L-茶氨酸对H_2O_2致L02细胞损伤的保护作用及其机制研究

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摘要 研究L-茶氨酸对肝细胞损伤的保护作用及其机制。利用H_2O_2诱导的L02肝细胞损伤模型, 分别用MTT法检测细胞存活率、测定LDH、流式细胞术检测细胞凋亡率、Western blot法检测Caspase-3和PARP蛋白表达及Bax/Bcl-2比值的变化, 评价L-茶氨酸是否能保护H_2O_2诱导的肝细胞损伤。结果表明, L-茶氨酸能提高H_2O_2损伤的L02细胞存活率, 减少LDH的渗漏, 降低肝细胞凋亡, 且L-茶氨酸通过抑制Caspase-3的激活和PARP的切割及Bax/Bcl-2比值的升高而发挥抗凋亡的作用。L-茶氨酸对肝细胞损伤有一定的治疗和保护作用。

关键词 L-茶氨酸; H_2O_2; L02细胞; 保护作用

Protective Effect and the Mechanism of L-theanine Against H_2O_2 Induced Injury in L02 Cells

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Abstract The object of this study was to explore the protective effect and the mechanism of L-theanine against hepatocyte injury. The human hepatic L02 cells injured by H_2O_2 were used as a cell model. The protective effect of L-theanine against H_2O_2-induced hepatocyte injury was evaluated by MTT determination for cell viability, LDH assay, using flow cytometry for apoptosis rate and Western blot for protein expression of Caspase-3, PARP and the ratio of Bax/Bcl-2. The results showed that L-theanine attenuated the cell viability loss and LDH release induced by H_2O_2, and prevented L02 cells from H_2O_2-induced cell apoptosis. In addition, L-theanine mediated the anti-apotosis role by decreasing Caspase-3 activation, PARP cleave and Bax/Bcl-2 ratio. In conclusion, L-theanine helps to protect and cure the hepatocyte injury.

Key words L-theanine; H_2O_2; L02 cells; protective effect

氧化应激与肝脏疾病密切相关, 在氧化应激过程中由活性氧(reactive oxygen species, ROS)引起的脂质过氧化反应是非酒精性脂肪肝和肝癌发病机理中重要的因素之一[1-2]。在介导肝纤维化的众多机制中, ROS也发挥着重要的作用[3]。体内ROS积累到一定程度可对机体细胞产生损伤作用。H_2O_2作为ROS家族成员之一, 不仅能直接氧化细胞膜上的脂质和蛋白质, 而且能自由穿过细胞膜和细胞内的铁离子反应生成羟基自由基(•OH)等活性更强的自由基, 并通过多种途径诱导细胞凋亡。研究发现, H_2O_2可以通过氧化应激引发肝细胞凋亡[4]。

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L-茶氨酸是绿茶中的一种天然氨基酸, 具有保护心脑血管、抗肿瘤、抗糖尿病和减轻酒精对肝脏损伤等多种生物学活性[5-8], 并具有安全、无副作用的优点。研究表明, L-茶氨酸对多种刺激引起的神经细胞或肝脏损伤有保护作用[8-10], 其保护机制与其抗氧化活性和抑制凋亡等相关。肝细胞损伤的过程常常伴随细胞凋亡, 抑凋亡因子Bcl-2和促凋亡因子Bax在细胞凋亡中发挥着重要作用。本研究将用H_2O_2刺激的L02细胞模型来研究L-茶氨酸对H_2O_2诱导的肝细胞损伤的保护作用, 为阐明L-茶氨酸的肝保护作用机理提供理论依据。

1 材料与方法

1.1 主要试剂

Polyclonal antibody against pro-caspase3、PARP、Bax、β-Actin购自Cell Signaling Technology公司(Beverly, MA, USA); antibody against Bcl-2购自Signaling Antibody Biotechnology公司; Rabbit monoclonal antibody against GAPDH购自南京Bioworld生物科技有限公司; Western blot所用二抗均购自美国Calbiochem公司。L-茶氨酸(L-theanine, CAS: 3081-61-6, 纯度≥99%, HPLC)、3-(4,5-噻唑-2)-2,5-二苯基四氮唑溴盐(MTT)购自美国Sigma公司; LDH测定试剂盒购自南京建成生物工程有限公司。

