

· 基础研究 ·

阿托伐他汀诱导的MIN6细胞铁死亡及相关机制研究

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[摘要] 目的: 探讨阿托伐他汀(atorvastatin, Ator)是否可诱导小鼠胰岛 β 细胞株MIN6细胞发生铁死亡, 并探讨其可能的作用机制。方法: 将MIN6细胞分为对照组、Ator组、Ator+凋亡抑制剂(Z-VAD-FMK)组、Ator+坏死抑制剂(necrostatin-1, Nec-1)组和Ator+铁死亡抑制剂(ferrostatin-1, Fer-1)组。采用CCK-8法检测细胞活力; 透射电镜观察细胞超微结构; 荧光显微镜观察活性氧(reactive oxygen species, ROS)和 Fe^{2+} 水平; 酶联免疫吸附试验(enzyme-linked immuno sorbent assay, ELISA)检测丙二醛(malondialdehyde, MDA)和还原型谷胱甘肽(glutathione, GSH)含量; 实时荧光定量PCR法(quantitative real-time PCR, RT-qPCR)检测凋亡基因半胱氨酸蛋白酶3(caspase-3)、坏死基因受体结合丝氨酸苏氨酸激酶3(receptor-interacting serine threonine kinase 3, Ripk3)、铁死亡相关基因长链酯酰辅酶A合成酶4(acyl-coA synthetase long-chain family member 4, Acsl4)、前列腺素内过氧化物合酶2(prostaglandin-endoperoxide synthase 2, Ptgs2)和谷胱甘肽过氧化物酶4(glutathione peroxidase 4, Gpx4)的mRNA表达水平; Western blot检测4-羟基壬烯醛(4-hydroxynonenal, 4-HNE)和GPX4的蛋白表达水平。结果: 与Ator组相比, Ator+Z-VAD-FMK组和Ator+Fer-1组细胞存活率更高($P < 0.01$)。透射电镜下Ator组细胞可见凋亡、铁死亡和自噬相关的形态学特征。与对照组相比, Ator组细胞 Fe^{2+} 相对荧光强度、MDA水平和ROS相对水平均升高, GSH含量下降; caspase-3、Acsl4、Ptgs2的mRNA及4-HNE的蛋白表达增加($P < 0.05$), GPX4的mRNA和蛋白表达减少($P < 0.05$)。与Ator组相比, Ator+Fer-1组 Fe^{2+} 相对荧光强度、MDA水平和ROS相对水平均下降, GSH含量上升; Acsl4的mRNA表达减少, Gpx4的mRNA表达增加($P < 0.05$); 4-HNE的蛋白表达减少而GPX4的蛋白表达增加, 但差异无统计学意义。结论: Ator可能通过抑制甲羟戊酸途径下调GPX4表达, 诱导MIN6细胞发生铁死亡。

[关键词] 阿托伐他汀; 铁死亡; 新发糖尿病; MIN6细胞**[中图分类号]** R587.1**[文献标志码]** A**[文章编号]** 1007-4368(2024)08-1044-07**doi:** 10.7655/NYDXBNSN240215

Study of atorvastatin induced ferroptosis in MIN6 cells and its related mechanisms

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[Abstract] **Objective:** To explore whether atorvastatin (Ator) can induce ferroptosis in pancreatic β -cell line MIN6 cells and its possible mechanism. **Methods:** MIN6 cells were divided into control group, Ator group, Ator+apoptosis inhibitor (Z-VAD-FMK) group, Ator+necrostatin-1 (Nec-1) group and Ator+ferrostatin-1 (Fer-1) group. Cell viability was detected by cell counting kit-8 (CCK-8) method. The ultrastructure of cells was observed by transmission electron microscopy. The levels of reactive oxygen species (ROS) and Fe^{2+} were observed by fluorescence microscopy. The contents of malondialdehyde (MDA) and glutathione (GSH) were detected by enzyme-linked immunosorbent assay (ELISA) method. Real-time quantitative PCR was used to detect the mRNA levels of caspase-3, receptor-interacting protein kinase 3 (Ripk3), acyl-CoA synthetase long-chain family member 4 (Acsl4), prostaglandin endoperoxidase synthase 2 (Ptgs2) and glutathione peroxidase 4 (Gpx4). Western blot was used to detect the proteins levels of 4-hydroxynonenal (4-HNE) and GPX4. **Results:** Compared with the Ator group, cell viability of MIN6 was higher in Ator+Z-VAD-FMK group and Ator+Fer-1 group ($P < 0.01$). MIN6 cells, which were treated with Ator, exhibited the characteristic morphologic features associated with apoptosis, ferroptosis and autophagy under transmission electron microscopy. Compared with the control group, the levels of the intracellular Fe^{2+} , MDA and ROS in the Ator group were increased and GSH was decreased. The mRNA relative expression levels of

