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老年性痴呆发病过程中内源性甲醛慢性损伤机制 *

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摘要 通过原子力显微镜、荧光标记、Congo 染色等方法，观察到低浓度甲醛可以诱导人类神经 Tau 蛋白错误折叠并形成具有细胞毒性的似球状聚积物；气相色谱和液相色谱等分析结果表明，神经鞘磷脂 N-Acyl-4-sphingoine-1-phosphocholine (myelin)的过氧化能够产生甲醛分子；脂质过氧化的代谢产物丙二醛(malondialdehyde)在修饰蛋白质(BSA)的过程中，亦可产生甲醛分子．以上结果为内源性甲醛的产生揭示了新的途径．值得注意的是，在生理条件下，血液中内源性甲醛的水平维持在一个动态平衡((0.087±0.004) mmol/L)，与体外培养神经细胞时甲醛产生毒性的浓度(～0.1 mmol/L)十分接近，甚至已经达到产生一定细胞毒性的水平．随着机体的衰老，内源性甲醛的调节机能下降，在氧化应激等相关因素的诱导作用下，内源性甲醛浓度可能升高，对中枢神经系统一定部位的神经细胞造成慢性损伤，这可能是散发性老年痴呆发病的机制之一．

关键词 内源性甲醛，脂质过氧化，丙二醛，能量代谢失衡，散发性老年痴呆，甲醛慢性损伤
学科分类号 Q5，R74

甲醛是化学结构最简单的醛类分子，广泛存在于人类的生活环境中．甲醛作为重要的有机交联剂被应用于涂料、木材加工、纺织品染色、消毒剂、固定剂、防腐剂等各个方面．甲醛具有较高的毒性，当空气中甲醛含量达到 0.1 mg/m^3 时，人就会感觉到异味和不适，达到 30 mg/m^3 时，就可能危及生命．

然而，人类机体自身也产生甲醛，不同细胞其甲醛含量不同[1]．细胞核内产生的甲醛主要来自于 DNA 甲基化和脱甲基化，另外细胞器如线粒体、内质网等也产生甲醛．一些胺类(甲胺、多胺等)能通过氨基脲敏感性胺氧化酶 (semicarbazide-sensitive amine oxidase, SSAO)代谢，生成甲醛[2, 3]．脂质过氧化是体内产生甲醛的另一途径．体内甲醛含量的异常升高，可以造成重要系统，特别是中枢神经系统的损伤．甲醛对神经系统造成的损害可表现为学习、记忆、情感状态等方面的障碍。小鼠的急性和亚急性实验都肯定了甲醛的神经毒性作用[4]，甲醛暴露会引起小鼠脑的形态发生改变[5]．大鼠甲醛暴露后，寻找迷宫路线的能力发生障碍[6]．代谢失衡、脂质过氧化、应激状态等异常情况下，血液中的甲醛浓度会显著升高．

神经系统特定蛋白质的异常修饰、错误折叠及其在脑内的聚积是神经退行性疾病共同的病理特征[7]．侧链基团的异常修饰，如磷酸化[8, 9]、糖基化[10, 11]等能导致蛋白质的聚积，并多见于散发性老年痴呆[12]．另外，基因突变会导致相关蛋白或降解片段的错误折叠和聚积，多见于家族性痴呆。近年来的研究表明[13]，每一个活细胞都能产生甲醛，而且内源性甲醛的产生与代谢可能是多途径的．在此，本文作者就神经鞘磷脂的过氧化产生甲醛，以及脂质过氧化产物丙二醛在修饰蛋白质的过程中产生甲醛的现象进行报道，同时认为，内源性甲醛含量的长期升高，将造成中枢神经系统的慢性损伤，可能是散发性老年痴呆的重要发病因素之一．

1 材料与方法

1.1 材料

表达人类神经 tau23 异构体的 BL21 菌株，由英国剑桥大学 Geodert 教授馈赠；Tau-1 单克隆抗

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体由龚成新教授(New York Institute for Basic Research in Developmental Disabilities，USA)惠赠；malondialdehyde、N-Acyl-4-sphingoine-1-phosphocholine及IPTG购于Sigma公司；P-Sepharose、Q-Sepharose为Amersham Biosciences公司产品；BCA蛋白质定量试剂盒购于Pierce公司；DNPH为北京化工厂生产．所用试剂全部为分析纯，使用之前没有进一步纯化．

