

CaMKII γ 和CaMKII δ 通过PI3K/Akt/Erk信号通路减轻小鼠神经元缺血再灌注损伤

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摘要:目的 观察钙/钙调蛋白依赖性激酶 II (CaMK II) 的同工型 CaMK II γ 和CaMK II δ 对鼠神经元细胞缺血缺氧再灌注损伤的影响,并探究其作用机制。方法 分离胚胎期第18天胎鼠大脑用于提取原代神经元,在5% CO₂、37 °C条件下培养,分为常规培养的对照组、空白对照组 (si-NT)、CaMKII γ 敲除组 (si-CAMK2G)和CaMKII δ 敲除组 (si-CAMK2D)。研究组神经元转染处理后,更换为无糖培养基,并将其置于缺氧环境中模拟氧糖剥夺 (OGD/R) 条件,持续1 h,随后复原标准培养环境。通过对神经元裂解物用免疫蛋白印迹检测磷脂酰肌醇-3-激酶/细胞外信号调节激酶 (PI3K/Akt/Erk) 信号通路构件的表达量,并建立小鼠大脑中动脉闭塞 (MCAO) 模型,通过对比假手术组 (Sham) ($n=25$) 和 MCAO 组 ($n=25$) PI3K/Akt/Erk 信号通路表达来进行验证。结果 si-CAMK2G 组的胎鼠神经元细胞在 OGD/R 12、24、48、72 h 的生存率明显低于 si-NT 组 ($P<0.01$ 或 0.001),并可逆转 OGD/R 介导的胎鼠神经元细胞 CaMK II γ 表达上调。si-CAMK2G 组和 si-CAMK2D 组与 si-NT 组相比,PI3K/Akt/Erk 信号通路表达受到明显抑制 ($P<0.01$)。在 MCAO 模型中,MCAO 组小鼠脑 CaMKII δ 和 CaMK II γ 的表达显著增加并激活了 PI3K/Akt/Erk 信号通路,CaMKII δ 和 CaMKII γ , Erk、磷酸化 Erk、Akt 和磷酸化 Akt 在 MCAO 造模成功再灌注后 24、48、72 和 96 h 的表达显著高于 Sham 组再灌注 24 h ($P<0.05$, 0.01 或 0.0001)。结论 CaMK II γ 和 CaMKII δ 在神经元细胞发生缺血缺氧损伤时的神经保护作用可能是通过 PI3K/Akt/Erk 信号通路介导的。

关键词:脑缺血再灌注损伤;钙/钙调蛋白依赖性激酶 II;神经保护;磷脂酰肌醇-3-激酶;细胞外信号调节激酶;信号通路

PI3K/Akt/Erk signaling pathway mediates neuroprotection of CaMKII γ and CaMKII δ against ischemic reperfusion injury in mice

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Abstract: Objective To observe neuroprotective effects of Ca²⁺/calmodulin-dependent kinase II (CaMK II) γ and CaMKII δ against acute neuronal ischemic reperfusion injury in mice and explore the underlying mechanism. **Methods** Primary cultures of brain neurons isolated from fetal mice (gestational age of 18 days) were transfected with two specific siRNAs (si-CAMK2G and si-CAMK2D) or a control sequence (si-NT). After the transfection, the cells were exposed to oxygen-glucose deprivation/reperfusion (OGD/R) conditions for 1 h followed by routine culture. The expressions of phosphatidylinositol-3-kinase/extracellular signal-regulated kinase (PI3K/Akt/Erk) signaling pathway components in the neurons were detected using immunoblotting. The expressions of the PI3K/Akt/Erk signaling pathway proteins were also detected in the brain tissues of mice receiving middle cerebral artery occlusion (MCAO) or sham operation. **Results** The neuronal cells transfected with si-CAMK2G showed significantly lower survival rates than those with si-NT transfection at 12, 24, 48, and 72 h after OGD/R ($P<0.01$), and si-CAMK2G transfection inhibited OGD/R-induced upregulation of CaMKII γ expression. Compared to si-NT, transfection with si-CAMK2G and si-CAMK2D both significantly inhibited the expressions of PI3K/Akt/Erk signaling pathway components ($P<0.01$). In the mouse models of MCAO, the expressions of CaMKII δ and CaMKII γ were significantly increased in the brain, where activation of the PI3K/Akt/Erk signaling pathway was detected. The expression levels of CaMKII δ , CaMKII γ , Erk, phosphorylated Erk, Akt, and phosphorylated Akt were all significantly higher in MCAO mice than in the sham-operated mice at 24, 48, 72, and 96 h after reperfusion ($P<0.05$). **Conclusion** The neuroprotective effects of CaMKII δ and CaMKII γ against acute neuronal ischemic reperfusion injury are mediated probably by the PI3K/Akt/Erk pathway.

