

白桦脂酸通过抗氧化应激作用抑制脂多糖诱导的血管收缩功能损伤

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摘要 白桦脂酸(betulinic acid, BA)有良好的抗心血管氧化应激损伤作用。然而,BA对脂多糖(lipopolysaccharide, LPS)诱导血管收缩功能损伤是否具有保护作用,该保护作用是否与抗氧化应激有关,尚不清楚。本研究给予雄性SD大鼠白桦脂酸灌胃(25 mg/kg/d, 3 d)预处理,于第4 d腹腔注射LPS(10 mg/kg),4 h后麻醉处死,分离血浆及胸主动脉,测定大鼠胸主动脉环收缩性,测定炎症因子白细胞介素6(interleukin-6, IL-6)及氧化应激指标。结果显示,白桦脂酸明显抑制LPS诱导的血浆及胸主动脉IL-6水平($P < 0.01$),降低LPS对苯肾上腺素、KCl及 Ca^{2+} 血管收缩反应的抑制作用($84.8\% \pm 9.09\%$ vs $42.80\% \pm 9.00\%$, $P < 0.01$; $127.48\% \pm 12.02\%$ vs $99.78\% \pm 6.02\%$, $P < 0.01$; $52.07\% \pm 13.48\%$ vs $20.83\% \pm 5.04\%$, $P < 0.01$),减少LPS诱导的胸主动脉丙二醛水平($P < 0.01$)及诱导型一氧化氮合酶(inducible nitric oxide synthase, iNOS)活性($P < 0.01$),缓解LPS对超氧化物歧化酶(superoxide dismutase, SOD)的抑制作用($P < 0.01$)。上述结果提示,白桦脂酸抑制LPS诱导血管收缩功能障碍的机制可能与增加机体SOD活性,抑制氧化应激及iNOS活性有关。

关键词 白桦脂酸; 脂多糖; 血管收缩; 氧化应激

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Betulinic Acid Reduces the Impairment of Aortic Contraction Induced by Lipopolysaccharide via Reducing Oxidative Stress

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Abstract Betulinic acid (BA) can reduce the level of cardiovascular oxidative stress. However, the roles of BA in lipopolysaccharide (LPS)-induced aortic contraction injury and its molecular mechanism remain unknown. In present study, male Sprague-Dawley rats were pretreated with BA (25 mg/kg/d, i. g.) for 3 days and intraperitoneally injected with LPS (10 mg/kg) at the 4th day. The rat was anesthetized and sacrificed by cervical dislocation after it was treated with LPS for 4 hours. The thoracic aorta was immediately dissected out to determine the contraction using the organ bath system. The inflammatory factor interleukin-6 (IL-6) and oxidative stress were measured in the aortic tissue and plasma. Results showed that IL-6 was significantly decreased by BA in plasma and thoracic aorta from LPS-treated rats ($P < 0.01$). The inhibition of phenylephrine, KCl and Ca^{2+} -produced aortic contraction

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by LPS was reduced by pretreatment with BA ($84.8\% \pm 9.09\%$ vs $42.80\% \pm 9.00\%$, $P < 0.01$; $127.48\% \pm 12.02\%$ vs $99.78\% \pm 6.02\%$, $P < 0.01$; $52.07\% \pm 13.48\%$ vs $20.83\% \pm 5.04\%$, $P < 0.01$). Malondialdehyde level and inducible nitric oxide synthase (iNOS) activity were all markedly increased ($P < 0.01$), while superoxide dismutase (SOD) activity was significantly decreased in the thoracic aorta from LPS-treated rats compared with the normal control group ($P < 0.01$). All these alterations were reversed by pretreatment with BA. These findings suggest that BA lessens the impairment of aortic contraction induced by LPS, which may associate with activating SOD and inhibiting oxidative stress and iNOS activity.

