PO Box 2345 Beijing 100023, China
Fax: +86-10-85381893
Email: wcjd@wjgnet.com www.wjgnet.com

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• 基础研究 BASIC RESEARCH •

ALA-PDT 诱导 SW480 细胞凋亡和胞内 Ca^{2+} 浓度变化的关系

郑江华, 时 德, 陈祖林

郑江华, 时德, 重庆医科大学附属第一医院血管外科 重庆市 400016
陈祖林, 第三军医大学新桥医院普外科 重庆市 400037
郑江华, 男, 1975-12-23生, 四川省广安市人, 汉族, 博士研究生, 主要从事血管外科及肿瘤综合治疗的临床与基础研究.
国家自然科学基金资助项目, No. 30271481
通讯作者: 陈祖林, 400037, 重庆市, 重庆市第三军医大学新桥医院普外科. chenzulin@163.com
电话: 023-68774405 传真: 023-68774605
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Relationship between intracellular Ca^{2+} and ALA-PDT induced apoptosis in SW480 cells

Jiang-Hua Zheng, De Shi, Zu-Lin Chen

Jiang-Hua Zheng, De Shi, Department of Vascular Surgery, the First Affiliated Hospital of Chongqing University of Medical Sciences, Chongqing 400016, China
Zu-Lin Chen, Department of General Surgery, Xinqiao Hospital, the Third Military Medical University, Chongqing 400037, China
Supported by National Natural Science Foundation of China, No. 30271481
Correspondence to: Dr. Zu-Lin Chen, Department of General Surgery, Xinqiao Hospital, the Third Military Medical University, Chongqing 400037, China. chenzulin@163.com
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Abstract

AIM: To investigate relationship between intracellular Ca^{2+} and apoptosis induced by aminolaevulinic acid-photodynamic therapy (ALA-PDT) in SW480 cells.

METHODS: SW480 cells were divided into control, light, ALA and ALA-PDT group. The corresponding treatment was performed in each group. The apoptosis of SW480 cells was detected by DNA fragment assay and TUNEL assay. The changes of intracelluar Ca^{2+} concentration in each group were observed by confocal laser scanning microscopy.

RESULTS: DNA ladder formation of apoptotic features was demonstrated 1 and 2 h after ALA-PDT treatment. The apoptosis index (AI) of 30 and 60 min after ALA-PDT treatment was 25.26 ± 5.04% and 50.45 ± 7.85%, respectively, which were significantly higher than those in the other 3 groups (all AI <10%, P <0.01). The concentration of intracelluar Ca^{2+} 20 min after ALA-PDT treatment was markedly increased than that at 10 min (fluorescent intensity: 185.40 ± 18.90 *vs* 100.00 ± 19.83, P <0.01), and then gradually decreased. However, the concentrations of intracellular Ca^{2+} had no significant changes in the other 3 groups.

CONCLUSION: The increases of intracellular Ca^{2+} may play an important role in the ALA-PDT-induced apoptosis in SW480 cells.

Key Words: Aminolaevulinic acid; Photodynamic therapy; SW480 cell; Intracellular Ca^{2+}; Apoptosis

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摘要

目的: 探讨ALA-PDT诱导人结肠癌细胞SW480凋亡和游离钙浓度变化关系.

方法: 将SW480细胞分为四组: 空白对照组、激光照射照组、ALA组和ALA-PDT组, 用DNA片段分析和TUNEL法检测细胞凋亡; 用激光共聚焦显微镜观测各组细胞内游离钙离子浓度的变化.

结果: ALA-PDT组的人结肠癌细胞在1、2 h有大量的DNA片段, TUNEL法显示ALA-PDT组的人结肠癌细胞在PDT后30 min AI为25.26±5.04%, PDT后60 min AI为50.45%±7.85%, 均高于其他3组 (AI均<10%, P <0.01); 激光共聚焦结果为ALA-PDT组细胞内游离钙离子浓度在20 min达高峰 (荧光强度: 185.40±18.90), 与10 min (荧光强度: 100.00±19.83) 相比有显著差异 (P <0.01), 之后又逐渐下降.

结论: 细胞内Ca^{2+}浓度的逐渐增加在PDT诱导的细胞凋亡过程中可能起着重要作用.

