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PP2 对大鼠面神经缺血损伤的保护作用*

申宝玺¹ 陈 静² 梁文学³ 陈慧珍¹ 李爱民^{1△}

(1 徐州医学院附属连云港市第一人民医院神经外科 江苏 连云港 222002; 2 邳州市人民医院 江苏 邳州 221300;

3 徐州医学院附属连云港市第一人民医院中心实验室 江苏 连云港 222002)

摘要 目的:探讨 PP2(4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine)在大鼠面神经缺血损伤模型中的保护作用,为进一步探索面神经缺血损伤的治疗方法提供实验依据。**方法:**实验选取 40 只雄性 SD 大鼠,分为四组:假手术组,损伤组,DMSO 溶剂对照组和 PP2 干预组。手术组沿耳廓外上缘行弧形切口,分离暴露出岩动脉,双极电凝灼断。假手术组暴露出岩动脉但不予灼断。PP2 干预组和 DMSO 溶剂对照组于术前 30 min 分别脑室注射 15 μ g(总体积 10 μ L)PP2 或相同体积 DMSO。于 3 d 后观察各组大鼠的行为学变化,同时运用免疫共沉淀和免疫印迹的技术分析其中分子机制。**结果:**(1)PP2 干预组的大鼠相对于手术组和 DMSO 溶剂对照组,瞬目反射和触须动度明显好转(2)大鼠面神经缺血损伤时,损伤组相对于假手术组,NMDAR-PSD95-Src 的结合明显增加 ($P<0.05$)(3)PP2 干预组 NR2A-PSD95-Src 的结合相对于手术组和 DMSO 溶剂对照组明显降低 ($P<0.05$)。**结论:**大鼠面神经缺血损伤时,存在 NMDAR-PSD95-Src 形成的信号通路,PP2 可以有效的抑制 NMDAR-PSD95-Src 信号通路的形成,从而起到面神经保护作用。

关键词:面神经;缺血损伤;NMDA 受体;PSD95;Src 激酶;PP2

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The Protective Effect of PP2 on Ischemic Injury of Facial Nerve in a Rat Model *

SHEN Bao-xi¹, CHEN Jing², LIANG Wen-xue³, CHEN Hui-zhen¹, LI Ai-min^{1△}

(1 Department of Neurosurgery, the first people's Hospital of Lianyungang affiliated to Xuzhou Medical College, Lianyungang, Jiangsu, 222002, China; 2 The people's Hospital of Pizhou, Xuzhou, Jiangsu, 221300, China; 3 Central Laboratory, the first people's Hospital of Lianyungang affiliated to Xuzhou Medical College, Lianyungang, Jiangsu, 222002, China)

ABSTRACT Objective: To investigate the protective effect of PP2 on the facial nerve ischemia injury in a rat model and to furnish the theoretic foundation and experimental evidence for further researching on the facial nerve ischemia injury. **Methods:** 40 male SD rats were selected and divided into four groups: sham group, Injury group, DMSO group, and PP2 group. All animals were anesthetized. The petrosal artery was interrupted by the bipolar electrocoagulation. The same operation procedures were taken without interrupting the petrosal artery in the sham group. 15 μ g PP2 (total volume 10 μ L) were infused into the rat left cereboventricle by a stepper-motorized microsyringe in the PP2 group and the same dose of DMSO was infused into the rat left cereboventricle in DMSO group. Behavioral changes were observed after 3 days and the techniques of co-immunoprecipitation and immunoblotting were used to analysis the Molecular mechanism. **Results:** (1) Blink reflex and whisker movement of rats in the PP2 group improved significantly. (2) The interactions of NMDAR with PSD95 and Src increased immediately in the injury group ($P<0.05$). (3) The increased interactions of NMDAR with PSD95 and Src were simultaneously suppressed in PP2 group compared with injury and DMSO group. **Conclusion:** There is a signal pathway assembled by NMDAR-PSD95-Src in the rat model of facial nerve ischemia. PP2 can inhibit the formation of NMDAR-PSD95-Src signal pathway effectively and play the role of protection in the rat model of facial nerve ischemia.

