP 组 ($P < 0.05$)。2. RT-PCR 结果显示 MDP 组 Klf4 的表达显著高于空白对照组和 HNK 组 ($P < 0.05$)。3. 倒置相差显微镜观察空白组胶质细胞形态呈静息状态; MDP 组小胶质细胞的胞体变圆或者椭圆, 突起消失, 呈“阿米巴”状; HNK 组活化状态的小胶质细胞明显减少。**结论:** 线粒体可溶蛋白可以激活小胶质细胞, 促进白细胞介素 IL-6 和 TNF- α 的释放, 和厚朴酚能够有效地抑制线粒体可溶蛋白诱导的小胶质细胞活化, 其机制可能是通过下调 Klf4 的表达发挥作用。

关键词: 小胶质细胞活化; 线粒体可溶蛋白; Klf4; 和厚朴酚

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Honokiol Inhibiting Microglia Activation Induced by MDP in vitro Experimental Study*

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ABSTRACT Objective: To investigate the activation of microglia induced by MSP and the activated process affected by Honokiol.

Methods: Mice BV2 microglia in logarithmic growth phase were randomly divided into 3 groups. (1) control group: the cells were cultured with serum free medium; (2) MDP group: the cells were treated with MDP after cultured for 24 h. (3) HNK group: after pretreated with Honokiol for 30 min, MDP was added in the cells. ELISA: The concentration of IL-6 and TNF- α in culture supernatant of these group were determined; Semi-quantity RT-PCR method was used to analyze the dynamic expression of transcription factor Klf4 in different groups; Cellular morphological changes were observed under phase-contrast microscope. **Results:** ELISA: MSP (10 μ g/ml, 100 μ g/ml) treated for 7h. Compared with the control group, IL-6 and TNF- α content in cell supernatant have statistical significance ($P < 0.05$), suggesting microglia activation have certain concentration dependent manner; MSP (100 μ g/ml) treated for 1, 3, 5, 7 h. Compared with control group, IL-6 and TNF- α content in cell supernatant have statistical significance at each time point ($P < 0.05$), suggesting microglia activation have certain time dependent manner; MPS (100 μ g/ml) treated for 7 h. Compared with the HNK group, IL-6 and TNF- α content in cell supernatant have statistical significance ($P < 0.05$), suggesting Honokiol could decrease the expression of IL-6 and TNF- α . (2) RT-PCR: Klf4 expression level raised obviously after stimulating of microglia by MSP, and honokiol could downregulate Klf4 expression. (3) Morphological observation: Under phase-contrast microscope, microglia in the control group were in resting state; microglia in the MDP group, the cell body became round, neurites disappeared, presenting "Amoeba" shape; The activation of microglia in HNK group was reduced obviously. **Conclusion:** 1. MSP can induce microglia activation, and promote IL-6 and TNF- α release, and its activation effect is in the manner of concentration and time dependence. 2. Honokiol can effectively inhibit the microglia activation induced by MSP, the possible mechanism is to down-regulate Klf4 expression of the microglia.

Key words: Microglia activation; MSP; Klf4; Honokiol

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前言

小胶质细胞是中枢神经系统(CNS)重要的固有免疫细胞,小胶质细胞通过与神经元突触的接触监测突触的功能状态,轻微的病理变化即能引起小胶质细胞活化^[1,2]。小胶质细胞的活化广泛参与了神经系统疾病的病理过程,Takeuchi H 等^[3]研究发现,增加小胶质细胞刺激因子,其周围的死亡神经细胞数目上升。严重损伤或损伤因素持续存在时,持续激活的小胶质细胞释放大炎症介质产生损伤作用从而参与神经功能紊乱的病理过程^[4]。因此抑制小胶质细胞过度活化对神经功能的修复与重建有重要意义。CNS 损伤后会释放大炎症相关分子模式刺激因子(DAMPs),包括半胱氨酰白三烯类物质、ATP 及线粒体等,刺激小胶质细胞活化。Mustafa Raoof 等^[5]报道线粒体可溶蛋白(MSP)作为一种损伤后释放的重要 DAMPs,可以激活中性粒细胞,而线粒体成分是否参与 CNS 损伤后小胶质细胞的激活,目前未见文献报道。小胶质细胞的激活是一个逐级进展、多种调节机制、多条途径参与的过程,目前还未完全明了。2010 年 Deepak Kumar Kaushik 等^[6]研究显示 Krüppel 样因子 4(Krüppel-like factor 4, Klf4)参与脂多糖(lipopolysaccharide, LPS)诱导的小胶质细胞的激活,通过和厚朴酚(Honokiol)调控 Klf4 表达,可以减少促炎因子的释放,减轻炎症反应,可能对神经系统起到保护作用。本实验首次以 MSP 刺激小胶质细胞,在该模型中给予 Honokiol 处理,检测细胞炎症因子和 Klf4 的表达,观察小胶质细胞活化情况,分析 Honokiol 和 Klf4 在 MSP 诱导小胶质细胞活化中的作用。

