论著·神经科学

胰岛淀粉样多肽抑制PI3K/Akt通路而加重阿尔茨海默病小鼠认知损伤、神经炎症及海马Aβ斑块沉积

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[摘要] **目的** 胰岛淀粉样多肽(islet amyloid polypeptide,IAPP)是由胰岛β细胞合成分泌的活性多肽,糖尿病时在胰岛形成淀粉样斑块。本研究探讨了IAPP对阿尔茨海默病(Alzheimer's disease,AD)小鼠认知功能障碍和神经炎性反应等病理损伤的作用。**方法** 选用6月龄雌性C57BL/6J野生型(wild type,WT)及APP/PS1转基因AD小鼠,初始体质量20~23 g,按随机数字表法分为(n=5):WT组(等体积PBS腹腔注射)、WT+IAPP组(200 μg/kg IAPP腹腔注射)、AD组(等体积PBS腹腔注射)、AD+IAPP组(200 μg/kg IAPP腹腔注射),每日给药1次,连续干预10周。Morris水迷宫实验评估小鼠的认知功能;免疫组化检测小鼠海马Aβ斑块数量及面积;透射电镜观察小鼠海马神经元超微结构;生化试剂盒检测小鼠海马组织氧化应激指标H_2O_2水平、超氧化物歧化酶(superoxide dismutase,SOD)和谷胱甘肽过氧化物酶(glutathione peroxidase,GSH-Px)活性;ELISA检测促炎因子IL-1β、IL-6、TNF-α水平以及血清β淀粉样蛋白42(amyloid β-protein 42, $Aβ_{42}$)水平。基因芯片检测筛选差异表达mRNA,开展GO和KEGG分析,RT-qPCR验证差异mRNA的表达水平。Western blotting检测小鼠海马PI3K/Akt蛋白的相对表达水平。**结果** Morris水迷宫实验结果显示,与WT+IAPP组相比,WT组小鼠学习记忆能力无显著性差异;AD组比WT组小鼠学习记忆能力显著下降(P<0.05),AD+IAPP组比AD组小鼠学习记忆能力显著下降(P<0.05)。免疫组化提示,与WT组相比,WT+IAPP组小鼠海马的Aβ斑块数量与面积无显著性差异,AD组小鼠海马的Aβ斑块数量与面积明显增加(P<0.01);AD+IAPP组比AD组小鼠的Aβ斑块数量与面积显著增加(P<0.01)。透射电镜结果显示,AD+IAPP组比AD组小鼠海马神经元线粒体损伤加重,突触数量明显减少(P<0.05)。AD+IAPP组比AD组小鼠海马H_2O_2水平显著升高,SOD、GSH-Px活性显著降低(P<0.05)。AD+IAPP组比AD组小鼠海马IL-1β、IL-6、TNF-α水平明显上升,血清$Aβ_{42}$水平明显下降(P<0.05)。基因芯片筛选出555个差异mRNA(236个上调、319个下调);RT-qPCR验证结果与芯片结果一致;GO分析提示差异基因参与跨膜信号传导、神经发育过程;KEGG分析显示差异基因集中于PI3K/Akt通路。Western blotting显示,AD+IAPP组比AD组小鼠海马神经元PI3K和Akt蛋白及p-PI3K和p-Akt蛋白的表达明显降低(P<0.05)。**结论** IAPP可以加重AD小鼠的认知功能障碍,增加脑组织神经炎性反应水平,增加海马Aβ斑块沉积,其作用机制可能与抑制PI3K/Akt信号通路的活性有关。

[关键词] 阿尔茨海默病;胰岛淀粉样多肽;氧化应激;炎性反应;蛋白激酶B

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Islet amyloid polypeptide exacerbates cognitive impairment, neuroinflammation, and hippocampal Aβ plaque deposition in Alzheimer's disease mice by inhibiting the PI3K/Akt pathway

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[**Abstract**] **Objective** Islet amyloid polypeptide (IAPP) is an active peptide synthesized and secreted by pancreatic β-cells, which forms amyloid plaques in the islets under diabetic conditions. This study aims to determine the effect of IAPP on cognitive impairment, neuroinflammatory response, and other pathological injuries in Alzheimer's disease (AD) mice. **Methods** Six-month-old female C57BL/6J wild-type (WT) and APP/PS1 transgenic AD mice, with an initial body weight of 20 to 23 g, were randomly divided into 4 groups (n=5): WT group (intraperitoneal injection of equal volume of PBS), WT+IAPP group (intraperitoneal injection of 200 μg/kg IAPP), AD group (intraperitoneal injection of equal volume of PBS), and AD+IAPP group (intraperitoneal injection of 200 μg/kg IAPP). Intraperitoneal injection of IAPP was given to the corresponding mice for 10 consecutive weeks. Morris water maze test was performed to evaluate cognitive function. Immunohistochemistry was applied to detect the number and area of Aβ plaques in the hippocampus. Transmission electron microscopy (TEM) was used to observe the ultrastructural changes in hippocampal neurons. Biochemical kits were employed to detect the levels of hydrogen peroxide (H_2O_2) and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in the hippocampal tissues. ELISA was utilized to measure the levels of pro-inflammatory cytokines, including IL-1β, IL-6 and TNF-α, as well as serum amyloid β-protein 42 ($A\beta_{42}$) concentration. Microarray analysis was conducted to screen differentially expressed mRNAs, followed by GO and KEGG enrichment analyses. RT-qPCR was performed to measure the expression of differential mRNAs. Western blotting was carried out to detect the relative expression levels of proteins related to the PI3K/Akt signaling pathway in hippocampal tissues. **Results** Morris water maze test demonstrated that the learning and memory abilities were no statistical difference between the WT+IAPP group and the WT group, significantly decreased in the AD group than the WT group(P<0.05), and obviously decreased in the AD+IAPP group than the AD group (P<0.05). Immunohistochemical staining revealed that compared with the WT group, the number and area of hippocampal Aβ plaques in the WT+IAPP group showed no significant difference, while those in the AD group were significantly increased(P<0.01); compared with the AD group, the number and area of Aβ plaques in the AD+IAPP group were significantly increased (P<0.01). TEM demonstrated aggravated mitochondrial damage and a remarkable reduction in synaptic number in hippocampal neurons of the AD+IAPP group compared with the AD group (P<0.05). The level of H_2O_2 in the hippocampus was significantly increased, while the activities of SOD and GSH-Px were significantly decreased in the AD+IAPP group compared with the AD group (P<0.05). The hippocampal levels of IL-1β, IL-6 and TNF-α were significantly elevated, whereas serum $A\beta_{42}$ concentration was notably reduced in the AD+IAPP group compared with the AD group (P<0.05). A total of 555 differentially expressed mRNAs (236 upregulated and 319 downregulated) were screened out by microarray, and RT-qPCR verification results were consistent with the results of microarray. GO enrichment analysis indicated that these differentially expressed genes were involved in transmembrane signal transduction and neural development. KEGG analysis revealed that the differential genes were enriched in the