Keywords: cerebral ischemia/reperfusion injury; Ca²⁺/calmodulin-dependent kinase II; neuroprotection; phosphatidylinositol-3-kinase; extracellular signal-regulated kinase; signaling pathway

缺血性脑卒中是全球范围内致残率最高的疾病之一,也是导致患者死亡的第3大原因^[1]。由于人口基数大以及老龄化,我国缺血性脑卒中患者数居全球首位^[2]。

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尽快恢复脑卒中患者缺血区域血流灌注有助于神经细胞功能修复,但会诱发脑缺血再灌注损伤,造成继发性神经功能恶化。目前针对脑缺血再灌注损伤的确切治疗手段尚有待开发^[3]。

钙/钙调节蛋白依赖蛋白激酶 II (CaMKII) 家族是一种丝氨酸/苏氨酸特性的多功能蛋白激酶,通过钙离子信号通路调节多种细胞的生存与活性,参与调节突触可

塑性,影响行为、海马学习及记忆功能;在脑缺血中,CaMKII表达和/或激活的改变是缺血诱导神经细胞死亡的关键因素^[4-6]。但既往关于CaMKII对缺血性脑卒中神经保护的研究结果颇具争议。有报道指出CaMKII具有促进凋亡的神经损伤作用;CaMKII过表达可增加谷氨酸诱导的缺血损伤后神经元死亡率,使用CaMKII抑制剂则可减轻缺血后神经损伤^[7-9]。另一些研究表明,CaMKII可以通过磷酸化和抑制促凋亡蛋白一氧化氮合酶、Bax等发挥神经保护作用;激活CaMKII可提高缺血缺氧神经元的存活率,敲除CaMKII基因可导致更严重的神经细胞死亡^[4,10-12]。造成上述对立观点的原因除了研究使用了非特异性CaMKII抑制剂、神经元来源于不同脑区或不同生长时期、缺血后损伤的观察时点不一之外^[4,13],更重要的是既往研究并未区分CaMKII各同工型对缺血靶点的影响。

CaMK II 包括 α 、 β 、 γ 、 δ 四类同工型,课题组前期研究证实缺血再灌注神经元细胞对CaMKII各同工型的表达差异,在针对单个同工型的探讨中发现:CaMKII δ 和CaMKII γ 通过激活核转录因子 κ B(NF- κ B)信号通路产生神经保护作用^[14,15]。然而在不同的缺血再灌注神经元研究中,NF- κ B信号通路会表现出促进炎症凋亡作用或者保护作用^[10,14-16]。因此我们猜测存在其他途径参与该通路介导CaMKII δ 和CaMKII γ 的神经保护作用。

细胞外信号调节激酶(Erk)信号途径和磷脂酰肌醇-3-激酶/蛋白激酶B(PI3K/Akt)信号途径两个通路均可因细胞缺血缺氧而被激活,影响并调控下游的NF- κ B信号通路^[17]。但目前尚无研究探讨CaMKII δ 和CaMKII γ 的神经保护作用与PI3K/Akt/Erk通路的内在联系。本研究拟在前期研究基础上,通过氧糖剥夺/复氧(OGD/R)和大脑中动脉闭塞(MCAO)模拟缺血再灌注,探讨PI3K/Akt/Erk是否参与调控CaMKII γ 和 δ 对脑缺血再灌注损伤的保护机制。

1 材料和方法

1.1 材料

1.1.1 实验试剂 GAPDH抗体(Badrila Inc);CaMKII(pan)(D11A10)抗体(Cell Signaling);p44/42MAPK(Ek1/2)抗体、Phospho-p44/42MAPK(Erk1/2)抗体、Akt抗体、Phospho-Akt(Ser473)抗体(Cell Signaling);胎牛血清(Gib-ol);0.05%和0.25%胰酶、2,3,5氯化三苯基四氮唑(Sigma);碘化丙啶、DharmaFECT转染试剂(ThermoFisher);钙黄绿素(Invitrogen);CAMK2G和CAMK2D-小干扰RNA(si-RNA)和非靶向siRNA(si-NT)(Integrated DNA Technologies);P3 Primary Cell 4d-nucleofector kits(Lonza)。

1.1.2 动物 SPF级雄性的BALB/e小鼠18~22 g 8~10周、孕18 d的野生型SD大鼠50只,购自南方医科大学

动物实验中心[质量合格许可证号SCXK(粤)2016-0041],洁净级饲养环境,在恒温26℃条件下,以12 h光照-黑暗交替循环。所有实验步骤均按南方医科大学实验动物伦理委员会批准开展(伦理批号:LAEC-2021-074)。

1.2 方法

1.2.1 胎鼠皮质神经元细胞分离培养

1.2.1.1 神经元提取 把SD孕鼠放入吸入麻醉诱导装置,以3%七氟醚与空气混合充入诱导箱中。待其进入麻醉状态后,迅速将胚胎剖出,并浸泡在预先上预冷的75%酒精内消毒。使用灭菌消毒后的剪刀迅速将胎鼠断头,分离胎鼠大脑并迅速将其置于事先预冷好的装有DMEM-F12的培养皿中。剪碎大脑组织后加入0.125%的胰酶,等待15 min后加入含有10%胎牛血清的DMEM-F12完全培养基。使用移液枪轻柔吹打3次,后静置1 min后弃掉上清。加入含有10 μ m/mL DNA酶的完全培养基,反复吹打使神经元分离到培养基中,静置1 min后吸取上层清液,滴入盖有孔径40 μ m滤膜的40 mL离心管中。加入DMEM-F12,重复以上操作2次。将收集到的所有细胞于4℃、1000 r/min离心5 min,吸出上层清液,用神经元接种液(DMEM-F12基础培养基+10%胎牛血清+1%的青霉素链霉素溶液)重悬细胞^[18]。