Key words betulinic acid; lipopolysaccharide; aortic contraction; oxidative stress

败血症是感染和创伤等诱发的严重炎症反应综合征,若不进行积极干预,将发展成持续低血压、全身器官灌注紊乱和衰竭的败血症休克,是导致急危重症患者死亡的首要因素之一^[1]。败血症休克的系统性炎症反应,通常是由革兰氏阴性细菌的细胞壁成分脂多糖(lipopolysaccharide, LPS)引起的。LPS 通常在细菌裂解后释放出来,被称为内毒素,可以刺激体内的巨噬细胞、中性粒细胞及内皮细胞等产生一系列的炎症细胞因子和活性氧自由基(reactive oxygen species, ROS),这些内源性介质参与了不可逆转血管低反应性的发生和发展,最终发展成为多器官功能衰竭^[2]。LPS 引起的血管功能障碍机制至今尚未完全阐明,大量的研究开始关注氧化应激在其中所起的作用。白桦脂酸(betulinic acid, BA)是一种五环三萜酸,存在于白桦树等多种植物中,具有抗氧化应激、抗炎、免疫调节和抗菌等多种生物活性^[3, 4]。以往研究发现,BA 有良好的抗心脑血管氧化应激损伤作用^[5, 6],能显著改善败血症引发的急性氧化应激肺损伤和肾损伤^[7-9]。然而,BA 对 LPS 诱导血管收缩功能损伤是否具有防治作用,该保护作用是否与抗氧化应激有关,尚不清楚。本文用腹腔注射 LPS 建立内毒素血症大鼠模型,研究 BA 预处理抗 LPS 诱导胸主动脉收缩障碍的氧化应激损伤机制。

1 材料与方法

1.1 材料

白桦脂酸(BA)购于上海同田生物有限公司,纯度 $\geq 98\%$;苯肾上腺素(phenylephrine hydrochloride, PE)、LPS 购于美国 Sigma-Aldrich 公司;超氧化物歧化酶(superoxide dismutase, SOD)、诱导型一氧化氮合酶(inducible nitric oxide synthase, iNOS)、丙二醛(malondialdehyde, MDA)测定试剂盒购于南京建成生物工程研究所;白细胞介素 6(interleukin-6, IL-6) ELISA 试剂盒购于武汉云克隆科技股份有限公司。

其余试剂为市售分析纯。

Krebs-Henseleit(K-H)液(mmol/L): NaCl 118, KCl 4.7, KH_2PO_4 1.2, MgSO_4 1.2, NaHCO_3 25, glucose 5.5, CaCl_2 2.5, pH 7.4。无钙 K-H 液中不加 CaCl_2 , 并加入 $50 \mu\text{mol/L}$ EGTA。

1.2 实验动物

清洁级雄性 Sprague-Dawley(SD)大鼠(体重 $220 \sim 300 \text{ g}$)购自上海斯莱克实验动物有限责任公司,所有实验动物方案均符合实验动物使用规范,并获得校实验动物伦理委员会批准。大鼠随机分为 4 组($n = 5$): (1)正常对照组(Ctrl),正常大鼠每天灌胃生理盐水(10 mL/kg),3 d 后腹腔注射生理盐水(10 mL/kg); (2)白桦脂酸对照组(BA),正常大鼠每天灌胃 BA(25 mg/kg)^[10, 11],3 d 后腹腔注射生理盐水(10 mL/kg); (3)LPS 损伤组,正常大鼠每天灌胃生理盐水(10 mL/kg),3 d 后腹腔注射 LPS(10 mg/kg)^[12]; (4)BA 治疗组,正常大鼠每天灌胃 BA(25 mg/kg),3 d 后腹腔注射 LPS(10 mg/kg)。所有大鼠在腹腔注射 LPS 或生理盐水 4 h 后,戊巴比妥钠(60 mg/kg)麻醉,肌注肝素钠(5000 IU),腹主动脉取血静置 15 min, 3500 r/min ,离心 10 min,取上层血浆备用,分离胸主动脉检测收缩性。

