# 白细胞介素-6通过AMPK/SIRT1通路下调eNOS Thr495/497

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**摘要** 该文探讨了白细胞介素-6(interleukin-6, IL-6)对牛主动脉内皮细胞(bovine aortic endothelial cells, BAECs)的内皮型一氧化氮合成酶(endothelial nitric oxide synthase, eNOS)的影响及其可能的发生机制。在原代BAECs细胞培养基础上, 选取IL-6不同浓度(10、20、40 ng/mL)、不同时间(0.5、1、2 h)处理BAECs; AMPKα1 siRNA预处理BAECs后加入IL-6共处理2 h。用Western blot方法检测BAECs细胞中eNOS、Thr497、腺苷酸活化蛋白激酶(AMPK)、p-AMPK、沉默信息调节因子2相关酶1(SIRT1)的表达情况, 用一氧化氮(NO)检测试剂盒检测BAECs细胞中NO含量。结果显示, 与正常对照组相比, IL-6显著降低Thr497的表达($P$<0.05), 同时伴有p-AMPK、SIRT1上调($P$<0.05), NO含量升高。而IL-6对Thr497的下调作用被AMPKα1 siRNA和EX-527阻断($P$<0.05)。总之, IL-6可下调BAECs中Thr497表达进而影响eNOS活性, 其作用机制可能涉及到AMPK/SIRT1通路的参与。

**关键词** 白细胞介素-6; 内皮型一氧化氮合成酶; 一氧化氮; 磷酸化; AMPK/SIRT1通路

# Interleukin-6 Down-Regulates eNOS Thr495/497 through AMPK/SIRT1 Pathway

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**Abstract** This is a study on the effects of IL-6 (interleukin-6) on eNOS (endothelial nitric oxide synthase) of BAECs (bovine aortic endothelial cells) and its mechanism. BAECs were treated with IL-6 in different concentrations (10, 20, 40 ng/mL) and different times (0.5, 1, 2 h). Beside, BAECs were pre-treated with AMPKα1 siRNA and then were treated with IL-6 for 2 h (kept with AMPKα1 siRNA). The expression of eNOS, Thr497, AMPK (adenylate-activated protein kinase), p-AMPK, and SIRT1 (silence information regulator 2 related enzyme 1) in BAECs were detected by Western blot. The NO (nitric oxide) content in BAECs was measured by NO detection kit. The results showed that IL-6 significantly reduced the expression of Thr497 ($P$<0.05), accompanied by up-regulation of p-AMPK, SIRT1 ($P$<0.05), and increased NO content compared with the normal control group. The down-regulation of Thr497 by IL-6 was blocked by AMPKα1 siRNA and EX-527 ($P$<0.05). In short, IL-6 could down-regulate the expression of Thr497 in BAECs and then affect eNOS activity. Its mechanism may involve the participation of AMPK/SIRT1 pathway.

**Keywords** interleukin-6; endothelial nitric oxide synthase; nitric oxide; phosphorylation; AMPK/SIRT1 pathway

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血管内皮作为循环血液与内皮下组织的屏障, 在调节血管通透性与免疫炎症反应等方面具有关键性作用。血管内皮细胞中的内皮型一氧化氮合成酶(endothelial nitric oxide synthase, eNOS)产生的一氧化氮(nitric oxide, NO)含量的异常会导致内皮功能障碍, 这在糖尿病、动脉粥样硬化以及脓毒血症等血管炎性病变的早期发病机制中尤为重要[1]。eNOS对维持血管稳态具有重要的生理意义, 除了保持脉管系统扩张外, 还可以防止内膜上血小板聚集和白细胞黏附, 并防止平滑肌增殖[2]。在生理条件下, eNOS是血管NO的主要产生来源, 内皮NO扩散到平滑肌细胞并引起血管舒张, 能够减缓血管疾病的发生[3]。eNOS受到多种翻译后修饰的调控, 其中激酶介导的蛋白磷酸化起着关键作用[4]。Ser1179和Thr497是eNOS的两个主要磷酸化调控位点, 分别起正性和负性调控作用。

以eNOS改变为特征的内皮功能障碍是慢性炎症性血管疾病的常见表现。导致损伤的慢性炎症有多种形式, 从单纯的血管病变, 例如高血压和动脉粥样硬化, 到全身性自身免疫性疾病。IL-6是一种多功能性细胞因子, 在调节免疫系统中具有重要功能。研究证明, 作为一种有效的促炎和抗炎细胞因子, IL-6在宿主防御病原体和急性应激中起着关键作用[5]。