PI3K/Akt signaling pathway. Western blotting showed that the protein expression levels of PI3K, Akt, p-PI3K, and p-Akt in hippocampal neurons were significantly decreased in the AD+IAPP group compared with the AD group (P<0.05). **Conclusion** IAPP can aggravate cognitive dysfunction, enhance inflammatory responses, and promote hippocampal Aβ-plaque deposition in AD mice. Its underlying mechanism may be related to the inhibition of PI3K/Akt signaling-pathway activity.

[**Key words**] Alzheimer's disease; islet amyloid polypeptide; oxidative stress; inflammatory response; protein kinase B

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阿尔茨海默病(Alzheimer's disease,AD)是一种主要发生于老年人群脑部的神经退行性疾病[1]。2020年中国有AD患者约983万[2],随着全球人口老龄化持续加剧,AD发病率呈快速上升趋势[3]。AD患者的认知功能障碍给家庭与社会带来了沉重的负担。AD主要的病理变化为脑内Aβ的过度沉积以及Tau蛋白的过度磷酸化。AD的发病机制主要包括神经炎性反应、氧化应激、线粒体功能障碍等[4]。

近年来糖尿病可以增加AD发生的风险成为研究的热点问题。有研究证实,胰岛淀粉样多肽(islet amyloid polypeptide,IAPP)在糖尿病增加AD的发生风险中起到了重要作用[5]。IAPP是由胰岛β细胞合成分泌的活性多肽,与胰岛素协同调节血糖,抑制胰高血糖素释放。糖尿病时IAPP水平相对升高,在胰岛形成淀粉样斑块,促进了糖尿病的发展。IAPP除调节外周糖代谢外,研究还证实其在脑组织中呈高表达,提示其可能参与AD的病理过程[6]。IAPP与Aβ具有高度相似的淀粉样结构,在脑组织中与Aβ相结合,可能对Aβ的过度沉积和AD的认知功能障碍有影响[7]。在不伴有糖尿病的AD小鼠中血清IAPP的水平也是升高的,但是IAPP对AD发生的作用机制是不清楚的。

磷脂酰肌醇3-激酶(phosphatidylinositol 3-kinase,PI3K)是一种重要的脂质激酶,在调控细胞氧化应激与炎症反应中发挥关键作用。PI3K可以激活下游蛋白激酶B(protein kinase B,PKB/Akt)信号通路,这一过程对细胞生长、存活、代谢及迁移等生命活动至关重要[8]。既往研究证实,PI3K/Akt通路可在神经元损伤中发挥重要保护性作用,能够缓解氧化应激损伤,在AD及外伤性脑损伤等多种神经系统疾病中得到证实[9]。然而,在IAPP促进AD发生的病理状态下,PI3K/Akt通路的活性及其调控机制尚不明确。

根据以上的研究结果,本研究假设IAPP促进了AD的发生发展。为此,本研究通过对转基因AD小鼠腹腔注射IAPP,检测小鼠的学习记忆功能、脑内Aβ斑块沉积和神经元结构的变化,同时检测氧化应激和炎性反应水平,以及PI3K/Akt信号通路活性的变化,探讨IAPP对AD小鼠认知功能和相关病理变化的影响,有助于阐明AD发生的病理机制,为制定AD的防治策略提供新的实验依据。

1 材料与方法

1.1 实验动物分组及给药

选用6月龄雌性C57BL/6J野生型(wild type,WT)与APP/PS1转基因AD小鼠,初始体质量20~23 g,同批次购入,年龄及体质量匹配,由南京君科生物有限公司提供。小鼠饲养于SPF级环境,12 h光照和黑暗循环,自由摄食饮水。本研究操作已通过第三军医大学实验动物福利伦理审查委员会批准。每日早晚监测小鼠状态,实验期间无意外不良事件发生。当动物体质量减轻到原体质量的20%~25%或呈濒死状态设为人道终点。采用随机数字表法将小鼠分为4组(n=5):WT组、WT+IAPP组、AD组、AD+IAPP组。WT+IAPP组、AD+IAPP组小鼠每日单次腹腔注射IAPP(200 μg/kg),WT组与AD组腹腔注射等体积PBS,连续干预10周,给药方案参照文献[10]。干预周期结束后开展Morris水迷宫行为学检测。水迷宫实验全部完成后次日进行样本取材,小鼠经腹腔注射戊巴比妥钠(50 mg/kg)麻醉,确认麻醉充分后行脱颈安乐死,确认小鼠死亡后取脑组织,并分离双侧海马组织,左右海马分开留存,同时采集小鼠外周血清样本。WT组、WT+IAPP组、AD组、AD+IAPP组的右侧海马组织均用于免疫组化染色检测Aβ数量和面积,每组纳入3只小鼠;仅选取AD组、AD+IAPP组小鼠左侧海马组织进行透射电镜观察神经元超微结构,生化法检测氧化应激指标,ELISA检测炎症因子及血清β淀粉样蛋白42(amyloid

β-protein 42, $A\beta_{42}$)水平,基因芯片检测差异mRNA,RT-qPCR验证基因,差异基因功能注释与通路富集分析以及Western blotting检测PI3K/Akt通路蛋白表达水平,每组纳入3只小鼠。每组额外预留2只同批动物,仅在样本制备、检测失败时替补。