1.3 血管收缩性测定

胸主动脉环的制备参照文献[4], Medlab 生物信号处理系统记录血管环张力,初始张力设为 2 g ,平衡 1 h,加入 60 mmol/L KCl,重复 3 次,以激发血管环最适活性。待动脉环稳定后,浴槽中加入 $1 \mu\text{mol/L}$ PE,收缩达峰值稳定后,加入 $10 \mu\text{mol/L}$ ACh,若血管舒张 $60\% \sim 90\%$,可认为内皮完整,若血管舒张 $< 30\%$,则认为内皮被破坏。分别记录各组大鼠胸主动脉环对累积浓度的 $10^{-9} \sim 10^{-5} \text{ mol/L}$ PE、 $5 \sim 120 \text{ mmol/L}$ KCl 及 $0.25 \sim 5 \text{ mmol/L}$ CaCl_2 引起的收缩效应。血管收缩性以各浓度收缩剂引发的张力与之前 $1 \mu\text{mol/L}$ PE 或第 3 次 60 mmol/L KCl 诱发张力的百分比表示。

1.4 生化指标的测定

处理完各组动物后分离胸主动脉,去除血管内血凝块,滤纸吸干并称重,在冰匀浆液(含 10 mmol/L Tris-HCl,0.1 mmol/L EGTA,10 mmol/L sucrose,0.8% NaCl,pH 7.4)中制成 10% 的组织匀浆,离心(3 000 r/min,10 min,4℃),取上清。按 ELISA 试剂盒要求检测血浆及组织匀浆上清液中 IL-6 水平,严格按照试剂盒说明书检测血浆及组织匀浆上清 SOD 活性,检测组织匀浆上清 MDA 水平及 iNOS 活性。

1.5 统计学方法

数据以平均值 ± 标准差 ($\bar{x} \pm s$) 表示,采用

One-way ANOVA 和 Newman-Keuls 法进行显著性检验, $P < 0.05$ 认为差异有显著性。

2 结果

2.1 白桦脂酸明显抑制 LPS 诱导的 IL-6 水平

由 Fig. 1 可知,LPS 组大鼠血浆及胸主动脉 IL-6 水平与对照组相比有显著升高($P < 0.01$),分别是对照组的 4.3 倍和 3.2 倍。BA 预处理明显降低了 LPS 诱导的 IL-6 水平($P < 0.01$),血浆及胸主动脉 IL-6 水平分别是单纯 LPS 组的 66% 和 60%,但仍显著高于对照组($P < 0.05$)。BA 预处理对正常大鼠胸主动脉与血浆 IL-6 无明显影响(Fig. 1)。

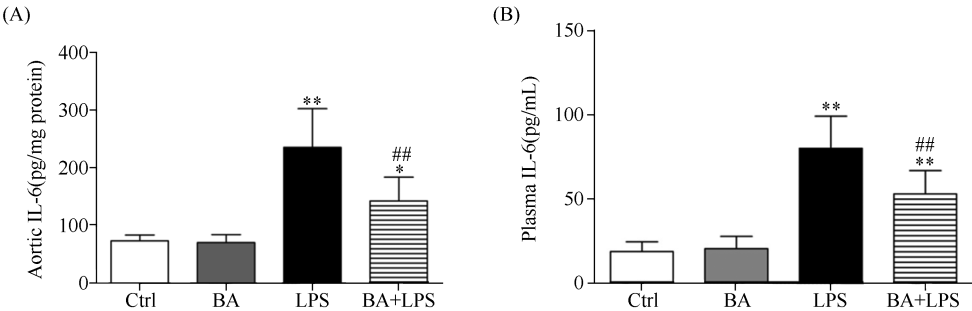


Fig. 1 Effect of betulinic acid (BA) on the levels of IL-6 in rat Male Sprague-Dawley rats were pretreated with BA (25 mg/kg/d, i. g.) for 3 days and intraperitoneally injected with lipopolysaccharide (LPS, 10 mg/kg) at the 4th day for 4 hours. The plasma was collected and the thoracic aorta was immediately dissected out from the anesthetized rat. The levels of aortic (A) and plasma (B) IL-6 were determined by ELISA assay in rats from the normal control group (Ctrl), BA-pretreated group (BA), LPS-treated group (LPS), and BA plus LPS-treated group (BA + LPS). Values are expressed as mean ± SD, $n = 5$, * $P < 0.05$, ** $P < 0.01$ vs Ctrl; ## $P < 0.01$ vs LPS