IL-6对血管损伤的调节多有文献报道[6]。然而IL-6如何参与调控血管内皮细胞eNOS磷酸化尚不清楚。本研究以体外培养的牛主动脉内皮细胞(bovine aortic endothelial cells, BAECs)为实验对象, 探讨炎症因子IL-6刺激对血管内皮细胞eNOS Thr497磷酸化位点的影响及其可能的相关机制。

## 1 材料与方法

### 1.1 材料

DMEM培养基、DMSO、胎牛血清(fetal bovine setum, FBS)、人重组肿瘤坏死因子-α、人重组白细胞介素-1β、人重组白细胞介素-6购自Sigma-Aldrich公司。AMPKα1 siRNA购自Biomics公司。Lipofectamine™3000、Opti-MEM购自Invitrogen公司。 RIPA裂解液、BCA蛋白浓度测定试剂盒、总一氧化氮测试试剂盒购自Beyotime Biotechnology公司。 eNOS(1:2 000)、p-eNOS-Ser1177(1:1 000)、p-eNOS-Ser1177(1:1 000)、AMPK(1:1 000)、p-AMPK-Thr172(1:1 000)、SIRT1(1:1 000)抗体购自Cell Signaling Technology公司。 β-actin抗体、羊抗兔二抗(1:10 000)购自Proteintech Group公司。EX-527购自MedChemExpress公司。PVDF膜购自德国Schleicher and Schuell公司。其他相关但未提及的化合物购于Sigma公司或Gibco公司。

### 1.2 方法

1.2.1 牛主动脉内皮细胞的培养与鉴定 无菌条件下取长度为8~10 cm的牛胸主动脉, 并将一端结扎, 翻转主动脉, 使内膜朝外, 灭菌生理盐水反复冲洗数次后收集消化液, 后置入含0.25%的I型胶原酶中, 于37 °C水浴20 min后, 去除动脉, 收集消化液, 放入离心管中1 000 r/min离心10 min, 弃上清, 收集内皮细胞。在DMEM培养基中于37 °C、5% $CO_2$条件下培养, 2 h和12 h时分别换液1次, 然后每2~3天换液1次。3~5天后, 待细胞生长成均匀单层细胞并达90%以上汇合时, 用0.05%胰蛋白酶和0.02%乙二胺四乙酸(ethylene diamine tetraacetic acid, EDTA)消化传代。显微镜下可见数个成团的内皮细胞呈鹅卵石状贴壁生长, 利用内皮细胞VIII因子相关抗原, 采用免疫荧光法进行内皮细胞鉴定。

1.2.2 Western blot 冰上裂解60 min提取BAECs细胞总蛋白, BCA法测定蛋白浓度。每孔蛋白上样量约为30 μg, 选用8%的分离胶进行SDS-PAGE电泳(浓缩胶80 V, 分离胶120 V), 在200 V、120 min的条件下将其湿转至PVDF膜上, 室温下用3%~5%的脱脂奶粉封闭1.5 h, 分别加入相应一抗4 °C孵育过夜。一抗孵育完成后, TBST洗3次, 每次10 min, 于常温孵育二抗1.5 h, 重复洗涤3次。ECL化学发光显色, 用Image Lab 6.0分析条带灰度值, 以磷酸化蛋白与总蛋白灰度值的比值表示磷酸化表达量水平高低。

1.2.3 siRNA转染 取倍增期BAECs细胞, 以$5\times10^5$个/孔的密度接种于6孔板中, 常规培养。实验分成四组: 空白对照组、IL-6组、AMPKα1 siRNA组和AMPKα1 siRNA+IL-6组。转染时用终浓度为100 nmol/L的AMPKα1 siRNA和Opti-MEM轻柔混匀, 再将Lipofectamine™ 3000和Opti-MEM混匀, 室温静置5 min。将上述两者混匀, 室温放置20 min。更换培养液, 将转染复合物加到培养皿中, 轻柔摇匀, 转染6 h后换新鲜的含10% FBS的DMEM(低糖)培养基继续培养24 h。