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caspase-3, Acsl4 and Ptg2 were increased, as well as the protein relative expression level of 4-HNE (all $P < 0.05$). The mRNA and protein relative expression levels of GPX4 were decreased ($P < 0.05$). Compared with the Ator group, the levels of the intracellular Fe^{2+} , MDA and ROS in the Ator+Fer-1 group were decreased and GSH was increased. The mRNA relative expression level of Acsl4 was decreased and Gpx4 was increased (all $P < 0.05$). The protein relative expression levels of 4-HNE was decreased and GPX4 was increased, though the changes were not statistically significant. **Conclusion:** Atorvastatin may induce ferroptosis in MIN6 cells by down-regulating GPX4 expression through inhibiting mevalonate pathway.

[Key words] atorvastatin; ferroptosis; new-onset diabetes mellitus; MIN6 cell

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他汀类药物是3-羟基-3-甲基戊二酰辅酶A还原酶抑制剂,通过抑制甲羟戊酸通路的限速酶降低胆固醇合成,被广泛用于预防心血管疾病^[1-2]。尽管他汀类药物可有效降低心脑血管事件发生率,但越来越多的证据表明,他汀类药物的使用可使新发糖尿病(new-onset diabetes mellitus, NODM)的风险增加10%~12%^[3]。他汀类药物引起NODM的可能机制包括:诱导胰岛 β 细胞发生凋亡、坏死及自噬^[4-6],减少胰岛素分泌,增加胰岛素抵抗以及干扰外周组织摄取葡萄糖等^[7],但其确切机制仍需进一步研究。

铁死亡是一种由铁依赖的脂质过氧化物过度积累导致的新程序性细胞死亡^[8]。已有研究证实他汀类药物可以诱导肿瘤细胞、肝星状细胞(hepatic stellate cell, HSC)、脂肪细胞及肌细胞发生铁死亡^[9-12]。本课题组前期研究发现棕榈酸可诱导MIN6细胞发生铁死亡^[13]。然而,他汀类药物能否会诱导胰岛 β 细胞发生铁死亡,参与NODM的发生尚未见报道。

1 材料和方法

1.1 材料

小鼠胰岛 β 细胞MIN6细胞由本实验室保存。DMEM高糖培养基、胎牛血清、青霉素-链霉素、 β -巯基乙醇、胰酶(Gibco公司,美国);阿托伐他汀(atorvastatin, Ator)、铁死亡抑制剂(ferrostatin-1, Fer-1)、凋亡抑制剂(Z-VAD-FMK)、坏死抑制剂(necrostatin-1, Nec-1)、CCK-8试剂盒(MCE公司,美国);活性氧(reactive oxygen species, ROS)荧光探针、丙二醛(malondialdehyde, MDA)试剂盒、还原型谷胱甘肽(glutathione, GSH)检测试剂盒、蛋白裂解液、蛋白酶抑制剂PMSF、BCA蛋白浓度测定试剂盒、辣根过氧化物酶标记山羊抗小鼠二抗、辣根过氧化物酶标记山羊抗兔二抗(上海碧云天公司); Fe^{2+} 荧光探针(FerroOrange)(Dojindo公司,日本);