1.2 细胞株和培养液

人正常肝细胞株L02(购自中国科学院上海生命科学研究院细胞库)在37 °C、5% CO_2条件下, 用含10%胎牛血清(fetal bovine serum, FBS)和抗生素(100 U/mL青霉素和100 μg/mL链霉素)的DMEM完全培养液(Hyclone公司)培养。

1.3 MTT法检测L-茶氨酸对H_2O_2损伤的L02细胞存活率的影响

将细胞按每孔200 μL接种入96孔细胞培养板中, 置于37 °C培养箱中培养, 待细胞覆盖板底60%~70%时, 取对数生长期的L02细胞进行实验分组, 分为空白对照组、L-茶氨酸组(1 mmol/L)、H_2O_2组(0.4 mmol/L)及L-茶氨酸(500, 750, 1 000 μmol/L)与H_2O_2(0.4 mmol/L)联合处理组。按照分组先加入L-茶氨酸预孵育1 h, 再加入H_2O_2(0.4 mmol/L)。培养12 h后, 将96孔板中的培养液吸弃, 用无菌PBS洗涤3次后, 每孔加MTT溶液20 μL(5 mg/mL), 放置37 °C温箱继续培养4 h, 弃上清液。每孔加150 μL二甲基亚砜(DMSO), 振荡10 min, 酶标仪上在570 nm波长处测定各孔的吸光度值, 以正常对照为100%计算细胞存活率(%)。

1.4 流式细胞术检测L-茶氨酸对H_2O_2损伤的L02细胞凋亡的影响

取L02细胞以5×10^5/孔接种于6孔板, 待细胞覆盖底部达到60%以上时进行试验, 必要时更换培养液。对细胞进行分组和处理, 分为空白对照组、H_2O_2组(0.4 mmol/L)及L-茶氨酸(1 000 μmol/L)与H_2O_2(0.4 mmol/L)联合处理组。按照分组先加入L-茶氨酸预孵育1 h, 再加入H_2O_2(终浓度为0.4 mmol/L), 培养12 h。反应完成后, 胰酶消化细胞, 轻轻吹打转移至已标记好的流式离心管中。室温下, 1 000×*g*离心5 min, 弃上清, 以去除胰酶, 沉淀用PBS洗2~3次。弃上清, 用200 μL含有Annexin V-FITC和PI的缓冲液重悬, 轻轻混匀细胞, 避光室温作用15 min。上流式细胞仪检测, FITC的激发波长为488 nm, 发射波长为575 nm, 每个样品采集至少10 000个细胞, 数据由流式细胞仪Cell Quest(Becton Dickinson, Mountain view, CA, USA)软件进行分析。

1.5 L-茶氨酸对H_2O_2损伤的L02细胞LDH渗漏的影响

将细胞按每孔5×10^3/200 μL接种入96孔细胞培养板中, 然后置于37 °C温箱中培养, 待细胞覆盖板底60%~70%时进行试验, 分组同1.3, 作用完成后, 取培养液上清按照试剂盒说明在420 nm处测定LDH的值, 其中以正常对照为100%计算LDH(% of control)。

1.6 Western blot测定L-茶氨酸对H_2O_2损伤的L02细胞Caspase-3、PARP及Bax/Bcl-2的影响

将细胞按每孔2×10^5/mL接种入12孔细胞培养板中, 然后置于37 °C温箱中培养, 待细胞覆盖板底60%~70%时进行试验。分组同1.4, 按照分组先加入L-茶氨酸预孵育1 h, 再加入H_2O_2(终浓度为0.4 mmol/L), 培养12 h。收取细胞并进行免疫印迹。反应完成后, 将培养的L02细胞用预冷的PBS漂洗2次, 加入预冷的细胞裂解液在冰上孵育30 min。细胞裂解物于4 °C, 15 000×*g*离心15 min, 收取离心上清液, 进行SDS-PAGE(12%)并转移至NC膜(Whatman公司)上。用含5%脱脂奶粉的TBS封闭1 h, 加入一抗4 °C孵育过夜, 以荧光偶联的羊抗鼠或羊抗兔二抗室温孵育1 h, 用TBST洗3次, 每次5 min, 然后用LI-COR Odyssey Infrared Imaging System扫描, 并用 LI-COR