关键词: δ氨基酮戊酸; 光动力疗法; SW480细胞; 细胞内游离钙; 凋亡

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0 引言

δ氨基酮戊酸 (aminolaevulinic acid, ALA)-光动力疗法 (photodynamic therapy, PDT) 是指ALA诱导肿瘤细胞产生原卟啉Ⅸ (protoporphyrin,Pp Ⅸ), Pp Ⅸ 接受光照后在细胞内产生活性氧物质, 而导致肿瘤细胞凋亡或坏死的一种治疗方法,又称为内源性光动力疗法[1]. ALA代谢快, 毒副作用小, 是一种很有前途的治疗肿瘤的方法[2]. 细胞分子生物学的发展促进了PDT诱导细胞死亡机制的研究, 1991年, Agarwal *et al* 首次证实了PDT能通过诱导细胞凋亡导致小鼠L5178细胞死亡, 自此, PDT诱导许多不同细胞系细胞发生凋亡等到了证实[3-6]. 我们探讨ALA-PDT诱导SW480发生细胞凋亡期间细胞内Ca^{2+}浓度变化, 分析其作用机制.

1 材料和方法

1.1 材料 ALA(Sigma公司); 培养液RPMI1640(Hyclone公司); D-Hanks(Hyclone公司); Hepes(Sigma公司); 胰蛋白酶(北京鼎国生物技术公司);小牛血清 (成都哈里公司); TUNEL试剂盒 (Roche公司); Fluo-3/AM(Sigma公司); SW480(第四军医大学动物实验中心提供).

1.2 方法

1.2.1 PDT处理[7] 用含有青霉素和链霉素 (分别100 kU/L) 和100 mL/L小牛血清的RPM1640培养基培养人结肠癌细胞 (SW480), 37 ℃, 50 mL/L CO_2, 饱和湿度, 单层贴壁生长. 实验分为4组: 空白对照组, 激光照射组, ALA组及ALA-PDT组. 光敏剂ALA用PBS配制成400 mg/L的储存液, 过滤待用, 工作液用无血清培养基稀释为40 mg/L (终浓度). ALA组和ALA-PDT处理组在暗室按预定时间加入ALA, 严格避光条件下继续孵育4 h, 激光照射组和ALA-PDT处理组使用半导体激光仪 (西南师范大学激光所, 波长532 nm, 距光斑3 cm处输出功率12 mw)垂直照射培养板30 min. 空白对照组不加ALA且不受激光照射, ALA组加入ALA不受激光照射.

1.2.2 DNA片断分析 SW480细胞 (2-6×10^9/L)经ALA-PDT作用0、1、2 h后收获细胞, PBS(pH7.4) 洗2遍, 沉淀加入50 μL细胞裂解液 (10 mmol/L Tris-HCl, 10 mmol/L NaCl, 10 mmol/L EDTA, 10 g/L SDS pH8.0) 和终浓度为0.5 g/L的蛋白酶K, 50 ℃水浴2 h, 5 000 r/min离心10 min, 上清移入新Eppendorf管, 以等体积的苯酚/氯仿(1∶1)、苯酚/氯仿/异丙醇 (25∶24∶1) 和氯仿各抽提1次, 取上层水相加1/10体积3 mol/L醋酸钠(NaAc)、2倍体积冷无水乙醇, 颠倒混匀, 置 -20 ℃ 30 min, 13 000 *g*离心10 min, 烘干乙醇和NaAc, 沉淀加20 μL TE缓冲液 (50 mmol/L Tris-HCl, 10 mmol/L EDTA)溶解, 加RNase A(200 mg/L)于37 ℃ 1 h, 取20 μL样品加6×上样缓冲液4 μL混合上样, 另取一道加入DNA Marker, 15 g/L琼脂糖凝胶电泳 (凝胶含0.5 mg/L EB), 紫外灯下观察并摄影.

1.2.3 末端标记法(TUNEL)检测细胞凋亡[8] 采用盖玻片培养法, 取对数生长细胞1×10^8/L, 接种到含有高压灭菌盖玻片 (22 mm×22 mm, 100 g/L多聚赖氨酸处理)的6孔培养板中, 48 h后行ALA-PDT处理, 分别在PDT后30 min和60 min后, 取出盖玻片, 用PBS冲洗3次, 16 g/L多聚甲醛固定30 min, 其余步骤按试剂盒说明进行,光镜下分别记数明显的5个高倍视野内的凋亡细胞数和细胞总数, 计算凋亡指数 (apoptosis index, AI). AI=凋亡细胞数/细胞总数×100%.

