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线粒体大麻素受体 1 在大鼠海马神经元缺氧复氧损伤中对线粒体分裂的影响 *

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摘要 目的:探讨线粒体 CB1 受体(mitochondrial cannabinoid receptor1,mtCB1)在大鼠海马神经元缺氧复氧损伤中对线粒体分裂的影响。**方法:**原代培养新生的 Wistar 大鼠海马神经元,将培养至第 8 天的海马神经元采用随机数字表分为 5 组(n=60):正常组(N 组):正常培养,不做任何处理;缺氧复氧组(H/R 组):采用氧糖剥夺法构建海马神经元缺氧复氧损伤模型,缺氧 6h,复氧 20 h;缺氧复氧组 +ACEA+AM251 组 (H/R+ACEA+AM251 组):缺氧 6 h 结束后立即加入 ACEA 和 AM251,终浓度分别为 1 μmol/L、10 μmol/L,复氧 20 h;缺氧复氧 +ACEA+ Hemopressin(H/R+ ACEA+ Hemo 组):缺氧 6h 结束后立即加入 ACEA 和 Hemopressin,终浓度分别为 1 μmol/L、10 μmol/L,复氧 20 h;缺氧复氧 + 赋形剂组 (H/R+V 组):同样于缺氧 6h 结束后立即加入二甲基亚砜(DMSO),终浓度 <0.1 %,复氧 20 h。使用激光共聚焦显微镜检测细胞内 Ca^{2+} 的浓度,流式细胞仪检测细胞凋亡率,Western blot 检测凋亡诱导因子 (AIF)、线粒体分裂相关蛋白 Drp1、Fis1,细胞凋亡相关蛋白细胞色素 C (Cytc) 和 Rho 相关的卷曲蛋白激酶 1 (ROCK1)的表达。**结果:**与 N 组相比,H/R 组、H/R+ACEA+AM251 组、H/R+ACEA+Hemo 组和 H/R+V 组的细胞内 Ca^{2+} 浓度、细胞凋亡率、以及 AIF、Drp1、Fis1、Cytc、ROCK1 蛋白的表达水平均明显增加(P<0.05);与 H/R 组相比,H/R+ ACEA+Hem 组上述各检测指标明显降低 (P<0.05),H/R+ACEA+AM251 组和 H/R+V 组各指标比较差异无统计学意义 (P>0.05)。**结论:** 线粒体 CB1 受体(mtCB1 受体)可能通过降低细胞内 ROS 的含量来减少细胞内 Ca^{2+} 浓度和 ROCK1 的表达,进而抑制线粒体分裂,并最终减轻海马神经元缺氧复氧损伤。

关键词:线粒体 CB1 受体;线粒体分裂;海马神经元;缺氧复氧损伤

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Effect of Mitochondrial CB1 Receptor on the Mitochondrial Fission in a Rat Hippocampal Neuron Model of Hypoxia/Re-oxygenation Injury*

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ABSTRACT Objective: To evaluate the effect of mitochondrial CB1 receptor (mtCB1) on mitochondrial fission in a rat hippocampal neuron model of hypoxia/re-oxygenation (H/R) injury. **Methods:** Primarily cultured hippocampal neurons obtained from Wistar rats were divided into 5 groups (n=60) using a random number table: normal group (N group):were cultured in normal medium, without any administration; H/R group: the hippocampal neurons were subjected to oxygen-glucose deprivation (OGD) for 6h followed by re-oxygenation for 20h; H/R+ACEA+AM251 group: ACEA and AM251 were add with respectively final concentration 1 umol/L, 10 umol/L during the 20h re-oxygenation; H/R+ACEA+ Hemopressin group: ACEA and Hemopressin were add into culture medium with respectively final concentration 1 umol/L, 10 umol/L during the re-oxygenation for 20h; H/R + Vehicle group (H/R+V group): DMSO was add into culture medium with final concentration <0.1 % during the 20h re-oxygenation. Laser scanning confocal microscope was used to measure Ca^{2+} concentration in cytoplasm. The apoptosis rate was tested by flow cytometry. Western blot was adopted to examine the expression of apoptosis-inducing factor (AIF), dynamin-related protein 1(Drp1), fission 1(Fis1), apoptosis-related protein cytochrome c (Cytc), and Rho-associated coiled-coil containing protein kinase (ROCK1). **Results:** Compared to the N group, the apoptosis rate, Ca^{2+}concentration, the expression of AIF, Drp1, Fis1, Cytc and ROCK1were significantly increased in the other four groups (P<0.05); Compared to the H/R group, those detection indexes mentioned above were significantly decreased in H/R+ ACEA+ Hemopressin group (P<0.05), and there were no significant differences were observed in H/R+ACEA+AM251 group and H/R+ Vehicle group (P>0.05); **Conclusion:** The reduction of ROS induced by mtCB1 can alleviate the expression of ROCK1 and Ca^{2+} concentration in cytoplasm, and

