

甲状腺素联合多奈哌齐对成年期甲减大鼠海马内超微结构及 synaptotagmin-1 表达的影响

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摘要 目的 观察甲状腺素(T4)联合多奈哌齐(DON)治疗对成年期甲状腺功能减退症(简称甲减)大鼠海马内超微结构及突触结合蛋白 synaptotagmin-1 (syt-1) 表达的影响。方法 饮 0.05% 丙基硫氧嘧啶(PTU)水建立成年期大鼠甲减模型共 6 周,第 5 周起,T4 治疗组给予腹腔注射 T4 6 μg/100 g 体重、DON 治疗组饮 0.005% DON 水,联合治疗组给予 T4 + DON 治疗,对照组及甲减组每日腹腔注射等量生理盐水。采用放射免疫法测定血清甲状腺素水平,透射电镜观察海马内超微结构,Western blot 方法分析海马内 syt-1 的表达水平。结果 甲减组、DON 治疗组大鼠血清甲状腺激素水平明显降低($P < 0.01$),T4 治疗组、联合治疗组与对照组差异无统计学意义;电镜下甲减大鼠海马内神经元中线粒体呈现明显空泡变性、游离核糖体稀疏、突触结构受损、突触小泡数量减少,T4 或 DON 治疗后上述损伤有所改善,而联合治疗恢复后表现最接近对照组;甲减大鼠海马内 syt-1 蛋白表达量明显降低($P < 0.01$),单独 T4 或 DON 治疗后表达仍下降($P <$

0.05) 联合治疗后 syt-1 表达恢复正常。结论 成年期甲减可致大鼠海马内超微结构发生病理学损害,syt-1 蛋白表达下降,T4 + DON 治疗有利于上述损伤的修复,作用优于单一药物治疗。

关键词 甲状腺功能减退症; 海马; 超微结构; synaptotagmin-1; 甲状腺素; 多奈哌齐

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成年期甲状腺功能减退症(简称甲减)可引起一系列中枢神经系统结构和功能的损害^[1]。海马作为中枢神经系统中与认知、情感等功能密切相关的区域,其神经元是甲状腺素作用的靶点^[2]。成年期甲减可导致海马形态结构和功能的损害^[3],其机制可能涉及神经元间联系和突触可塑性,是多种突触蛋白参与的复杂生理过程。Synaptotagmin-1 (syt-1) 是突触囊泡包膜蛋白,在神经元内表达丰富,主要分布于小突触囊泡和大致密囊泡表面,通过调控突触囊泡的循环再利用参与突触传递的全过程,促进神经递质的释放,与学习和记忆有关^[4]。以往的研究^[5]表明成年期甲减能导致 syt-1 蛋白在海马内表达下降,然而给予生理剂量甲状腺素(thyroxine, T4)替代治疗至血清甲状腺激素水平正常后,syt-1

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(RACK1) mutants using molecular cloning techniques and observe the expression and cellular localization of RACK1 mutants and their co-localization with chloride intracellular channel protein 1 (CLIC1) for further study on the cellular functions of RACK1 protein. **Methods** RACK1 mutants were amplified by PCR with the template including the full length cDNA fragment of RACK1. Eukaryotic plasmids pcDNA3.1-RACK1-N(1-138)-FLAG, pcDNA3.1-RACK1-C(130-317)-FLAG were constructed respectively. The cellular expression and localization of RACK1 and its mutants in mammalian cells were detected by Western blot and confocal fluorescence microscopy respectively. **Results** All the plasmids of RACK1 mutants were successfully constructed. Western blot and confocal fluorescence microscopy results indicated that RACK1 and its mutants localized both in cytoplasm and nucleus, mainly in cytoplasm. The co-localization existed between RACK1 and CLIC1, and the mutants of RACK1 had similar co-localization with CLIC1. **Conclusion** Recombinant plasmids of RACK1 mutants are constructed successfully and express effectively in eukaryotic cells. RACK1 protein and its mutants appear to co-localize with CLIC1 respectively, which implies that RACK1 and its mutants may interact with CLIC1 respectively in mammalian cells. The study is very important for exploring the function of RACK1.