1.2.4 NO含量测定 收集各组BAECs细胞培养液, 按照NO检测试剂盒说明书进行操作, 检测样品中的硝酸盐与亚硝酸盐含量推算出总的一氧化氮的量。通过比色法在全波长酶标仪上测定吸光度值, 根据

标准品测得标准曲线, 将待测品吸光度值代入标准曲线公式算得培养基中的NO(μmol/L)含量。

1.2.5 统计 所有的实验数据来源于至少3次独立实验结果, 所得数据用平均值±标准差($\bar{x}\pm s$)表示。采用8.0版本的GraphPad软件作图统计, 两组间均数比较使用双尾$t$检验。$P$<0.05表示差异具有统计学意义。

## 2 结果

### 2.1 牛主动脉内皮细胞鉴定和培养

显微镜形态学显示: BAECs呈多角形, 核仁显著, 呈圆形或椭圆形。细胞融合成单层, 如"铺路石"样镶嵌排列。使用VIII因子对细胞进行鉴定, 蓝色荧光提示表达VIII因子(图1)。

### 2.2 TNF-α、IL-1β、IL-6处理BAECs后Thr497表达水平的变化

与对照组相比, IL-6干预BAECs 2 h, Thr497显著下降($P$<0.05)。TNF-α或IL-1β处理BAECs 2 h, Thr497与对照组相比均无统计学差异($P$>0.05, 图2)。

### 2.3 不同浓度的IL-6处理BAECs后Thr497表达水平的变化

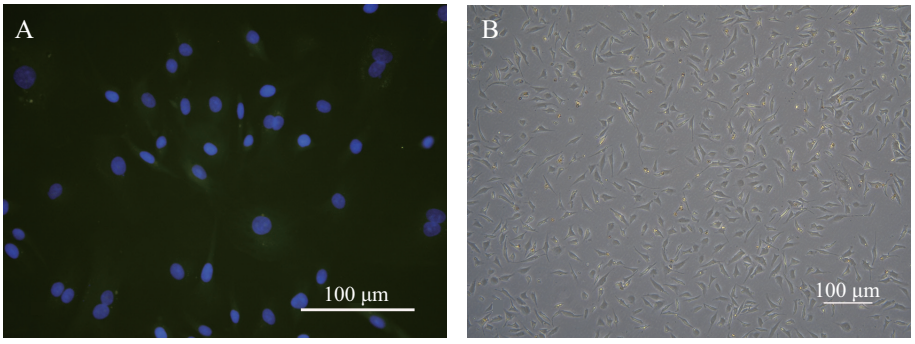
不同浓度(10、20、40 ng/mL)的IL-6干预BAECs 2 h。与对照组相比, 20、40 ng/mL浓度IL-6处理细胞后, Thr497的蛋白表达水平显著降低, 结果具有统计学差异($P$<0.05, 图3)。

### 2.4 IL-6处理BAECs不同时间后Thr497、PKC-α的表达水平和NO含量的变化

用IL-6(40 ng/mL)干预 BAECs 0.5、1、2 h。与对照组相比, 1、2 h处理组的Thr497表达水平均显著降低, 其NO含量较对照组增加, 结果具有统计学差异($P$<0.05, 图4)。

### 2.5 IL-6处理BAECs不同时间段后p-AMPK和SIRT1表达水平的变化

IL-6(40 ng/mL)干预BAECs 0.5、1、2 h。结果提示, 1、2 h处理组p-AMPK和SIRT1的表达量较对照组显著上升, 结果具有统计学差异($P$<0.05, 图5)。

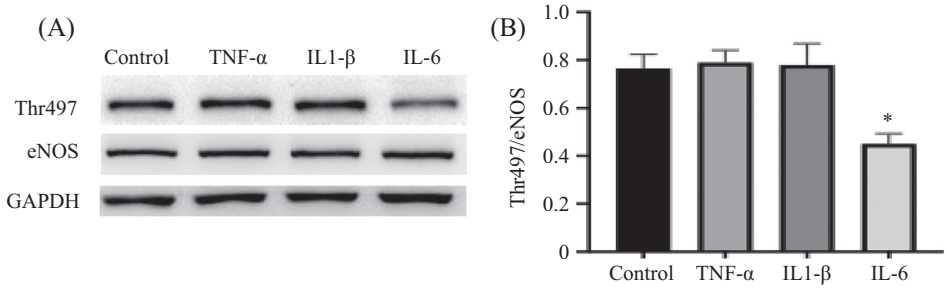



A: BAECs VIII因子免疫荧光染色, 蓝色为VIII因子表达阳性; B: BAECs原代培养细胞。

A: BAECs factor VIII immunofluorescence staining. Blue is positive for factor VIII expression; B: BAECs primary cultured cells.