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inhibit the mitochondrial fission, and eventually attenuate the H/R injury of hippocampal neurons.

Key words: Mitochondrial CB1 receptor; Mitochondria fission; Hippocampal neuron; Hypoxia/ re-oxygenation injury

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前言

脑缺血再灌注损伤可引起包括感觉、知觉、运动在内的多种神经功能的损伤,严重危害机体的健康。脑缺血再灌注损伤普遍存在细胞凋亡,而线粒体分裂和融合介导的线粒体凋亡通路和细胞凋亡关系密切[1,2]。CB1 受体是内源性大麻素受体,在缺血再灌注的情况下,内源性大麻素由炎性细胞、内皮细胞和实质性细胞合成[3]。在脑缺血再灌注损伤时,激活细胞膜上的 CB1 受体可以减轻脑梗死的面积,促进线粒体的生物发生,改变线粒体的形态[4]。有研究表明神经元线粒体膜上也存在 CB1 受体,即线粒体 CB1 受体(mtCB1 受体),在生理及缺血再灌注的状态下激活 mtCB1 受体可通过调节线粒体的氧化呼吸链降低 ROS 的产生、增加线粒体膜电位、抑制神经元的凋亡[5-7]。然而,mtCB1 受体激活后是否可以通过影响线粒体分裂来发挥脑保护作用,目前尚不清楚。本研究以 mtCB1 受体为切入点,探讨了 mtCB1 受体在大鼠海马神经元缺氧复氧损伤中对线粒体分裂的影响,以期为脑缺血再灌注损伤提供新的理论依据。

1 材料与方法

1.1 材料

选择出生 24h 之内的 Wistar 大鼠,购于青岛实验动物中心。DMEM/F12、0.25%的胰酶购于美国 Hyclone 公司;血清、B27、神经细胞基础培养基 Neurobasal 以及 Earle's 液购于美国 Gibco 公司;多聚赖氨酸购自美国 Sigama 公司;透膜性 CB1 受体激动剂 ACEA 购于英国 Tocris 公司;透膜性 CB1 受体抑制剂 AM251 购于美国 Selleckchem 公司;非透膜性 CB1 受体抑制剂 Hemopressin 购于美国 Cayman 公司;BCA 蛋白试剂盒购于碧云天公司;兔抗大鼠 Drp1、AIF 单克隆抗体以及小鼠抗大鼠 Fis1 单克隆抗体和绵羊抗大鼠 Cytc 单克隆抗体均购于美国 Millipore 公司;兔抗大鼠 ROCK1 单克隆抗体购于台湾的 Arigo 公司;流式细胞凋亡试剂盒购于美国的 BD 公司。