1.2 主要试剂与设备

IAPP购自美国Sigma-Aldrich公司,Morris水迷宫行为学检测系统购自上海欣软信息科技有限公司。免疫组化使用的兔抗Aβ一抗和HRP标记的羊抗兔二抗均购自美国Thermo Fisher Scientific公司,DAB显色试剂盒购自北京中杉金桥生物技术有限公司。H_2O_2、超氧化物歧化酶(superoxide dismutase,SOD)和谷胱甘肽过氧化物酶(glutathione peroxidase,GSH-Px)生化检测试剂盒均购自南京建成生物工程研究所。IL-1β、IL-6和TNF-α ELISA检测试剂盒购自中国ABclonal公司,小鼠$A\beta_{42}$ ELISA试剂盒购自江苏酶标生物科技有限公司。gDNA Erase的PrimeScript™ RT试剂盒购自北京宝日医生物技术有限公司。兔抗PI3K、p-PI3K、Akt和p-Akt一抗抗体购自美国CST公司,GAPDH抗体购自英国Abcam公司。

1.3 水迷宫检测小鼠学习记忆能力

小鼠经IAPP腹腔连续干预10周后,采用Morris水迷宫实验检测空间学习与记忆功能。圆形水池均等划分为4个象限,于第三象限水面下1 cm处固定透明逃生平台;水中添加奶粉使水体呈乳白色,遮挡水下平台以消除视觉线索。定位航行实验连续开展5 d,每日4次训练,每日随机选取4个象限作为入水点,将小鼠面向池壁放入水中。以小鼠入水后登上平台并停留10 s以上的时长为逃避潜伏期,单次游泳时限90 s,若小鼠90 s内未找到平台,由操作人员轻柔引导至平台并停留10 s,该次潜伏期统一记为90 s,相邻2次训练间隔不少于4 min。全部定位航行训练结束后,次日开展空间探索实验,移除水下逃生平台,统一从同一非目标象限投放小鼠,记录120 s内小鼠穿越原平台区域的次数及目标象限总停留时长。

1.4 免疫组化检测小鼠海马组织Aβ斑块数量与面积

海马组织经4%多聚甲醛固定、石蜡包埋,制备5 μm切片,二甲苯脱蜡水化、高温抗原修复、PBS洗涤后,3%H_2O_2避光孵育20 min阻断内源性过氧化物酶,山羊血清封闭非特异性结合。加入兔抗Aβ一抗4 ℃孵育过夜,PBS洗涤后加HRP标记的羊抗兔二抗室温孵育30 min,按照DAB显色试剂盒说明书配制显色工作液,临用前新鲜配制,DAB缓冲液、DAB底物液、3%H_2O_2溶液按照体积比(20∶1∶1)充分混匀,滴加至切片进行显色反应。苏木精复染3 min,分化返蓝、脱水透明后中性树胶封片。使用Image-Pro Plus 6.0软件分析,每张切片随机选取多个视野,统计Aβ阳性斑块数量并计算阳性染色面积,以斑块平均数、阳性面积占比量化海马Aβ沉积水平。

1.5 透射电镜观察小鼠海马神经元超微结构

采用荷兰飞利浦CM100型透射电镜观察神经元及突触超微结构,设备最大放大倍数45万倍,分辨率0.34 nm。小鼠海马组织经0.1 mol/L PBS充分漂洗后,加入1%锇酸后固定120 min,梯度乙醇逐级脱水过夜,每级10~15 min,脱水完成后将组织块放入丙酮-环氧树脂混合液(体积比1∶1),37 ℃孵育60 min;更换为丙酮-环氧树脂混合液(1∶2)渗透120 min;最后更换为纯环氧树脂渗透120 min,置于硅橡胶模具恒温包埋聚合12 h。包埋块静置4 d后进行切片,上机观察并比较各组海马神经元超微结构差异。

1.6 生化方法及ELISA检测小鼠海马氧化应激、炎症因子与血清$A\beta_{42}$水平

取小鼠海马组织,经预冷生理盐水漂洗、吸干、称量后,按1∶9比例加入提取液并在冰上匀浆,在4 ℃下以3 000 r/min离心10 min,获取上清。分离小鼠血清后低温保存,用于$A\beta_{42}$检测。采用BCA法测定样本总蛋白浓度以校正上样误差。依据试剂盒说明书检测海马组织H_2O_2、SOD、GSH-Px氧化应激指标,采用ELISA方法检测海马组织IL-1β、IL-6、TNF-α及血清$A\beta_{42}$水平。所有样本设置平行复孔,结果经蛋白浓度归一化校正,用于评估脑组织氧化应激、炎性反应及Aβ代谢情况。

1.7 基因芯片检测小鼠海马差异表达mRNA

基因芯片检测委托上海数谱生物科技有限公司完成。严格按照RNAiso Plus试剂说明书提取各组海马组织总RNA,采用NanoDrop ND-1000分光光度计检测RNA浓度与纯度,结合琼脂糖凝胶电泳鉴定RNA完整性;使用Arraystar Flash RNA标记试剂盒对RNA样本进行荧光标记,标记产物通过Agilent SureHyb系统完成芯片杂交。杂交后洗涤芯片并扫描采集探针信号,配套软件对原始信号进行标准化处理,基于R语言limma包筛选差异表达mRNA。筛选阈值设定为$|\log_2FC|>1.0$、$P<0.05$;差异mRNA分布采用火山图可视化,分层聚类热图展示样本间差异基因表达模式。

1.8 RT-qPCR验证小鼠海马差异表达mRNA

采用RNAiso Plus分别提取AD组和AD+IAPP组小鼠海马组织总RNA。大约1 000 ng总RNA用gDNA Erase的PrimeScript™ RT试剂盒反转录。按生产商说明书,用20 μL RT-qPCR重复3次。以内参基因GAPDH检测mRNA,以$2^{-\Delta\Delta Ct}$方法为参照。表1为所用引物序列。

表1 目的基因引物序列

Table 1 Primer sequences for the target genes

Gene	Primer sequence(5′→3′)
Gpr139	Forward:GTTTACCAGATATCATGAAGCCG
	Reverse:ATCTCAGTCCCTCCTCTGG
Pcp4	Forward:GCGATTCCTTAAATGGACAG
	Reverse:TGAACTGAGACTGAATGGC
Ccl3	Forward:CGGTGTCATCTTCCTAACC
	Reverse:GTCGCTGACATATTTCTGGA
Leap2	Forward:CTATGCAGAAAGGACAGCAG
	Reverse:CTTCACTTGGGTGTCTCCA
GAPDH	Forward:CACTGAGCAAGAGAGGCCCTAT
	Reverse:GCAGCGAACTTTATTGATGGTATT