1 材料和方法

1.1 主要材料和仪器

小胶质细胞 BV2 购自中国医学科学院北京协和细胞资源中心。胎牛血清(FBS)购自杭州司机青生物工程有限公司;基础培养基(Hyclone DMEM/High Glucose)为赛默飞世尔生物化学制品有限公司(中国北京)产品;TNF- α ELISA 试剂盒和 IL-6 ELISA 试剂盒均为美国 R&D 公司产品;和厚朴酚购于美国 sigma 公司。RT-PCR 试剂盒和 DNA MarkerI 购于北京 TIANGEN 公司。

1.2 线粒体可溶性蛋白制备和浓度测定

取成年小鼠脑、肝组织,剪碎放入小容量玻璃匀浆器内,按 0.1 ml:100-200 mg 比例加入冰预冷的 Lysis Buffer, 0 °C 冰浴下研磨组织,4 °C 离心,得到线粒体沉淀。应用 Bradford 比色法测定线粒体可溶蛋白浓度。

1.3 小胶质细胞培养与分组

BV-2 细胞培养于完全培养基(10 %FBS-DMEM)内,置含 5 % CO₂ 的 37 °C 内培养。取处于对数生长期的 BV-2 细胞接种于培养板中培养 24 h,随机分为 3 组:(1)空白对照组:加无血清培养基;(2)MDP 组:接种 24 h 后分别行 1 μ g/ml、10 μ g/ml、100 μ g/ml 浓度 MSP 按 1、3、5、7 h 处理。(3)HNK 干预组:和厚朴酚孵育 30 min 后行 MSP 处理。

1.4 ELISA 检测

收集各组上清液,去除漂浮细胞等杂质,根据白细胞介素 IL-6 和 TNF- α 定量 ELISA 试剂盒说明书进行操作,检测 IL-6

和 TNF- α 浓度。

1.5 RT-PCR 方法半定量检测目的基因的表达

按 Trizol 法提组织总 RNA, RNA 逆转录生成 cDNA, PCR 扩增,从 NCBI EST Database 中下载大鼠 Klf-4 基因序列,采用 Primer5-Cracked 软件设计引物序列, Klf-4 基因引物:Forward primer: 5'-ATGAGGCAGCCACCTGGCGA-3'; Reverse primer: 5'-TTAAAAGTGCCTCTTCATGTGTAA-3'; Product length: 142bp。引物由北京鼎国昌盛生物技术有限公司合成(121218A66-93)。琼脂糖凝胶电泳,在 BIO-RAD UVP 凝胶成像分析系统下拍照保存,用该系统自带软件对 PCR 条带产物进行区带灰度分析,计算目的基因与其内参条带灰度比。

1.6 形态学观察

采用 100 μ g/ml MDP 处理细胞 7 h 时,将空白组、MDP 组、HNK 组培养板置于倒置相差显微镜下观察各组细胞形态变化,各组均取板孔正中视野进行观察并拍照。

1.7 结果统计及分析

数据以均数 $\pm$ 标准差($\bar{x} \pm s$)表示,采用 SPSS19.0 软件包进行统计学分析,各组数据采用单因素方差分析,两组间比较采用 t 检验,以 P<0.05 为差异有统计学意义。

2 实验结果

2.1 ELISA 检测各组细胞上清液中白细胞介素 IL-6 和 TNF- α 浓度结果:

MSP(1 μ g/ml, 10 μ g/ml, 100 μ g/ml)干预 7h 时上清液中白细胞介素 IL-6 和 TNF- α 含量:空白组和 1 μ g/ml 组间差异无统计学意义(P>0.05), 10 μ g/ml、100 μ g/ml 组与对照组差异有统计学意义(P<0.05);提示线粒体可溶蛋白激活小胶质细胞呈现一定的浓度依赖性(图 1、2)。MSP(100 μ g/ml)刺激 1、3、5、7h 时上清液中白细胞介素 IL-6 和 TNF- α 含量:随着时间延长呈逐渐升高的趋势,各组与对照组比较组间差异均有统计学意义(P<0.05);提示随着刺激时间的延长,小胶质细胞活化程度增强,说明线粒体可溶蛋白激活小胶质细胞呈现一定的时间依赖性(图 3、4)。MPS(100 μ g/ml)刺激 7h 时 MDP 组与 HNK 组上清液中白细胞介素 IL-6 和 TNF- α 含量差异有统计学意义(P<0.05);提示和厚朴酚能够抑制小胶质细胞的活化,降低了炎症介质的释放。(图 5、6)。