**图1 BAECs细胞的鉴定与培养**

**Fig.1 Identification and culture of BAECs cells**

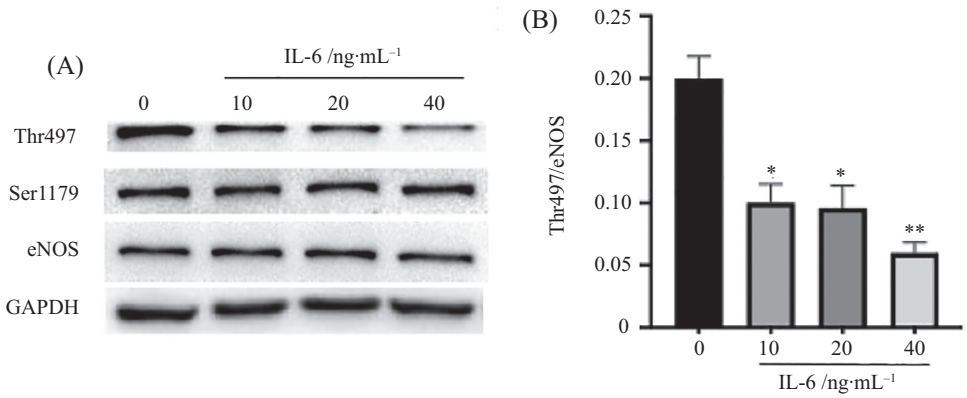



A: Western blot检测到IL-6处理细胞后Thr497表达降低; B: IL-6处理细胞后Thr497表达降低, *$P$<0.05, 与Control组比较。

A: the expression of Thr497 decreased after treated with IL-6 measured by Western blot; B: the expression of Thr497 after treated with IL-6, *$P$<0.05 compared with Control group.

**图2 TNF-α、IL-1β、IL-6对BAECs Thr497的影响**

**Fig.2 The effects of TNF-α, IL-1β, and IL-6 on BAECs Thr497**

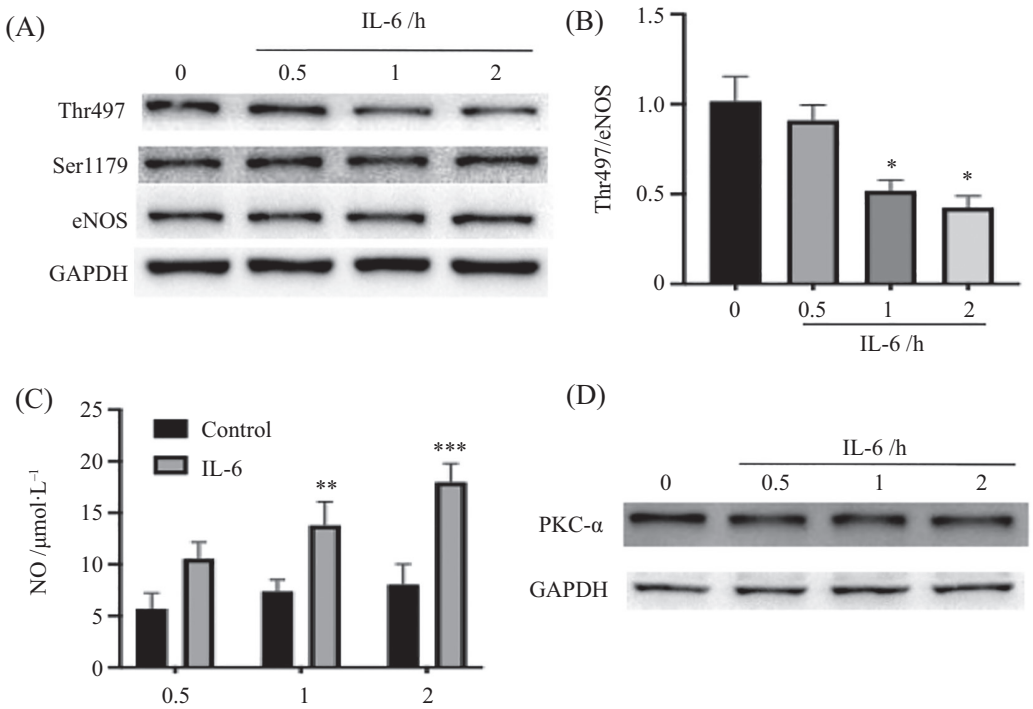



A: Western blot检测IL-6以10、20、40 ng/mL浓度处理细胞后Thr497表达降低; B: IL-6处理细胞后Thr497表达降低, *$P$<0.05, **$P$<0.01, 与0 ng/mL组比较。