1.9 差异基因功能注释与通路富集分析

GO从三维维度标准化注释基因功能,包含生物学过程(biological process,BP)、分子功能(molecular function,MF)、细胞组分(cellular component,CC)三大模块;KEGG数据库可注释基因参与的代谢通路与信号调控网络,直观解析基因在病理状态下的调控机制。针对筛选得到的差异表达基因(differentially expressed genes,DEGs)开展GO功能注释与KEGG通路富集分析,采用了STRING在线数据库(https://cn.string-db.org)中的KEGG通路富集分析及GO功能模块进行分析。

1.10 Western blotting检测小鼠海马PI3K/Akt通路蛋白表达水平

海马组织加入RIPA裂解液提取总蛋白,蛋白样本混入SDS-PAGE上样缓冲液煮沸变性;经SDS-PAGE电泳分离后,转印至PVDF膜。5%BSA室温封闭2 h,加入兔抗PI3K、兔抗p-PI3K、兔抗Akt和兔抗p-Akt一抗(1∶1 000),4 ℃孵育过夜;TBST充分洗膜后,加入HRP标记山羊抗兔源二抗(1∶5 000)室温孵育1~2 h。将ECL发光工作液均匀覆盖PVDF膜,室温孵育2 min,置于化学发光成像系统中采集条带图像并进行灰度定量分析。以GAPDH作为内参蛋白校正上样量,以目的蛋白与内参蛋白积分光密度比值代表蛋白相对表达量。

1.11 统计学分析

所有实验数据使用GraphPad Prism 10.0软件进行统计学分析。多组间整体比较采用单因素方差分析(One-way ANOVA);若组间差异具有统计学意义,后续采用Tukey多重比较检验进行任意两组间比较。两组单独比较时采用独立样本t检验。以$P<0.05$表示差异具有统计学意义。

2 结果

2.1 IAPP加重AD小鼠学习记忆能力障碍

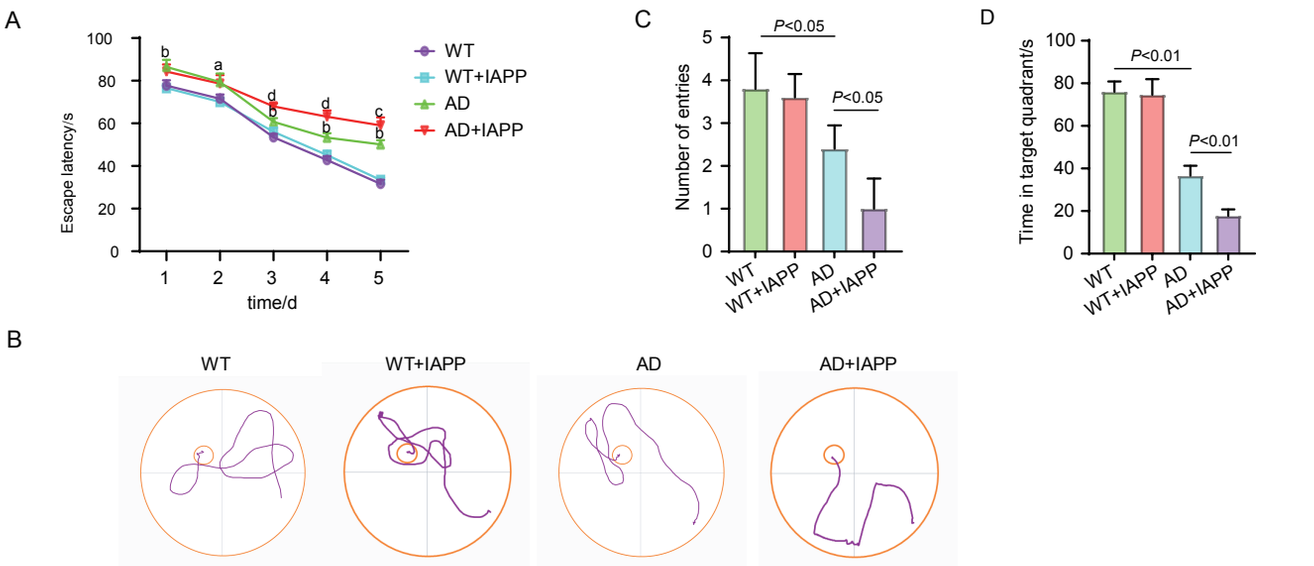
Morris水迷宫检测4组小鼠学习记忆能力。在前2 d,WT组与WT+IAPP组小鼠逃避潜伏期未见统计学差异,AD组小鼠逃避潜伏期长于WT组小鼠($P<0.01$,$P<0.05$),且AD组与AD+IAPP组小鼠组间无显著区别;从第3天开始,WT组与WT+IAPP组小鼠逃避潜伏期仍无明显差异,但AD组小鼠逃避潜伏期较WT组小鼠显著延长($P<0.01$),AD+IAPP组小鼠逃避潜伏期较AD组小鼠延长($P<0.01$),且在第4、5天时,AD+IAPP组小鼠逃避潜伏期较AD组小鼠进一步延长($P<0.01$,$P<0.05$,图1A)。空间探索实验结果可见代表性游泳轨迹(图1B)。WT组与WT+IAPP组小鼠的穿越平台象限的次数和平台象限停留时间无显著差异;AD组与WT组小鼠比较,穿越平台象限的次数和平台象限停留时间减少($P<0.05$,$P<0.01$);AD+IAPP组与AD组小鼠比较,穿越平台象限的次数和平台象限停留时间显著减少($P<0.05$,$P<0.01$,图1C、D)。这些结果表明,AD组与WT组小鼠相比学习记忆能力下降,IAPP可以加重AD小鼠的学习记忆能力障碍。