1.2.1.2 接种神经元 预先按每孔1 mL的量向6孔板中加入多聚赖氨酸(0.1 mg/mL)。消毒后放置于37℃培养箱中过夜。次日稀释神经元悬液到需要浓度(1×10^5 、 5×10^5 、 1×10^6 细胞/mL)分别加入1 mL六孔板的上样孔中,培养环境为37℃、5% CO₂、饱和湿度,等待原代神经元充分贴壁。

1.2.2 si-RNA转染 根据产品说明使用P3 Primary Cell 4D-Nucleofector试剂对原代神经元细胞进行Myc-DDK-CaMKII δ 质粒(OriGene Technologies)转染。使用Dharma FECT转染试剂和Cell Line Nucleofector V试剂将CAMK2G siRNA(si-CAMK2G)、CAMK2D siRNA(si-CAMK2D)和非靶向siRNA(si-NT)转染到原代神经元细胞中。转染48 h后,原代神经元接受OGD/R处理,然后在常氧培养条件下复氧不同时间,并观察Erk、磷酸化Erk(p-Erk)、Akt、磷酸化Akt(pS473-Akt)的表达。

1.2.3 OGD/R模型 在对37℃、5%CO₂条件中培养的神经元细胞更换无糖培养液后,将其置入1% O₂+5% CO₂+94%N₂的37℃缺氧箱内培养1 h;之后以原培养方式继续观察72 h。对照组细胞则一直放置于37℃、5% CO₂培养箱内,不更换无糖培养液。通过钙黄绿素AM/碘化丙啶染色评估细胞生存率^[19]。

1.2.4 小鼠MCAO模型建立^[19] 随机将小鼠分为假手术组(Sham)和MCAO组,每组各25只,使用3%七氟醚

混合空气诱导小鼠进入麻醉状态后用1%七氟醚维持,在显微镜下分离颈部组织暴露左侧颈总动脉、颈外动脉及颈内动脉,Sham组只进行动脉分离。MCAO组使用4-0号缝合线行颈总动脉的近心端和颈外动脉的远心端结扎;由颈总动脉将头端直径0.23 mm、主干直径0.18 mm的尼龙栓线插入至大脑中动脉,深度约120 mm,阻断1 h后抽出线栓,缝合并消毒术口后将小鼠放入笼中饲养,术后24 h后对两组小鼠进行神经行为学评分,以此判断MCAO模型的效果,神经功能缺陷评分标准如下^[19]:0=无可观察的缺陷;1=前肢屈曲;2=前肢屈曲和侧推抗力减弱;3=前肢屈曲、侧推抗力减弱和单侧盘旋;4=前肢屈曲和行走能力受损或丧失。取大脑组织行2,3,5-三苯基氯化四氮唑染色,应用Image J软件评估脑梗死面积。并于再灌注后24、48、72和96 h观察梗死灶周围脑组织的CaMKII γ 和CaMKII δ 、Erk、p-Erk、Akt和pS473-Akt的表达。

1.2.5 Western blot检测 将200 μ L RIPA细胞裂解液分别加入神经元样品中,30 min后将裂解好的神经元碎片混合液离心5 min(4 $^{\circ}$ C 1000 r/min)。收集上清液使用BCA蛋白测定试剂盒定量蛋白裂解物。各取30 μ g样品经过SDS-聚丙烯酰胺凝胶电泳分离后转移至PVDF膜上。PVDF膜取出放置在配好的封闭液中(10 mL TBST+脱脂奶粉0.2 g),在室温下封闭2 h。一抗(1:1000稀释)4 $^{\circ}$ C孵育过夜,二抗(1:1000稀释)常温孵育1 h。结果通过化学发光进行可视化。使用Imaging J软件对图像进行定量分析。

1.3 统计学分析

计量资料采用均数 \pm 标准差表示,多组间的比较采用ANOVA检验。使用SPSS26.0统计软件进行统计分析, $P<0.05$ 被认为差异有统计学意义。

2 结果

2.1 敲减CaMK II γ 显著降低大鼠神经元细胞的生存率

与转染si-NT的对照细胞相比,转染si-CAMK2G的神经元细胞在OGD/R 12、24、48、72 h后,多数细胞形态呈现出梭形或圆形,细胞体积缩小,突触数目减少(图1)。细胞生存率明显低于si-NT(图2)。

2.2 敲减CAMK2G或CAMK2D可抑制PI3K/Akt/Erk信号通路表达上调

与对照组细胞相比,转染si-CAMK2G可特异性逆转OGD/R介导的鼠神经元细胞CaMKII γ 的表达上调,转染si-CAMK2D可特异性逆转CaMKII δ 的表达上调($P<0.01$)。与对照组细胞相比,转染si-CAMK2G或si-CAMK2D可使Erk、p-Erk和Akt的蛋白表达明显受到抑制;转染si-CAMK2D1可抑制pS473-Akt的蛋白表达($P<0.01$)。与转染了si-NT的胎鼠原代神经元细胞

