Drp1抑制剂Mdivi-1对脂多糖/D-半乳糖胺诱导的急性肝损伤的影响及机制研究

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摘要 线粒体动力相关蛋白1(dynamin-related protein 1, Drp1)是细胞凋亡调控的新靶点, 而凋亡是急性肝损伤的重要特征, 该研究采用选择性Drp1抑制剂Mdivi-1, 探讨了Drp1在急性肝损伤中的可能病理生理学作用及其潜在的药靶价值。该研究动物实验采用6~8周龄雄性BAlB/c小鼠, 在腹腔内注射脂多糖(lipopolysaccharide, LPS)/D-半乳糖胺(D-galactosamine, D-Gal), 诱导急性肝损伤模型。实验分为四组: 正常对照组(A)、Mdivi-1单独处理组(B)、模型组(C)和Mdivi-1干预组(D)。Mdivi-1在LPS/D-Gal暴露前30 min经腹腔注入, 在LPS/D-Gal注射后1.5 h或6.0 h处死动物, 采集肝组织和血浆标本。实验通过组织病理学染色、比色法检测血浆转氨酶活性、细胞因子TNF-α检测、casepase-3、casepase-8和casepase-9的活性检测和TUNEL技术探讨Mdivi-1介导下肝损伤的改善情况。结果显示, 通过H&E染色观察肝组织病理学改变, 正常对照组和Mdivi-1单独处理组小鼠肝组织结构无异常, LPS/D-Gal暴露后可引起一系列显著的组织学异常改变, Mdivi-1干预组肝组织学异常明显减轻。通过检测血浆谷丙转氨酶(alanine aminotransferase, ALT)、谷草转氨酶(aspartate aminotransferase, AST)活性评估肝脏功能损害程度, Mdivi-1干预组ALT和AST的活性明显降低(P<0.05)。采用ELISA法检测血浆中肿瘤坏死因子α(tumor necrosis factor alpha, TNF-α)水平以评估炎症反应程度, Mdivi-1干预组的炎性细胞因子表达降低明显(P<0.05)。通过半胱氨酸蛋白酶-3(casepase-3)、半胱氨酸蛋白酶-8(casepase-8)、半胱氨酸蛋白酶-9(casepase-9)和TUNEL染色评估组织凋亡程度, Mdivi-1干预组的凋亡水平降低显著(P<0.05)。所以得出结论, Mdivi-1干预可减轻 LPS/D-Gal诱导的肝组织病变、降低血浆转氨酶、下调血浆中 TNF-α水平、降低肝内casepase-3、casepase-8和casepase -9活性并减少TUNEL阳性细胞的数量。以上数据表明, Drp1抑制剂Mdivi-1可减轻LPS/D-Gal诱导的急性肝损伤。

关键词 动力相关蛋白1; 线粒体; 急性肝损伤; 脂多糖; 凋亡

Effects of Drp1 Inhibitor Mdivi-1 on Lipopolysaccharide/D-galactosamine-Induced Acute Liver Injury and Its Mechanism

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Abstract The mitochondrial protein Drp1 (dynamin-related protein 1) is a new target for the regulation of apoptosis, and apoptosis is an important feature of acute liver injury. In this study, the selective Drp1 inhibitor Mdi-