2.2 IAPP加重AD小鼠大脑海马区Aβ斑块沉积

免疫组化检测各组小鼠大脑海马区Aβ斑块沉积,结果显示:WT组与WT+IAPP组小鼠大脑海马区Aβ斑块未见显著差异,AD组与WT组小鼠相比Aβ斑块的数量与面积显著增加($P<0.01$,图2A),AD+IAPP组小鼠Aβ斑块数量和面积较AD组显著增加($P<0.01$,图2B、C),表明IAPP可以增加大脑海马区Aβ的聚集。

2.3 IAPP加重AD鼠大脑海马区神经元的超微结构损害

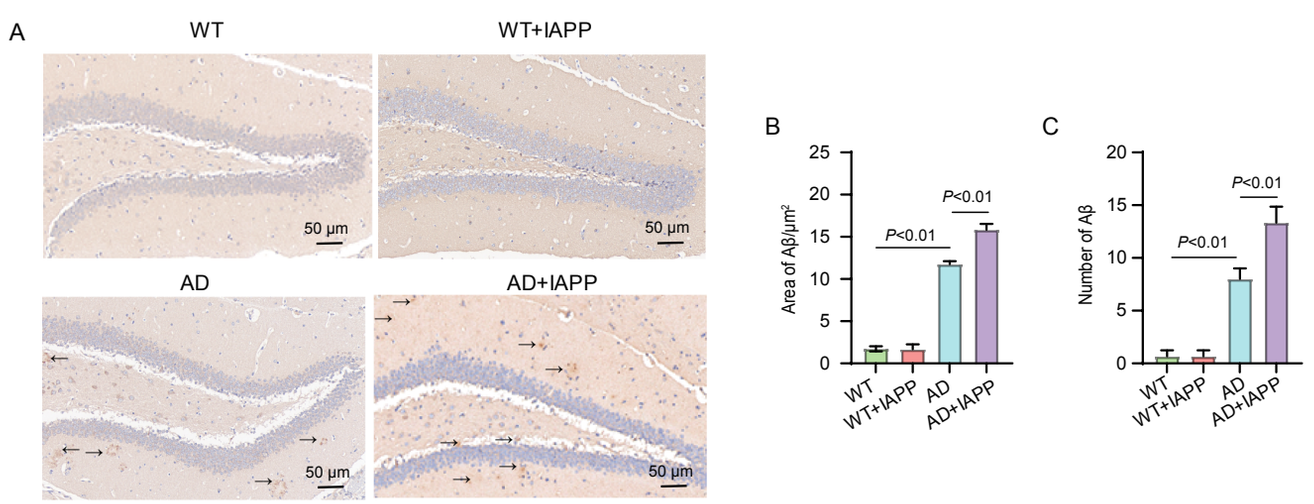
透射电镜检测2组小鼠海马神经元超微结构。结果显示,与AD组比较,AD+IAPP组小鼠神经元线粒体空泡化增加(图3A),神经元突触数量减少($P<0.05$,图3B、C),表明IAPP可以损害AD小鼠大脑海马区神经元的超微结构。



A: Duration of escape latency across groups. a: $P<0.05$, b: $P<0.01$, compared with the WT group; c: $P<0.05$, d: $P<0.01$, compared with the AD group; B: Representative swimming trajectories in the spatial probe test; C: Number of platform crossings across groups; D: Dwelling time in the target quadrant across groups

图1 Morris水迷宫检测小鼠空间学习记忆能力 (n=5, $\bar{x}\pm s$)

Figure 1 Spatial learning and memory in 4 groups of mice using Morris water maze test (n=5, $\bar{x}\pm s$)



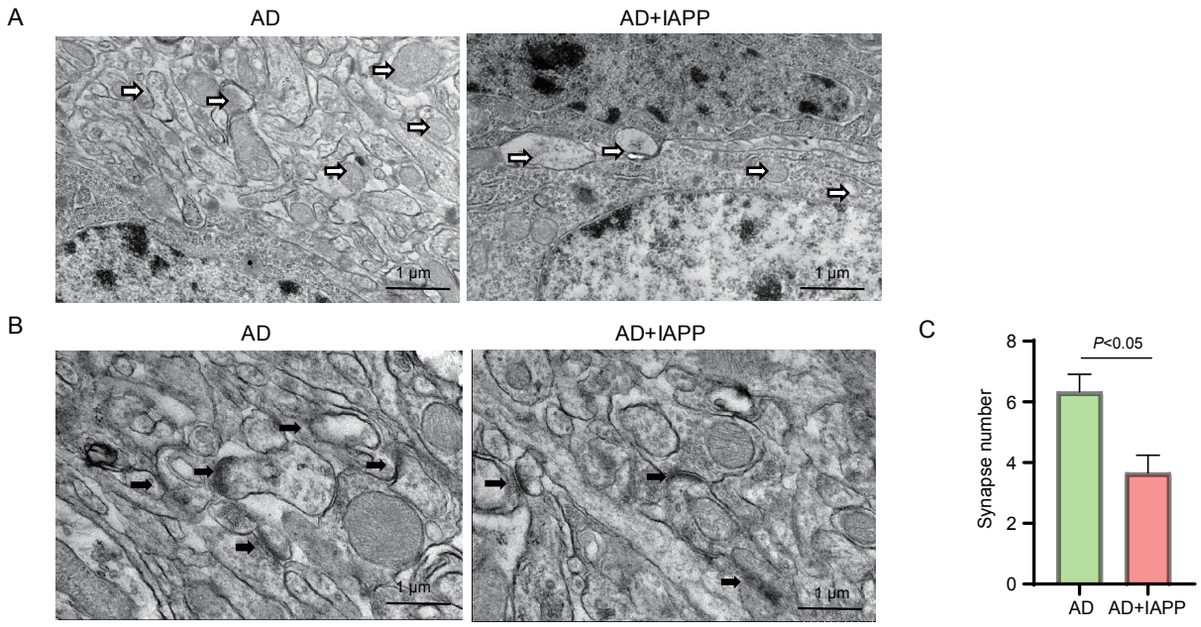
A: Representative images of hippocampal Aβ plaques in each group. Arrows indicate Aβ plaques; B: Quantitative analysis of hippocampal Aβ plaque area in each group; C: Quantitative analysis of hippocampal Aβ plaque number in each group

图2 免疫组化染色检测小鼠大脑海马区Aβ斑块 (n=3, $\bar{x}\pm s$)

