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四乙胺络合铁蛋白亚基质谱特性和诱导人宫颈癌
细胞凋亡的蛋白质组学研究

Mass Spectrometry Characteristics of Ferritin Subunits
Binding to TEA and Proteomics of Hela Cells Apoptosis
Exposed to TEA

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中文摘要

大量研究指出, 铁蛋白主要生理功能是释放铁给细胞, 合成含铁的酶蛋白, 储存细胞中的过量铁, 避免产生铁中毒现象。哺乳动物铁蛋白由 H 和 L 亚基组成, 其中 H 亚基负责络合亚铁离子和氧化亚铁形成高铁组分, L 亚基负责铁核形成与矿化。有关铁蛋白亚基类型、亚基之间的相互作用强度和执行新生理功能还存在着争议。本文以猪胰脏铁蛋白 (pig pancreas ferritin, PPF) 为实验材料, 优化铁蛋白分离技术, 小批量制备高纯度的 PPF。选用 SDS-PAGE 方法分析了 PPF 由 H (21.0kDa), L 亚基 (19.0 kDa) 和 SH (small H subunits) 小亚基组成。选用基质辅助激光解吸电离飞行时间质谱仪 (MALDI-TOF/TOF MS) 直接分析 PPF 中含有不稳定的 H 和 SH 亚基, 其中 L-L、H-L、L-SH 和 H-SH 亚基之间的相互作用强度较高。采用反相高压液相色谱仪 (RP-HPLC) 技术也发现, PPF 由 H、L 和少量 SH 亚基组成, 推算 L/H 亚基之间的比值约为 2:1。采用 MALDI-TOF MS 技术分析 PPF 中不稳定的 H 和 SH 亚基, 其分子量分别为 20135.015 Da 和 12925.221 Da。采取混合基质, 增强质谱仪的激光强度和反应介质酸度等措施, MALDI-TOF MS 技术获得 PPF 对应 4 个亚基质谱峰, 其 m/z 值分别为 9981.82, 10649.16, 19960.71 和 20835.96 Da, 对应的亚基分子式为 $[M^{2+}]_L$ 、 $[M^{2+}]_H$ 、 $[M^+]_L$ 和 $[M^+]_H$, 但未获得 SH 亚基的质谱信息, 说明 PPF 亚基之间的相互作用强度, 稳定性随亚基结构的变化而变化。分别采用 ESI-Q-TOF MS 技术和 RP-HPLC-ESI-Q-TOF MS 分别鉴定 PPF 亚基类性、相互作用强度和计算它们同源性信息。酶解实验结果表明, 与野猪相比, 其 H 和 L 亚基同源性分别达到 85% 和 100% (部分序列), 并同样发现 PPF 中的部分 H 亚基不稳定, 易被解离成准亚基离子, 供质谱分析。

钾离子通道阻滞剂四乙胺 (TEA) 和四氨基吡啶 (4-AP) 均能高效阻断细胞膜上的钾离子通道。TEA 直接络合于铁蛋白 H 亚基上, 形成 PPF_H-TEA 复合物, 未发现络合于 L 亚基的 PPF_L-TEA 复合物。选用化学强还原剂 Na₂S₂O₄ 和生物还原剂 Vc, 分别研究 PPF 释放铁的动力学全过程, 指出 PPF 释放铁的全过程可分为快速释放铁 (一级反应动力学) 和慢速释放铁 (零级反应动力学) 的过程。其

变化趋势和规律与 PPF_H-TEA 复合物释放铁全过程很相似。

TEA 和 4-AP 也能阻断癌症细胞细膜上的钾离子通道,从而起到抑制细胞生长效果。紫杉醇、TEA 和 4-AP 抑制肿瘤细胞生长已有详细的研究报道,但用于分别或协同抑制肿瘤细胞生长或揭示其凋亡分子机制的研究报道甚少。本论文选用 MTT、单染/双染流式细胞术分别研究诱导人宫颈癌 (Hela) 细胞凋亡速率和变化趋势,证实了这三种抑制剂能协同增强抑制 Hela 细胞凋亡速率,且死亡的主要原因为细胞凋亡和细胞坏死。运用细胞膜片钳技术在全细胞模式下测定 Hela 细胞膜上的钾电流,在 TEA 处理组中,电流抑制率高达 80%。MALDI-TOF MS 技术证实 TEA 能改变 Hela 细胞膜表层的多肽分布与组成,可能是 TEA 能诱导细胞表达差异蛋白质或多肽。选用顺铂-人血清转铁蛋白诱导 HepG2 肝癌细胞凋亡过程中所表达的 26 种差异蛋白质为目标蛋白,设计对应的引物,研究在 TEA 和 4-AP 分别诱导 Hela 细胞凋亡过程中所产生 mRNA 表达情况与趋势,并发现这 26 种蛋白质均得到差异表达,可见 TEA 和 4-AP 类似其他抗肿瘤药物,均通过相同或相似的途径和机制诱导肿瘤细胞凋亡。