2.2 白桦脂酸明显减轻 LPS 对苯肾上腺素及 Ca²⁺ 诱导胸主动脉收缩功能的抑制作用

由 Fig. 2A 可知,与对照组相比,LPS 组大鼠胸

主动脉对苯肾上腺素(PE)的收缩反应性明显降低($P < 0.01$)。BA 预处理明显减轻了 LPS 对 PE 收缩血管的抑制作用($P < 0.05$),最大收缩张力从

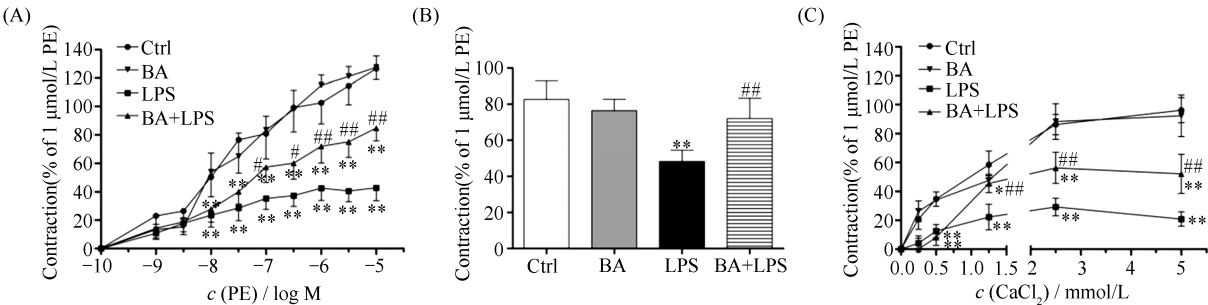


Fig. 2 Effect of BA on aortic contraction in rat Male Sprague-Dawley rats were pretreated with BA (25 mg/kg/d, i. g.) for 3 days and intraperitoneally injected with LPS (10 mg/kg) at the 4th day for 4 hours. The thoracic aorta was immediately dissected out from the anesthetized rat. (A) The aortic contraction produced by different concentrations of phenylephrine (PE, 0.001-10 μmol/L). (B) The aortic contraction produced by 1 μmol/L PE without Ca²⁺. (C) The aortic contraction produced by 1 μmol/L PE and different concentrations of Ca²⁺ (0.25-5 mmol/L) was determined using organ bath system in the normal control group (Ctrl), BA-pretreated group (BA), LPS-treated group (LPS), and BA plus LPS-treated group (BA + LPS). Values are calculated as percentage of the contraction in response to PE (1 μmol/L) and expressed as mean ± SD, $n = 5$, ** $P < 0.01$ vs Ctrl; # $P < 0.05$, ## $P < 0.01$ vs LPS

42.80% ± 9.00% 上升为 84.8% ± 9.09%。在无 Ca²⁺ K-H 液中,LPS 组的 PE 收缩反应性明显低于对照组 ($P < 0.01$),该作用被 BA 预处理所逆转 (Fig. 2B)。由 Fig. 2C 可知,PE (1 μmol/L) 预刺激后,与对照组相比,LPS 组大鼠胸主动脉对细胞外 Ca²⁺ 的收缩反应性明显降低 ($P < 0.01$)。BA 预处理明显抑制了 LPS 对细胞外 Ca²⁺ 收缩血管的抑制作用 ($P < 0.01$),最大收缩张力从 20.83% ± 5.04% 上升为 52.07% ± 13.48%。BA 预处理对正常大鼠胸主动脉收缩性无明显影响 (Fig. 2)。