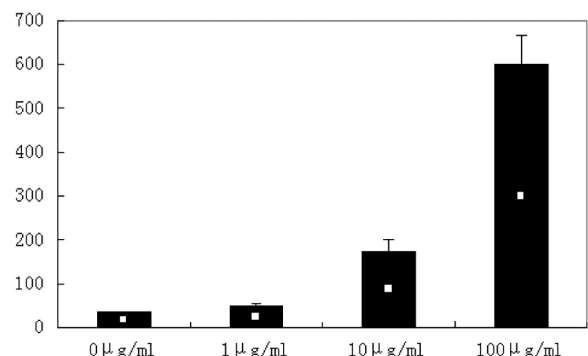
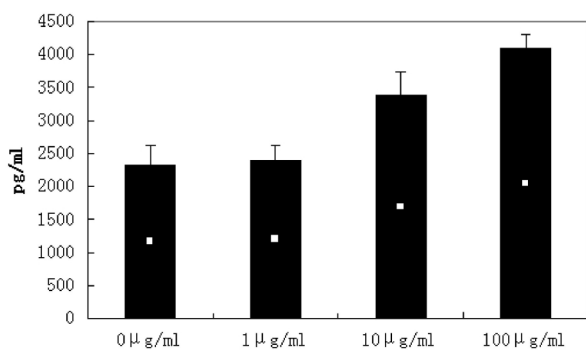
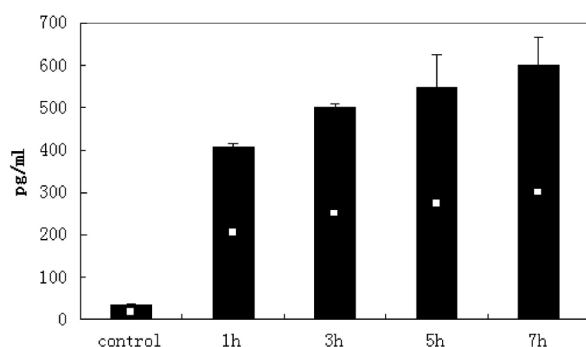
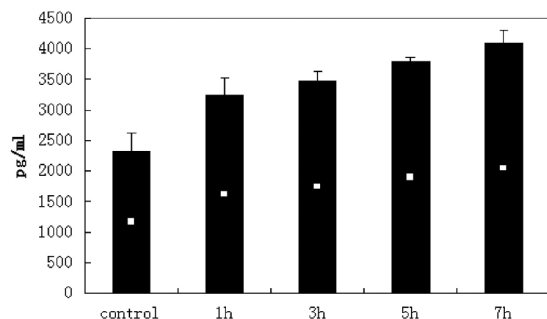
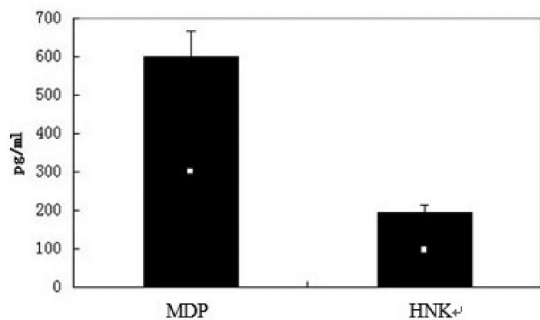
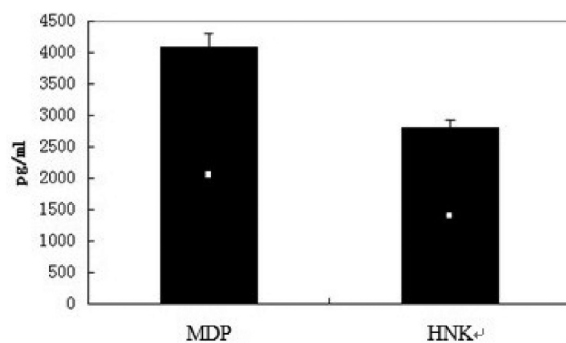