人成神经瘤细胞SH-SY5Y，由美国Sloan Kettering癌症中心的Biedler博士馈赠；SDMEM、F12培养基、新生牛血清、马血清、青霉素、四环素、羊抗鼠IgG购自Hyclone公司；吸收光谱通过Hitachi UV-2010紫外分光光度计测定．

神经Tau蛋白的表达、菌体匀浆、可溶性蛋白的收集等，以及采用SP-sepharose、Q-sepharose柱层析对Tau23进行纯化的过程，参考曲梅花等使用的方法[14]．如图1a所示，分离纯化后的Tau23蛋白在SDS-PAGE和(单克隆抗体Tau-1)蛋白质印迹膜上均为单一条带．通过“微管蛋白聚集”法测定显示，神经tau的活性与国际同行所报道的一致[15]．

1.2　低浓度甲醛诱导神经Tau错误折叠分子聚积

Tau蛋白(1 g/L)溶解在50 mmol/L磷酸缓冲液(pH 7.2)中，与不同浓度(0.001%～0.5%)甲醛保温(37℃)，12 h后取样，采用Congo红染色，同时在原子力显微镜下观察蛋白质聚积的情况．

1.3　细胞培养与蛋白毒性聚积物诱导细胞死亡

神经Tau与甲醛保温聚积后，采用Amicon Ultra-4(Millipore)柱超滤除去样品中残留的甲醛．在5% CO_2孵箱中(37℃)，培养人成神经瘤细胞SH-SY5Y (DMEM/F12培养基，含青霉素100 U/ml，四环素100 mg/L)．培养基中含有10%新生牛血清和5%马血清。将甲醛诱导聚积的Tau (0.1～100 μmol/L)加入到细胞培养液中，通过离心(5 000 r/min，4℃，10 min)分离细胞，并将细胞悬浮在磷酸盐缓冲液(pH 7.2)中，加入10 mg/L Hoechst 33258 (37℃)保温10 min，在Nikon Microphot-FXA荧光显微镜下观察，对细胞核的形态进行分析．

1.4　神经鞘磷脂的过氧化及其甲醛的测定

将1 g/L神经鞘磷脂(N-Acyl-4-sphingoine-1-phosphocholine or sphingomyelin)溶解在50 mmol/L磷酸缓冲液(pH 7.2)中，与1 mmol/L过氧化氢(H_2O_2)混合，37℃保温24 h，取样与DNPH试剂进行反应，反应后的产物通过气相色谱-质谱法(GC-MS HP6890 GC，USA)进行测定．DNPH的测定参考Yu等测定甲醛的方法[16]．在相同条件下，以10 mg/L甲醛作为对照．

1.5　丙二醛修饰蛋白质及其甲醛的测定

在50 mmol/L磷酸缓冲液(pH 7.2)中，将丙二醛(10 mmol/L)与50 μmol/L BSA混合保温(37℃，24 h)，其后，将DNPH试剂加入到保温体系中，取样进行气相色谱-质谱分析．在相同条件下，10 mg/L甲醛作为对照．

1.6　原子力显微镜的观察

通过50 mmol/L磷酸缓冲液(pH 7.2)稀释甲醛诱导聚积后的神经Tau蛋白，10 μl滴加到云母膜表面[17]．放置1 min，用200 μl双蒸水冲洗3次，经氮气吹干．通过原子力显微镜(Nanoscope MultiMode Ⅲa系统)，以tapping模式进行观察．在相同条件下，未经甲醛诱导的神经Tau蛋白作为对照．

2　结果与讨论

2.1　低浓度甲醛诱导神经Tau蛋白形成似球状细胞毒性产物

关于异常磷酸化[15]以及相关基因突变导致Tau在脑内聚积的工作已经有了大量报道，但是对于醛类诱导Tau蛋白错误折叠形成细胞毒性产物对神经系统危害[2]的研究相对较少．如图1所示，低浓度甲醛(0.01%～0.1%)可导致人类神经Tau蛋白错误折叠并形成似球状的聚积物，在云母膜上其平均半高宽(大小)为(22.4±3.5) nm．在相同情况下，没有与甲醛保温的Tau蛋白为单体，其平均大小为(8.2±1.2) nm．将0.1%甲醛与Tau蛋白保温2周以上，未观察到纤维状结构的形成，Congo红染色揭示所形成的聚积物为淀粉样沉积．