TRIZol试剂盒(TaKaRa公司,日本);逆转录试剂盒(HIScript III All-in-one RT SuperMix试剂盒)、荧光定量PCR试剂盒(南京诺唯赞公司); β 肌动蛋白(β -actin)单克隆抗体(武汉Proteintech公司);4-羟基壬烯醛(4-hydroxynonenal, 4-HNE)单克隆抗体(Thermo Scientific公司,美国);谷胱甘肽过氧化物酶4(glutathione peroxidase 4, GPX4)单克隆抗体(武汉AB clonal公司);二甲基亚砜(dimethyl sulfoxide, DMSO)(Sigma公司,美国)。

1.2 方法

1.2.1 细胞培养与分组

将细胞置于含15%胎牛血清、1%青霉素-链霉素和0.1% β -巯基乙醇的高糖培养基中,在37℃、5% CO_2 的恒温培养箱中培养,待细胞密度达70%~80%时进行后续操作。将MIN6细胞分为对照组(control)、Ator组(Ator 25 μ mol/L)、Ator+Fer-1组(Ator 25 μ mol/L+Fer-1 5 μ mol/L)、Ator+Z-VAD-FMK组(Ator 25 μ mol/L+Z-VAD-FMK 10 μ mol/L)、Ator+Nec-1组(Ator 25 μ mol/L+Nec-1 10 μ mol/L),干预细胞48 h。

1.2.2 CCK-8法检测细胞活力

将细胞以 2×10^4 个/孔接种至96孔板,按上述实验分组分别干预48 h后,按照CCK-8检测试剂盒操作说明,每孔加入10 μ L CCK-8溶液,37℃避光孵育2 h。酶标仪测定每孔450 nm处的吸光度并计算细胞存活率。

1.2.3 透射电子显微镜观察细胞超微结构

将细胞以 1.3×10^6 个/孔接种至6孔板,按上述实验分组干预48 h收集细胞,加入2.5%戊二醛4℃固定过夜,1%锇酸固定细胞2 h,乙醇梯度脱水后环氧树脂包埋、超薄切片,3%醋酸铀、柠檬酸铅双重染色,漂洗后透射电镜观察。

1.2.4 ROS测定

将细胞以 1.3×10^6 个/孔接种至6孔板,按上述实

验分组干预48 h后,弃上清。按1:1 000稀释DCFH-DA荧光探针,每孔加入1 mL稀释液,37 °C避光孵育30 min。PBS洗涤3次后,使用荧光显微镜进行观察拍照。用Image J软件进行荧光强度分析。

1.2.5 MDA测定

将细胞以 1.3×10^6 个/孔接种至6孔板,按上述实验分组干预48 h。随后收集细胞,按照MDA检测试剂盒的说明,使用酶标仪测定每孔532 nm处的吸光度,根据标准曲线计算MDA水平。

1.2.6 GSH测定

按说明书配制所需试剂,收集细胞上清,去除内源性GSH;混匀反应试剂,室温反应5 min,再加入50 μ L 0.5 mg/mL NADPH溶液,室温反应25 min,用酶标仪测定每孔412 nm处的吸光度。单点法绘制标准曲线并计算出GSH含量。

1.2.7 细胞内 Fe^{2+} 检测

将细胞以 1.3×10^6 个/孔接种至6孔板,按上述实验分组干预48 h。去除培养液用PBS洗涤后,每孔加入1 μ mol/L的FerroOrange工作液1 mL,37 °C避光孵育30 min,使用荧光显微镜获得荧光图像。用Image J软件进行荧光强度分析。