相比,转染si-CAMK2G或si-CAMK2D可抑制pS473-Akt的蛋白表达($P<0.01$,图3、4)。

2.3 MCAO增加小鼠脑CaMKII δ 和CaMK II γ 的表达,激活PI3K/Akt/Erk信号通路

MCAO组小鼠缺血后出现100%运动减少、90%侧倾姿势、84%扁平姿势、转圈68%、低反应性52%、前肢屈曲100%、肌力下降100%、运动失调100%神经行为学总体评分增加,而sham组无明显变化MCAO诱导的脑组织梗死会在24 h内显现,缺血脑区的神经元进行性坏死会在72 h达到峰值,随后在96 h出现一定恢复(图5)。从梗死灶周围的半暗区域收集的脑组织,使用CaMKII(pan, D11A10)抗体通过Western blot可检测到CaMKII δ 和CaMKII γ 在24、48和72 h持续上调,并在96 h回调,CaMKII δ 显著高于sham组再灌注24 h($P<0.0001$),CaMKII γ ($P<0.05$ 或0.01);sham组内各观察时点的CaMKII δ 和CaMKII γ 表达与再灌注24h比较差异无统计学意义($P>0.05$,图6、7)。

在MCAO造模成功再灌注24、48、72和96 h后,Erk、p-Erk、Akt、pS473-Akt的表达均高于sham组再灌注24 h的表达($P<0.05$ 或0.01),上调变化趋势与CaMKII δ 和CaMKII γ 相似;sham组内各观察时点Erk、p-Erk、Akt、pS473-Akt的表达与再灌注24 h比较差异无统计学意义($P>0.05$,图8、9)。

3 讨论

课题组前期研究使用能识别所有CaMKII同工型的pan CaMKII抗体,首次证明在原代神经元中,缺血再灌注损伤可选择性地诱导CaMKII δ 和CaMKII γ ^[14,15]。课题组发现在缺血再灌注后,小鼠来源神经瘤母细胞中主要上调的同工型CaMKII δ ,而非CaMKII α 或CaMKII β 。相比之下,在小鼠原代皮质神经元中,CaMKII γ 是上调程度最高的同工型,其次是CaMKII δ ^[15]。CaMKII α 和CaMKII β 在离体缺氧损伤神经元中的表达远低于CaMKII δ 和CaMKII γ ,且缺血再灌注损伤诱导的CaMKII γ 高表达及持续上调是一个新的发现,暗示了这种同工型在神经元中的潜在重要作用。此外,CAMK2G在DNA水平的峰值上调达7倍,远高于CAMK2D的3~4倍,表明CaMKII γ 可能对大脑中的神经元具有重要的功能。此外,我们还证实缺血再灌注的神经元细胞对CaMKII各同工型的表达差异,并通过质粒转染过表达以及si-RNA敲减CAMK2D和CAMK2G证明其通过激活NF- κ B信号通路产生神经保护作用,显示了NF- κ B信号通路对缺血再灌注神经元不仅具有促进炎症凋亡作用,还存在保护作用。