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vi-1 was used to explore the role of Drp1 in the possible pathophysiology of liver injury and its potential drug target value. In this study, 6-8 week-old male BAlB/c mice were injected intraperitoneally with LPS (lipopolysaccharide)/D-Gal (D-galactosamine) to induce an acute liver injury model. The experiment was divided into four groups: normal control group (A), Mdivi-1 alone treatment group (B), model group (C), and Mdivi-1 intervention group (D). Mdivi-1 was injected intraperitoneally 30 minutes before LPS/D-Gal exposure. Animals were sacrificed after LPS/D-Gal injection in 1.5 h or 6.0 h, liver tissue and plasma samples were collected. Histopathological staining, colorimetric detection of plasma transaminase activity, detection of cytokine TNF-α, activity of casepase-3, casepase-8, and casepase-9 and TUNEL technology were used to explore the improvement of liver injury induced by Mdivi-1. The results showed that the pathological changes of liver tissue were observed by HE staining. There was no abnormal liver tissue structure in normal control group and Mdivi-1 alone treatment group. LPS/D-Gal exposure could cause a series of significant histological abnormalities. The degree of liver damage was assessed by measuring the activity of plasma ALT (alanine aminotransferase) and AST (aspartate aminotransferase). The activity of ALT and AST in the Mdivi-1 intervention group was significantly reduced (P<0.05). TNF-α (tumor necrosis factor alpha) levels in plasma were used to assess the degree of inflammatory response. The expression of inflammatory cytokines in the Mdivi-1 intervention group was significantly reduced (P<0.05). Casepase-3, casepase-8, casepase-9 and TUNEL staining were used to assess the degree of tissue apoptosis, and the level of apoptosis in the Mdivi-1 intervention group decreased significant (P<0.05). Therefore, it was concluded that Mdivi-1 intervention could reduce LPS/D-Gal-induced liver tissue lesions, reduce plasma transaminase, down-regulate plasma TNF-α levels, reduce liver casepase-3, casepase-8, casepase-9 activities and reduce the number of TUNEL positive cells. The above data indicated that the Drp1 inhibitor Mdivi-1 could reduce LPS/D-Gal-induced acute liver injury.

Keywords dynamin-related protein 1; mitochondrion; acute liver injury; lipopolysaccharide; apoptosis

急性肝炎是由感染了自身免疫因子、药物、毒素等致病因子引起的以炎症/免疫反应为基础的肝脏损害, 可导致肝功能障碍甚至死亡[1-3]。脂多糖(lipopolysaccharide, LPS)是革兰氏阴性菌的主要毒性成分[4], 可强烈激活炎症反应[5-6]; D-半乳糖胺(D-galactosamine, D-Gal)可在肝细胞中被选择性代谢, 显著增强肝脏对LPS致炎效应的敏感性[7]。LPS/D-Gal诱导的小鼠肝损伤被广泛用于研究肝炎的发病机制及护肝新药开发[8-9]。

动力相关蛋白1(dynamin-related protein 1, Drp1)是一种介导线粒体分裂的蛋白, 位于细胞质中, 属于大鸟苷三磷酸氢酶(GTPases)蛋白超家族, 参与调控线粒体的结构重塑[10-11]。在凋亡期间, Drp1可与Bax相互作用并促进细胞色素c释放, 因而, Drp1可作为细胞凋亡调控的新靶点[12]。Drp1的选择性抑制剂Mdivi-1是一种喹唑啉酮衍生物[13]。Mdivi-1可通过变构作用防止Drp1寡聚化、从而抑制Drp1的功能[14]。体内外的实验研究表明, Mdivi-1可有效阻断Bax/Bak依赖性的细胞色素c释放和细胞凋亡[15]。凋亡是LPS/D-Gal模型的重要特征[16], 那么, Drp1抑制剂是否可在LPS/D-Gal诱导的急性肝损伤中发挥保护效果呢?

为研究Drp1抑制剂在急性肝损伤中的可能保护作用, 本实验参照文献和我们先前报道的方法复制了LPS/D-Gal诱导的急性肝损伤小鼠模型[17-18], 探讨了Drp1抑制剂Mdivi-1对肝组织损伤程度、炎症反应水平、肝细胞凋亡的影响, 本研究旨在探讨线粒体蛋白Drp1在急性肝损伤发生发展中的可能作用, 为急性肝损伤的防治提供新的实验资料。

1 材料与方法

1.1 动物及实验材料

雄性BAlB/c小鼠(6~8 周龄, 18~20 g)购自重庆医科大学实验动物中心。LPS购自Sigma公司; D-Gal购自Sigma公司; Drp1抑制剂Mdivi-1购自Cayman公司; 小鼠TNF-α ELISA检测试剂盒购自欣博胜生物科技公司; 血浆谷丙转氨酶(alanine aminotransferase, ALT)和谷草转氨酶(aspartate aminotransferase, AST)测定试剂盒购自南京建成生物工程研究所; casepase-3、casepase-8、casepase-9试剂盒购自碧云