1.2.8 实时荧光定量PCR法检测目的基因mRNA的表达水平

应用TRIzol试剂盒提取MIN6细胞中的总RNA,采用HIScript III All-in-one RT SuperMix试剂盒将RNA逆转录为cDNA。通过荧光定量PCR系统和SYBR Green试剂盒进行qRT-PCR。反应条件:95 °C预变性5 min,95 °C 10 s,退火延伸60 °C 30 s,40个循环。 β -actin作为内参基因,通过 $2^{-\Delta\Delta CT}$ 法计算基因的相对表达量。相关引物序列如下: β -actin(Mouse)上游5'-GGCTGTATTCCCCTCCATCG-3',下游5'-CCAGTTGGTAACAATGCCATGT-3'; caspase-3(Mouse)上游5'-TGACTGGAAAGCCGAAACTC-3',下游5'-AGCCTCCACCGGTATCTTCT-3'; Ripk3(Mouse)上游5'-CAGTGGGACTTCGTGTCCG-3',下游5'-CAAGCTGTGTAGGTAGCACATC-3'; PtgS2(Mouse)上游5'-GGGAGTCTGGAACATTGTGAA-3',下游5'-GTGCACATTGTAAGTAGGTGGA-3'; Acs14(Mouse)上游5'-CTCACCATTATATTGCTGCCTGT-3',下游5'-TCTCTTTGCCATAGCGTTTTTCT-3'; Gpx4(Mouse)上游5'-GCCTGGATAAGTACAGGG-GTT-3',下游5'-CATGCAGATCGACTAGCTGAG-3'。

1.2.9 Western blot检测蛋白的相对表达量

各组干预结束后收集细胞,使用蛋白裂解液和

PMSF提取总蛋白,BCA法测定蛋白浓度后进行定量、变性处理。随后进行电泳、转膜、封闭, β -actin(1:20 000)、4-HNE(1:1 000)、GPX4(1:1 000)一抗孵育过夜,次日室温孵育二抗(1:1 000)1.5 h后ECL显影。使用Image J软件进行灰度分析。

1.3 统计学方法

采用Graph Pad Prism 9.0软件进行数据分析及作图。符合正态分布的计量资料以均数 \pm 标准差($\bar{x} \pm s$)表示,进行方差齐性检验,显示方差齐,两组间比较采用独立样本 t 检验,多组间比较采用单因素方差分析,两两比较采用Tukey's检验, $P < 0.05$ 为差异有统计学意义。

2 结果

2.1 Ator抑制MIN6细胞增殖

预实验结果显示Ator以时间和剂量依赖性方式抑制MIN6细胞的增殖。当Ator浓度为25 μ mol/L,作用48 h时,细胞存活率为 $(68.30 \pm 0.53)\%$ ($P < 0.01$,图1)。与Ator组相比,Ator+Z-VAD-FMK组细胞存活率 $(78.89 \pm 1.09)\%$ 和Ator+Fer-1组细胞存活率 $(82.06 \pm 1.87)\%$ 显著提高($P < 0.01$,图1)。

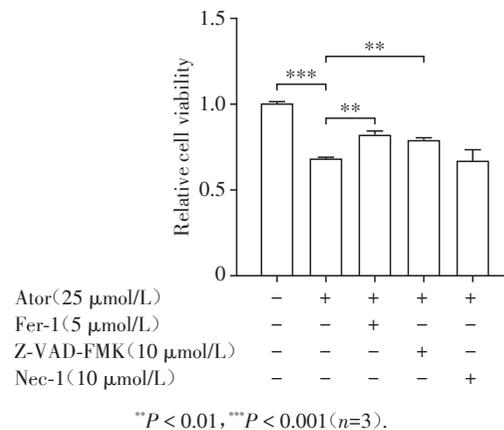


图1 Ator对MIN6细胞存活率的影响

Figure 1 Effects of Ator on the cell viability of MIN6 cells

2.2 透射电镜下MIN6细胞超微结构

电镜观察结果如图2所示,对照组MIN6细胞线粒体结构基本正常,未见明显线粒体皱缩和嵴断裂,而Ator组MIN6细胞中出现染色质浓缩的凋亡特征,线粒体肿胀、空化,数量减少,线粒体嵴断裂或减少,双层膜密度增加的铁死亡特征(红色箭头)。细胞膜破裂,可见大量溶酶体,有自噬小体形成(黑色箭头)。

2.3 Ator对MIN6细胞 Fe^{2+} 及脂质过氧化的影响

如图3所示,与对照组相比,Ator组细胞内 Fe^{2+}

相对荧光强度、ROS水平和MDA含量更高($P < 0.01$), 而GSH水平明显降低($P < 0.01$), 补充Fer-1能缓解上述变化($P < 0.05$)。