2.3 白桦脂酸明显减轻 LPS 对 KCl 及 Ca²⁺ 诱导胸主动脉收缩功能的抑制作用

由 Fig. 3A 可知,与对照组相比,LPS 组大鼠胸主

动脉对 KCl 的收缩反应性明显降低 (128.07% ± 12.02% vs 99.78% ± 6.02%, $P < 0.05$),BA 预处理明显抑制 LPS 对 KCl 收缩血管的抑制作用 ($P < 0.05$),最大收缩张力从 99.78% ± 6.02% 上升为 127.48% ± 12.02%。在无 Ca²⁺ K-H 液中,LPS 组的 KCl 收缩反应性明显低于对照组 ($P < 0.01$),该作用被 BA 预处理所逆转 (Fig. 3B)。由 Fig. 3C 可知,KCl (60 mmol/L) 预刺激后,与对照组相比,LPS 组大鼠胸主动脉对细胞外 Ca²⁺ 的收缩反应性明显降低 ($P < 0.01$)。BA 预处理明显抑制了 LPS 对细胞外 Ca²⁺ 收缩血管的抑制作用 ($P < 0.05$),最大收缩张力从 73.67% ± 17.16% 上升为 92.91% ± 13.48%。BA 预处理对正常大鼠胸主动脉收缩性无明显影响 (Fig. 3)。

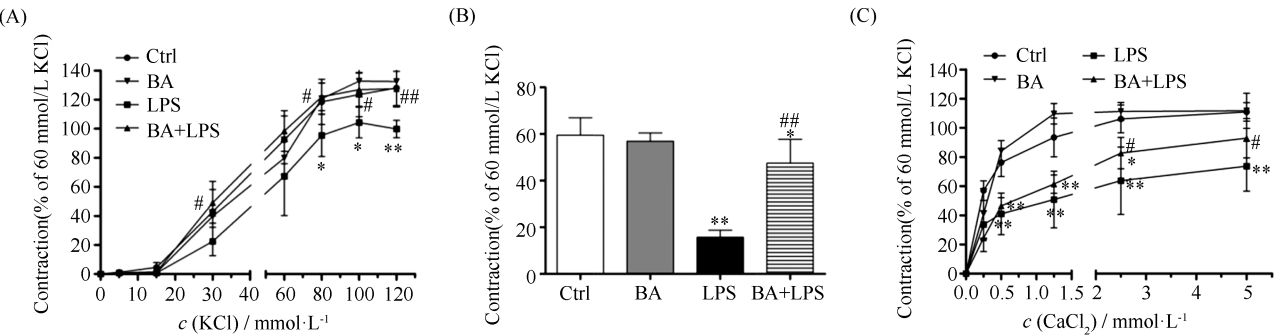


Fig. 3 Effect of BA on aortic contraction in rat Male Sprague-Dawley rats were pretreated with BA (25 mg/kg/d, i. g.) for 3 days and intraperitoneally injected with LPS (10 mg/kg) at the 4th day for 4 hours. The thoracic aorta was immediately dissected out from the anesthetized rat. (A) The aortic contraction produced by different concentrations of KCl (5-120 mmol/L). (B) The aortic contraction produced by 60 mmol/L KCl without Ca²⁺. (C) The aortic contraction produced by 60 mmol/L KCl and different concentrations of Ca²⁺ (0.25-5 mmol/L) was determined using organ bath system in the normal control group (Ctrl), BA-pretreated group (BA), LPS-treated group (LPS), and BA plus LPS-treated group (BA + LPS). Values are calculated as percentage of the contraction in response to KCl (60 mmol/L) and expressed as mean ± SD, $n = 5$, * $P < 0.05$, ** $P < 0.01$ vs Ctrl; # $P < 0.05$, ## $P < 0.01$ vs LPS

2.4 白桦脂酸明显改善 LPS 诱导的氧化应激

由 Fig. 4A 可知,LPS 组大鼠胸主动脉 MDA 水平与对照组相比有显著升高 ($P < 0.01$),达到对照组的 2.3 倍。BA 预处理明显降低 LPS 诱导的 MDA 水平 ($P < 0.01$),降低为单纯 LPS 组的 64%,但仍显著高于对照组 ($P < 0.05$)。如 Fig. 4B 和 C 所示,LPS 组大鼠胸主动脉与血浆 SOD 活性均明显低于对照组 ($P < 0.01$),分别是对照组的 61% 和 63%;BA 预处理明显抑制 LPS 导致的 SOD 活性降低 ($P < 0.01$),血浆及胸主动脉 SOD 活性分别是单纯 LPS 组的 1.4 倍和 1.5 倍。BA 预处理对正常大鼠胸主动脉及血浆氧化应激无明显影响 (Fig. 4)。