对PI3K/Akt/Erk信号通路与心脑缺血再灌注损伤的研究提示,该通路的激活与缺血再灌注损伤密切相关

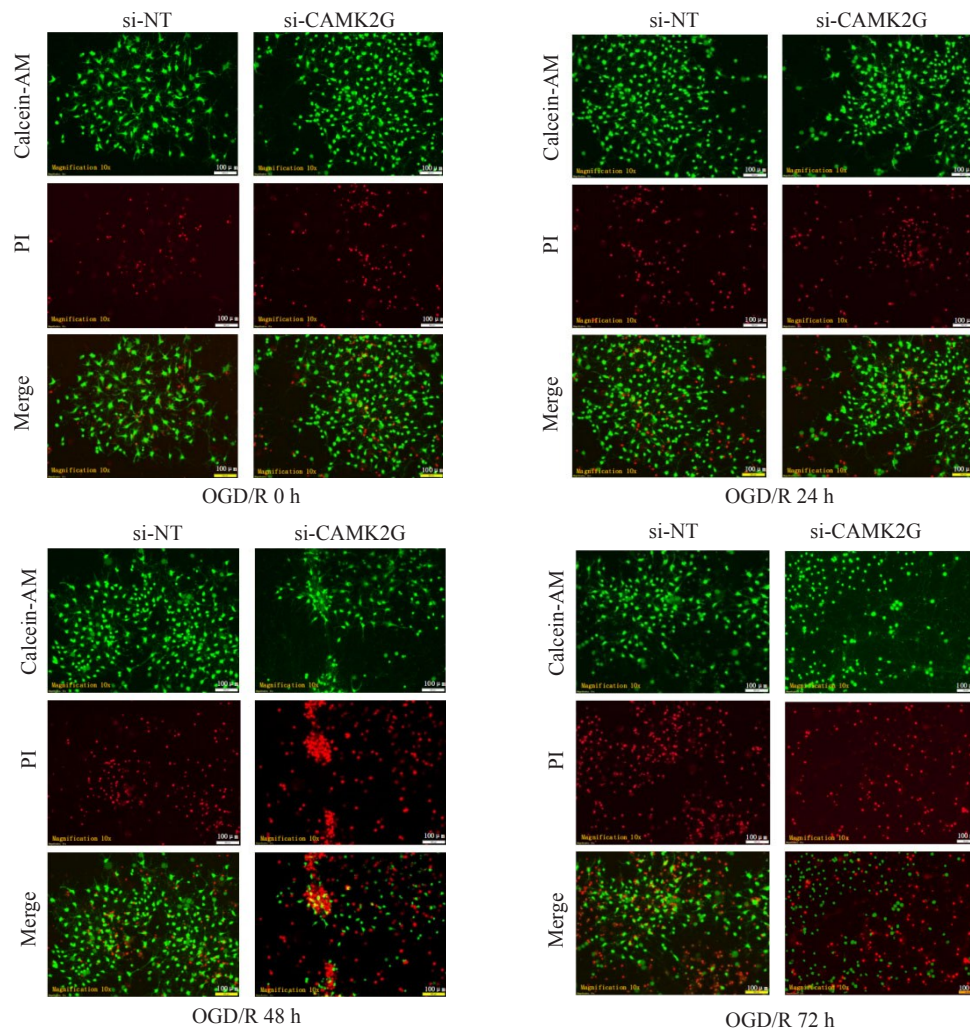


图1 OGD/R后0~72 h钙黄绿素AM/碘化丙啶染色的原代神经元图像

Fig.1 Representative images of calcein-AM/ propidium iodide (PI) staining of the primary neurons from 0 h to 72 h following oxygen-glucos deprivation/reperfusion (OGD/R) (Original magnification: ×200). The red channel shows PI staining for cell death, and the green shows calcein-AM staining of live cells.

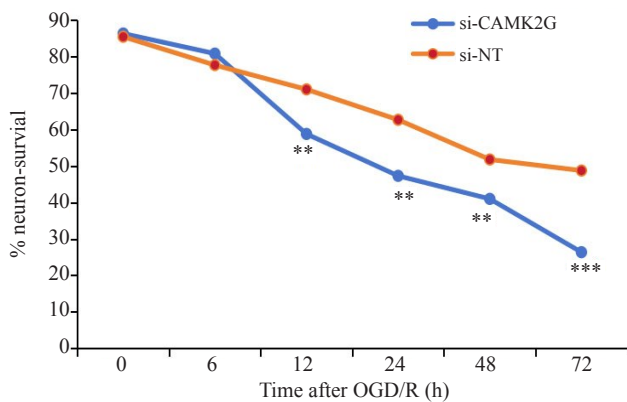


图2 敲减CAMK2G可增加OGD/R诱导的原代神经元细胞死亡

Fig.2 Knockdown of CAMK2G exacerbates OGD/R-induced neuronal cell death. Cell survival was assessed by calcein-AM/PI staining. The total PI-positive or calcein-AM-positive cells were counted from 10 random fields in each image. The neuron survival rate was calculated as the ratio of calcein-AM-positive cell number over the total cell number by Image J. ** $P < 0.01$, *** $P < 0.001$.

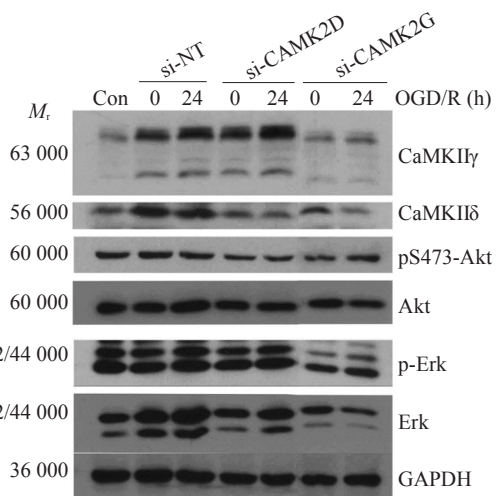


图3 OGD/R对si-NT、si-CAMK2D、si-CAMK2G转染后的大鼠原代神经元细胞中PI3K/Ark/Erk通路组件的影响

Fig.3 Knockdown of CAMK2D or CAMK2G inhibits upregulation of CaMKII δ or CaMKII γ , respectively, and suppresses the expressions of Erk, p-Erk, Akt and pS473-Akt induced by OGD/R. GAPDH was blotted as the loading control. Representative images from 3 independent experiments are shown.