甲醛可与氨基酸基团中的α/ε氨基、巯基以及羟基等发生反应，使蛋白质分子构象发生改变(图2)[10]．甲醛与氨基发生反应减少了溶液中蛋白质的正电荷，也可导致蛋白质分子间的交联而发生聚积．甲醛与巯基的反应在蛋白质聚积中也具有重要作用，0.01%甲醛可以诱导神经Tau蛋白聚积，但对于不含有巯基的α-synuclein的聚积作用却相对不明显．神经细胞内Tau蛋白的浓度约为15 μmol/L，细胞学实验显示[18]，在去除残留甲醛后，1～10 μmol/L Tau蛋白球状聚积物对SH-SY5Y、原代培养大鼠海马细胞以及HEK293

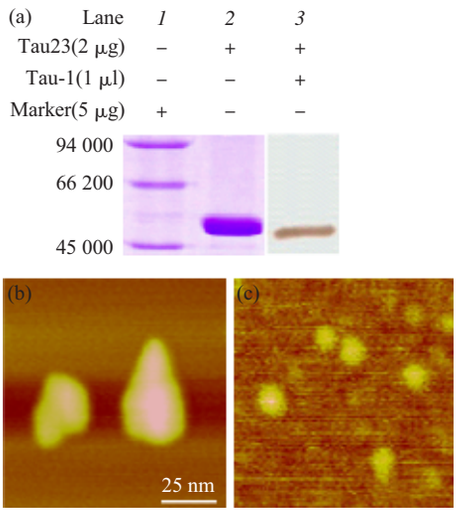

Fig. 1　AFM images of neuronal Tau in the presence of formaldehyde

Neuronal Tau was expressed and purified as described before[15]. After purification, Tau protein was electrophoresed on SDS-PAGE and analyzed by Western blot (a). Tau protein (20 μmol/L) was incubated in 50 mmol/L phosphate buffer (pH 7.2) containing 0.1% formaldehyde as indicated at 37℃ over night (b). Aliquots were taken and diluted to the desired concentration using the phosphate buffer, and the samples were dropped onto mica surfaces and dried in air before observed under atomic force microscope. Protein tau in the absence of formaldehyde was used as control (c).

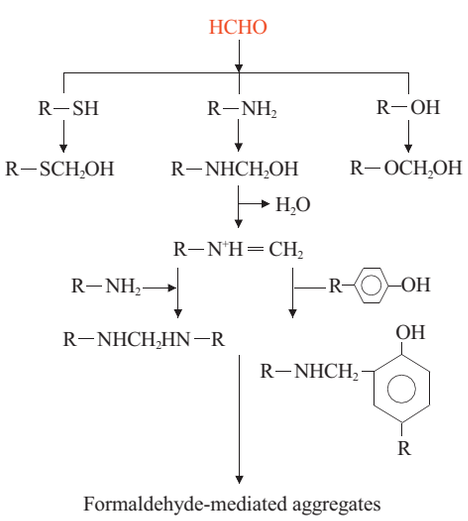

Fig. 2　Scheme for formaldehyde-mediated protein aggregates

Formaldehyde reacts with the side chains of amino acid residues such as (α/ε)-amino, thiol and hydroxyl groups. In the presence of formaldehyde, amino groups are blocked, leading to a decrease in positive charges of protein molecules. Further reaction between formaldehyde-modified proteins leads to molecular crosslinking and aggregation.