天生物科技公司。

1.2 模型及处理

实验小鼠随机分4组(n=8)。(A)正常对照组: 腹腔注射溶剂; (B)Mdivi-1单独处理组: 腹腔注射Drp1抑制剂Mdivi-1, 剂量为 50 mg/kg, 溶于10%的DMSO(用食用油稀释); (C)模型组: 腹腔注射 LPS/D-Gal, LPS剂量为10 μg/kg, D-Gal剂量为700 mg/kg, 溶解于生理盐水; (D)Mdivi-1干预组: Mdivi-1注入后30 min再注入LPS/D-Gal, 剂量同前。小鼠在LPS/D-Gal处理后6 h断颈处死, 采集血浆和肝组织标本。建模成功与否由肝脏转氨酶活性和组织病理学结果判断。以上动物实验得到了重庆医科大学医学伦理委员会的批准。

1.3 组织病理学观察

将小鼠的右肝叶固定在4%多聚甲醛中, 标本脱水后包埋于石蜡中。切片, 随后用苏木精和伊红(H&E)染色, 使用光学显微镜(Olympus, Tokyo, Japan)进行组织病理学观察。

1.4 血浆ALT和AST检测

小鼠模型建立后6 h处死, 取其眼球血, 12 000 r/min离心15 min, 取上层血清, 按照说明书, 将血浆样品加入96孔板中。加入ALT或AST基质液, 将板在37 ℃下孵育30 min。将2-4-二硝基苯肼加入各孔中, 将板在37 ℃下孵育20 min。加入NaOH溶液, 并根据标准曲线, 计算出在490 nm吸光度下ALT和AST的水平。

1.5 细胞因子检测

采用ELISA试剂盒、参照说明书中的方法, 所有试剂在使用前将平衡到室温水平。将样品加入到包被有抗体的96孔板中, 并将板在37 ℃水浴箱中孵育90 min。用洗涤缓冲液洗板5次, 在吸收纸上轻拍。将检测抗体溶液添加到每个孔中, 并将板在37 ℃下孵育60 min。洗涤缓冲液洗板5次。随后, 将酶结合物工作液加入到每个孔中, 并将板在37 ℃下孵育30 min。洗涤缓冲液洗涤板5次。加入显色底物TMB, 并将板避光孵育15 min。最后, 通过添加终止溶液来终止反应, 并在450 nm下测量标准曲线, 根据吸光度值计算TNF-α的浓度。

1.6 Casepase-3、casepase-8和casepase-9活性检测

采用casepase-3、casepase-8、casepase-9检测试剂盒, 按照说明书, 将肝脏样品在4 ℃下12 000 r/min离心15 min。将上清液转移到新管中, 通过Bradford蛋白测定试剂盒(Beyotime)测定蛋白质浓度。检测酶活性时, 依次加入检测缓冲液、待测样本, 混匀后再加入casepase-3、casepase-8或casepase-9的底物, 37 ℃下孵育90 min后, 在405 nm处测量吸光度值, 根据标准曲线计算半胱氨酸蛋白酶的活性, 再将各样本的蛋白酶活性与蛋白浓度比值进行标准化处理。

1.7 TUNEL检测

使用原位细胞死亡检测试剂盒(Roche), 按照说明书, 使用一系列二甲苯和乙醇脱蜡和再水合的组织切片, 将组织切片在37 ℃下孵育30 min, 与TUNEL反应混合物在37 ℃下孵育60 min。将切片与每个样品的转化器-POD一起孵育30 min。用苏木精轻微复染切片。在光学显微镜(Olympus, Japan)下观察组织。

1.8 统计学分析

所有数值均表示为$\bar{x}\pm s$。使用GraphPad Prism软件6.0版(GraphPad Software, San Diego, CA), 使用单向ANOVA(Dunnett t检验)检验来分析正态分布数据的平均值之间的差异。P<0.05表示差异具有统计学意义。