Key words RACK1; recombinant plasmids; transfection; fluorescence; Western blot

蛋白的表达却未能完全恢复。多奈哌齐(donepezil, DON)是一种胆碱酯酶抑制剂,能有效改善认知和记忆障碍^[6],对于甲减引起的海马损害可能也具有一定的治疗效果。该研究采用丙硫氧嘧啶(propylthiouracil, PTU)水制备甲减大鼠模型,观察大鼠海马内超微结构及syt-1蛋白表达的情况,并评价L-T4联合DON的治疗效果。

1 材料与方法

1.1 主要试剂 PTU、四碘甲状腺原氨酸(tetraiodothyronine, T4)、DON(美国Sigma公司);三碘原氨酸(triiodothyronine, T3)、T4放射免疫试剂盒(北方生物技术研究);兔抗大鼠抗syt-1蛋白多克隆抗体、兔抗大鼠抗GAPDH单克隆抗体(美国Abcam公司);辣根过氧化物酶(HRP)标记的山羊抗兔IgG(北京中杉金桥有限公司);ECL化学发光试剂盒(美国Pierce公司)。

1.2 动物模型复制 健康雄性SD大鼠40只,3月龄,SPF级,230~260g,购于安徽医科大学实验动物中心。所有大鼠给予标准饲料喂养,自由饮水,温度21~23℃,湿度(50±5)%,昼夜均衡。适应性喂养1周后,将SD大鼠随机分为5组:正常对照组(CON组)、甲减组(Hypo组)、甲状腺素治疗组(T4组)、多奈哌齐治疗组(DON组)、甲状腺素联合多奈哌齐治疗组(T4+DON组),每组8只。造模总时间6周,CON组饮用正常水,其他4组每天饮用0.05%PTU水^[3]。4周后,T4组开始腹腔注射T4(溶解在生理盐水中,6μg/100g体重),DON组在饮用水中加0.005%DON,T4+DON组既饮用0.005%DON也腹腔注射T4。同时,余下3组大鼠以等量生理盐水替代注射。治疗时间共2周,各组大鼠根据每周称量的体重结果,调整给药剂量。

1.3 标本制备 称量5组大鼠体重,给予水合氯醛(0.3ml/100g体重)腹腔注射,麻醉后打开腹腔,腹主动脉取血待测血清T3、T4水平。取血后将大鼠取脑,冰上迅速分离背侧海马组织,左侧海马放于-80℃冰箱待做Western blot实验。

1.4 甲状腺激素水平测定 采用放射免疫法进行大鼠血清T3、T4水平测定。

1.5 电镜观察 将组织切成1mm³小块,2.5%戊二醛4℃固定4~6h后,再以1%锇酸固定1h,经乙醇脱水,环氧树脂(Epon812)包埋,进行超薄切片,在醋酸铀及枸橼酸铅溶液中浸泡染色,常规冲洗后,用

日产JEM-1230型透射电镜观察超微结构并射片。

1.6 Western blot 法分析 取背侧海马组织置于玻璃匀浆器,加入RIPA裂解液和PMSF,冰上匀浆,提取总蛋白。4℃、15000r/min离心15min,吸取上清液。用Lorry法测定总蛋白浓度,定量后向样品中加入2×上样缓冲液(1:1),98℃10min将蛋白变性处理,分装,-80℃冻存。各组取20μg样品进行SDS-聚丙烯酰胺凝胶电泳2.5h,转膜,5%脱脂牛奶封闭过夜。次日加入一抗syt-1(1:1000)及GAPDH(1:4000)孵育2h,0.05%PBST溶液洗膜3次,每次10min,洗涤后用HRP标记的IgG二抗(1:10000)室温孵育1~2h,再用0.05%PBST溶液洗膜,最后加入ECL增强发光液,在Fine-do X6显影仪(上海天能公司)下拍摄,以GAPDH作为内参对照,计算目的蛋白与GAPDH的相对光密度比值。

1.7 统计学处理 运用SPSS 16.0统计软件进行单因素方差分析,两两间比较采用LSD法检验,数据以 $\bar{x} \pm s$ 表示。

2 结果

2.1 大鼠体重和血清甲状腺素水平 各组大鼠造模前体重差异无统计学意义;造模结束后,CON组体重增加了93.0%,而Hypo组、T4组、DON组、T4+DON组与造模前相比体重分别增加38.6%、63.2%、35.6%、63.6%,与CON组相比差异均有统计学意义($F=156.215, P<0.01$)。在血清放射免疫试验中,与CON组相比,Hypo组及DON组T3、T4水平显著降低,差异有统计学意义($F=15.236, 357.516, P<0.01$);T4组及联合治疗组大鼠血清T3、T4水平接近正常水平,差异无统计学意义,见表1。