Key words: Facial nerve; Ischemia; NMDA receptor; PSD95; Src; PP2

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前言

面神经损伤而导致的面瘫给病人带来了沉重的经济和心理负担。目前面神经损伤的治疗主要有面神经减压^[1]、面神经吻

合^[2]、面神经移植^[3]的方法。但是治疗效果并不理想^[4]。有研究指出无论是面神经的完整解剖保留后的面神经功能缺失,还是颞骨骨折造成的面神经功能损伤,都牵涉到了面神经血流供应的损害^[5],最终导致面神经的缺血直至面神经功能的缺失。

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作者简介:申宝玺(1986-),男,硕士研究生,主要研究方向:颅底外科基础与临床,电话:15996949767, E-mail: bxshen@outlook.com

△通讯作者:李爱民,教授,博士, E-mail: liaimin6529@163.com

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NMDA 受体是离子型谷氨酸受体,在中枢神经系统广泛的表达^[6]。NMDA 受体是由功能亚基 NR1,调节亚基 NR2 和 NR3 构成^[7]。当受到病理刺激后,NMDA 受体对钙离子的通透性大幅度提高, Ca^{2+} 大量内流,导致细胞 Ca^{2+} 超载^[8],从而激活下游的 Ca^{2+} 依赖的信号级联反应,最终导致神经元的损伤和死亡^[9]。PSD95 是一种胞质内蛋白质,其分子结构是由 N-末端的 PDZ1,PDZ2,PDZ3 序列和一个 SH3 结构域以及 C-末端的 GK 结构域构成,是突触后的重要骨架蛋白^[10]。Src 激酶是一种膜结合的非受体酪氨酸蛋白激酶,在发育成熟的中枢神经系统,Src 家族的一个重要功能就是调节离子通道的活性^[11]。研究认为,当海马的 CA1 区缺血的时候,由 PSD95 介导,NMDA 受体和 PSD95,Src 激酶组装成 NR2A-PSD95-Src 信号模块^[12]。Src 激酶在第 416 位酪氨酸发生自身的酪氨酸磷酸化^[13],活化的 Src 激酶又促进了 NMDA 受体的 NR2A 亚基的磷酸化,从而活化了 NMDA 受体^[14],使得 NMDA 受体对 Ca^{2+} 通透性大幅提高, Ca^{2+} 大量内流,激活了下游的 Ca^{2+} 依赖的信号通路,最终导致兴奋性毒性的发生,海马 CA1 区的细胞的死亡^[15]。

但是,在面神经缺血损伤时,是否也存在由 NR2A-PSD95-Src 构成的信号通路,以及 Src 激酶抑制剂 PP2 是否可以对面神经缺血损伤起到保护作用,均未见报道。因此,本研究拟探讨在面神经缺血所导致的面神经元损伤的过程中,是否也存在 NMDAR-PSD95-Src 的信号通路,同时,将给予 Src 激酶的特异性抑制剂 PP2,以观察 PP2 在面神经缺血损伤中是否具有保护作用。

1 材料和方法

1.1 大鼠面神经缺血模型的制备

成年雄性 Sprague-Dawley 大鼠(徐州医学院实验动物中心)40 只,体重 200-250 g。随机分成 sham 组, injury 组, DMSO 组, PP2 组。自由饮食,室温,昼夜节律下饲养。腹腔注射 10% 的医用水合氯醛,注射量为 0.35 ml/100 g。大鼠右侧卧位,面神经管内面神经及岩动脉走行,暴露出岩动脉。继续向前分离暴露岩动脉直至面神经伴行,双极电凝灼断。假手术组接受同样的手术操作,但是暴露岩动脉后并不电凝。

1.2 Src 激酶抑制剂 PP2 的干预

DMSO 溶剂对照组和 PP2 干预组的大鼠,分别于术前 30 min,脑室注射 15 μg (总体 10 μL)PP2 或相同体积的 DMSO。术后 7 天,大鼠在麻醉状态下被断头,在大鼠脑立体定位仪下取出手术侧面神经核团。然后迅速放置于液氮中备用。

1.3 大鼠的行为学评价

瞬目反射:用 5 mL 注射器在距离大鼠角膜 2 cm 处快速推出空气,用气流刺激大鼠角膜,观察眼睑闭合动作的频率,强度。触须运动:在安静的环境中,观察大鼠触须运动,包括触须细微的颤动和节律性拂动,时间长度为 3 min。

1.4 组织准备

在预冷的 1 mL 裂解液中,添加 10 μL PMSEF,将组织和裂解液加入预冷的玻璃匀浆器中,在冰浴条件下充分研磨。将组织研磨液转移到 1.5 mL 的离心管中,于 4 $^{\circ}\text{C}$, 12 000 rpm 离心 10 min,离心完毕,吸取上清液转移到新的 1.5 mL 离心管中,即为组织蛋白提取液。采用 Lowry 法^[16]测量组织蛋白提取液的浓

度,储存在 -80 $^{\circ}\text{C}$ 冰箱备用。

1.5 免疫共沉淀

向含相同蛋白量的样品中,加入 5 倍体积的免疫沉淀缓冲液(IP buffer),加入 2 μg 一抗,在 4 $^{\circ}\text{C}$ 冷藏箱内用摇床摇动过夜。再加入 25 μL Protein A/G-琼脂糖,4 $^{\circ}\text{C}$ 摇动 1 h。10000 $\times$ g 离心 2 min,弃上清,沉淀用免疫沉淀缓冲液(IP buffer)洗三遍。得到等体积(约 25 μL)免疫沉淀复合物,加 1/4 体积 5 $\times$ SDS-PAGE 样品缓冲液,沸水浴 5 min,离心取上清,做免疫共沉淀分析。