细胞株等可以产生明显的毒性，而作为对照的 Tau 蛋白纤维状聚积物的细胞毒性却不显著(图 3)．另外，在低浓度甲醛的诱导下，SH-SY5Y 细胞产生的 Aβ 明显增加，细胞出现变性死亡[19]．同时，甲醛代谢产物甲酸在局部的累积可形成微环境酸中毒，也是造成中枢神经系统局部，包括脑白质等慢性损伤的原因[20]．

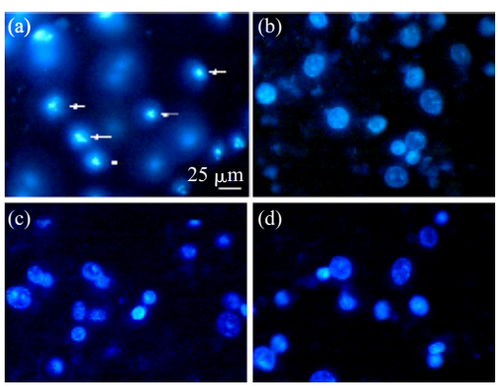

Fig. 3　Hoechst staining of cells treated by amyloid-like Tau

SH-SY5Y cells were cultured for 72 h in the presence of 0.1% formaldehyde-induced tau deposits (100 mg/L, panel a), self-aggregated tau (panel b), native tau (panel c) and control culture in DMEM without serum (panel d). The cells were collected and stained with Hoechst 33258 and nuclei were visualized by fluorescence microscopy. The arrows designate the presence of apoptotic nuclear profiles.

2.2　内源性甲醛产生新途径

甲醛可以通过血脑屏障，在生理条件下，其血液中的含量维持在一个动态平衡．不同方法测定血液中甲醛的浓度所得到的数值不同，采用气相色谱等方法，测定人类血液中甲醛的含量为(0.087±0.004) mmol/L[21~23]，Szarvas 等[24]采用放射性标记测得人血中甲醛浓度为 0.01～0.02 mmol/L．不同方法测定的血甲醛浓度范围在 0.01～0.09 mmol/L．那么，这个数值范围与甲醛达到细胞毒性的浓度相差多少呢？图 4a 给出了在含有不同浓度甲醛的培养基中，SH-SY5Y 细胞株生长的实验结果。当培养液中的甲醛浓度约为 0.1 mmol/L 时，细胞生长和繁殖受到了明显的抑制．类似的结果也在其他细胞株的甲醛毒性实验中观察到(在此不再赘述)．值得注意的是，在生理条件下，血液中所维持的甲醛水平非常接近于甲醛对细胞产生损伤的浓度，甚至在 0.08～0.09 mmol/L 甲醛浓度下，对体外培养的 SH-SY5Y 细胞已经可以产生一定的毒性(图 4b)．该现象暗示，在氧化应激、醛酮应激等诱导因素的

作用下，特别在衰老过程中，机体对甲醛的清除机能下降，内源性甲醛在血液中的浓度升高而危害性增大，从而可能导致中枢神经系统的细胞损害．

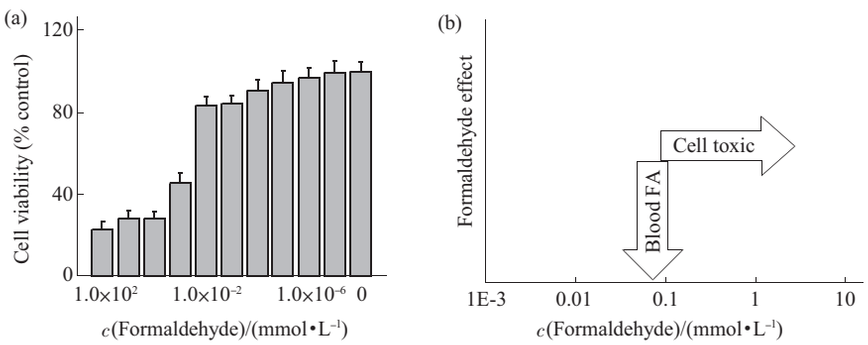

Fig. 4　MTT assay of cell viability and relationship between blood formaldehyde and cell toxicity

Cells were grown in 96-well plates for 24 h and treated with different concentrations of formaldehyde for 72 h. After adding MTT (0.5 g/L final concentration) to the culture medium of the cells, plates were incubated at 37℃ for 30 min. The assay was stopped by replacement of the MTT-containing medium with 100 μl dimethysulfoxide (DMSO). Absorbance at 595 nm was read by means of an ELISA plate reader. Each experiment was repeated at least three times (a). A schematic showed the relationship between blood formaldehyde and the cell (SH-SY5Y) toxicity *in vitro* (b).