1.2.4 激光共聚焦显微镜动态观察[9] 取对数生长细胞1×10^8/L, 接种到含有高压灭菌盖玻片 (22 mm×22 mm, 100 g/L多聚赖氨酸处理) 的6孔培养板中, 孵育48 h后, 用D-Hanks液漂洗3次, PDT处理后, 各组细胞均用 D-Hanks液漂洗三次, 用终浓度4.4 μm/L的Fluo-3/AM溶液于37 ℃避光条件下染色30 min, D-Hanks液漂洗3次, 最后再用D-Hanks液酯化15 min, 激光照射后, 将盖玻片置于激光共聚焦显微镜载物台上, 选择激发波长488 nm、发射波长526 nm, 动态观察各组细胞的荧光强度, 每5 min扫描1次, 共60 min. 因为Fluo-3/AM与细胞内游离钙离子结合显示荧光, 其荧光强度与所结合的Fluo-3/AM细胞内游离钙离子浓度成正比, 所以测得的荧光强度可作为反映细胞内游离钙离子浓度的指标.

统计学处理 数据以mean±SD表示, 显著性检验采用χ^2检验, 采用SPSS10.0统计软件进行数据处理.

2 结果

2.1 DNA片断分析 SW480细胞经ALA-PDT分别处理1、2 h后可见明显的DNA梯形条带 (DNA ladder), PDT即刻、激光照射组、空白对照组以及ALA组未见DNA梯形条带 (图1).

2.2 TUNEL分析 光镜下阳性细胞明显皱缩, 呈圆形, 核

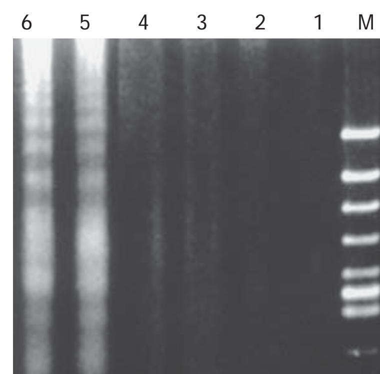

图 1 DNA片断分析. M: Maker; 1: 空白对照组; 2: 激光照射组; 3: ALA组; 4: ALA-PDT后即刻; 5: ALA-PDT后1 h; 6: ALA-PDT后2 h.

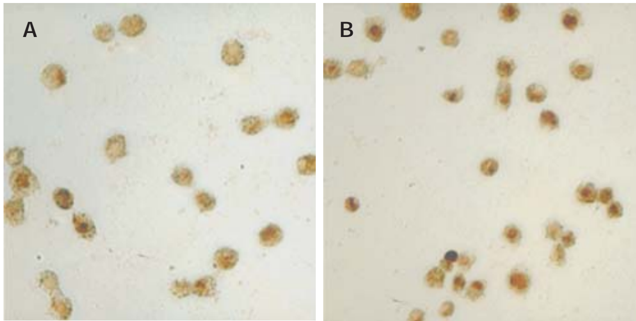

图 2 ALA-PDT诱导SW480细胞凋亡分析. A: 30 min; B: 60 min.

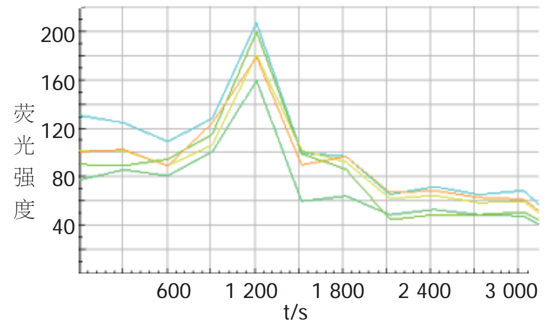

图 3 SW480细胞内游离钙浓度变化曲线图.

染色质致密, 染成棕色. ALA-PDT后细胞有明显凋亡, PDT后30 min AI为25.26+5.04%, PDT后60 min AI为50.45+7.85%, 其余各组, 包括激光照射组, 空白对照组以及ALA组AI均<10%, 有显著差异 ($P<0.01$, 图2).