1.6 电泳和免疫印迹

组织蛋白提取液混合 5 $\times$ 蛋白上样缓冲液。煮沸 5 min,上样。等量的蛋白样品经 8% 的 SDS-聚丙烯酰胺凝胶电泳分离后^[17],以湿转法电转移至 PVDF 膜上,再经 3%BSA 封闭 1 h 后,加入适量稀释的一抗,4 $^{\circ}\text{C}$ 过夜。用洗涤液(washing buffer)洗膜三遍,换入相应的二抗,室温孵育 1 h,洗膜三遍,以 NBT/BCIP 显色,显色后,蒸馏水洗涤终止反应。扫描条带,并且进行分析。

1.7 试剂

一抗:兔抗大鼠 anti-NMDAR2A(sigma 公司, G9038),小鼠抗大鼠 anti-PSD95 (santa cruz 公司, sc-32290),兔抗大鼠 anti-Src(cell signaling 公司, 36D10),二抗:碱性磷酸酯酶标记山羊抗兔 IgG(H+L)(碧云天, A0239),碱性磷酸酯酶标记山羊抗小鼠 IgG (H+L) (碧云天, A0258),PP2 (4-amino-5-(4-chlorophenyl)-7- (t-butyl)pyrazolo [3,4-d]pyrimidine)(sigma, P0042),DMSO(sigma, 276855),免疫共沉淀试剂盒(Bio Vision, K286-25),SDS-PAGE 凝胶配制试剂盒(碧云天, P0012A),BCIP/NBT 碱性磷酸酯酶显色试剂盒(碧云天, C3206)等。

1.8 统计分析

用于分析统计的软件有 Sigma STAT32 统计学软件,实验数据以均值 $\pm$ 标准差(mean $\pm$ SD)表示。统计分析采用单因素方差分析(ANONA),多组均数与同一对照组均数的比较采用最小显著差法(LSD), $P < 0.05$ 为统计学差异有显著性。

2 结果

2.1 PP2 的应用对面神经缺血损伤有保护作用

术前,大鼠鼻端朝前,居于正中,触须向前散开并在活动时可表现为两侧对称的节律性摆动,瞬目反射明显,能完全闭眼。假手术组:大鼠在术后 72 h 瞬目反射和触须运动均恢复正常。与术前行为学观察比较没有明显差别。手术组:术后 72 h,手术侧瞬目反射消失,触须运动消失。DMSO 组:术后 72 h,手术侧瞬目反射消失,触须运动消失。于手术组无明显差别。PP2 干预组:术后 72 h,手术侧瞬目反射有一定程度的好转,有轻微触须运动。相对手术组和 DMSO 组,瞬目反射和触须运动都有一定程度的改善。说明 PP2 的干预处理,对于面神经缺血损伤有一定程度的保护作用。

2.2 大鼠面神经缺血损伤时存在 NR2A-PSD95-Src 信号通路

通过免疫共沉淀和免疫印迹的实验方法,结果表明相对于假手术组,手术组中 NR2A, PSD95, Src 三者的结合明显增加($aP < 0.05$ versus sham 组)。说明在大鼠面神经缺血损伤时,存在 NR2A-PSD95-Src 的信号通路(图 1)。

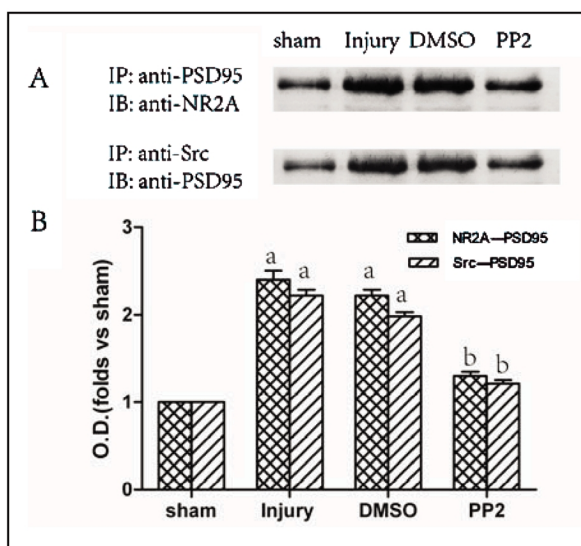


图1 运用免疫共沉淀的方法分析面神经缺血损伤后 NR2A-PSD95-Src 的偶联情况以及 PP2 对面神经缺血损伤所诱导的 NR2A-PSD95-Src 偶联的抑制。蛋白样品分别用 anti-PSD95, anti-Src 免疫沉淀, 再分别用 anti-NR2A 和 anti-PSD95 做免疫印迹检测。PP2 组和 DMSO 组的大鼠分别于术前 30 min 注射 15 μ g PP2 (总体积 10 μ L) 和相同体积的 DMSO。数值用均值 $\pm$ 标准差表示。并且表示为 sham 组的倍数。

aP<0.05 versus sham 组, bP<0.05 versus injury 组 (n=5 rats)