2 结果与分析

2.1 Mdivi-1减轻LPS/D-Gal诱导的急性肝损伤

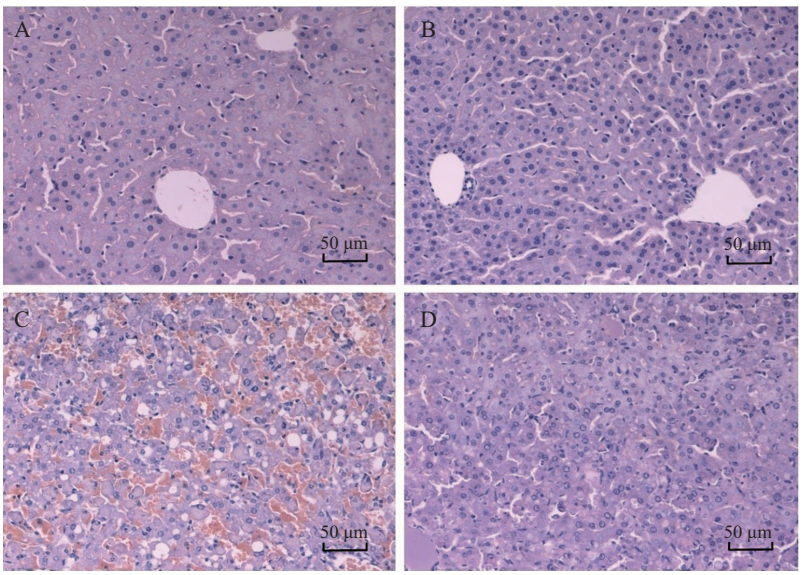
肝组织切片(图1)显示, 正常对照组和药物对照组小鼠肝组织结构无异常, LPS/D-Gal暴露可引起一系列显著的组织学异常, 镜下, 肝小叶大面积充血, 肝细胞广泛变性, 肝小叶内可有散在的点状坏死, 坏死区域可见中性粒细胞浸润。这些异常改变在Mdivi-1干预后得到了明显缓解, 提示Mdivi-1处理可减轻LPS/D-Gal诱导的急性肝损伤。与此结果相一致, 肝脏谷丙转氨酶和谷草转氨酶在Mdivi-1处理组显著降低(图2)和(图3)。

2.2 Mdivi-1抑制LPS/D-Gal诱导的促炎因子产生

TNF-α等促炎细胞因子是参加炎症反应及肝组织损伤的关键介质。我们采用酶联免疫吸附法检测了各组小鼠血浆中的TNF-α的水平, 结果显示, LPS/D-Gal可诱导TNF-α水平的显著上升, 而Mdivi-1处理则可显著抑制TNF-α水平的升高(图 4)。这些结果提示, Mdivi-1在急性肝损伤中的保护作用可能与其对炎症反应的抑制作用有关。

2.3 Mdivi-1抑制 LPS/D-Gal诱导的肝细胞凋亡

如图 5所示, LPS/D-Gal暴露小鼠肝组织中casepase-3、casepase-8和casepase-9活性明显增加,

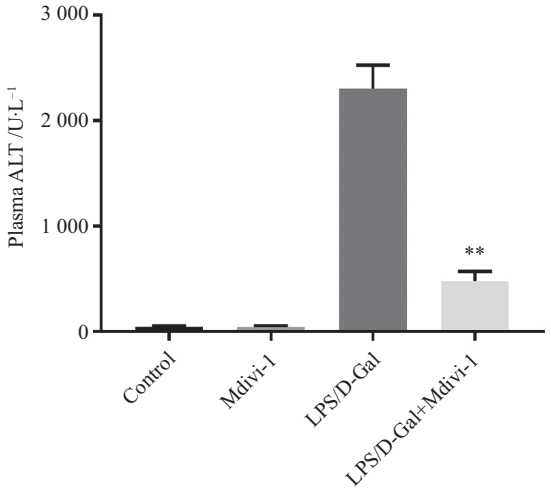


A: 正常对照组; B: Mdivi-1单独处理组; C: LPS/D-Gal单独处理组; D: LPS/D-Gal+Mdivi-1处理组。

A: control group; B: Mdivi-1 injected group C: LPS/D-Gal injected group D: LPS/D-Gal+Mdivi-1 injected group.

图1 Mdivi-1减轻LPS/D-gal诱导的肝组织损伤

Fig.1 Mdivi-1 reduces LPS/D-Gal-induced liver tissue injury



Control: 正常对照组; Mdivi-1: Mdivi-1单独处理组; LPS/D-Gal: LPS/D-Gal单独处理组; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1处理组。n=8, $^{**}P$<0.01, 与LPS/D-Gal组比较。

Control: Control group; Mdivi-1: Mdivi-1 injected group; LPS/D-Gal: LPS/D-Gal injected group; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1 injected group. n=8, $^{**}P$<0.01 compared with LPS/D-Gal.