A: the expression of Thr497 decreased after treated with IL-6 in 10, 20, 40 ng/mL measured by Western blot; B: the expression of Thr497 decreased after treated with IL-6, *$P$<0.05, **$P$<0.01 compared with 0 ng/mL group.

**图3 不同浓度IL-6处理BAECs后Thr497表达水平的变化**

**Fig.3 Changes of the expression level of Thr497 after treating BAECs with different concentrations of IL-6**




A: Western blot检测到IL-6以0.5、1、2 h时间梯度处理细胞后Thr497表达降低; B: IL-6处理细胞后Thr497表达降低, *$P$<0.05, 与0 ng/mL组比较; C: IL-6处理BAECs后培养基中NO含量明显增加, **$P$<0.01, ***$P$<0.001,与Control组比较; D: IL-6处理BAECs不同时间后PKC-α无明显变化。

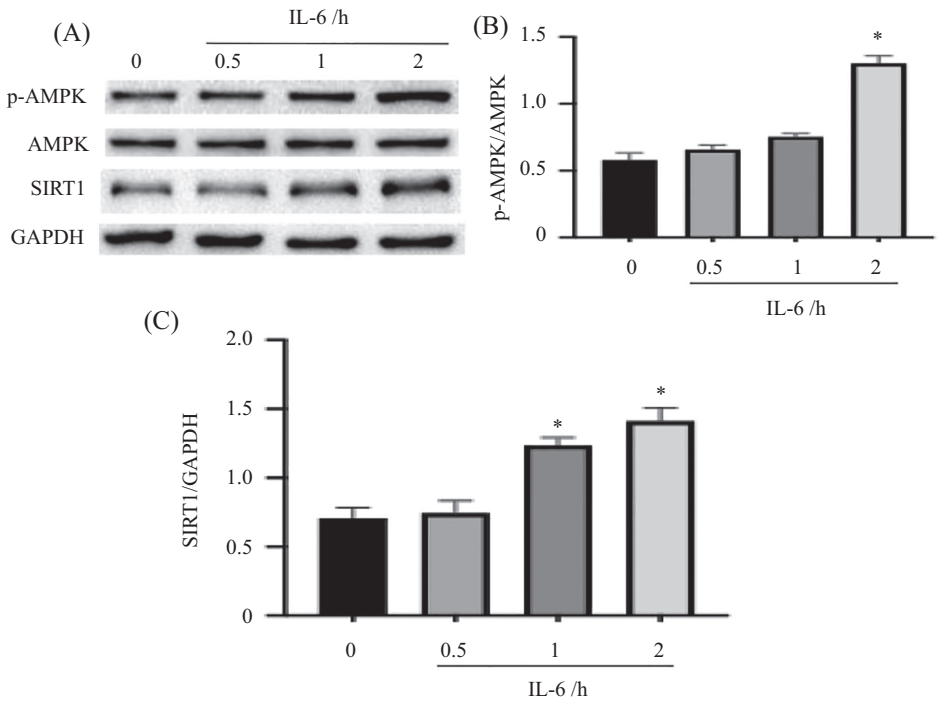
A: the expression of Thr497 decreased after treated with IL-6 in 0.5, 1, 2 h measured by Western blot; B: the expression of Thr497 decreased after treated with IL-6, *$P$<0.05 compared with 0 ng/mL group; C: the content of NO in the medium after treated with IL-6 was significantly increased, **$P$<0.01, ***$P$<0.001 compared with Control group; D: there was no significant change in PKC-α after treating with IL-6 at different time.