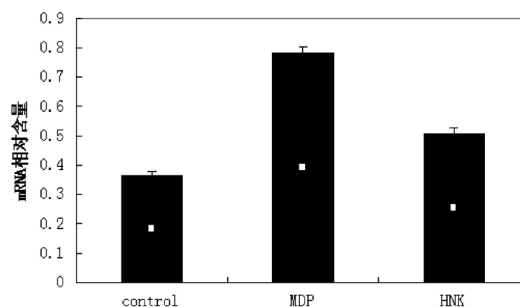
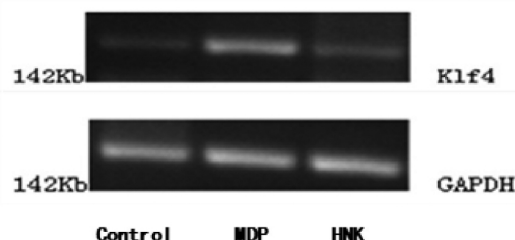
图 1 不同浓度 MSP 刺激上清液中 IL-6 浓度($\bar{x} \pm s$, n=3)

Fig. 1 IL-6 concentration in different MPS concentrations in the supernatant fluid ($\bar{x} \pm s$, n=3)

图2 不同浓度MSP刺激上清液中TNF-α浓度($\bar{x} \pm s$, n=3)Fig. 2 TNF alpha concentration in different MPS concentrations in the supernatant fluid($\bar{x} \pm s$, n=3)图3 不同时间点上清液中白细胞介素IL-6浓度($\bar{x} \pm s$, n=3)Fig. 3 IL-6 concentration at each time point in the supernatant fluid($\bar{x} \pm s$, n=3)图4 不同时间点上清液中TNF-α浓度($\bar{x} \pm s$, n=3)Fig. 4 TNF alpha concentration at each time point in the supernatant fluid($\bar{x} \pm s$, n=3)图5 MDP组与HNK组上清液中IL-6浓度($\bar{x} \pm s$, n=3)Fig. 5 IL-6 concentrations in the supernatant of MDP and HNK group($\bar{x} \pm s$, n=3)图6 MDP组与HNK组上清液中TNF-α浓度($\bar{x} \pm s$, n=3)Fig. 6 TNF alpha concentrations in the supernatant of MDP and HNK group($\bar{x} \pm s$, n=3)

2.2 QT-PCR 检测转录因子Klf4的表达情况

MSP(100 μg/ml)刺激7h时MDP组与control组比较差异有统计学意义,说明线粒体可溶蛋白刺激小胶质细胞后Klf4明显上调,而与HNK组比较组间差异也有统计学意义,提示和厚朴酚能下调活化的小胶质细胞Klf4表达(如图7)。

图7 各组细胞Klf4表达量($\bar{x} \pm s$, n=3)Fig. 7 The expression quantity of each cell Klf4($\bar{x} \pm s$, n=3)

2.3 BV2小胶质细胞形态变化

倒置相差显微镜观察到control组小胶质细胞呈相对静息状态,胞体较小,突起细而长;MDP组明显表现为突起回缩、突起少而短的灌木样细胞,胞体变圆,相对增大,数量增多,呈现激活状态,而HNK组活化状态的小胶质细胞明显减少。(如图8)。

3 讨论

机体主要通过病原体相关分子模式(pathogen associated-molecular patten, PAMP)和损伤相关分子模式(damage associated molecular pattern, DAMP)来识别各种内、外环境变化的刺激,其中PAMP识别病原微生物的刺激,由免疫系统负责,DAMP主要是识别除病原微生物以外的刺激信号,由感觉系统感知,神经系统、内分泌系统和免疫系统共同完成。以往大量的

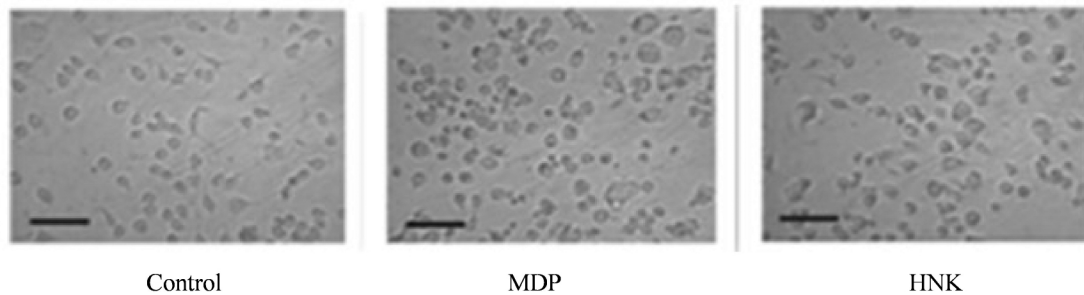


图 8 各组小胶质细胞形态变化(200×)