Odyssey的分析软件直接对条带进行定量分析。

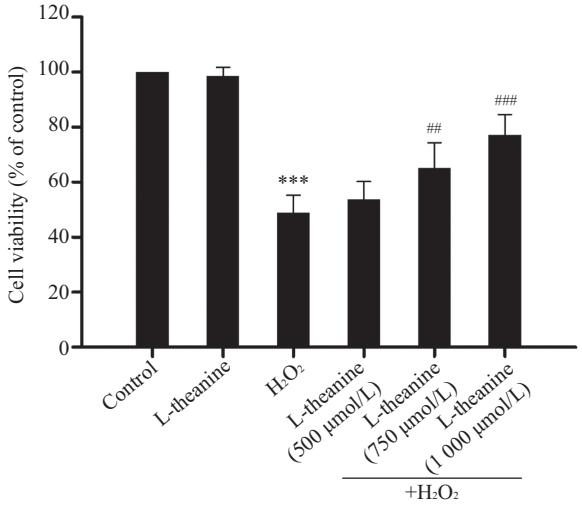
1.7 数据统计分析

采用Stat6.0软件进行统计学分析, 所有数值均表示为mean±S.D.并设置多个重复, 经单因素方差分析后用Student's *t*检验统计分析, $P<0.05$、$P<0.01$或$P<0.001$被认为有统计学意义。

2 结果

2.1 L-茶氨酸对H_2O_2损伤的L02细胞存活率的影响

为了检测L-茶氨酸对H_2O_2损伤的L02细胞活性的影响, 用不同剂量(250, 500, 750, 1 000 μmol/L)的L-茶氨酸预孵细胞1 h, 再加入H_2O_2(0.4 mmol/L)共同培养12 h。结果如图1所示, H_2O_2能降低L02细胞活性($P<0.01$)。当L-茶氨酸达到750 μmol/L以上时可以拮抗H_2O_2的作用, L02细胞活性明显升高, 预孵750 μmol/L和1 000 μmol/L的L-茶氨酸, L02细胞存活率分别为(65.28±9.10)%和(77.17±7.39)%, 与H_2O_2损伤组比较, 差异具有统计学意义($P<0.01$)。



用不同剂量(500, 750, 1 000 μmol/L)的L-茶氨酸预孵细胞1 h, 再加入H_2O_2(0.4 mmol/L)共同培养12 h。结果用mean±S.D.表示(n=6)。***$P<0.001$, 与对照组相比。$^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, 与单独用H_2O_2处理的L02细胞组相比。

Cells were pretreated with L-theanine (500, 750, 1 000 μmol/L) for 1 h, then incubated with H_2O_2 (0.4 mmol/L) in the presence of L-theanine for a further 12 h. Each bar represented the mean±S.D. (n=6). ***$P<0.001$ compared with the control group; $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ compared with the group of H_2O_2-treated L02 cells alone.