**图4 IL-6处理BAECs不同时间后Thr497表达水平和NO含量的变化**

**Fig.4 Changes of Thr497 expression level and NO content after treating with IL-6**

### 2.6 AMPKα1 siRNA预处理后, IL-6对BAECs Thr497表达水平和NO含量的影响

AMPKα1 siRNA能显著阻断IL-6下调BAECs Thr497的作用, AMPKα1 siRNA+IL-6组与IL-6处理组相比具有统计学差异($P$<0.01), 单纯AMPKα1 siRNA组较Control组无统计学差异($P$>0.05)。且AMPKα1 siRNA能显著阻断IL-6调节BAECs培养液中NO含量的作用, AMPKα1 siRNA+IL-6组与IL-6处理组相比具有统计学差异($P$<0.05), 单纯AMPKα1 siRNA组较Control组无统计学差异($P$>0.05, 图6)。




A: Western blot检测到IL-6以0.5、1、2 h处理细胞后p-AMPK、SIRT1表达明显升高; B: IL-6处理细胞后p-AMPK表达明显升高, *$P$<0.05, 与0 h组比较; C: IL-6处理细胞后SIRT1表达明显升高, *$P$<0.05, 与0 h组比较。

A: the expression of P-AMPK and SIRT1 was significantly increased treated with IL-6 in 0.5, 1 and 2 h measured by Western blot; B: the expression of P-AMPK was significantly increased after treated with IL-6, *$P$<0.05 compared with 0 h group; C: SIRT1 expression was significantly increased after treated with IL-6, *$P$<0.05 compared with 0 h group.

**图5 IL-6处理BAECs不同时间段p-AMPK和SIRT1表达水平的变化**

**Fig.5 Changes of p-AMPK and SIRT1 expression in BAECs treated with IL-6 at different time**

### 2.7 SIRT1抑制剂EX-527预处理后, IL-6对BAECs Thr497表达水平和NO含量的影响

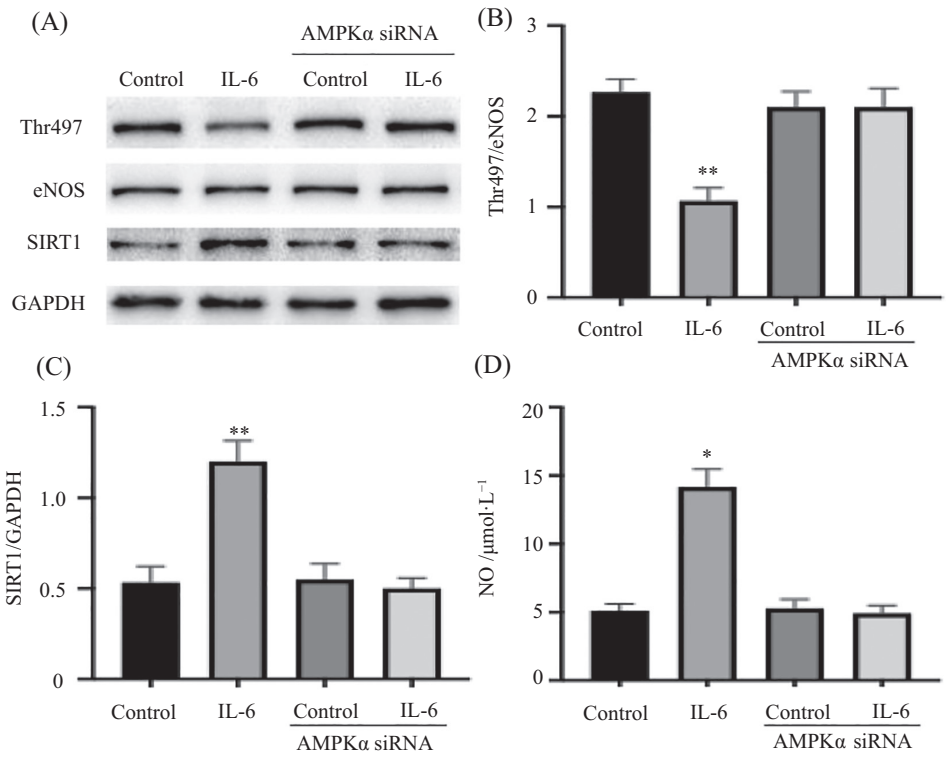
EX-527能显著阻断IL-6下调BAECs Thr497的作用, EX-527+IL-6组与IL-6处理组相比具有统计学差异($P$<0.01), 单纯EX-527组较Control组无统计学差异($P$>0.05)。且EX-527能显著阻断IL-6提高BAECs培养液中NO含量的作用, EX-527+IL-6组与IL-6处理组相比具有统计学差异($P$<0.05), 单纯EX-527组较Control组无统计学差异($P$>0.05, 图7)。