2.2.1　神经鞘磷脂过氧化．

在衰老进程中，不但相关脑细胞发生变性死亡，而且白质也出现退行性变[25]，并且与神经鞘磷脂的过氧化相关．在神经鞘磷脂过氧化中是否有甲醛的产生呢？如图 5 所示，通过气相色谱 - 质谱技术对过氧化氢与神经鞘磷脂的反应产物进行分析，可以观察到在神经鞘磷脂的过氧化过程中，其产物中有甲醛的产生．在相同条件下，未检测到作为对照的神经鞘磷脂中含有甲醛．

动物实验表明[26]，在脂质过氧化状态下，机体内甲醛水平可大幅增高，从而与中枢神经系统的重要生物功能蛋白质发生反应[20]，产生细胞毒性聚积物，致使细胞变性死亡，造成脑局部的慢性损伤．值得注意的是，甲醛还可能与某些神经递质，如甘氨酸、γ- 氨基丁酸等发生反应，从而影响神经系统的相关功能 (另文叙述)．

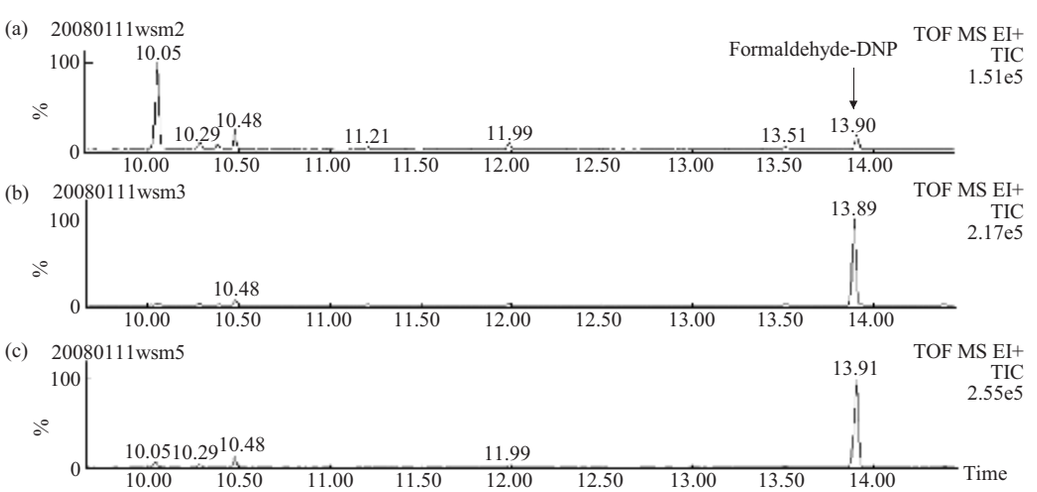

Fig. 5　Detection of formaldehyde in the reaction of BSA with malondialdehyde and sphingomyelin with hydrogen peroxide

N-Acyl-4-sphingoine-1-phosphocholine (1 g/L) was incubated with 1mmol/L H_2O_2 in 50 mmol/L phosphate buffer (pH 7.2) at 37℃ for 24 h (b). Under the same conditions, malondialdehyde (10 mmol/L) was incubated to BSA (0.05 mmol/L) (c). Formaldehyde (10 μg/L) alone was used as control (a). DNPH was added to reaction mixtures as described [17], before aliquots were performed on a GC-MS (HP6890 GC, USA).

2.2.2 丙二醛化学修饰蛋白质.