2.5 白桦脂酸明显抑制 LPS 诱导的 iNOS 活性

由 Fig. 5 可知,与对照组相比,LPS 组大鼠胸主动脉 iNOS 活性显著升高 ($P < 0.01$),达到对照组的

5.5 倍。BA 预处理明显降低 LPS 诱导的 iNOS 活性水平 ($P < 0.01$),降低为单纯 LPS 组的 46%,但仍显著高于对照组 ($P < 0.01$)。BA 预处理对正常大鼠胸主动脉 iNOS 活性无明显影响 (Fig. 5)。

3 讨论

败血症休克晚期的全身不可逆性低血压,与大面积的外周血管舒张和心肌收缩抑制相关^[13]。LPS 模型作为急性模型,可在短时间内使模型动物达到败血症晚期^[14]。本文发现,腹腔注射 LPS, 2 h 后,大鼠即表现出明显的内毒素血症症状,包括腹泻和活动力低下等;LPS 处理 4 h 内没有动物死亡,血浆及胸主动脉 IL-6 水平明显增高,胸主动脉收缩性明显减低,提示败血症系统性炎症及血管低反应性的存在。BA 预处理 3 d 明显抑制 LPS 诱导的 IL-6 水平

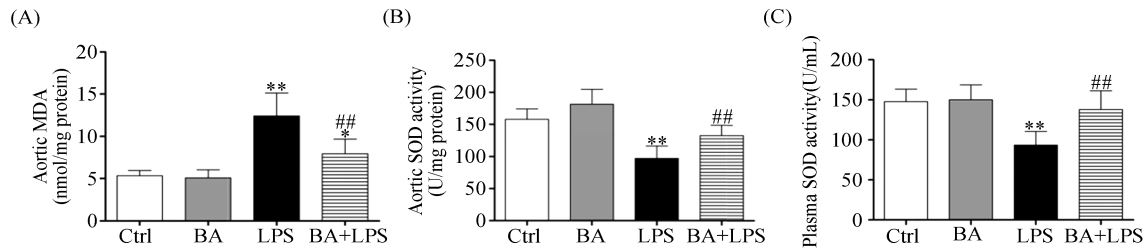


Fig. 4 Effect of BA on oxidative stress in rat Male Sprague-Dawley rats were pretreated with BA (25 mg/kg/d, i. g.) for 3 days and intraperitoneally injected with LPS (10 mg/kg) at the 4th day for 4 hours. The plasma was collected and the thoracic aorta was immediately dissected out from the anesthetized rat. (A) Aortic malondialdehyde (MDA), (B) aortic superoxide dismutase (SOD) activity and (C) plasma SOD activity were determined in rats from the normal control group (Ctrl), BA-pretreated group (BA), LPS-treated group (LPS), and BA plus LPS-treated group (BA + LPS). Values are expressed as mean ± SD, n = 5, * P < 0. 05, ** P < 0. 01 vs Ctrl; ## P < 0. 01 vs LPS

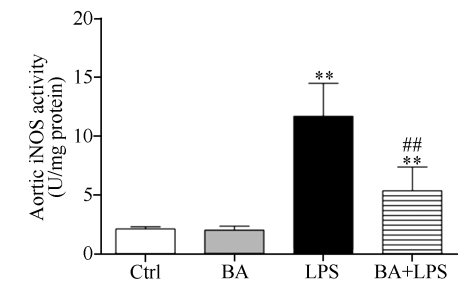


Fig. 5 Effect of BA on inducible nitric oxide synthase (iNOS) activity in the rat aorta Male Sprague-Dawley rats were pretreated with BA (25 mg/kg/d, i. g.) for 3 days and intraperitoneally injected with LPS (10 mg/kg) at the 4th day for 4 hours. The thoracic aorta was immediately dissected out from the anesthetized rat. (A) Aortic iNOS activity was determined in rats from the normal control group (Ctrl), BA-pretreated group (BA), LPS-treated group (LPS), and BA plus LPS-treated group (BA + LPS). Values are expressed as mean ± SD, n = 5, ** P < 0. 01 vs Ctrl; ## P < 0. 01 vs LPS