1.2 方法

1.2.1 原代海马神经元的培养 取出生 24 h 之内的 Wistar 大鼠,75 %乙醇消毒冰上断头取脑,减去皮肤和硬脑膜,用镊子夹取两侧的海马组织放于 6 cm 的培养皿中,眼科剪剪碎后移于离心管中,加入 0.25 %的胰酶,用吸管轻轻吹打后,置于 37 ℃水浴锅中消化 20 min。用含 20 %胎牛血清的 DMEM/F12 终止消化,过 200 目筛网,1000 r/min 离心 5 min 后用含有 20 %胎牛血清的 DMEM/F12 重悬,并在倒置显微镜下进行细胞计数。25 cm× 25 cm 培养瓶提前用 0.1 mg/mL 多聚赖氨酸包被过夜,使用前用 PBS 冲洗 3 遍,以 7× 10^5 的密度接种于的培养瓶中,置于培养箱(37 ℃ 、5 %CO_2、95 %O_2、相对湿度 100 %)培养。24 h 后将培养基换为 Neurobasal-A 培养基 (含 1 %丙酮酸钠、1% L- 谷氨酰胺和 2 % B27),之后每 2-3 天根据培养基颜色半量换液。培养至第 8 天,用神经元特异性烯纯化酶染色鉴定神经元的纯度。

1.2.2 实验分组 随机数字表法随机分为 5 组 (1) 正常组(N 组):正常培养至第 8 天,不做任何处理;(2) 缺氧复氧组(H/R 组):缺氧 6 h,复氧 20 h,建立大鼠海马神经元缺氧复氧损伤模型;(3)缺氧复氧 +ACEA+AM251 组(H/R+ACEA+AM251 组)缺氧 6 h 后立即加入 ACEA 和 AM251,终浓度分别为 1 μmol/L、10 μmol/L,复氧 20 h;(4) 缺氧复氧 +ACEA+Hemopressin 组(H/R+ACEA+Hemo 组)于缺氧 6 h 后加入 ACEA 和 Hemopressin,终浓度分别为 1 μmol/L、10 μmol/L,复氧 20 h ;(5)缺氧复氧 + 赋形剂组(H/R+V 组)同样于缺氧 6 h 后加入二甲基亚砜(DMSO),终浓度 <0.1 %,复氧 20 h。药物的使用剂量参照文献[6]。

1.2.3 海马神经元缺氧复氧损伤模型建立 培养至第 8 天的细胞吸尽培养基,PBS 冲洗两遍,换成无糖的 Earle's 培养基置于三气培养箱中培养(2 %O_2、5 %CO_2、93 %N_2)6 h 后,换成原来的培养基在 95 %O_2-5 %CO_2 培养箱中继续培养 20 h。

1.2.4 Western blot 检测 Drp1、Fis1、AIF、Cytc、ROCK1 的表达 提取各组蛋白,用 BCA 法测定蛋白浓度。配制 6 %、10 %、15 % 的分离胶和 5 %的浓缩胶,蛋白质上样电泳 1.5 h 后将蛋白转至甲醛活化后的 PVDF 膜(300 MA 1.5 h),5 %的脱脂奶粉封闭 2 h 后,分别加入小鼠抗大鼠单克隆一抗 Fis1(1:1000)、兔抗大鼠单克隆一抗 Drp1 (1:2000)、兔抗大鼠单克隆一抗 AIF(1:2000)、绵羊抗大鼠单克隆一抗 Cytc(1:5000)以及兔抗大鼠单克隆一抗 ROCK1(1:1000)4 ℃ 孵育过夜,TBST 洗膜 3 次(15 min/ 次)后,用辣根过氧化物酶标记的山羊抗小鼠、山羊抗兔、兔抗绵羊 IgG 二抗 (1:10000)、常温下孵育 1 h,TBST 洗膜 3 次(15 min/ 次)后显影,GADPH 作为内参,利用 Image pro 软件对蛋白质条带进行灰度分析,目的蛋白灰度值和 GADPH 蛋白灰度值的比值反映目的蛋白的相对表达水平。

1.2.5 流式细胞仪检测细胞凋亡 胰酶消化后收集细胞,预冷的 PBS 冲洗一次 (1000 r/min 3 min),1× Binding buffer 100 ul 重悬并移至 1.5 mL EP 管中,向细胞悬液中分别加入 AV 、PI 各 5 μL,室温避光孵育 15 min,1× Binding buffer 补足至 500 μL,即可上机检测。Flowjo 软件分析细胞凋亡率。