在氧自由基的攻击下，体内 (包括中枢神经系统) 脂质可以发生过氧化产生丙二醛[27]. Preuss 等[28]在一组志愿者的实验过程中发现，当采用脂肪燃烧剂 CitriMax 对人体脂肪进行消耗时，尿中的脂质过氧化产物，包括丙二醛、乙醛、甲醛以及丙酮的水平明显增加 (125%～258%). 为了研究丙二醛在修饰蛋白质的过程中所释放出的产物，本文作者将丙二醛与 BSA 保温(37℃)，并在不同时间取样，采用 SDS-PAGE 进行检验，可以观察到在丙二醛的作用下，BSA 发生部分聚集形成了二聚体 (图 6). 通过气相色谱 - 质谱技术测定反应体系中的产物 (图 5c). 结果显示，丙二醛在修饰 BSA 的过程中产生了甲醛分子. 相同条件下，作为对照的丙二醛溶液不产生甲醛。体内脂质过氧化在不断发生，因此丙二醛在体内维持在一定的水平，大鼠肝脏含量约 26～47 nmol/g，小鼠血浆约 23.4 nmol/g，小鼠红细胞 47.2 nmol/g[29]，并且随时都可能与蛋白质发生相互作用. 丙二醛与蛋白质相互作用，可能是人体内甲醛产生的一个新的途径(图 7). 释放出的甲醛继续与其他蛋白质，或生物活性分子发生反应，或在酶促条件下被转化为甲酸. 高脂血症时，脂质过氧化增加，体内的丙二醛含量可显著升高，产生的甲醛也将增加. 由于人体内的 H4-folate 的含量有限，甲酸因其代谢速度较慢而在局部累积，导致微环境酸性增加，严重时可形成微环境酸中毒，而造成局部损伤.

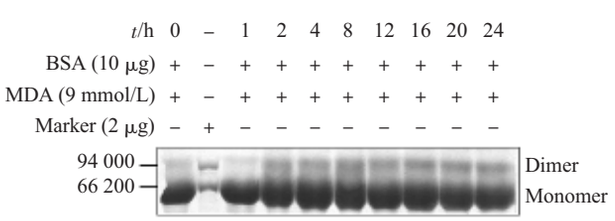

Fig. 6　BSA in the presence of MDA

BSA (3 g/L) was incubated with MDA (9 mmol/L) in PBS buffer (pH 7.2) at 37℃, and aliquots were taken at different time intervals for SDS-PAGE stained with Commassie brilliant blue.

2.3　内源性甲醛的产生与神经退行性疾病

内源性甲醛的存在是探索神经系统慢性甲醛损伤的重要证据. 早在 1956 年，Mackenzie 和 Abeles[30]就证明了甲基甘氨酸(monomethyl-Gly)在线粒体上进行代谢时可以产生甲醛. 磷酸肌酸是能量的一种储存形式，细胞在消耗能量的过程中，磷酸肌酸可以释放出高能磷酸键，为细胞提供能量. 在肌酸的分解代谢过程中，可以产生甲基甘氨酸. 同时，在血液循环系统和脂肪细胞中存在的氨基脲敏感性胺氧化酶(SSAO)，可以催化一些胺类如甲胺以及氨基丙酮脱氨基分别生成甲醛和甲基乙二醛[3]. 研究表明，细胞在甲基化和脱甲基化的过程产生甲醛[31, 32]. 因此，当线粒体等细胞器发生应激时，内源性甲醛可能升高. 在生理条件下，血液中的甲醛维持在一定的水平(正常人的尿甲醛为～3.25 μmol/L[33]). 当能量代谢失调，甲胺和氨基丙酮升高，甲醛在血液中的浓度也随之升高. Poortmans 等[34]给 20 位青年男性连续口服肌酸 (21g/ 天) 14 天，发现尿中的甲醛和甲胺含量分别较生理状态增高了 4.5 倍 (P = 0.002) 和 9.2 倍 (P = 0.001). 通过对 23 名老年性痴呆患者的临床调查，本文作者初步发现，尿甲醛随着衰老的进程而明显升高，并且与认知功能损害的程度有一定的关系(具体数据将有另文报道). 甲醛无时无刻不在我们体内生成，由于不同原因而造成内源性甲醛水平的长期增高，可能导致中枢神经系统的甲醛慢性损伤.