表1 各组大鼠体重及血清激素值比较($n=8, \bar{x} \pm s$)

组别	体重(g)		T3(nmol/L)	T4(nmol/L)
	造模前	造模后		
CON	231.25±9.54	446.25±11.10	0.35±0.07	76.19±6.07
Hypo	233.12±7.99	323.12±18.53**	0.19±0.05**	21.75±2.50**
T4	231.25±7.44	377.38±7.78**	0.36±0.05	75.03±4.95
DON	230.62±11.78	312.75±11.64**	0.18±0.07**	20.58±2.73**
T4+DON	233.75±2.31	382.38±8.48**	0.37±0.10	74.50±4.81

与CON组比较:** $P<0.01$

2.2 电镜观察

2.2.1 神经元变化 CON组神经元内细胞核膜光滑完整,染色质分布均匀;线粒体发达、内部嵴结构清晰;粗面内质网及核糖体丰富(图1A)。Hypo

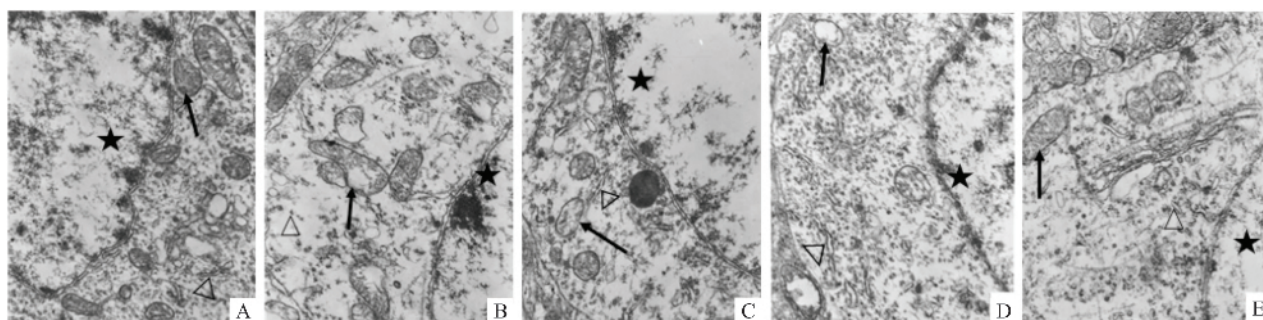


图1 电镜观察各组大鼠海马内神经元超微结构 轴-铅双染×15 000

A: CON组; B: Hypo组; C: T4组; D: DON组; E: T4+DON组; ★: 神经元细胞核; ↑: 线粒体; △: 粗面内质网或核糖体

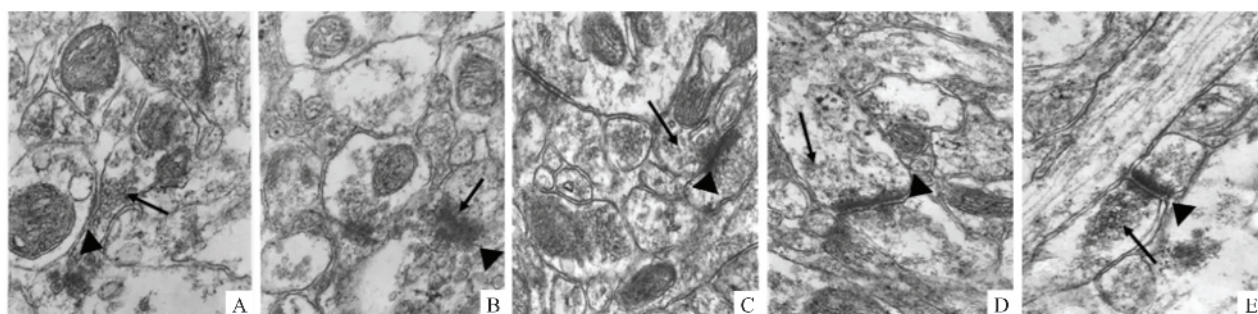


图2 电镜观察各组大鼠海马内突触超微结构 轴-铅双染×40 000

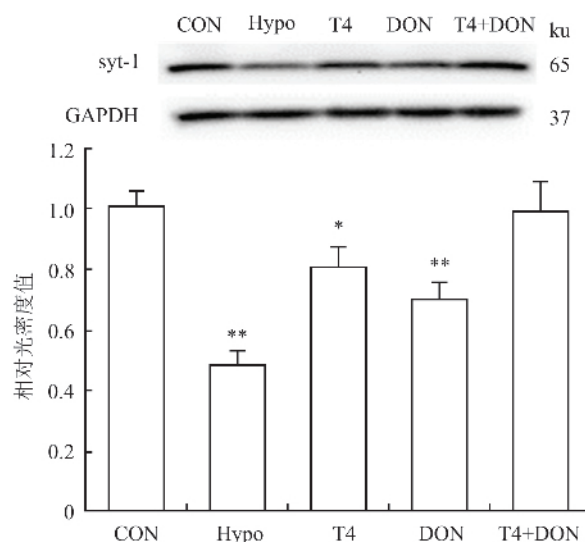
A: CON组; B: Hypo组; C: T4组; D: DON组; E: T4+DON组; ▲: 突触结构; ↑: 突触小泡

组神经元内细胞核染色质边集;大量线粒体肿胀,嵴断裂,内膜面积减少,呈现显著空泡样变性;粗面内质网及核糖体明显稀疏(图1B)。T4组、DON组神经元内细胞核核膜清晰,而细胞器较稀疏,少量线粒体内空泡形成,部分粗面内质网轻度扩张(图1C、D)。T4+DON组神经元内线粒体、粗面内质网及核糖体形态与CON组基本相似(图1E)。