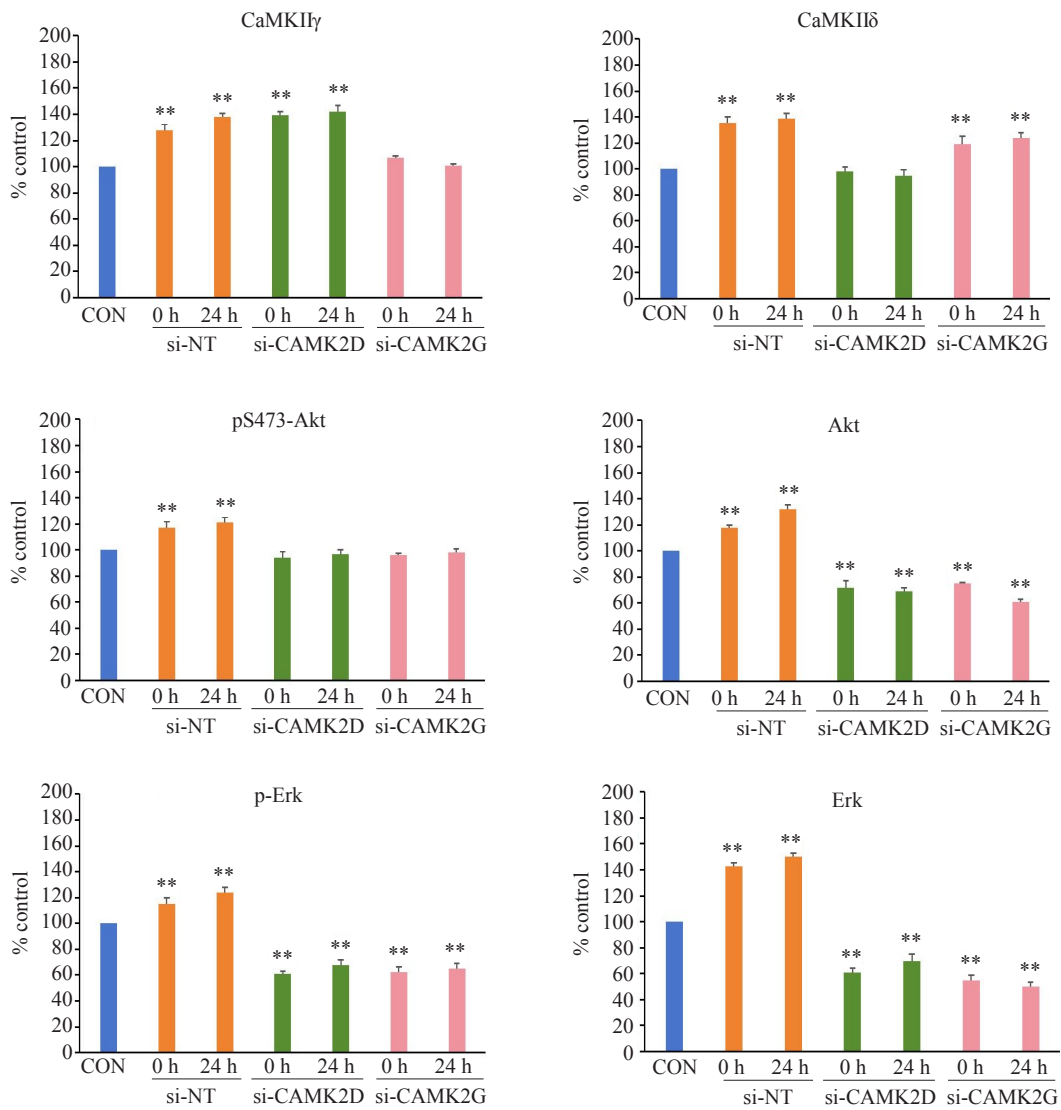


图4 各灰度值表达量化图

Fig.4 Quantification of the expression levels of CaMKII δ , CaMKII γ , Akt, phosphorylate-Akt, Erk and phosphorylate-Erk in the neurons with or without CAMK2G and CAMK2D knockdown by densitometry analysis. Percent control (y-axis) represents the expression of the target genes to that of the controls under normoxic condition (100%). Con: Control. p-Erk: Phosphorylate-Erk. Data are Mean \pm SD from 4 independent experiments. **P<0.01 vs control.

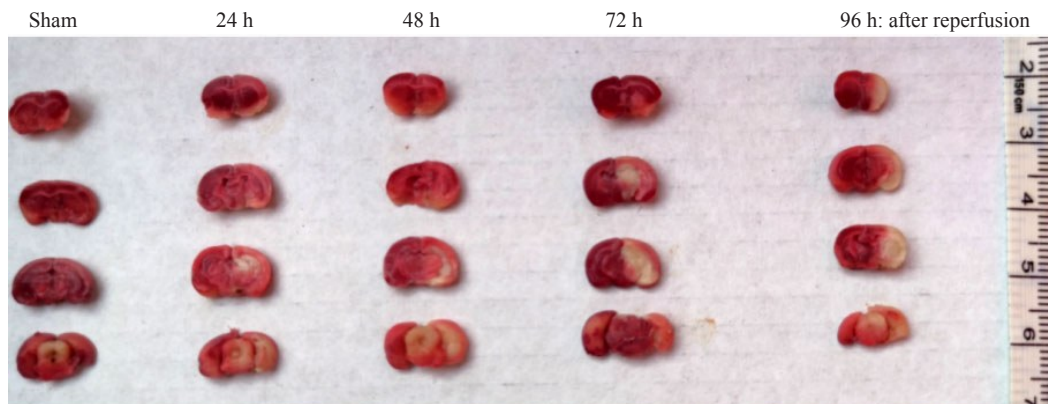


图5 MCAO 诱导小鼠脑梗死

Fig.5 Cerebral infarction in mice induced by MCAO observed at 24, 48, 72, and 96 h after reperfusion.