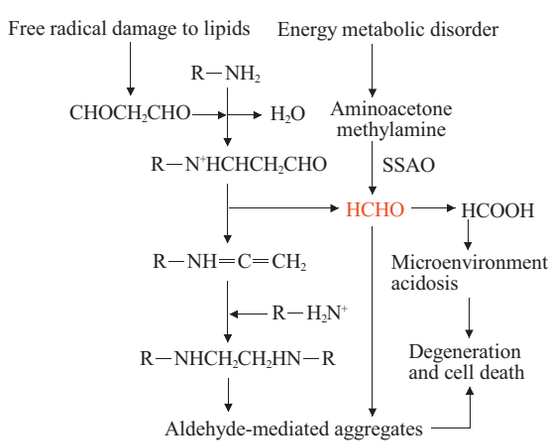

Fig. 7　Scheme for metabolism of lipid peroxidation-derived aldehyde such as malondialdehyde and formaldehyde

Free radical damage to lipids results in malondialdehyde which reacts with the amino groups of a protein and releases an intermediate product of formaldehyde. Consequently, formaldehyde-mediated protein aggregation and deposit may occur in vivo. The formaldehyde chronic damage is probably one of the most important factors involved in neurodegeneration.

2.4　中枢神经系统的甲醛慢性损伤机制

在生理情况下，血液中的甲醛浓度动态维持在

接近甲醛造成神经细胞毒性的水平，甚至该浓度已经可以对体外培养的神经细胞 (SH-SY5Y 等) 产生一定毒性．随着衰老的进程，机体调节内源性甲醛 (等醛类分子) 的机能下降，因此，在应激等诱导因素的作用下，内源性甲醛等醛类分子在体内的水平升高，对中枢神经系统产生的危害加大，造成神经细胞功能障碍、变性死亡，形成局部慢性损伤，这可能是散发性老年性痴呆的重要发病机制之一．

致谢 曲梅花在原子力显微镜观察神经 Tau 蛋白聚积方面提供了帮助．感谢中国科学院生物物理研究所阎锡蕴博士在细胞培养方面提供的帮助．

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Formaldehyde-mediated Chronic Damage May Be Related to Sporadic Neurodegeneration*

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Abstract Formaldehyde is directly toxic to cells and its intermediate metabolite formic acid leads to acidosis in microenvironment *in vivo*. According to recent literatures, endogenous formaldehyde production is related to several metabolic pathways such as amine oxidation (catalyzed by semicarbazide-sensitive amine oxidase, SSAO), methylation and demethylation. Under oxidation stress and energy metabolic imbalance, formaldehyde markedly increases in human circulation. Here, the authors found that formaldehyde is released from the reaction of malondialdehyde with a protein (BSA) in which protein side chains such as amino groups are chemically modified. Moreover, formaldehyde is also produced from the sphingomyelin solution in the presence of hydrogen peroxide as the myelin peroxidation occurs. Formaldehyde at low concentration induces neuronal Tau aggregation, resulting in formation of globular like aggregates which are toxic to SH-SY5Y cells, HEK-293 cells and hippocampus neurons in the primary culture. According to Chen *et al*. (2006), endogenous aldehydes are related to beta-amyloid misfolding, oligomerization and fibrillogenesis. Furthermore, formaldehyde is able to react with some neurotransmitters and thus impairs their structures and functions. Under physiological conditions, the human blood formaldehyde is dynamically kept approximately (0.087±0.004) mmol/L. Notably, this concentration is close to the half-lethal dose of formaldehyde (0.10～0.12 mmol/L) to neural cells in the *in vitro* cell culture such as SH-SY5Y cells. Furthermore, cell growth can be partially affected and inhibited in the presence of formaldehyde at (0.087± 0.004) mmol/L during the *in vitro* culture. This suggests that human body needs a strong degradation system to remove endogenous formaldehyde. As shown in clinical trials, the formaldehyde level in urine of Alzheimer's patients was markedly higher than the control subjects. The urine formaldehyde level was shown to be related to the cognitive impairment. Therefore, the level of blood (brain) formaldehyde is supposed to be changeable and increased under aging, leading to a higher risk chance to impair human brain, especially under stressing. The formaldehyde chronic damage to neural cells (grey mater) and neural fibers (white mater) may be one of the most

important pathological mechanisms for sporadic neurodegeneration for instance Alzheimer's disease, because hypofunction in scavengering endogenous formaldehyde occurs as aging.

Key words endogenous formaldehyde, lipids peroxidation, energy metabolic imbalance, sporadic AD, formaldehyde-mediated damage

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