图1 不同浓度的L-茶氨酸对H_2O_2诱导L02细胞活力损伤的保护作用

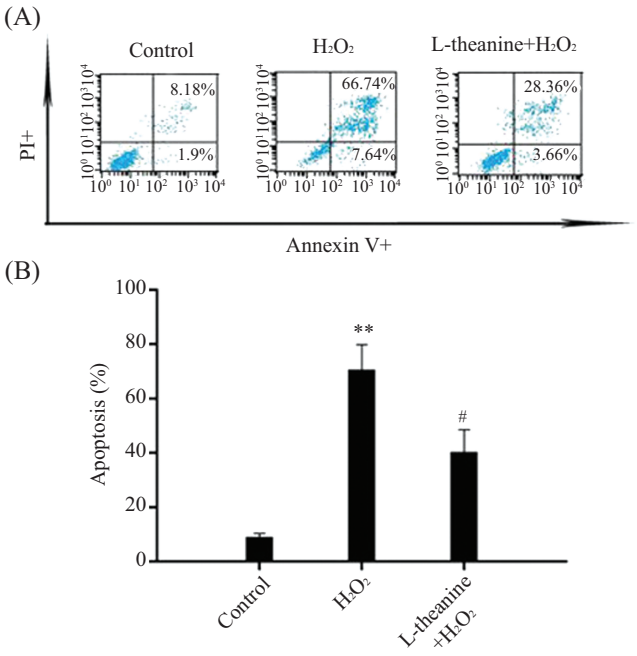
Fig.1 Protective effect of different doses of L-theanine against H_2O_2-induced cell viability loss in human hepatic L02 cells

2.2 L-茶氨酸抑制由H_2O_2损伤引起的L02细胞凋亡

由图2可知, 与空白对照组相比, H_2O_2刺激组细胞的凋亡率显著增加, 增加了74.38%($P<0.01$), 表明细胞受到明显损伤。L-茶氨酸能够抑制H_2O_2引起的L02细胞凋亡, 与H_2O_2刺激组相比, L-茶氨酸(1 000 μmol/L)与H_2O_2(0.4 mmol/L)联合处理组细胞的凋亡率降低至32.02%($P<0.05$)。结果表明, L-茶氨酸能抑制由H_2O_2导致的肝细胞凋亡。

2.3 L-茶氨酸降低H_2O_2损伤的L02细胞中LDH的渗漏

由图3可知, 与空白对照组相比, H_2O_2刺激组细胞培养液中LDH含量增加了25.92%($P<0.001$), 表明细胞受到明显损伤。L-茶氨酸能够抑制H_2O_2引起的



A: 细胞经L-theanine或者H_2O_2处理过后, 采用Annexin V-FITC和propidine iodide(PI)双染, 在FACScan(Becton Dickinson, Mountain view, CA, USA)上进行流式细胞分析; B: 采用荧光激活细胞分选术测定细胞凋亡率。结果用mean±S.D.表示(n=3)。**$P<0.01$, 与对照组相比。$^{\#}P<0.05$, 与单独用H_2O_2处理的L02细胞组相比。

A: after treated with L-theanine or H_2O_2 for indicated time, L02 cells were dyed with both of Annexin V-FITC and propidine iodide (PI). Flow cytometric analysis was performed with FACScan (Becton Dickinson, Mountain view, CA, USA) with the Cell Quest program; B: percentage of apoptotic cells, as determined by Annexin-V-FITC and PI staining followed by the fluorescence-activated cell sorting using flow cytometry. Each bar represents the mean±S.D. (n=3). **$P<0.01$ compared with the control group; $^{\#}P<0.05$ compared with the group of H_2O_2-treated L02 cells alone.

图2 L-茶氨酸抑制由H_2O_2诱导的L02细胞凋亡

Fig.2 L-theanine inhibited L02 cells from H_2O_2-induced apoptosis

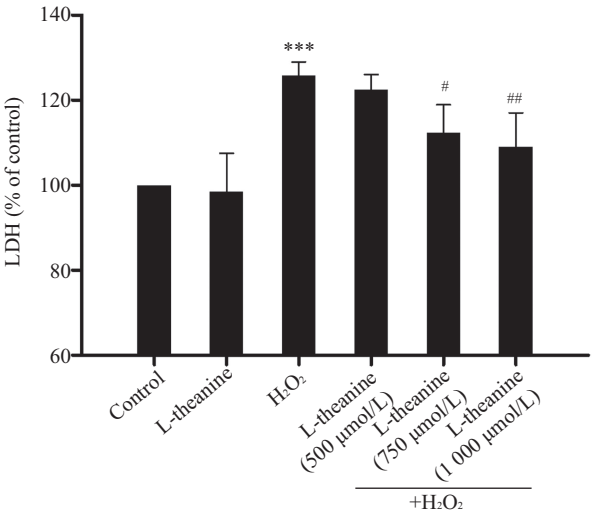
L02细胞培养液中LDH渗漏的增加, 与H_2O_2刺激组相比, L-茶氨酸(750, 1 000 μmol/L)与H_2O_2(0.4 mmol/L)联合处理组细胞培养液中的LDH活性分别降低了13.56%(P<0.05)和16.82%(P<0.01), 且呈现一定的剂量依赖性。结果表明, L-茶氨酸能降低H_2O_2损伤的L02细胞中的LDH渗漏。