2.4 Ator对MIN6细胞凋亡、坏死和铁死亡相关基因及蛋白表达的影响

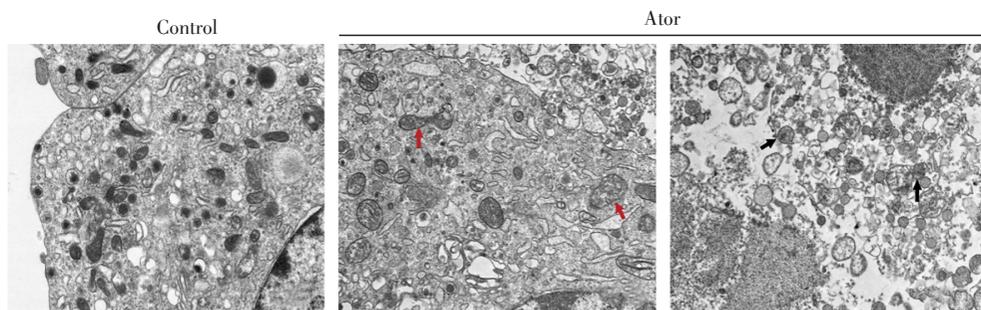
与对照组相比, Ator组MIN6细胞凋亡基因caspase-3表达增加($P < 0.05$, 图4A), 坏死基因Ripk3表达也增加, 但差异无统计学意义; 铁死亡相关基因Ptgs2和Acsl4表达上调, Gpx4表达下调($P < 0.05$, 图4A、B)。与Ator组相比, Ator+Fer-1组Acsl4基因表达下调, Gpx4表达上调($P < 0.05$, 图4B)。Western

blot检测结果显示, 与对照组相比, Ator组4-HNE蛋白表达水平升高, GPX4蛋白表达水平降低($P < 0.05$, 图4C~E); Fer-1干预后, 4-HNE蛋白表达水平降低, GPX4蛋白表达水平升高, 但差异无统计学意义。

3 讨论

本研究以小鼠胰岛β细胞株MIN6细胞为研究模型, 用Ator处理MIN6细胞, 结果显示Ator可诱导胰岛β细胞发生铁死亡。

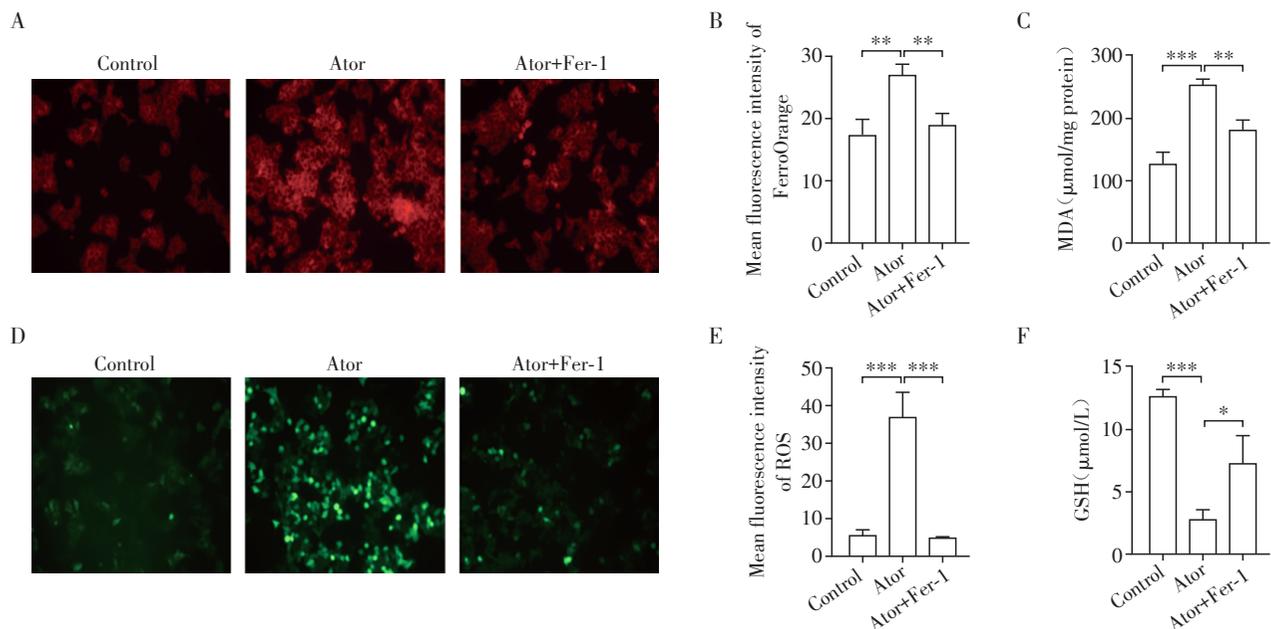
他汀类药物是防治心血管疾病的首选药物, 通过降低低密度脂蛋白和稳定斑块, 延缓动脉粥样硬