Figure 2 Immunohistochemical staining for Aβ plaques in the mouse hippocampus (n=3, $\bar{x}\pm s$)

2.4 IAPP增加AD小鼠脑氧化应激和炎性反应水平,同时降低血清$A\beta_{42}$的水平

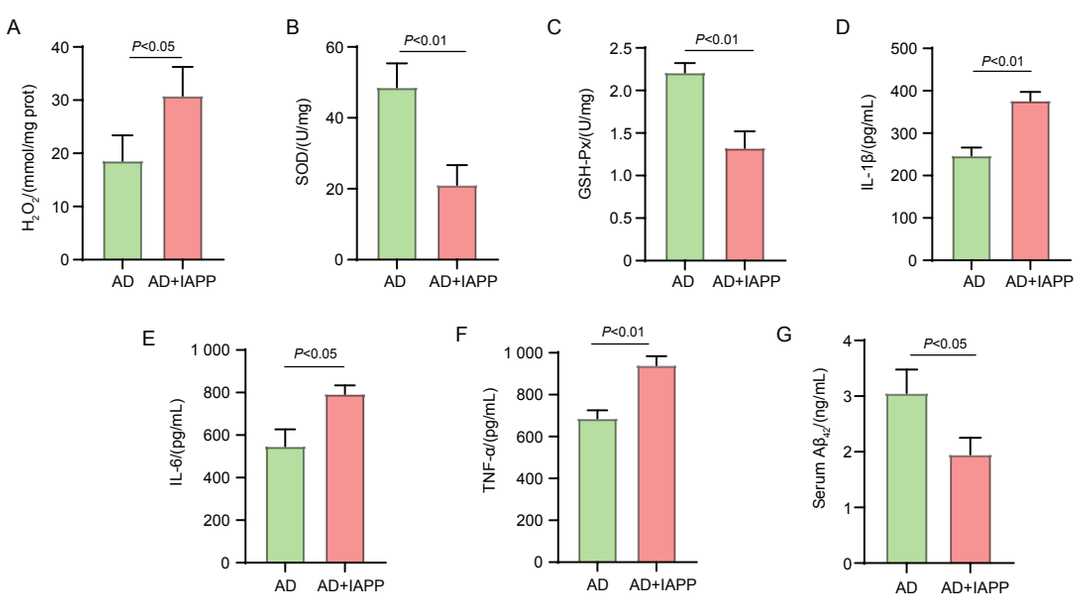
在2组小鼠中检测了氧化应激和炎性反应水平,IAPP增加了AD小鼠脑海马组织H_2O_2水平($P<0.05$),同时降低了SOD、GSH-Px活性($P<0.01$,图4A~C)。IAPP增加了AD小鼠脑组织IL-1β、IL-6和TNF-α的水平($P<0.05$,图4D~F),提示IAPP增加了AD小鼠脑组织氧化应激和炎性反应水平。此外,与AD组小鼠相比,AD+IAPP组小鼠血清$A\beta_{42}$水平显著降低($P<0.05$,图4G)。



A: Representative TEM images of neuronal mitochondrial ultrastructure in the hippocampus of each group, white arrows indicating damaged mitochondria; B: Representative TEM images of synaptic ultrastructure in the hippocampus of each group, black arrows indicating synapses; C: Quantitative analysis of hippocampal synaptic number in each group

图3 透射电镜观察IAPP对AD组小鼠大脑海马区神经元超微结构的影响 (n=3, $\bar{x}\pm s$)

Figure 3 Effects of IAPP on the hippocampal neuron ultrastructure in AD mice by transmission electron microscopy (n=3, $\bar{x}\pm s$)



A to C: Hippocampal oxidative stress markers (H_2O_2, SOD, and GSH-Px) in each group; D to F: Hippocampal inflammatory cytokines (IL-1β, IL-6, and TNF-α) in each group; G: Serum $A\beta_{42}$ level in each group

图4 IAPP对AD小鼠海马组织氧化应激水平和炎性反应水平及血清$A\beta_{42}$水平的影响 (n=3, $\bar{x}\pm s$)

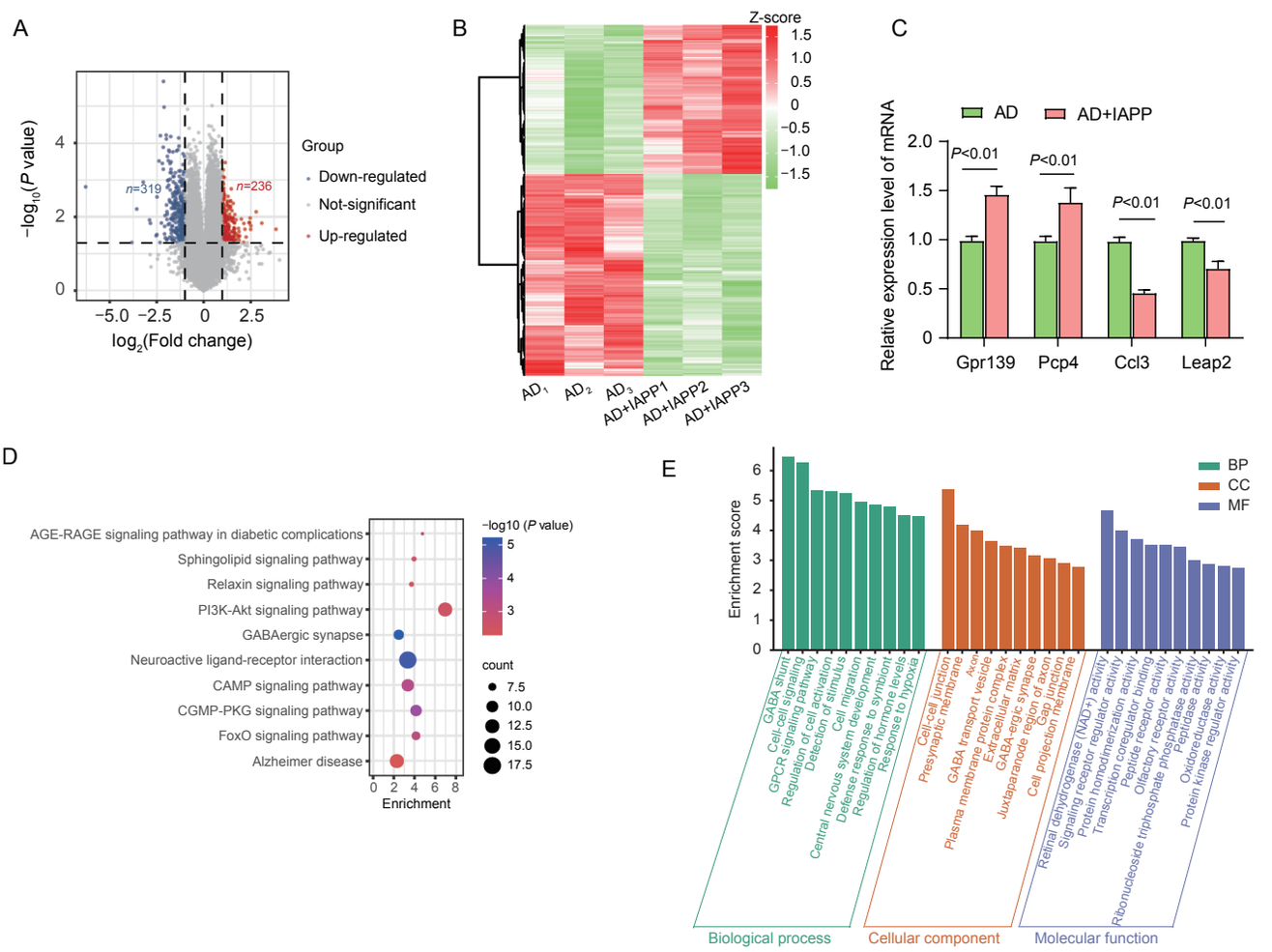
Figure 4 Effects of IAPP on oxidative stress and inflammatory responses in the hippocampus, as well as serum $A\beta_{42}$ level in AD mice (n=3, $\bar{x}\pm s$)