Fig.1 Co-IP analysis of interactions of NR2A and PSD95 with Src after ischemic injury of facial nerve and the effect of PP2 on the interactions of NMDAR-PSD95-Src induced by the ischemic injury of facial nerve. Sample proteins were IP with anti-PSD-95 or anti-Src antibodies and then IB with anti-NR2A or anti-PSD-95 respectively. Rats were pretreated with 15 μ g of PP2 (total volume 10 μ L) or the same dose of DMSO 30 min before ischemia injury in the PP2 group and the DMSO group. Data are the mean $\pm$ SD and are expressed as fold versus respective sham. aP<0.05 versus sham; bP<0.05 versus respective injury groups (n=5 rats)

3 讨论

本实验采用了免疫共沉淀和免疫印迹的试验方法, 并且通过 Src 激酶的抑制剂 PP2 进行干预, 结果显示: 在大鼠面神经缺血损伤时, 面神经元中存在 NR2A-PSD95-Src 的信号通路; 给予 PP2 干预, 可以有效的抑制 NR2A-PSD95-Src 的信号通路的装配, 从而对面神经的缺血损伤起到保护作用。

当前, 面神经缺血损伤的临床治疗并不理想。因此, 需要研究面神经缺血损伤的分子机制, 为以后的临床治疗提供实验依据。NMDA 受体是中枢神经系统广泛分布的离子型谷氨酸受体^[18], Ca^{2+} 通过激活的 NMDAR 离子通道时会产生兴奋性神经毒性^[19]。在谷氨酸能突触的突触后密集区, 受体蛋白、骨架蛋白和多种信号分子可直接或间接的结合到 NMDA 受体上, 形成一个以 NMDA 受体为核心的动态信号复合体^[20], PSD95 是此复合体中的关键蛋白。作为一种支架蛋白, PSD95 包括以下的 5 个结构域: N 端的 3 个 PDZ 结构域, 1 个 SH3 结构域, C 端的 GK 结构域^[21]。实验证实 PDZ 结构域可以介导蛋白质之间的互相偶联, SH3 能结合富含脯氨酸的序列, GK 结构域可以介导多种突触后膜蛋白的聚集^[22]。在 PSD95 锚定的众多蛋白中, 具有介导 NMDAR 酪氨酸磷酸化的 Src 激酶是调控 NMDAR 功

能的重要分子^[23]。有学者认为, 在大鼠的海马 CA1 区缺血损伤时, NMDA 受体通过骨架蛋白 PSD95 募集 Src 酪氨酸蛋白激酶。Src 可以使 NMDA 受体的 NR2A 亚基发生酪氨酸磷酸化, 从而提高了 NMDA 受体对 Ca^{2+} 的通透性, Ca^{2+} 大量内流, 激活了下游的信号通路, 从而引起了海马 CA1 区的神经元损伤^[24]。本实验结果显示, 在面神经缺血损伤时, injury 组中的 NMDAR-PSD95-Src 的结合明显增加, 说明在面神经缺血损伤时, 存在 NMDAR-PSD95-Src 的信号通路。同时, 在给予 Src 激酶的抑制剂 PP2 时, 相对于 injury 组和 DMSO 组, PP2 干预组大鼠的瞬目反射和触须运动都有一定程度的改善, 说明了 PP2 对于面神经缺血损伤具有保护作用。通过图 1 可以看出给予 PP2 干预后, 相对于 injury 组和 DMSO 组, PP2 组中的 NMDAR-PSD95-Src 的结合明显减少, 提示 PP2 在面神经缺血损伤时可以有效的抑制 NMDAR-PSD95-Src 信号通路的装配, 从而减少了 Src 激酶对 NMDAR 的酪氨酸磷酸化作用, 有效的抑制了 NMDA 受体的活化, 进而抑制了兴奋性毒性的发生。最终在大鼠面神经缺血模型中起到保护作用。

综上所述, 本实验结果提示大鼠面神经缺血损伤时, 存在由 NMDAR-PSD95-Src 形成的三聚体信号通路; 给予 PP2 干预, 可以有效的减少 NMDAR-PSD95-Src 三聚体信号通路的形成, 从而对大鼠面神经缺血损伤起到保护作用。

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