选用蛋白质组学及相关分析技术筛选与鉴定出 TEA 诱导 Hela 细胞凋亡过程中的差异蛋白质,发现 33 个蛋白表达量上存在显著差异,其中 13 个蛋白表达量上调,20 个蛋白表达量下调。差异蛋白质涉及 ATP 结合,分子伴侣,酶的活性,钾钙离子结合,细胞转运,氧化还原平衡,代谢等多种分子生物功能,通过差异蛋白的亚细胞定位发现,TEA 主要的靶向位点为细胞质蛋白。选用 Ingenuity IPA 软件分析得到差异蛋白涵盖细胞移动,肿瘤形成,细胞发育等蛋白网络。此外采用 western-blotting 分析谷胱甘肽 S 转移酶 (GSTO1) 差异蛋白的表达水平,其表达量随 TEA 浓度递增而降低。Real-time PCR 分析 Hela 表达的 33 种差异蛋白所对应的 mRNA 水平、变化趋势和规律基本上与 2D-PAGE 分离胶中蛋白质表达量相似。这些结果进一步佐证了采用蛋白质组学技术筛选的由 TEA 诱导 Hela 细胞表达的差异蛋白的可靠性,揭示 TEA 不仅阻断 Hela 钾离子通道,而且在蛋白合成的过程中,通过影响细胞的转录调控机制,诱导肿瘤细胞凋亡。TEA 属于带强毒性的抗肿瘤药物,诱导肿瘤细胞凋亡的分子机制类似于临床一线用药。本论文立意新颖,研究内容具有重要的科学意义,并潜在着应用价值。

关键词: 猪胰铁蛋白; 蛋白质组学; 四乙胺

Abstract

Many research studies indicate that main physiological function of ferritin is releasing iron to cell, iron-related proteins synthesis, excess iron storage within cell and avoid from iron-toxicity. Mammals' ferritins are composed of H (heavy) and L (light) subunits. The H subunit is responsible for Fe^{2+} binding and oxidizes Fe^{2+} into Fe^{3+} , L subunit is responsible for Fe^{3+} -nuclei formation and mineral. There is still no clear evidence to elucidate all subunits types, interaction intensity and their new biological functions. In this thesis, pig pancreas ferritin (PPF) was used as experimental materials, we optimized ferritin separation condition and prepared high purity PPF in small quantities. SDS-PAGE gel was selected to analysis subunits of ferritin, they were H (21k Da) and L (19 k Da) subunits, SH (small H subunit) was also found in protein shell. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/TOF MS) directly analyzed unstable H and SH subunits in PPF. The bond between L-L, H-L, L-SH and H-SH subunits are stronger. Reversed-phase high performance liquid chromatography (RP-HPLC) effectively separated H, L and SH subunits of PPF, the ratio between L/H was roughly 2:1. MALDI-TOF MS measured the molecular weights of H and SH subunits, which were 20135.015 Da and 12925.221 Da respectively. Increased laser intensity, mixed matrices and acidity of matrix assisted MALDI-TOF MS to obtain four PPF spectrum peaks, the m/z values were 9981.82, 10649.16, 19960.71 and 20835.96 Da. The corresponding ions are $[\text{M}^{2+}]_{\text{L}}$, $[\text{M}^{2+}]_{\text{H}}$, $[\text{M}^+]_{\text{L}}$ and $[\text{M}^+]_{\text{H}}$. However, no spectrum of SH subunit was obtained in this analysis. The results reveal that interactions stability among subunits is associated with composition of protein shell.

Electrospray ionization quadruple time of flight mass spectrometry (ESI-Q-TOF MS) and RP-HPLC-ESI-Q-TOF MS identified types of PPF subunits, their interaction intensities and calculated their homological information. Proteolytic digestion results infer that PPF is mainly composed with H and L subunits, which share 85% and 100% homology with *Sus Scrofa*. Moreover, partial H subunits of PPF were unstable and

they were readily ionized and detected by ESI-Q-TOF. Tetraethylammonium (TEA) and 4-amino pyridine(4-AP)are blockers of potassium ion current. TEA could modify H subunits directly and formed PPF_H-TEA complex, no PPF_L-TEA was found in L subunit. Strong chemical reducing reagent Na₂S₂O₄ and biological reducing reagent Vc were selected to study iron release mechanism of PPF. The results proved that the entire process of iron release contained fast-release iron (first-order reaction kinetics) and slow-release iron (zero-order reaction kinetics). The reaction kinetics was similar as PPF_H-TEA complex.

TEA and 4-AP are used to block potassium channel of tumor and cancer cells to inhibit their proliferation. There are strong evidence indicated that TEA, paclitaxel and 4-AP can reduce tumor cell viability. However, there is little report about the combined molecular mechanism of TEA and 4-AP inhibiting tumor cells growth or inducing apoptosis. MTT assay, PI staining and Annexin V-FITC/PI dual-staining flow cytometry analysis indicated these three inhibitors synergetic enhanced apoptosis rate of Hela cell, but the major mechanism of TEA-induced cell death was necrosis. In whole-cell mode, cell patch clamp measured the potassium current of Hela cell, the current could be inhibited up to 80% in TEA treatment group. MALDI-TOF MS proved that TEA inducement changed polypeptides distribution and composition on surface of cell membrane, it might due to different polypeptides and proteins expression. Differential proteins expressed under cisplatin-human serum transferritin induced HepG2 cell were circled out as target proteins, primers were designed as these selected differential proteins, mRNA assay was carried to study expression tendency under TEA and 4-AP exposure. The results indicated all 26 proteins had different expression levels. They suggest that, like other antitumor drugs, TEA and 4-AP share the similar or same pathways and mechanisms of tumor cells apoptotic inducement. Comparative proteomic technology was first applied to select and identify differential proteins expressed under TEA exposure, 33 significant changed proteins was identify by MALDI-TOF MS (13 were up-regulated and 20 were down-regulated). They are involved in ATP binding, molecular chaperon, enzyme activity, K/Ca ion binding, cell transportation, redox homeostasis and metabolism.

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