图2 Mdivi-1抑制LPS/D-Gal诱导的血浆ALT上升

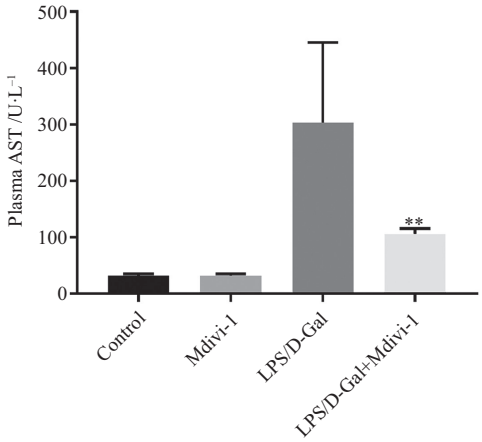
Fig.2 Mdivi-1 inhibits LPS/D-Gal-induced elevation of ALT in plasma

提示LPS/D-Gal可诱导肝细胞凋亡; 而Mdivi-1处理后, LPS暴露小鼠肝组织中casepase-3、casepase-8和casepase-9活性显著下降。此外, TUNEL染色分析表明(图6和图7), Mdivi-1处理可减少TUNEL阳性肝细胞数量。

3 讨论

本研究在LPS/D-Gal诱导的急性肝损伤小鼠的肝组织中发现, Drp1抑制剂Mdivi-1处理可显著减轻LPS/D-Gal诱导的肝组织损伤, 这一保护效应表现在血浆转氨酶升高受到抑制、组织病理学异常得到改善。这些结果表明, Drp1可能在急性肝损伤的发生发展中发挥了重要作用, 有望成为治疗急性肝损伤的新靶点。

实验证据表明, 促炎性细胞因子TNF-α在急性肝损伤的发生发展中发挥了关键作用[19]。在本研究

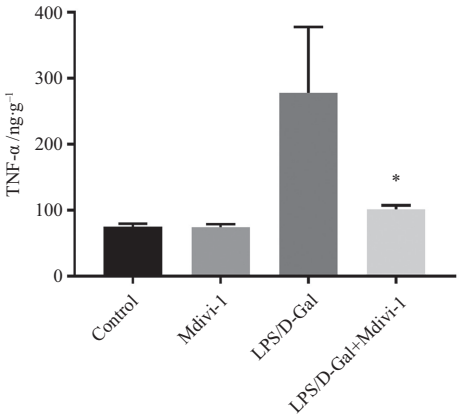


Control: 正常对照组; Mdivi-1: Mdivi-1单独处理组; LPS/D-Gal: LPS/D-Gal单独处理组; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1处理组。n=8, **P<0.01, 与LPS/D-Gal组比较。

Control: Control group; Mdivi-1: Mdivi-1 injected group; LPS/D-Gal: LPS/D-Gal injected group; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1 injected group. n=8, **P<0.01 compared with LPS/D-Gal.

图3 Mdivi-1抑制LPS/D-Gal诱导的血浆ALT上升

Fig.3 Mdivi-1 inhibits LPS/D-Gal-induced elevation of ALT in plasma

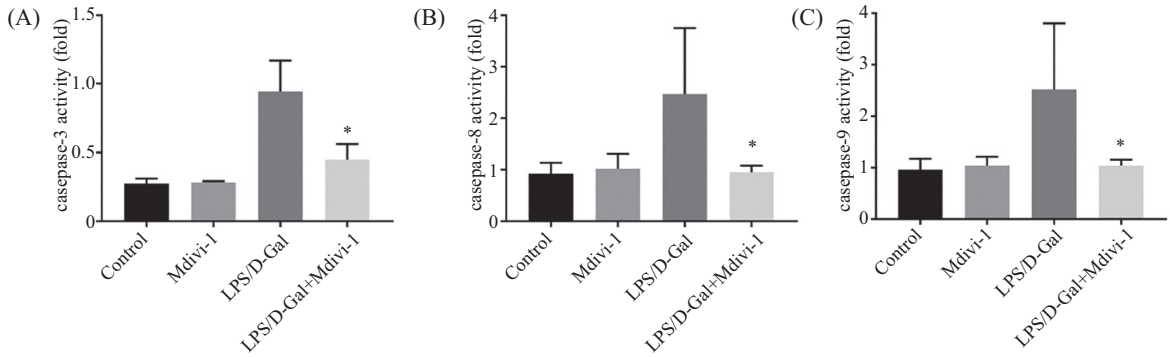


Control: 正常对照组; Mdivi-1: Mdivi-1单独处理组; LPS/D-Gal: LPS/D-Gal单独处理组; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1处理组。n=8, *P<0.05, 与LPS/D-Gal组比较。