2.5　IAPP干预AD小鼠海马差异基因富集于PI3K/Akt信号通路

对AD小鼠和AD+IAPP小鼠的海马组织进行基因芯片检测。筛选到差异表达基因555个,上调基因236个,下调基因319个(差异倍数的绝对值>2,$|\log_2FC|$>1.0且P<0.05)。并根据结果绘制热图和火山图(图5A、B)。为了验证芯片结果的可靠性,随机挑选具有潜在生物学功能的4个差异表达的mRNA进行RT-qPCR验证,结果显示:Gpr139、Pcp4基因上调,Ccl3、Leap2基因下调(P<0.01,图5C),与基因芯片结果一致。KEGG分析显示,AD+IAPP小鼠与AD小鼠的mRNA表达差异显著富集于PI3K/Akt等信号通路(图5D)。GO功能富集分析显示,BP集中于信号传导、中枢神经系统发育与细胞迁移;CC显著富集于突触前膜、轴突、GABA能突触、细胞连接及胞外基质;MF主要涉及氧化还原酶活性、转录共调节、信号受体调控与蛋白激酶调节活性(图5E)。

2.6　IAPP抑制AD小鼠大脑海马神经元PI3K/Akt信号通路的活性

Western blotting检测2组小鼠海马神经元PI3K和Akt的蛋白表达,结果显示,与AD组比较,AD+IAPP组小鼠海马区神经元PI3K和Akt蛋白及p-PI3K和p-Akt蛋白的表达均显著下调(P<0.05,图6),提示PI3K/Akt信号系统活性抑制可能与IAPP对AD小鼠认知障碍和形态结构损伤有关。



A: Volcano plot of differentially expressed mRNAs between AD and AD+IAPP mice; B: Clustering heatmap of differentially expressed mRNAs between AD and AD+IAPP mice; C: Relative expression levels of 4 significantly upregulated or downregulated mRNAs in AD and AD+IAPP mice; D: Bubble plot of KEGG pathway enrichment analysis for differentially expressed mRNAs between AD and AD+IAPP mice; E: Bar chart of GO functional enrichment for differentially expressed mRNAs between AD and AD+IAPP mice

图5　KEGG分析PI3K/Akt信号通路参与IAPP的作用(n=3, $\bar{x}\pm s$)

Figure 5　KEGG analysis showing PI3K/Akt signaling pathway is involved in the function of IAPP (n=3, $\bar{x}\pm s$)

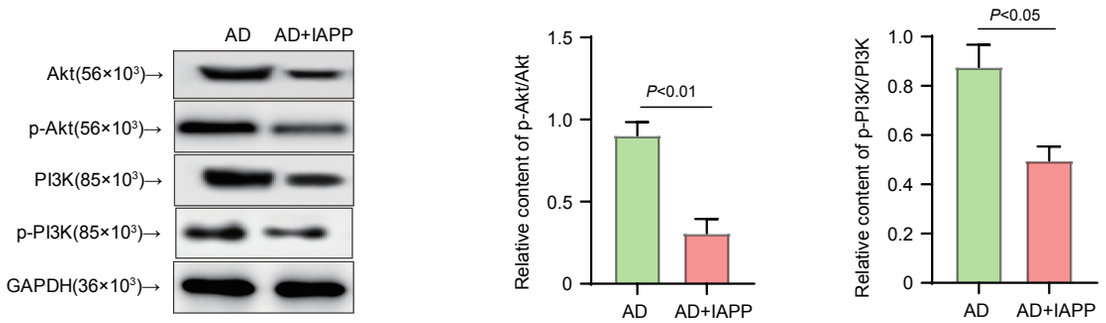


图6 Western blotting检测IAPP对AD小鼠PI3K、p-PI3K、Akt和p-Akt蛋白表达的影响 (n=3, $\bar{x}\pm s$)

Figure 6 Effects of IAPP on PI3K, p-PI3K, Akt and p-Akt protein expression in the hippocampus of AD mice detected by Western blotting (n=3, $\bar{x}\pm s$)