In the control group, the mitochondrial structure of MIN6 cells was basically normal, and no obvious mitochondrial shrinkage or ridge breakage was observed. In the Ator group, the red arrow indicated mitochondrial swelling and cavitation, and mitochondrial ridge breakage or reduction accompanied by increased double-layer membrane density. The black arrow represents the autophagosome ($\times 5000$).

图2 透射电镜下MIN6细胞超微结构图

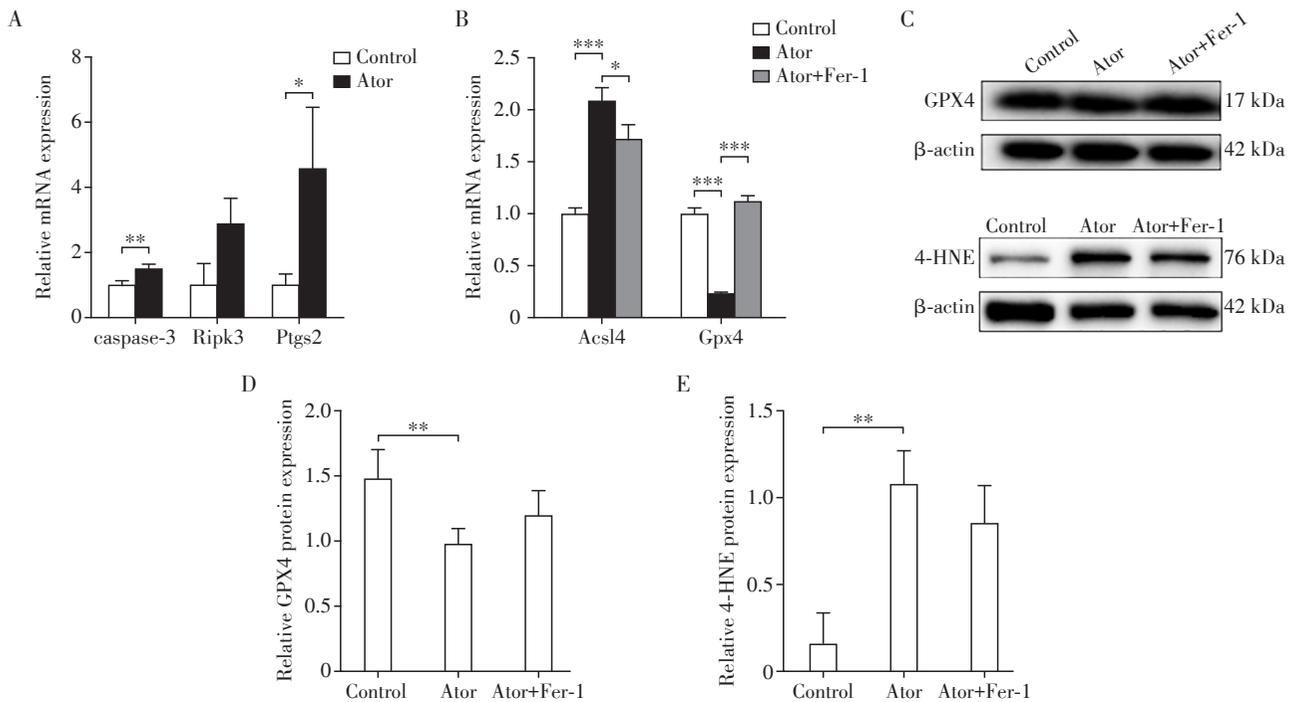
Figure 2 Ultrastructure of MIN6 cells under transmission electron microscopy