## 3 讨论

血管内皮细胞参与体内多种病理生理过程[7], 在生理状态下, 内皮细胞黏附分子的表达和趋化因子的分泌被抑制, 从而使内皮细胞和循环免疫细胞之间的相互作用减小。炎症刺激后, 内皮细胞转变为活化状态, 活化的内皮细胞和巨噬细胞分泌多种生长因子和趋化分子, 导致血管内皮损伤[8]。血管内皮损伤、功能障碍是高血压、动脉粥样硬化等疾病的始动环节[9]。

eNOS在内皮功能的调节中起着至关重要的作用, 并通过产生气体递质NO来充当血管张力和体内平衡的主要调节剂[10]。有研究发现, eNOS被抑制后血管形成减少[11], 血管张力减弱导致血管舒张功能受损[12]。而在BODIGA等[13]研究中, eNOS的激活产生NO, 可以减少内皮细胞渗漏, 明显改善由顺铂诱导的血管损伤。eNOS-Ser1179的磷酸化是eNOS激活的关键, 有文献报道在小血管中, 高脂饮食通过减少胰岛素介导的eNOS Ser1179磷酸化, 导致血管舒张功能受损。而eNOS Thr497的磷酸化会抑制NO的合成, 激活eNOS的内皮激动剂可以诱导eNOS Thr497的去磷酸化[14], 从而对内皮产生保护作用。在本实验中, IL-6早期可诱导Thr497的去磷酸化, 从而增加eNOS的活性, 提示IL-6早期引起eNOS释放NO, 对血管内皮细胞产生保护作用。

IL-6是维持体内平衡的重要细胞因子, 机体或组织受到损伤时, 会立即产生IL-6, 并通过激活急性期和免疫应答来帮助宿主抵抗这种紧急压力[15]。但在血管功能方面, 研究表明, IL-6的产生导致血管内




A: AMPKα1 siRNA预处理后, Western blot检测到IL-6处理组Thr497、SIRT1表达无明显变化; B: IL-6处理细胞后Thr497蛋白表达量上升, AMPKα1 siRNA预处理后其无明显变化, $^{**}P<0.01$, 与Control组比较; C: IL-6处理细胞后SIRT1表达明显升高, AMPKα1 siRNA预处理后其无明显变化, $^{**}P<0.01$, 与Control组比较; D: IL-6处理细胞后NO含量明显升高, AMPKα1 siRNA预处理后其无明显变化, $^{*}P<0.05$, 与Control组比较。
A: after AMPKα1 siRNA pretreatment, Western blot detected no significant changes in Thr497 and SIRT1 expression in the IL-6 treatment group; B: Thr497 protein expression increased after IL-6 treatment, and there was no significant change after AMPKα1 siRNA pretreatment. $^{**}P<0.01$ compared with Control group; C: the expression of SIRT1 was significantly increased after IL-6 treated cells, and there was no significant change after AMPKα1 siRNA pretreatment, $^{**}P<0.01$ compared with Control group; D: after IL-6 treated cells, the NO content was significantly increased, and there was no significant change after AMPKα1 siRNA pretreatment, $^{*}P<0.05$ compared with Control group.

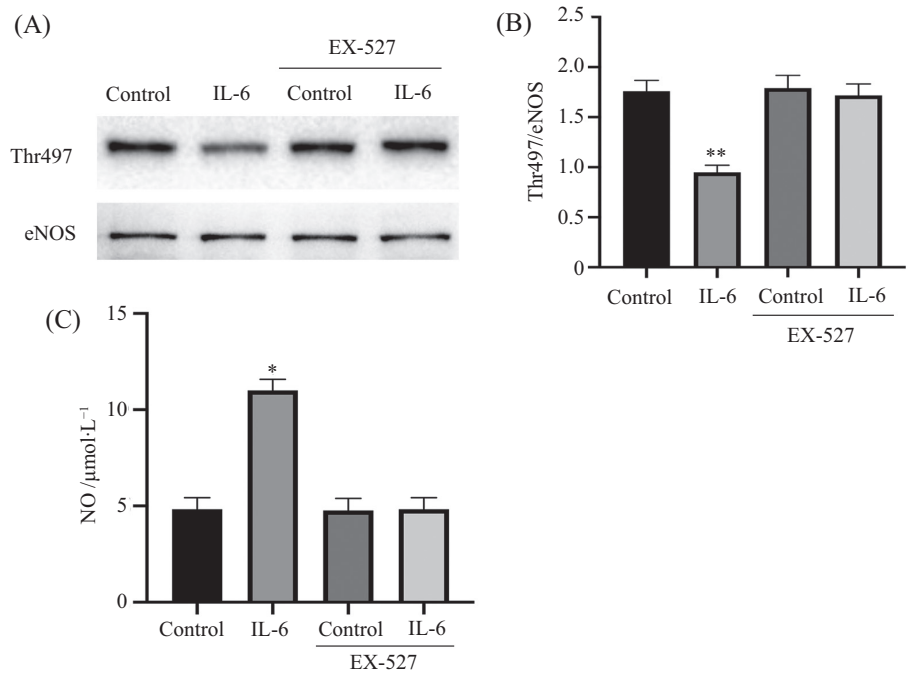
**图6 AMPKα1 siRNA预处理后Thr497、SIRT1表达水平和NO含量的变化**