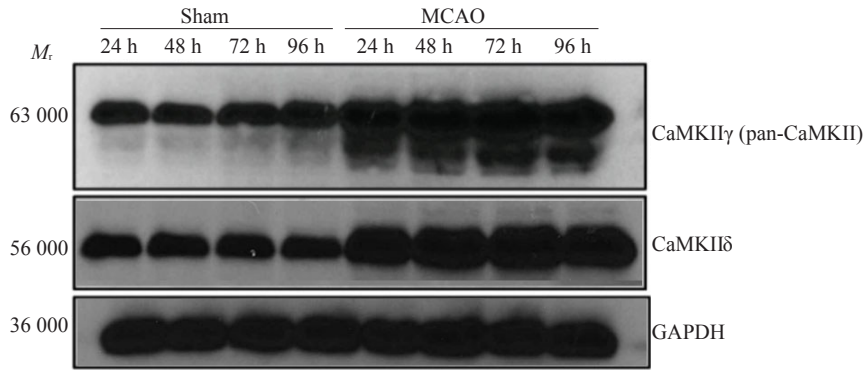


图6 通过 Western blotting 分析两组梗死灶周围脑组织的 CaMKII γ 和 CaMKII δ 的表达
Fig.6 Analysis of CaMKII γ and CaMKII δ expressions in the penumbra of sham-operated and MCAO mice at 24, 48, 72 and 96 h after reperfusion by Western blotting.

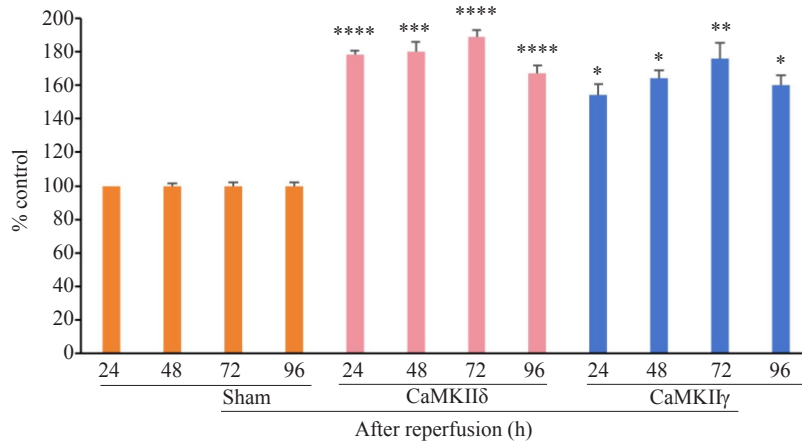


图7 通过灰度值分析将 CaMKII δ 和 CaMKII γ 的表达量化
Fig.7 Quantification of the levels of CaMKII δ and CaMKII γ by densitometry analysis. The value in sham-operated group at 24 h served as the control (100%). Data are Mean \pm SD from 4 independent experiments. * P <0.05; ** P <0.01; *** P <0.001; **** P <0.0001 vs sham 24 h.

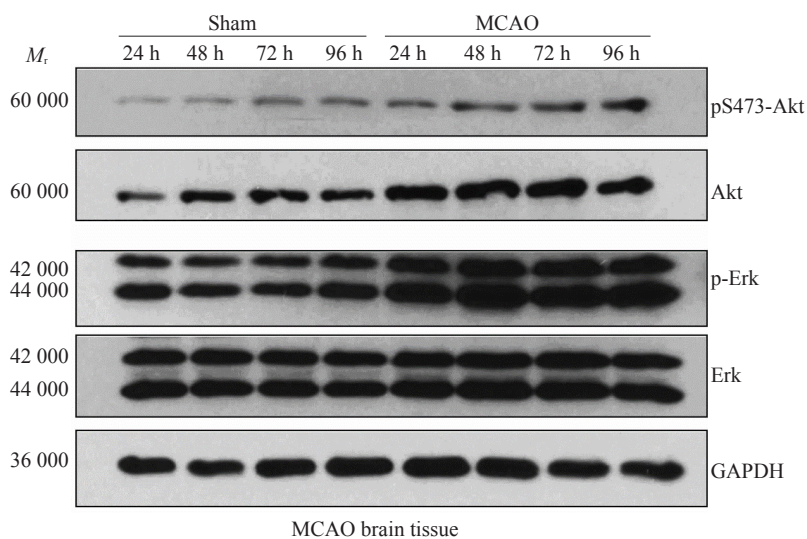


图8 Sham 组和 MCAO 组织梗死灶周围组织中 PI3K/Akt/Erk 信号通路的 Western blot 分析
Fig.8 PI3K/Akt/Erk signaling pathway in the penumbra of sham-operated and MCAO mice at 24, 48, 72 and 96 h after reperfusion by Western blotting. Representative images from 4 independent experiments are shown.