A: Fluorescence probe was used to detect Fe^{2+} expression in cells of each group ($\times 200$). B: Fe^{2+} relative fluorescence intensity of each group. C: MDA content in each group. D: Fluorescence probe was used to detect ROS expression in cells of each group ($\times 200$). E: Relative fluorescence intensity of ROS in each group. F: GSH content in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n=3$).

图3 Ator对MIN6细胞 Fe^{2+} 及脂质过氧化物的影响

Figure 3 Effects of Ator on Fe^{2+} and lipid peroxidation in MIN6 cells



A: Relative mRNA expression levels of apoptosis gene caspase-3, necrosis gene Ripk3 and ferroptosis gene Ptgs2 in MIN6 cells. B: Relative mRNA expression levels of ferroptosis related genes Acs14 and Gpx4 in MIN6 cells. C: GPX4 and 4-HNE protein detection. D-E: Relative expression of GPX4(D) and 4-HNE(E) proteins. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($n=3$).

图4 Ator对MIN6细胞铁死亡、凋亡和坏死相关基因及蛋白表达水平的影响

Figure 4 Effects of Ator on expression levels of genes and proteins related to ferroptosis, apoptosis and necrosis in MIN6 cells

化^[14]。近年来,越来越多的证据表明他汀类药物可以剂量依赖性增加NODM风险,但其具体机制尚未明确。体内及体外研究发现,他汀类药物降低胰岛素敏感性和胰岛 β 细胞功能,可能与影响L型 Ca^{2+} 通道、葡萄糖转运蛋白4易位、胰岛素受体底物1和胰岛素受体表达或磷酸化、脂肪细胞成熟或分化、异戊二烯和辅酶 Q_{10} 生物生成以及脂联素和瘦素水平有关^[15]。此外,研究证实,他汀类药物通过削弱胰岛mTOR信号转导减少功能性胰岛 β 细胞的数量^[16]。Sadighara等^[17]研究发现Ator可引起线粒体功能障碍,导致大鼠胰腺细胞凋亡。本课题组前期研究结果表明胰岛 β 细胞可发生铁死亡。Hong等^[18]的研究也发现镉通过引起铁积累和脂质过氧化导致胰岛 β 细胞铁死亡。因此他汀类药物是否会诱导胰岛 β 细胞发生铁死亡引起NODM值得进一步探索。

2012年Dixon等^[19]首次在肿瘤细胞中发现铁死亡,并证实其是一种 Fe^{2+} 依赖性的非凋亡、非坏死的细胞死亡方式。在形态学上,铁死亡不同于凋亡、坏死及自噬,主要表现为线粒体体积缩小,线粒体嵴减少或消失,双层膜密度增加^[20]。本研究观察到

在Ator干预下 β 细胞的线粒体呈现铁死亡电镜特征:线粒体嵴断裂或减少,双层膜密度增加。此外 β 细胞同时出现凋亡和自噬特征,电镜下表现为染色质浓缩,自噬小体形成。因此,本研究发现Ator在诱导 β 细胞凋亡、自噬的同时也能诱导细胞发生铁死亡。