1.2.6 激光共聚焦显微镜检测细胞内 Ca^{2+} 荧光强度 将接种于 24 孔板爬片中神经元培养至第 8 天,随机分组建立氧糖剥夺模型。Hank's 液冲洗细胞 3 次后,每孔加入 Fluo3-AM 与 Pluronic F127 工作液 100 μL,37 ℃避光孵育 45 min,加入 Hank's 液冲洗细胞 3 次,每孔加入 Hank's 液 100 μL,37 ℃避光孵育 30 min,移除 Hank's 液,4 %多聚甲醛固定 45 min,甘油防猝灭,封片后,激光共聚焦显微镜观察 Ca^{2+} 荧光强度,Image J 软件进行图片分析。

1.3 统计学分析

采用 SPSS 17.0 软件进行统计学分析,各组计量资料以均数± 标准差($\bar{x}\pm s$)表示,多组间比较采用单因素的方差分析,进一步两组间比较采用 SNK-q 检验,以 P<0.05 表示差异有统计

学意义。

2 结果

2.1 各组海马神经元 Ca^{2+} 浓度及细胞凋亡率的比较

与 N 组相比，其他四组细胞凋亡率和 Ca^{2+} 荧光强度均明显增高(P<0.05)；与 H/R 组相比，H/R+ ACEA+ Hemo 组上述各检测指标明显降低 (P<0.05)，H/R+ACEA+AM251 组和 H/R+V 组各指标差异无统计学意义(P>0.05)。见表 1。

表 1 五组海马神经元 Ca^{2+} 浓度及细胞凋亡率的比较($\bar{x}\pm s$)

Table 1 Comparison of the Ca^{2+} concentration and apoptosis rate among different groups ($\bar{x}\pm s$)

Groups	Ca^{2+}	Apoptosis rate (%)
N Group	0.0207± 0.0025	1.5± 0.4
H/R Group	0.0749± 0.0041[a]	42.4± 0.8[a]
H/R +ACEA+AM251 Group	0.0723± 0.0033[a]	40.44± 0.3[a]
H/R+ACEA+ Hemo Group	0.0576± 0.0021[ab]	25.49± 1.2[ab]
H/R+V Group	0.0783± 0.0046[a]	41.44± 0.6[a]

Note: [a] P<0.05, compared to the N group; [b] P<0.05, compared to the H/R group.

2.2 各组海马神经元线粒体分裂及凋亡相关蛋白表达的比较

与 N 组相比，其他四组 Drp1、Fis1、AIF、Cytc、ROCK1 的表达水平都明显增高(P<0.05)；与 H/R 组相，H/R+ACEA+ Hemo 组上述各检测指标显著降低 (P<0.05)，H/R+ACEA+AM251 组和 H/R+V 组各指标差异无统计学意义(P>0.05)，见表 2。

表 2 各组海马神经元线粒体分裂及凋亡相关蛋白表达水平的比较($\bar{x}\pm s$)

Table 2 Comparison of the expressions of mitochondrial fission and apoptosis related proteins among different groups ($\bar{x}\pm s$)

Groups	Drp1	Fis1	AIF	Cytc	ROCK1
N Group	0.469± 0.038	0.938± 0.064	0.612± 0.003	0.164± 0.004	0.731± 0.007
H/R Group	1.852± 0.055[a]	2.12± 0.041[a]	2.026± 0.013[a]	1.047± 0.045[a]	2.12± 0.019[a]
H/R+ACEA+AM251Group	1.885± 0.058[a]	2.136± 0.059[a]	2.056± 0.002[a]	0.971± 0.039[a]	2.099± 0.051[a]
H/R+ACEA+Hemo Group	1.451± 0.026[ab]	1.766± 0.029[ab]	1.551± 0.022[ab]	0.75± 0.014[ab]	1.775± 0.041[ab]
H/R+V Group	1.932± 0.049[a]	2.167± 0.003[a]	2.069± 0.009[a]	1.106± 0.045[a]	2.19± 0.025[a]

Note: [a] P<0.05 compared to the N group; [b] P<0.05, compared to the H/R group.