2.3 激光共聚焦显微镜动态观察 激光共聚焦显微镜动态观察到ALA-PDT后20 min达高峰, 之后又逐渐下降, 经激光共聚焦显微镜专用数据分析软件 (powerphys) 分析 (图3) 可知PDT后10 min时荧光强度为100.00+19.83, 而20 min时达185.40+18.90 ($P<0.01$). 而空白对照组、单独激光照射组、ALA组细胞内钙无明显变化 (图4).

3 讨论

PDT作用过程中, 光敏剂特异聚集在肿瘤细胞或组织中, 在适当波长 (通常是红光或近紫外光或与光敏剂的吸光谱相近的光) 的光照下, 光敏剂吸收特定波长的光后, 转变为激发三重态光敏剂, 此三重态光敏剂可经Ⅰ或Ⅱ型反应型反应, 产生光敏剂的自由基离子或单线态氧, 自由基离子进一步与周围的氧反应生成氧化产物; 单线态氧是一种高反应性物质, 能与脂肪酸、蛋白质及核酸等物质反应而产生损伤效应, 最终导致细胞凋亡或死亡[10]. 由于PDT处理的细胞可见膜去极化[11-13], 细胞内K^+外流, Na^+-K^+依赖式ATP酶抑制[14], 因此PDT诱导细胞凋亡主要靶点可能细胞膜[15-18]. 有作者认为位于细胞膜上的磷脂酶A_2 (PLA_2) 和磷脂酶C(PLC) 的激活是PDT诱导细胞凋亡的早期机制, 理由是Ca^{2+}作为细胞信号的第二信使受大量刺激因素的影响, 并与PLA_2和PLC激活有关[19]. 激活的PLC水解磷

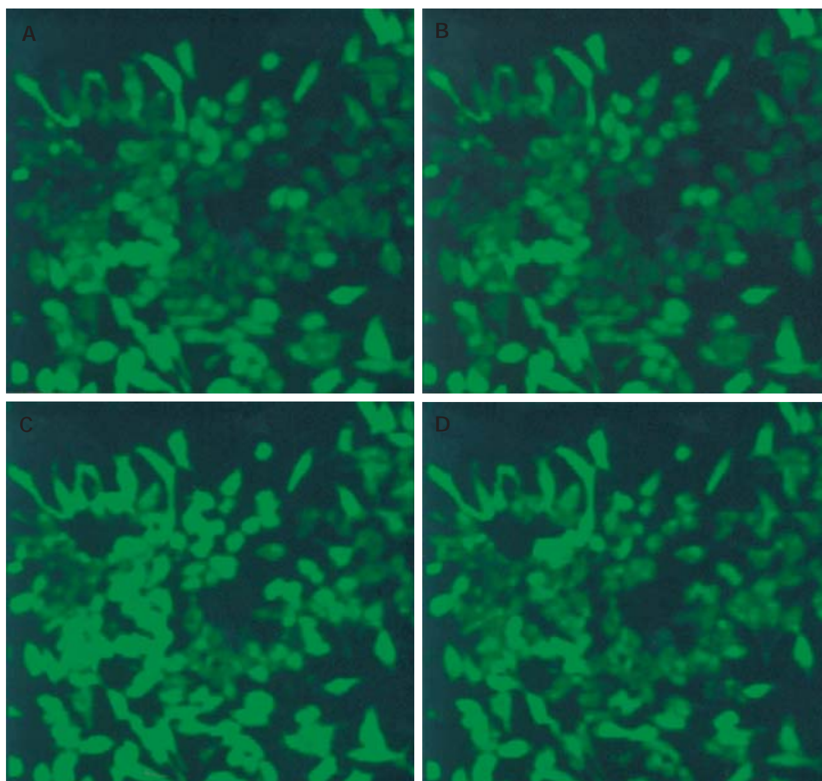

图 4 PDT后SW480细胞的激光共聚焦显微镜扫描图. A: 10 min; B: 15 min; C: 20 min; D: 25 min.

脂酰肌醇-4,5-二磷酸 (PIP_2) 产生三磷酸肌醇 (IP_3) 和二酰甘油 (DG), IP_3促进细胞内Ca^{2+}浓度增加, DG激活蛋白酶C(PKC), 从而分别形成IP_3/Ca^{2+}和DG/PKC信号传递途径[20-22]. 在IP_3/Ca^{2+}途径中, Ca^{2+}与钙调蛋白 (CaM) 结合激活calcineurin(CN), 由CN将磷酸化的NF-AT(nuclear factor of activated T cell) 脱磷酸[23], NF-AT因而进入核内, 并与转录因子AP-1(activator protein-1)结合, 从而诱导IL-2基因转录[24-26]. 因此, 本实验的目的是探讨PDT后细胞早期凋亡和细胞内钙浓度的关系.