铁死亡重要的生化特征是 Fe^{2+} 积累、脂质过氧化和GSH耗竭。当细胞内 Fe^{2+} 过多时,通过芬顿反应释放出具有极强氧化能力的羟基自由基,进而氧化多不饱和脂肪酸,生成ROS和大量的脂质过氧化物如MDA、4-HNE等毒性物质,破坏细胞结构及稳定性,促进铁死亡发生^[21]。本研究发现在Ator干预下MIN6细胞内 Fe^{2+} 积累、MDA和ROS含量增加及GSH水平降低符合铁死亡生化特征。已知ROS在Ator诱导的氧化应激中起主要作用,导致线粒体损伤、细胞膜氧化损伤及DNA损伤等^[22-23]。本文推测ROS是由Ator通过MIN6细胞中铁过载诱导的芬顿反应产生,而ROS的积累引起脂质过氧化,最终导致细胞发生铁死亡。

铁死亡的主要调控途径包括铁代谢、脂质代谢和谷氨酸/胱氨酸反向转运体(system Xc⁻)/GSH/

GPX4通路。研究发现参与铁结合、转运和储存的蛋白如转铁蛋白(transferrin, Tf)、转铁蛋白受体-1(transferrin receptor-1, TfR1)、核受体辅激活蛋白4(nuclear receptor coactivator 4, NCOA4)等功能紊乱会促进铁死亡发生^[24]。Agmon等^[25]发现ACSL4和溶血磷脂酰胆碱酰基转移酶3(lysophosphatidylcholine acyltransferase3, LPCAT3)是铁死亡中脂肪酸代谢和脂质重塑的关键酶, ACSL4将多不饱和脂肪酸掺入细胞膜中, LPCAT3将酰基插入溶血磷脂中促进铁死亡。研究表明, GPX4是关键的铁死亡防御基因, 编码细胞溶质、线粒体和核仁亚型, 其主要功能是利用GSH作为辅因子来抵抗脂质过氧化, 从而保护膜完整性^[26]。本研究发现Ator可引起MIN6细胞GPX4的基因及蛋白表达减少, Acsl4基因及4-HNE蛋白表达增加, 而加入Fer-1后均有不同程度的改善。以上结果提示Ator可能通过ACSL4和GPX4相关途径调控MIN6细胞内铁死亡过程。

研究表明, 甲羟戊酸途径代谢参与铁死亡, 其直接影响细胞对脂质过氧化和铁死亡的敏感性^[27]。在胆固醇合成途径中, 甲羟戊酸通路产生的异戊二酸焦磷酸酯(isopentenylpyrophosphate, IPP), 可以从多个方面影响铁死亡。IPP作为角鲨烯和辅酶Q₁₀的前体物质, 也是硒代半胱氨酸tRNA酶促异物烯基化的限制底物。使用他汀类药物阻断甲羟戊酸途径会影响硒代半胱氨酸tRNA的成熟和GPX4的合成, 从而使细胞对铁死亡敏感^[28]。体外研究证实Ator通过耗竭GSH并阻断Nrf2-xCT/GPX4通路诱导小鼠肌细胞铁死亡^[12]。Shu等^[11]发现Ator可增加ROS、MDA及Fe²⁺水平并降低GSH和GPX4表达诱导小鼠脂肪外植体铁死亡。Kitsugi等^[10]研究证明辛伐他汀降低HSC细胞活力并抑制细胞活化, 伴有铁积累、氧化应激、脂质过氧化和GPX4蛋白表达降低, 表明辛伐他汀通过促进铁死亡来抑制HSC的活化。本研究同样发现Ator可下调MIN6细胞GPX4表达, 推测他汀类药物可能通过抑制甲羟戊酸途径影响GPX4表达进而诱导胰岛β细胞发生铁死亡, 参与NODM的发生。

本研究存在以下不足: 仅从细胞层面探讨Ator对小鼠胰岛β细胞死亡方式的作用, 未在动物层面进行验证; 其次, 并未涉及GPX4相关通路具体的上下游调控机制。

综上所述, 本研究结果表明Ator不仅可诱导MIN6细胞发生凋亡, 同时发生铁死亡, 其共同参与胰岛β细胞的功能障碍和死亡, 并最终导致NODM

发生。靶向铁死亡可能为NODM的治疗提供新策略, 后续研究将进一步寻找潜在的干预药物。

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