2.4 L-茶氨酸抑制H_2O_2诱导的L02细胞中Caspase-3的激活和PARP的切割

与对照组相比, H_2O_2刺激组能引起L02细胞中Caspase-3的激活和PARP的切割; 与H_2O_2刺激组相比, L-茶氨酸预孵能降低由H_2O_2引起的Caspase-3的激活和PARP的切割, 差异具有统计学意义(图4)。

2.5 L-茶氨酸对H_2O_2诱导的L02细胞中BCL-2、Bax及Bax/Bcl-2的影响

如图5所示, 正常对照组和L-茶氨酸单刺激组中Bcl-2和Bax均有表达, 且两者之间差异无统计学意义。H_2O_2刺激后能引起L02细胞中Bcl-2蛋白表达下调和Bax蛋白表达上调, 使得Bax/Bcl-2比值升高, 且与对照组相比差异具有统计学意义(P<0.01)。L-茶氨酸作用后能抑制由H_2O_2引起的Bcl-2和Bax蛋白表达的变化, 使得Bax/Bcl-2比值下降, 与H_2O_2刺激组比较差异具有统计学意义(P<0.001)。

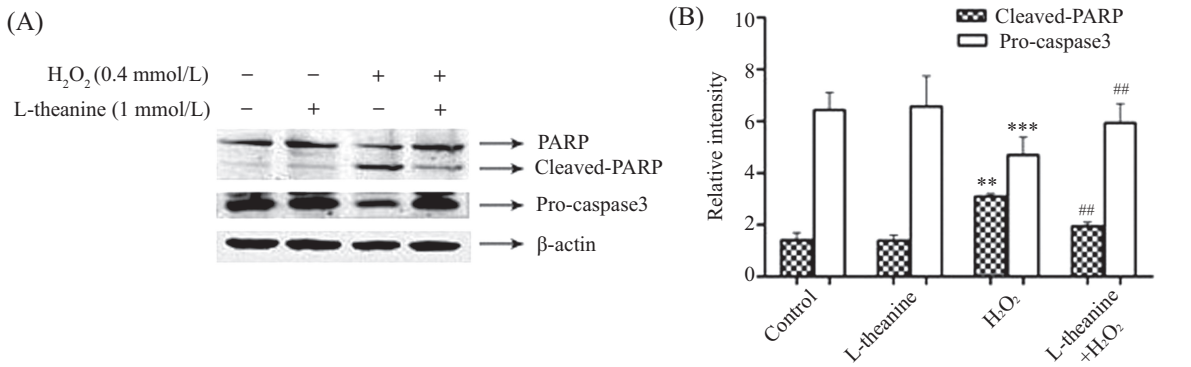


L02细胞经L-茶氨酸预处理或未处理, 再加入0.4 mmol/L的H_2O_2培养12 h, 测定细胞上清液中的LDH。结果用mean±S.D.表示(n=6)。***P<0.001, 与对照组相比。$^{\#}P$<0.05, $^{\#\#}P$<0.01, 与单独用H_2O_2处理的L02细胞组相比。

L02 cells were pretreated with or without L-theanine then treated with 0.4 mmol/L H_2O_2 for 12 h, LDH in cells supernatant was evaluated. Each bar represented the mean±S.D.(n=6). ***P<0.001 compared with the control group. $^{\#}P$<0.05, $^{\#\#}P$<0.01 compared with the group of H_2O_2-treated L02 cells alone.