及收缩功能障碍,显示极强的改善败血症休克血管低反应性的功能。

本研究发现,在 LPS 作用下,胸主动脉对 PE 的收缩反应性明显下降,且在无细胞外 Ca²⁺ 情况下,这种低反应性还存在,说明 LPS 不仅可抑制血管平滑肌细胞 α 受体功能,还对平滑肌细胞内质网 Ca²⁺ 释放产生抑制作用。此外,无论细胞外有无 Ca²⁺, LPS 组胸主动脉对 KCl 的收缩反应同样明显下降,进一步确认 LPS 抑制胸主动脉的收缩功能与阻碍内质网 Ca²⁺ 释放有关。随着细胞外 Ca²⁺ 浓度增加, LPS 明显降低 PE 或 KCl 预刺激的胸主动脉收缩反应,提示血管平滑肌细胞膜受体操控性 Ca²⁺ 通道与电压依赖性 Ca²⁺ 通道均被 LPS 所抑制。LPS 的上述血管收缩抑制作用被 BA 预处理显著降低,提示 BA 收缩力受到显著的影响。而在 BA 作用下,抑制

作用得到缓解,提示 BA 明显加强 LPS 处理大鼠胸主动脉收缩功能,该作用可能涉及电压依赖性钙通道、受体操控性钙通道、以及肌浆网钙释放功能的改善。

有研究证实,急性 LPS 处理在诱导系统性炎症反应的同时,还产生了过量的 ROS。氧化应激是败血症晚期多器官功能衰竭的重要促进因素,抑制氧化应激明显减轻 LPS 导致的血管功能损伤^[15, 16]。本研究发现,LPS 明显增加胸主动脉脂质过氧化产物丙二醛(MDA)水平,降低主动脉及血浆 SOD 活性。SOD 是机体第一道内源性抗氧化酶屏障,可将超氧阴离子还原为过氧化氢,后者再被过氧化氢酶分解^[8, 17]。上述结果提示,LPS 处理后,大鼠机体的氧化与抗氧化平衡失调,该结果与其他研究结果相一致^[18]。BA 预处理明显降低 LPS 大鼠胸主动脉 MDA 水平,上调 SOD 活性,提示 BA 减少 LPS 诱导的氧化应激可能是通过促进 SOD 活性,增加机体的抗氧化酶防御体系实现的。

有证据表明,LPS 及 LPS 诱导产生的细胞因子能够刺激 iNOS 的大量表达,从而产生高浓度的一氧化氮(nitric oxide, NO)^[16, 18]。由于 NO 能使平滑肌细胞肌浆网上的肌醇三磷酸(inositol trisphosphate, IP₃)敏感受体介导的内钙释放路径受到抑制,也就是说过量的 NO 可通过降低细胞内钙水平而介导主动脉的低反应性^[16]。此外,iNOS 催化产生的过量 NO 可与超氧阴离子快速结合,生成活性更高的过氧亚硝酸盐(ONOO⁻),加剧了败血症血管及其他器官的氧化应激损伤,这是 LPS 诱导败血症血管能量代谢障碍及收缩反应性下降的又一重要原因^[19]。本文发现,无细胞外 Ca²⁺ 时,LPS 明显抑制胸主动脉对 PE 的收缩反应性,且伴随有 iNOS

活性的急剧增高,而 BA 预处理改善 LPS 处理组大鼠胸主动脉的无细胞外 Ca^{2+} 收缩反应性并降低 iNOS 活性,这与先前的研究一致^[8, 20]。提示抑制 iNOS-NO 通路是 BA 发挥抗 LPS 氧化应激损伤,提高血管反应性的又一重要机制。

综上所述,BA 明显抑制 LPS 诱导的炎症及胸主动脉低反应性,该作用可能涉及电压依赖性钙通道、受体操控性钙通道、以及内质网钙释放功能的改善;BA 改善 LPS 大鼠血管收缩性的机制可能与增加机体 SOD 活性,抑制 iNOS 活性及氧化应激有关。本研究从抗氧化应激角度为寻求有效的败血症心血管损伤防治措施,提高败血症患者的心血管功能,为改善预后提供了有益的提示。

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