2.2.2 突触变化 CON组突触前膜、突触间隙、突触后膜3层结构清晰,特化带明显,突触小泡丰富,可见清亮型和致密芯型小泡(图2A)。Hypo组突触前、后膜融合,突触小泡明显减少,几乎不见清亮型(图2B)。T4组、DON组突触前、后膜模糊,突触间隙较清晰,突触小泡清亮型数量减少(图2C、2D)。T4+DON组突触三层结构较清晰,突触小泡较丰富,接近CON组(图2E)。

2.3 大鼠海马 *syt-1* 蛋白表达的变化 与CON组比较,*syt-1*蛋白在Hypo组海马内表达显著减少,为CON组的48.4% ($P < 0.01$)。在T4组和DON组,降低的*syt-1*蛋白表达量比Hypo组有所恢复,但与CON组比较仍有差异,分别为CON组的80.1% ($P < 0.05$)、69.9% ($P < 0.01$)。T4+DON组大鼠海马内*syt-1*蛋白表达量为CON组的98.9%,差异无

统计学意义。见图3。

图3 各组大鼠海马 *syt-1* 蛋白的表达
与CON组比较: * $P < 0.05$, ** $P < 0.01$

3 讨论

海马容易在成年阶段受到甲减的损害,本实验在形态学发现成年期甲减大鼠海马内神经元及突触

超微结构出现损害。透射电镜下神经元细胞中线粒体呈现肿胀、空泡变性,粗面内质网扩张,游离核糖体明显稀疏。David et al^[7]发现甲减大鼠神经元内的粗面内质网、高尔基复合体和线粒体形态都有改变,核糖体数目减少,与本实验结果相符。另外,本研究还观察到突触出现特化带融合,结构不清晰,突触小泡减少的形态改变。Cortes et al^[8]的研究结果表明成年甲减大鼠脑中出现多系统的神经元突触后变化,突触后密度减少。也有实验报道甲减导致神经元树突状超微结构的退化^[9]。众所周知,线粒体和核糖体是能量和蛋白质合成的活性位点^[10],突触作为神经元之间信息传递的结构基础,上述神经元内细胞器、突触超微结构的破坏可能会引起能量代谢的障碍,进而影响相关脑区蛋白的合成。

syt-1 是一种分子量为 65ku 的突触小体蛋白,其作为快速 Ca^{2+} 感受器,促进突触小泡的融合,在神经递质同步释放调控过程中起着重要作用。本实验运用 Western blot 方法测定的结果显示,与对照组相比,甲减组 syt-1 蛋白表达量明显减少。Wang et al^[11]发现在碘缺乏和甲减大鼠的小脑中, syt-1 蛋白表达下调,本研究结果与之一致,然而其受损机制仍未明确。研究^[12]显示 T4 可以调节脑中蛋白质的合成, T4 缺乏直接导致海马中甲状腺素受体表达下调并影响其作用的靶蛋白的表达,甲减时 syt-1 蛋白表达减少可能与此有关。