图3 L-茶氨酸对H_2O_2诱导的L02细胞中LDH漏出的影响

Fig.3 Effect of L-theanine against H_2O_2-induced LDH leakage in L02 cells



L02细胞经L-茶氨酸或H_2O_2处理后, 采用Western blot测定pro-caspase3的激活和PARP的切割, β-actin作为内参, 做3次重复。**P<0.01, ***P<0.001, 与对照组相比。$^{\#\#}P$<0.01, 与单独用H_2O_2处理的L02细胞组相比。

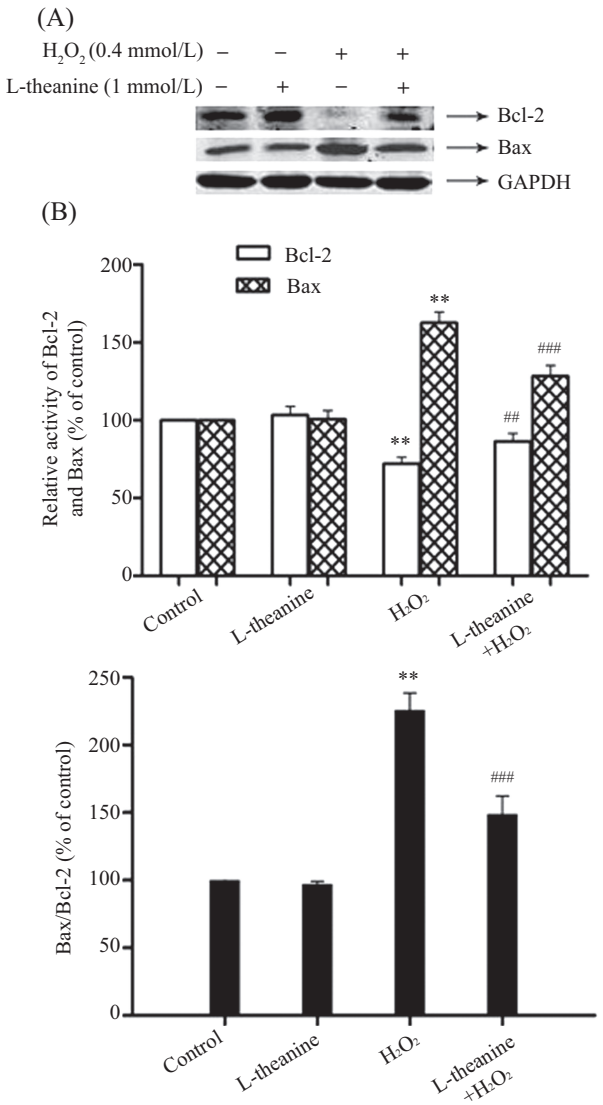
L02 cells were treated with L-theanine or H_2O_2 for indicated time, and the pro-caspase3 activation and PARP cleave were tested by Western blot. β-actin were used as the internal control. Values are averages of three independent experiments. **P<0.01, ***P<0.001 compared with the control group. $^{\#\#}P$<0.01 compared with the group of H_2O_2-treated L02 cells alone.

图4 L-茶氨酸抑制H_2O_2诱导的Caspase-3激活和PARP切割

Fig.4 L-theanine inhibited H_2O_2-induced Caspase-3 activation and PARP cleave

3 讨论

病毒感染、酗酒、代谢紊乱及药物影响等都能引起肝脏损伤[11], 严重威胁人类健康。无论急性或慢性肝脏疾病都经历一系列、较长的病理转变, 从肝脏脂肪变性、肝炎、肝纤维化、肝硬化到肝癌[12]。氧化应激是导致肝脏损伤的重要机制, 各种急性或慢性肝脏疾病过程都伴随着氧化应激引起的肝细胞损伤[13-14]。H_2O_2是一种重要的活性氧成分, 过量的



L02细胞经L-茶氨酸或H_2O_2处理后, 采用Western blot测定Bcl-2和Bax, GAPDH作为内参, 重复3次。$^{**}P$<0.01, 与对照组相比。$^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001, 与单独用H_2O_2刺激的L02细胞组相比。

L02 cells were treated with L-theanine or H_2O_2 for indicated time, Bcl-2 and Bax were tested by Western blot. GAPDH were used as the internal control. Values are averages of three independent experiments. $^{**}P$<0.01 compared with the control group. $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 compared with the group of H_2O_2-treated L02 cells alone.