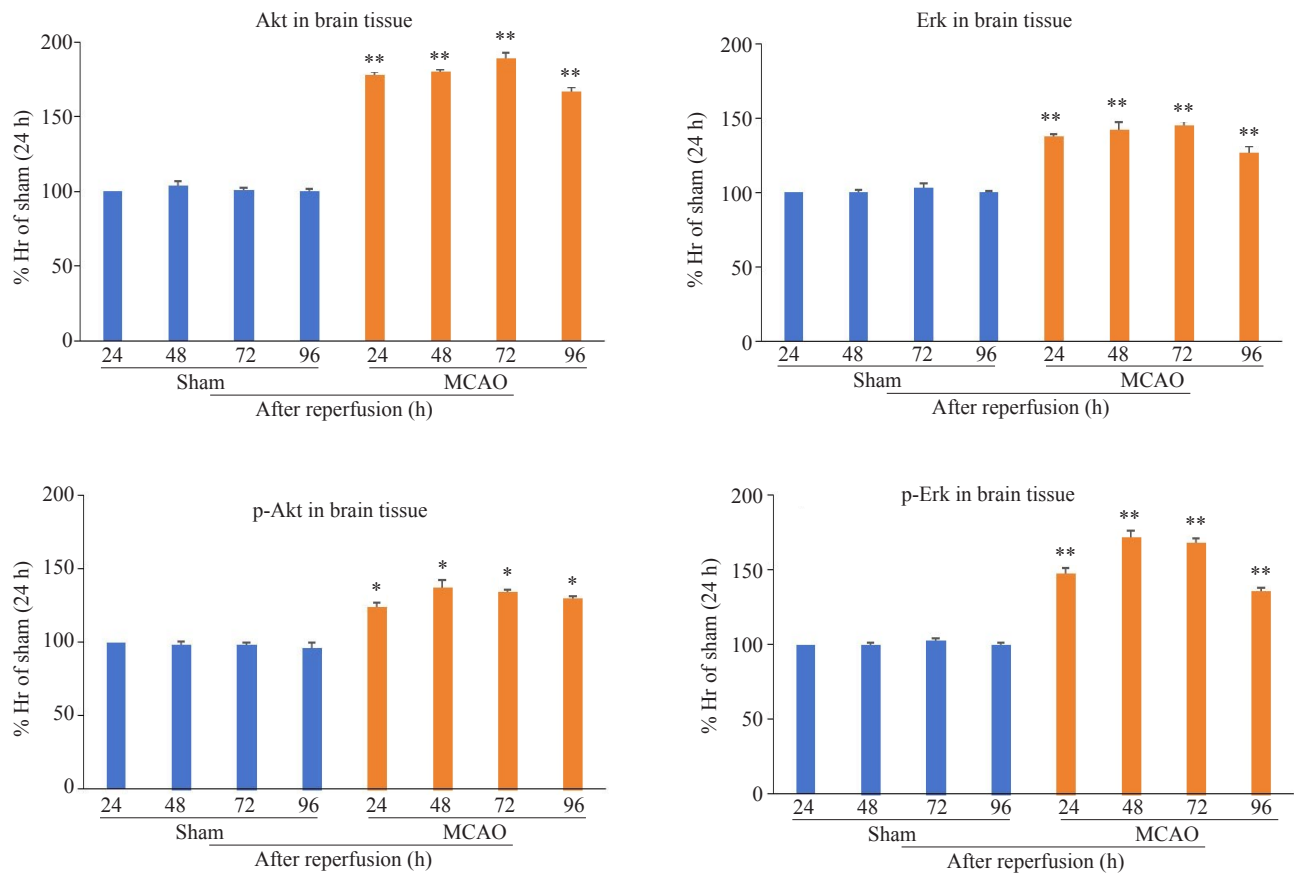


图9 各灰度值表达量化表

Fig.9 Quantification of the levels of Akt, phosphorylate-Akt, Erk and phosphorylate-Erk in the penumbra of sham-operated and MCAO mice by densitometry analysis. Data are $Mean \pm SD$ from 4 independent experiments. * $P < 0.05$; ** $P < 0.01$ vs sham 24 h.

关,由于Akt也可激活IkB激酶IKK α ,使NF- κ B的抑制蛋白IkB降解,进而导致NF- κ B从细胞质中释放,激活其靶基因而促进细胞的存活,调节Bcl-xL,发挥抗凋亡作用。是否因为PI3K/Akt和Erk通路参与其中,才使NF- κ B信号通路产生神经保护作用呢?

通过建立MCAO模型,我们发现缺血再灌注损伤可激活PI3K/Akt/Erk信号通路:Erk、p-Erk、Akt和pS473-Akt的表达上调。由此我们推断PI3K/Akt/Erk信号通路参与了神经缺血再灌注损伤的调控。而通过转染si-CAMK2D和si-CAMK2G敲减CAMK2D和CAMK2G,发现PI3K/Akt/Erk信号通路组件的表达明显受到抑制。因此,我们判断CaMKII δ 和CaMKII γ 通过激活NF- κ B信号通路产生神经保护作用的上游通路为PI3K/Akt/Erk信号通路,提示了CaMKII δ 和CaMKII γ 在缺血性损伤期间促进神经元存活的新机制,有助于开启脑缺血再灌注损伤治疗的新思路。

此外,CaMKII四个同工型在不同的脑区表达量各不相同^[20-23],而CaMKII γ 在海马和海马旁回、外侧缰核、前额叶皮质等脑区中,与学习记忆调节、认知功能、抑郁症、自闭症或精神分裂症病理生理密切相关^[24-27],由此我

们猜想CaMKII γ 可在脑缺血再灌注损伤神经保护的基础上,更精准地改善不同部位神经元的生存状态,促进神经功能恢复,改善相关脑区损伤后带来的并发症。由于本系列研究均聚焦于皮质神经元,我们后续将深入探讨CaMKII γ 及其子型对不同脑区脑缺血再灌注损伤的影响,以及作用机制。

综上所述,CaMKII γ 和CaMKII δ 在鼠皮质神经元细胞发生缺血缺氧损伤时,通过激活NF- κ B信号通路产生的神经保护作用,可能由PI3K/Akt/Erk信号通路介导。

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