T4 替代治疗是目前国际上公认的治疗甲减的标准方案。本实验中成年期甲减大鼠在给予常规剂量的 T4 治疗 2 周后,海马内神经元细胞器、突触超微结构的损伤及 syt-1 蛋白的表达部分恢复,但未达到正常水平。研究^[13]显示,即使 6 周的 T4 替代治疗也未能使表达减少的 PKC γ 蛋白恢复正常。上述均说明,在 T4 替代治疗到血清甲状腺激素恢复正常时,甲减引起的脑中分子障碍可能并没有完全恢复。研究^[14]表明,血清甲状腺激素浓度远远高于其在中枢神经系统中的浓度。T4 治疗后海马损伤未完全治愈的现象,可能与血清甲状腺激素水平恢复正常时,脑内剂量仍然不足有关。研究^[5]表明,当给予成年期甲减大鼠大剂量 T4 (20 μ g /100 g 体重) 冲击治疗时,海马内 syt-1 表达可恢复到正常水平,但同时血清 T3、T4 水平超过正常高值,有诱发甲亢的风险。

T4 联合 DON 治疗后,甲减大鼠海马内神经元线粒体、粗面内质网、核糖体形态与数量基本恢复,突触结构清晰、突触小泡数量丰富,突触小体内 syt-

1 的表达量达到正常水平,说明 DON 能改善甲减引起的海马损伤。DON 作为胆碱酯酶抑制剂,通过与胆碱酯酶结合,阻止脑中乙酰胆碱的水解,临床上主要用来治疗轻中度认知功能障碍,具有独立的神经保护作用。DON 被报道可以改善菌素引发的神经元线粒体功能障碍^[15];维护老龄大鼠椎体神经元的树突状分支、增加总树突长度和突触后密度^[16]。上述结果均提示 DON 可以通过改善海马内神经元细胞器、突触病变结构而发挥其神经保护作用。此外,有研究^[17]显示 DON 可以通过诱导乙酰胆碱的抗炎作用来改善 tau 病鼠海马中突触蛋白的表达;本课题组先前的免疫组织化学方法研究^[3]发现, DON 对甲减引起的突触蛋白 munc18、syntaxin-1 的损伤有益。这些突触蛋白的恢复可能也正是多奈哌齐发挥神经保护作用的体现。

综上所述,成年期甲减可造成大鼠海马内神经元细胞器、突触超微结构的损伤及突触小体内 syt-1 蛋白的表达减少,上述变化可以通过 T4 替代治疗得到部分恢复, T4 联合 DON 治疗使甲减导致的改变恢复至正常,比单独应用 T4 治疗更有效。

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Effects of thyroxine and donepezil on hippocampal ultrastructure and synaptotagmin-1 expression in hypothyroid adult rats

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Abstract Objective To investigate the effect of thyroxine (T4) and donepezil (DON) treatment on the hypothyroidism-induced alterations of ultrastructure and synaptotagmin-1 (syt-1) expression in the hippocampus of adult rats. **Methods** Administration of 0.05% propylthiouracil (PTU) to drinking water for 6 weeks was used to replicate the model of hypothyroidism. From the 5th week, T4 group was given thyroxine (6 µg/100 g body weight) by intraperitoneal injection, DON group was given 0.005% DON in the drinking water, T4 + DON group was given a combination of both, the control and hypothyroid groups were given the same volume of saline solution by intraperitoneal injection daily. The concentration of serum T3 and T4 was determined by radioimmunoassay kits, and the hippocampal ultrastructure was observed with transmission electron microscope (TEM) and the protein level of syt-1 was measured by Western blot. **Results** Compared to the control group, the concentration of serum T3 and T4 was significantly decreased in the hypothyroid group and the DON group ($P < 0.01$), but returned to the normal in the T4 group or combined with DON group. TEM revealed that significant degeneration was in mitochondria and rarefaction in free ribosomes of adult hypothyroid rats, synaptic structural damage and reduction of synaptic vesicles also appeared. The ultrastructural was partly restored by T4 or DON administration, but was completely restored by a combination of both treatments. Expression of syt-1 protein was significantly lower in hypothyroid rats compared with the controls ($P < 0.01$), and was still expressed at lower level in hypothyroid treated with T4 alone ($P < 0.05$), while was restored to normal values after co-administration of T4 and DON. **Conclusion** These observations indicate that adult hypothyroidism induces ultrastructural damage and a decrease of syt-1 protein in the hippocampus, and the alterations can not be restored by T4 monotherapy. In addition, the co-administration of T4 and DON result in more effective restoration than either alone.

Key words hypothyroidism; hippocampus; synaptotagmin-1; ultrastructure; thyroxine; donepezil