Control: Control group; Mdivi-1: Mdivi-1 injected group; LPS/D-Gal: LPS/D-Gal injected group; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1 injected group. n=8, *P<0.05 compared with LPS/D-Gal.

图4 Mdivi-1抑制LPS/D-Gal诱导的TNF-α的表达

Fig.4 Mdivi-1 inhibits LPS/D-Gal-induced TNF-α expression

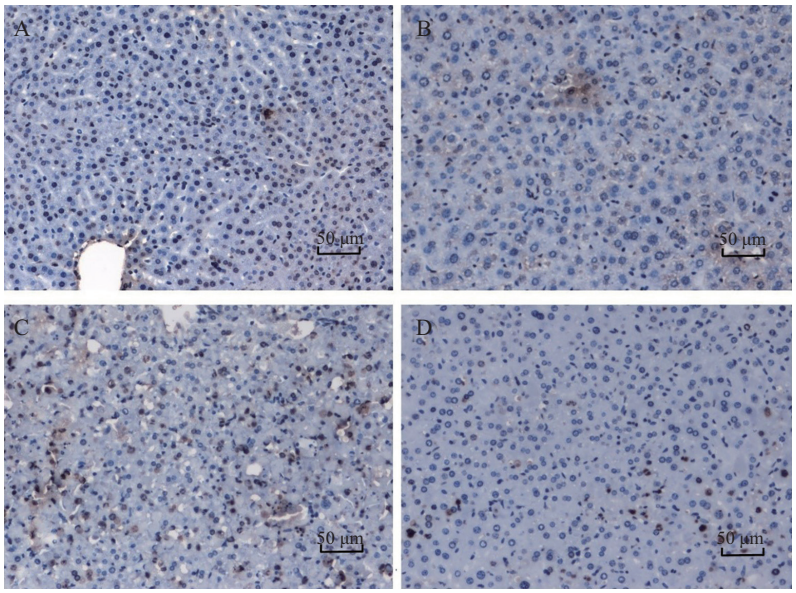


A: casepase-3活性检测; B: casepase-8活性检测; C: casepase-9 活性检测。 n=8, *P<0.05, 与LPS/D-Gal组比较。

A: the acitivity of casepase-3; B: the acitivity of casepase-8; C: the acitivity of casepase-9. n=8, *P<0.05 compared with LPS/D-Gal.

图5 Mdivi-1抑制LPS/D-Gal诱导的casepase-3、casepase-8和casepase-9激活

Fig.5 Mdivi-1 inhibits LPS/D-Gal-induced activation of casepase-3, casepase-8 and casepase-9

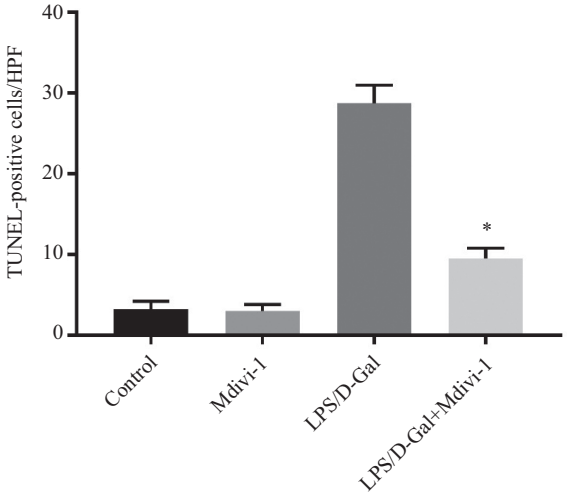


A: 对照组; B: Mdivi-1单独处理组; C: LPS/D-Gal单独处理组; D: LPS/D-Gal+Mdivi-1处理组。

A: control group; B: Mdivi-1 injected group C: LPS/D-Gal injected group D: LPS/D-Gal+Mdivi-1 injected group.