3 讨论

3.1 IAPP加重AD小鼠的认知功能障碍

许多研究已证实,IAPP可加重伴2型糖尿病的AD小鼠的认知功能障碍和相关病理损伤。在一项研究中,8月龄糖尿病AD小鼠脑内IAPP注射可显著促进脑内Aβ沉积,加重认知功能障碍[11]。此外,在不伴有糖尿病的AD小鼠中,IAPP同样被观察到有神经损伤作用。这项研究结果提示在IAPP转基因的AD小鼠中,脑内IAPP干预可与Aβ发生交叉聚集,加重Aβ斑块沉积和认知功能障碍[12]。也有一些研究得出与上述研究并不一致的结果。在3.5月龄雌性5XFAD小鼠中口服给予IAPP,可改善脑内Aβ沉积[13],给予IAPP受体激动剂能够改善APP/PS1小鼠的认知功能[14]。本研究以6月龄雌性APP/PS1转基因AD小鼠为研究对象,证实IAPP可增加AD认知损害,促进炎性反应水平,加重神经病理损伤。在既往的文献报道中,IAPP对AD认知功能及病理损害存在相互矛盾的结果,可能有以下主要原因:①AD小鼠模型的年龄及性别不同;②IAPP给药途径与剂量存在差异;③可能是干预方式不同,例如干预IAPP受体活性来证明IAPP的作用;④可能是缺乏对AD相关认知功能和神经病理做全面深入的检测,例如检测学习记忆能力的有Morris水迷宫和Y-迷宫,检测神经元损伤的相关信号通路。

3.2 IAPP增加AD小鼠Aβ在脑内的沉积

有研究发现,IAPP干预在AD小鼠中可以与脑内Aβ共同沉积形成淀粉样斑块,加重认知功能损伤[15-16]。这项研究证实,IAPP可能促进外周Aβ向脑内转运,导致血清$A\beta_{42}$降低、脑内Aβ沉积增加[17]。但也有实验提示IAPP在AD小鼠中可以减少Aβ在脑内的沉积,改善认知功能障碍[18]。本研究结果显示,IAPP可以促进大脑海马区Aβ的聚集,加重AD病理学改变。普兰林肽是IAPP的类似物,既保留了IAPP的生理功能,也不易形成淀粉样蛋白聚集,对AD可能存在潜在的治疗价值[19]。

3.3 IAPP加重AD小鼠神经元形态结构的损伤

脑部神经元形态结构的损伤是脑功能损伤程度的重要病理学标志,也是AD发生发展不同阶段的脑组织损伤的重要客观证据,表现为神经元形态异常和线粒体损伤、突触数量减少等特征[20]。既往研究显示,IAPP通过干扰神经元突触的稳定性,诱导AD小鼠神经元凋亡。以往关于IAPP对AD病理损伤的研究多聚焦于通过ELISA和Western blotting方法,检测神经元凋亡及炎症因子水平的变化,缺乏神经元超微结构损伤的证据。本研究通过透射电镜观察AD小鼠神经元的超微结构变化,证明IAPP干预后的AD小鼠神经元的线粒体损伤加重,突触数量减少。

3.4 IAPP增加AD小鼠脑组织的氧化应激和炎性反应水平

氧化应激失衡是AD认知功能障碍和脑神经元损伤的重要因素[21]。H_2O_2是脑内重要的活性氧中间产物,反映机体氧化应激水平。AD病理状态下,脑内H_2O_2异常累积,诱导神经元凋亡。过度沉积的Aβ可加重脑内H_2O_2蓄积,加重氧化应激[22]。SOD和GSH-Px是关键的抗氧化酶,其活性可直接反映氧化应激损伤程度[23]。神经炎性反应是AD发生发展的另一重要病理特征,与氧化应激共同促进AD的发展过程。AD发生中小胶质细胞过度活化,大量分泌IL-1β、IL-6和TNF-α等促炎因子[24]。促炎因子可进一步加重脑内Aβ沉积,加重神经元氧化损伤与凋亡[25]。既往研究证明,IAPP干预增加AD小鼠认知功能障碍的风险,但是检

测氧化应激和炎性反应水平的指标及相关病理变化较少[26]。本研究结果显示,IAPP干预可显著升高AD小鼠脑组织H_2O_2水平,同时降低SOD与GSH-Px活性,增加了IL-1β、IL-6和TNF-α水平,提示IAPP可以加剧脑组织氧化应激和炎性因子水平,加重病理损害与认知功能障碍。

3.5 PI3K/Akt信号通路可能参与IAPP对AD的作用

PI3K/Akt信号通路在细胞生长、增殖和氧化应激等生理及病理过程中发挥重要作用[27]。有动物实验研究证明,在AD发生发展中PI3K/Akt通路活性受到抑制,导致神经元抗氧化应激能力降低,脑内Aβ清除受阻,进而加速认知功能衰退[28]。此外,在AD发生中PI3K/Akt通路活性的变化也有不同的观点[29]。目前的研究尚未明确IAPP在促进AD发生发展中PI3K/Akt信号通路的作用。本研究结果显示,IAPP干预降低了AD小鼠海马神经元中PI3K和Akt的蛋白表达水平,提示PI3K/Akt通路活性受到抑制。同时采用生物信息学分析,证明PI3K/Akt信号通路可能参与了IAPP对AD发生的作用。

本研究仍存在一定局限性:第一,本研究仅选用雌性AD小鼠作为实验动物模型,未纳入雄性小鼠进行对照研究。第二,同时仅设置单一IAPP给药浓度,缺乏梯度浓度干预实验,难以阐明IAPP对AD损伤的剂量-效应关系。第三,本研究没有探讨IAPP对Aβ跨血脑屏障的转运作用,没有检测Aβ跨血脑屏障重要转运蛋白LRP和RAGE的表达,以及血脑屏障超微结构的损伤。第四,本研究没有开展临床研究,检测AD患者的脑脊液和血清的IAPP和Aβ水平变化,以及和认知功能障碍程度的相关性,探讨IAPP作为AD早期诊断的生物标志物的可行性。

综上所述,本研究对转基因AD小鼠通过腹腔注射IAPP,发现IAPP加重了AD小鼠的认知功能障碍,并揭示IAPP可以增加脑组织氧化应激水平,加重神经元线粒体损伤,减少神经元突触数量,进一步证实PI3K/Akt信号系统可能参与了IAPP对AD小鼠的损伤作用。本研究结果为AD发生机制和干预靶点提供了新的实验依据,为AD的防治提供了新的思路。

利益冲突 本文所有作者声明不存在利益冲突

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