3 讨论

线粒体被称为 " 能量工厂 "，通过氧化磷酸化过程产生 ATP 为细胞的分裂、分化等生命活动提供能量。此外，线粒体在调节细胞内钙离子浓度、ROS 的水平、细胞信息的传递以及细胞凋亡等方面也发挥重要作用[8,9]。在脑缺血再灌注过程中，线粒体结构和功能发生改变都会导致不可逆的损伤，众多改变中最主要的是线粒体分裂，而细胞能量代谢在线粒体分裂中发挥主要作用，能量代谢障碍必然会产生过多 ROS。

线粒体氧化呼吸链的活性是影响 ROS 产生的关键因素，研究表明 mtCB1 受体和线粒体氧化呼吸链的电子传递关系密切，并影响线粒体的生发[10]，mtCB1 受体激活后，线粒体氧化呼吸链的活性随之升高，从而抑制了 ROS 的产生[6]。细胞内 ROS 的含量又可以直接影响线粒体功能[11-13]，高浓度的 ROS 通过调节内质网使细胞内 Ca^{2+} 浓度增加[14-17]，细胞内钙超载则会通过调节 Drp1 磷酸化增加其活性[17,18]，促使其从细胞质转移至线粒体外膜并与 Fis1 结合引起线粒体的分裂。

此外，有研究表明 ROS 可以通过 Rho/Rho-Kinase 信号通路调节心肌的缺血再灌注损伤，调节线粒体的功能、动态变化及细胞凋亡的过程[19-22]，细胞内的 ROS 可以通过激活 Rho-A 提高下游的 ROCK1 的活性 [23]，而活化的 ROCK1 将引起 Drp1-ser600 位点丝氨酸的磷酸化，并促使其移位至线粒体外膜，与 Fis1 结合导致线粒体的分裂[24,25]。线粒体分裂又会反过来造成线粒体氧化呼吸链的活性降低，产生更多的 ROS，造成更严重的钙超载，从而形成一个恶性循环[26]。本研究结果显示，mtCB1 受体激活后，ROS、ROCK1、Ca^{2+} 均明显减少，提示 mtCB1 受体可能通过降低 ROS 的含量来影响 ROCK1 的表达，并降低细胞内 Ca^{2+} 浓度，最终抑制线粒体分裂。

线粒体分裂是介导细胞凋亡的关键通路[27,28]。线粒体分裂会进一步导致线粒体能量代谢障碍，过多的 ROS 造成线粒体膜电位去极化，并造成线粒体肿胀和结构破坏从而释放更多的凋亡介导因子如 Cytc 和 AIF，激活 Caspase3 启动下游的凋亡通路[29,30]。释放入胞质的 AIF 则会激活细胞核内的 Endonuclease G，导致 DNA 碎裂，启动非胱解酶依赖性的细胞程序性死亡[31]。高浓度的 ROS 也会作用于 Ca^{2+} 依赖性的线粒体通透性转换孔(mPTP)，诱发 mPTP 的开放，引起 Cytc 和 AIF 等凋亡诱导因子的释放，最终导致细胞凋亡[9]。

本研究将 ROCK1 和线粒体分裂与大麻素受体的脑保护作用联系起来，采用透膜性 CB1 受体激动剂 ACEA 和非透膜性 CB1 受体抑制剂 Hemopressin 相结合来激动 mtCB1 受体。

结果显示与 H/R 组相比，H/R+ACEA+ Hemopressin 组 Ca^{2+} 浓度、Drp1、Fis1、AIF、Cytc 和 ROCK1 的表达量以及神经元凋亡率均降低，表明激活 mtCB1 受体可能通过降低细胞内 ROS 的含量来减少细胞内 Ca^{2+} 浓度和 ROCK1 的表达，进而抑制线粒体分裂，并最终减轻海马神经元缺氧复氧损伤。

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