图6 Mdivi-1减轻LPS/D-Gal诱导的肝细胞凋亡

Fig.6 Mdivi-1 reduces LPS/D-Gal -induced hepatocyte apoptosis



Control: 正常对照组; Mdivi-1: Mdivi-1单独处理组; LPS/D-Gal: LPS/D-Gal单独处理组; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1处理组。n=8, *P<0.05, 与LPS/D-Gal组比较。

Control: Control group; Mdivi-1: Mdivi-1 injected group; LPS/D-Gal: LPS/D-Gal injected group; LPS/D-Gal+Mdivi-1: LPS/D-Gal+Mdivi-1 injected group. n=8, *P<0.05 compared with LPS/D-Gal.

图7 TUNEL阳性细胞计数

Fig.7 The count of TUNEL positive cells

中, LPS/D-Gal诱导的TNF-α表达上调可被Drp1抑制剂Mdivi-1阻断, 提示LPS/D-Gal诱导的全身性炎症反应被Mdivi-1所抑制。与此结果相一致, 在肝组织病理学检查中, LPS/D-Gal诱导的肝组织内炎性细胞浸润在Mdivi-1干预组也明显减轻。研究表明, 炎症的发生发展常伴随线粒体功能障碍[20-21], 而受损的线粒体又可通过释放大量活性氧、线粒体DNA(mtDNA)等进一步加重炎症反应和组织损伤[20,22]。在本研究中, Mdivi-1干预能显著抑制TNF-α的诱导表达，也说明线粒体在推动炎症反应的发生发展中发挥了重要作用。我们的实验也发现, 在抑制TNF-α表达的同时, Mdivi-1可显著降低血浆ALT和AST水平, 说明抑制炎症反应可能与Mdivi-1对肝损伤的保护效应密切相关。

除其强大的促炎效应外, TNF-α还具有强烈的促细胞凋亡活性[23-24], TNF-α信号传导最终导致caspase的级联切割和激活, 这是细胞凋亡的主要执行通路[25-26]。LPS/D-Gal诱发的急性肝损伤可分为两个阶段。在早期阶段, 炎症细胞被迅速激活并产生TNF-α, 这一反应通常在LPS/D-Gal刺激后1.5 h达到峰值[27]。而在晚期阶段, 高水平的TNF-α则通过死亡受体依赖途径诱导肝细胞大量凋亡[28]。因此, 抑制早期TNF-α的产生或抑制晚期TNF-α介导的促凋亡信号都可有效减轻LPS/D-Gal诱导的急性肝损伤[29]。实际上, 大量的实验研究也已经观察到抗炎药物或抗凋亡试剂可在LPS/D-Gal模型中发挥保护效应[30-32]。此研究中, LPS/D-Gal诱导的casepase-3、casepase-8和casepase-9升高被Mdivi-1抑制。与此一致, 经Mdivi-1处理的LPS/D-gal模型小鼠中TUNEL阳性细胞较少, 表明Mdivi-1抑制LPS/D-Gal诱导的细胞凋亡。因而, 抑制肝细胞凋亡可能也是Mdivi-1发挥保肝效应的重要环节。

近年来的研究表明, 线粒体处于分裂(fission)和融合(fusion)的动态平衡之中，这种动力学调节对线粒体的形态和功能有极为重要影响[33]。线粒体动力学调节在细胞凋亡中也发挥了至关重要的调节作用, 细胞凋亡过程往往伴随线粒体的分裂, 而线粒体的分裂往往是细胞凋亡的早期变化[34]。Drp1是促进线粒体分裂的关键蛋白, Mdivi-1通过抑制Drp1调节了线粒体分裂, 可能是本研究中Mdivi-1抑制肝细胞凋亡的主要机制。

综上所述, 研究表明, 采用Drp1抑制剂Mdivi-1干预可抑制肝内炎症反应, 减轻凋亡反应, 从而缓解肝组织损伤。虽然Mdivi-1发挥抗炎、抗凋亡保护效应的详细下游分子机制仍有待进一步的研究, 但研究初步表明, Drp1可能是肝组织损伤的重要干预靶点, 而Drp1抑制剂Mdivi-1可能在防治肝损伤中具有潜在应用价值。

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