图5 L-茶氨酸对H_2O_2诱导的Bcl-2和Bax的影响

Fig.5 Effects of L-theanine on H_2O_2-induced Bcl-2 and Bax

H_2O_2因氧化应激损伤可以导致许多类型的细胞发生凋亡, 包括诱导肝细胞凋亡[15-16]。细胞凋亡在肝脏疾病的发病机制中发挥重要作用, 因此, 抑制肝细胞凋亡有利于肝脏疾病的治疗。

L-茶氨酸是绿茶中的呈味物质, 是一种天然的氨基酸, 在食品行业已被广泛应用。已有研究表明, L-茶氨酸具有抗氧化活性, 它能保护神经细胞免受各种刺激引起的凋亡[9,17]。但L-茶氨酸是否能抑制肝细胞凋亡, 从而拮抗氧化应激对肝细胞的损伤, 尚未见文献报道。本研究以H_2O_2诱导的L02细胞建立了肝细胞损伤反应模型, 加入L-茶氨酸干预后, 与H_2O_2模型组相比, L-茶氨酸能显著恢复L02细胞的存活率, 减少LDH的渗漏, 降低细胞凋亡率, 提示L-茶氨酸能通过抗氧化作用和抑制细胞凋亡, 有效地保护由H_2O_2导致的肝细胞损伤。

研究表明, 线粒体凋亡通路参与介导肝细胞凋亡[18-19]。Caspase-3是线粒体凋亡通路上的一个重要的执行分子, 它在凋亡信号传导的许多途径中发挥作用。Caspase-3正常以酶原的形式存在于胞浆中, 在凋亡的早期阶段, 它能够被活化, 裂解相应的胞浆、胞核底物, 最终导致细胞凋亡。PARP作为Caspase-3的重要底物在细胞凋亡过程中可以被剪切, 这种剪切可使PARP在凋亡过程中失去DNA结合域结合DNA片段的功能, 阻碍PARP在维持基因组恒定中的DNA修复协调作用。故PAPR可通过Caspase-3的裂解剪切参与凋亡的发生[20-21]。本研究发现L-茶氨酸能抑制由H_2O_2引起的Caspase-3的激活和PARP的切割, 提示L-茶氨酸可能通过抑制由H_2O_2引起的Caspase-3的激活和PARP的切割来抑制L02细胞的凋亡。

在线粒体凋亡调控体系中, Bcl-2家族中抗凋亡基因(*Bcl-2*、*Bcl-xL*)或促凋亡基因(*Bax*、*Bad*和*Bid*)主要参与调节细胞凋亡[22]。Bcl-2蛋白是线粒体膜上的一种凋亡调控蛋白, 可以与线粒体膜上的电压依赖性阳离子通道结合来调节促凋亡因子细胞色素c的释放, 也可以和Bax蛋白结合, 阻断其插入到线粒体膜上来维持线粒体的膜电位, 而Bax是一种促凋亡蛋白, 故Bax/Bcl-2的比值反映了细胞损伤的程度。研究发现, H_2O_2能诱导Bax表达的增加而引起Bcl-2蛋白表达的降低从而使Bax/Bcl-2比值升高, L-茶氨酸能显著抑制H_2O_2刺激后L02细胞中Bax和Bcl-2蛋白表达的变化, 使Bax/Bcl-2的比值降低, 说明L-茶氨酸对抗H_2O_2诱导的肝细胞凋亡是通过抑制Bax/Bcl-2比值的升高来实现的。

综上所述, L-茶氨酸能通过抑制细胞凋亡来有效地保护由H_2O_2导致的肝细胞损伤, 促进Bcl-2蛋白表达和抑制Bax蛋白表达, 抑制Bax/Bcl-2比值的升高是L-茶氨酸发挥抗凋亡作用的重要途径之一。由此表明, L-茶氨酸可用于相关肝脏疾患的防治, 相关研究也有待进一步的深入。

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