在我们的实验中, DNA断裂片段出现在PDT后1-2 h, 凋亡形态学 (TUNEL) 结果显示: PDT后20 min AI为25.26+5.04%, PDT后40 min AI为50.45+7.85%, 在其他细胞系, PDT后细胞早期也发生凋亡, Kuzalova *et al* [27]用 ALA-PDT 处理K562细胞, 发现2 h后细胞出现凋亡特征. 另外, 激光共聚焦显微镜动态观察结果表明PDT后10 min时荧光强度为100.00+19.83, 之后逐渐增加, 20 min时达185.40+18.90, 之后又逐渐下降, 30 min后继续降至基线水平以下, 而空白对照组、激光照射组以及ALA组细胞内钙无明显变化, 在实验过程中, 采用D-hanks液平衡SW480细胞, 避开细胞外钙离子内流途径, 说明ALA-PDT对SW480细胞内的钙库有促进其开放的作用, 从而引起细胞内钙离子浓度升高; 30 min后游离钙继续降至基线水平以下可能是由于ALA-PDT后细胞质膜系统受到破坏, 细胞内钙离子流向细胞外造成的. Tajiri *et al* [28]用卟菲尔钠 (porfimer sodium)-PDT处理人鳞状细胞癌HSC-2, 发现细胞内钙离子在PDT后2 h达高峰. Zhou *et al* [29]用竹红菌素A-PDT处理人胃癌细胞MGC803, 结果表明细胞内钙离子浓度在PDT后60 s达高峰. 造成实验结果不一致的原因可能是由于光敏剂、细胞类型、光照剂量以及观察时间不同[30]. PDT后20 min, 细胞出现凋亡, 而PDT后10-20 min细胞内Ca^{2+}浓度逐渐增加, 20 min达高峰, 提示了细胞内Ca^{2+}浓度的逐渐增加在PDT诱导的细胞凋亡过程中可能起重要作用, 达高峰时立即引起 PDT诱导细胞凋亡. 为了证实ALA-PDT诱导SW480细胞凋亡中是否存在IP_3/Ca^{2+}信号传递途径, 有必要对CaM, CN, NF-AT以及AP-1等蛋白表达进一步研究.

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•消息•

首届北京地坛感染病学术会议

本刊讯 为庆祝建院60周年, 北京地坛医院决定于2006-03-02/04在北京召开全国性的"首届北京地坛感染病学术会议", 预计全国的同行500人参加这次重要的会议. 会议邀请了40余位我国德高望重、年富力强的感染病专家作专题学术讲演.

1 会议征稿内容

这次感染病学术会议征文的内容包括病毒性肝炎、HIV/AIDS、各种传染性疾病和感染性疾病, 抗生素的合理使用, 也包括新发/复燃的传染病. 论文全文和摘要请发到电子信箱: cj@genetherapy.com.cn; 或hy@genetherapy.com.cn.

2 与会专家名单

首届北京地坛感染病学术会议邀请的专题报告专家(按照汉语拼音排序)有: 白雪帆, 陈智, 陈志海, 成军, 段钟平, 窦晓光, 范小玲, 高志良, 郭利民, 侯金林, 贾继东, 郎振为, 李长青, 李兰娟, 李太生, 李兴旺, 刘沛, 刘庄, 伦文辉, 毛羽, 缪晓辉, 穆毅, 宁琴, 牛俊奇, 任红, 施光峰, 斯崇文, 谭德明, 唐红, 唐小平, 万谟彬, 王凤水, 王福生, 王贵强, 王磊, 王玲, 王宇明, 王宪波, 魏红山, 魏来, 翁心华, 谢青, 谢雯, 谢尧, 邢卉春, 徐道振, 杨东亮, 杨钧, 袁正宏, 赵红心, 庄辉

3 联系方式

首届北京地坛感染病学术会议组织委员会主席为成军教授. 地址: 北京市东城区安外大街地坛公园13号, 邮编: 100011; 电话: 010-64481639; 传真: 010-64481639. Email: cj@genetherapy.com.cn

欢迎全国感染病学界的各位专家和同仁来北京参加这次盛会.