**Fig.6 Changes of Thr497 and SIRT1 expression levels and NO content after AMPKα1 siRNA pretreatment**

皮细胞功能紊乱[16-17], WASSMANN等[18]观察到外源性IL-6可引起内皮细胞通过血管紧张素II 1型受体的上调而使主动脉产生功能障碍, SCHRADER等[19]报道, IL-6缺乏可抵抗血管紧张素II诱导的颈动脉功能障碍。但本研究发现, IL-6在炎性早期可增加血管内皮细胞eNOS的活性, 从而对内皮细胞产生保护作用。

实验证明, 蛋白激酶C(protein kinase C, PKC)可使eNOS Thr497磷酸化[14,20], PKC的激活剂(如PMA)可刺激Thr497磷酸化增加[21]。但本研究中IL-6作用内皮细胞后, PKC-α的蛋白表达量无明显变化。AMPK的激活具有许多潜在的抗动脉粥样硬化作用, 包括减少炎症细胞与血管内皮的黏附, 减少脂质积累和由氧化脂质引起的炎症细胞的增殖和促进NO的形成[22]。据文献报道, AMPK可激活内皮细胞中的eNOS[23-24], 小鼠体内研究表明, 药理激活的AMPK(例如AICAR、二甲双胍)可预防内皮功能障碍和氧化应激[25]。本实验发现, IL-6处理后, AMPK的磷酸化激活。为了进一步验证IL-6对AMPK的影响, 我们使用AMPKα siRNA进行预处理, 发现AMPK被抑制后, eNOS Thr497磷酸化水平下降, NO活性恢复至对照组水平, 这进一步证实了IL-6可以通过AMPK刺激内皮细胞NO产生, 从而发挥细胞保护作用。AMPK/SIRT1途径在与糖尿病相关的微血管损伤中起重要作用[26], 而SIRT1通过多种机制在心脏病的发病机理和治疗中起关键作用, 包括减少细胞凋亡、改善内皮功能和防御氧化应激[27]。我们发现, SIRT1的抑制剂EX-527可以抵抗IL-6诱导的内皮细胞eNOS Thr497表达降低, 进一步证明了IL-6可以通过AMPK/SIRT1发挥作用。

总而言之, IL-6是一种具有广泛而复杂的生物学活性的细胞因子, 我们的研究发现在细胞损伤早




A: EX-527(10 μmol/L)预处理24 h后, Western blot检测到IL-6处理组Thr497表达无明显变化; B: IL-6处理细胞后Thr497下降, EX-527预处理后其无明显变化, **$P$<0.01, 与Control组比较; C: IL-6处理细胞后NO含量明显升高, EX-527预处理后其无明显变化, *$P$<0.05, 与Control组比较。

A: the level of Thr497 in IL-6 treatment group was no significant change after pretreatment with EX-527 (10 μmol/L) for 24 h. B: Thr497 decreased after IL-6 treatment, but there was no significant change after EX-527 pretreatment. **$P$<0.01 compared with Control group; C: NO content was significantly increased after IL-6 treatment, and there was no significant change after EX-527 pretreatment. *$P$<0.05 compared with Control group.

**图7 EX-527预处理后Thr497表达水平和NO含量的变化**

**Fig.7 Changes of Thr497 expression level and NO content after EX-527 pretreatment**

期, IL-6可通过激活AMPK/SIRT1通路, 增加细胞内eNOS的活性, 从而发挥细胞保护作用。

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