Fig. 8 Morphological changes of microglial cells(200×)

CNS 系统免疫性疾病、神经退行性疾病、感染性疾病体外实验研究中广泛应用以 LPS 诱导小胶质细胞活化。LPS 是革兰氏阴性细菌细胞壁中的一种成分,主要启动的是 PAMP,是研究小胶质细胞活化和 CNS 退行性、免疫性、感染性病变的典型激动剂。

小胶质细胞广泛分布在大脑和脊髓中,占其胶质细胞总数的 5%-20%,密切监控 CNS 中微环境的变化^[79],CNS 系统外伤性病变尤其是脊髓的原发性损伤能够在数分钟后造成局部释放大量的 DAMPs,刺激小胶质细胞活化,激活 MG 会释放 IL-1、TNF- α 、IL-6 等细胞因子,趋化因子,还可以发挥抗原提呈作用,有效的诱导 CNS 浸润 T 细胞增殖和分化,引起 CD⁴⁺ 的炎症免疫反应而加重神经系统损伤^[10]。并且激活的小胶质细胞产生的趋化因子和周围的星形胶质细胞及免疫系统之间直接的或间接地相互的信息交流,而他们所产生的细胞因子又进一步促进小胶质细胞活化,从而呈现瀑布效应^[11]。小胶质细胞的激活是一个逐级进展、多种调节机制参与的过程,可能通过以下几个途径被激活:(1) IKK (I- κ B kinase) NF- κ B 途径,可由炎性应激反应激活^[12];(2) JAK-STAT (Janus kinase-signal transducer and activation of transcription)途径,是一个 IL-6 下游靶信号经典的激活通路^[13];(3) MAPK (mitogen-activated protein kinase)途径等。目前认为由 IKK (I- κ B kinase)-NF- κ B 介导炎性应激激活是其重要激活途径^[12]。如何平衡参与小胶质细胞活化的作用点,充分发挥其保护功能,尽可能降低其损害作用,有助于提高 CNS 病变后的治疗效果。

Deepak K Kaushik 等^[6]证明, Klf4 基因敲除能够下调 NF- κ B 的表达,降低了 LPS 诱导的小胶质细胞活化程度,减少了炎症因子 IL-6、TNF- α 的释放。Klf4 是一个具有双重功能转录因子,既可以激活又可以抑制转录作用。2009 年,Moore 等人首次在体外证实了 Klf4 对神经元的轴突再生有很强的抑制作用^[14]。除调节神经元自身的再生能力外, Klf4 可能还参与了神经系统的炎症反应^[15]。通过和厚朴酚(Honokiol)调控 Klf-4 表达,减轻炎症反应,可能对神经系统起到保护作用。和厚朴酚,中药厚朴中提取分离而得的木脂酚类化合物^[16],近来的药理学研究表明其具有抗炎、抗氧化、抗抑郁和促进轴突再生的特性。

本实验证明线粒体可溶蛋白可以激活小胶质细胞,促进炎症细胞因子 TNF- α 、IL-6 的表达,并可明显上调 KLF4;和厚朴酚抑制了小胶质细胞的活化,可能的机制是通过下调转录因子 KLF4 的表达,降低了促炎细胞因子 TNF- α 、IL-6 的表达。这样 CNS 外伤性损伤早期释放线粒体成分,可能启动了 DAMP,产

生无菌性炎症,激活了小胶质细胞。所以线粒体可溶蛋白诱导小胶质细胞活化更为接近外伤性 CNS 疾病的病理变化过程。通过调控线粒体对小胶质细胞的过度活化,在一定程度上减轻受损神经炎症反应,可能是 CNS 病变早期控制炎症反应的一种策略。和厚朴酚可以抑制 CNS 病变后小胶质细胞的过度激活,减轻活化的小胶质细胞对神经组织造成的损害,且容易通过血脑屏障,提示在临床治疗 CNS 病变具有一定应用前景。

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