α-酮戊二酸减轻脂多糖/*D*-半乳糖胺诱导的小鼠急性肝损伤

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摘要 该文旨在探讨α-酮戊二酸对脂多糖(lipopolysaccharide, LPS)及*D*-半乳糖胺(d-galactosamine, *D*-Gal)诱导的急性肝损伤发生发展的影响及其可能机制。实验分组: 正常对照组、AKG单独处理组、LPS/*D*-Gal组、LPS/*D*-Gal+AKG组。在雄性BALB/c小鼠中, 经腹腔注射LPS/*D*-Gal诱导急性肝损伤, α-酮戊二酸在LPS/*D*-Gal注射前0.5 h经腹腔注入。6 h后, 处死动物, 采集动物血浆及肝脏, 采用比色法检测血浆转氨酶水平以评估肝损伤情况; 采用HE染色观察肝组织病理学改变; 采用TUNEL染色和比色法检测caspase-3、caspase-8和caspase-9的活性; Western blot检测切割型caspase-3(cleaved caspase-3)片段含量, 反映肝细胞凋亡情况。结果显示, α-酮戊二酸干预显著下调LPS/*D*-Gal暴露小鼠血浆中天冬氨酸氨基转氨酶(aspartate transaminase, AST)与丙氨酸氨基转氨酶(alanine transaminase, ALT)水平, 有效降低肝组织中caspase-3、caspase-8和caspase-9的活性以及切割型caspase-3片段的含量, 明显减轻TUNEL阳性肝细胞数目以及肝组织病理学改变。以上结果提示, α-酮戊二酸可减轻LPS/*D*-Gal诱导的肝细胞凋亡及肝组织损伤。

关键词 α-酮戊二酸; 急性肝损伤; LPS/*D*-Gal; 凋亡; caspase-3

Alpha-Ketoglutarate Attenuates Endotoxin/*D*-Galactosamine-Induced Acuteliver Injury in Mice

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Abstract The paper aimed to explore the effect of alpha-ketoglutarate on the occurrence and development of acute liver injury induced by LPS (lipopolysaccharide) and *D*-Gal (*D*-galactosamine) and the underlying mechanism. Experimental groups: Control, AKG group, LPS/*D*-Gal group, LPS/*D*-Gal+AKG group. In male BALB/c mice, acute liver injury was induced by intraperitoneal injection of LPS/*D*-Gal. Alpha-ketoglutarate was injected intraperitoneally 0.5 h prior LPS/*D*-Gal. The mice were sacrificed at 6 h after LPS/*D*-Gal challenging, and plasma samples were collected for measuring the level of plasma transaminase by colorimetry to evaluate the liver injury. The pathological changes of liver tissue were observed by HE staining. The activities of caspase-3, caspase-8 and caspase-9 were detected by colorimetry. The apoptotic cell was estimated by TUNEL assay, and the level of cleaved caspase-3 was detected by Western blot to imply hepatocyte apoptosis. The results showed that alpha-ketoglutarate-treated LPS/*D*-Gal-exposed mice had significantly down-regulated incidence of histologic lesions, lower plasma

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AST (aspartate aminotransferase) and ALT (alanine aminotransferase). Treatment with alpha-ketoglutarate also reduced cleaved caspase-3 and caspase-3, caspase-8, caspase-9 activities and reduced the count of TUNEL-positive hepatocytes. Alpha-ketoglutarate can attenuate the pathological changes of acute liver injury induced by LPS/*D*-Gal and exert the protective effect of anti-apoptosis.

Keywords alpha-ketoglutarate; acute liver injury; LPS/*D*-Gal; apoptosis; caspase-3

肝脏是人体的重要代谢器官, 对维持机体代谢和内环境稳态至关重要; 肝脏也是重要的解毒器官, 具有强大的清除病原微生物和毒素的能力, 在宿主防御反应中发挥关键作用[1]。但肝脏也是酒精、药物、毒素、感染等损伤因子攻击的主要部位, 这些致病因子诱发的急性肝损伤是全球高发疾病, 严重者可发生肝功能衰竭, 成为重大的健康威胁[2]。因而, 探索急性肝损伤的防治药物具有重要的意义。

脂多糖(lipopolysaccharide, LPS)是革兰氏阴性菌细胞壁的组成成分, 也被称为内毒素, 具有强烈的致炎、致损伤效应[3]。LPS可在*D*-半乳糖胺(d-(+)-galactosamine hydrochloride, *D*-Gal)致敏的小鼠中选择性诱导急性肝组织损伤, 大量的肝细胞凋亡是其重要的病理特征[4]。这一模型被广泛应用于急性肝损伤发病机制及防治药物的研究[5]。

α-酮戊二酸(alpha-ketoglutarate, AKG)是三羧酸循环的中间代谢产物, 除可参与供能代谢外, 近年来的研究发现, AKG还具有抗氧化、抗肿瘤及免疫调节功能[6-8]。有研究表明, AKG可在H_2O_2诱导的肠上皮细胞损伤中发挥抗凋亡保护作用[9], AKG也可抑制氰化物诱导的神经细胞凋亡[10], 而含有AKG的氨基酸缓冲液可在大鼠脑缺血再灌注损伤中发挥抗凋亡保护作用[11]。那么, AKG是否也可在LPS/*D*-Gal诱导的肝损伤中发挥保护作用呢? 本文在LPS/*D*-Gal诱导的急性肝损伤小鼠模型中, 观察了AKG对肝组织损伤程度及肝细胞凋亡的调节效应。

1 材料和方法

1.1 材料

1.1.1 *动物* 雄性BALB/c小鼠(18~22 g, 6~8周龄), 购于重庆医科大学动物实验中心。动物饲养在标准动物室[室温: (20~25) °C, 湿度: (50±5)%]特定的无病原体环境中, 按12 h:12 h的光/暗周期进行饲养, 自由饲喂标准实验室食物和水。所有实验均按重庆医科大学医学动物保护委员会批准的规程进行(批准号: SYXK(渝)2018-0003)。

1.1.2 *试剂* LPS(生产批号: 039M4004V), *D*-Gal(生产批号: 091M1372V)和AKG(生产批号: 1002753196)均购于美国Sigma公司; 天冬氨酸氨基转移酶(aspartate aminotransferase, AST)和丙氨酸氨基转移酶(alanine aminotransferase, ALT)试剂盒(生产批号: 20200611)均购于中国南京建成生物工程研究所; Bradford蛋白定量测定试剂盒(生产批号: P0006-1), caspase-3活性检测试剂盒(生产批号: 062619191107), caspase-8活性检测试剂盒(生产批号: 040119191213), caspase-9活性检测试剂盒(生产批号: 060618200706)均购于中国碧云天生物工程研究所; TUNEL检测试剂盒(生产批号: 42134700)购于英国Roche公司; 切割型caspase-3兔单克隆抗体(生产批号: 21)、辣根过氧化物酶标记的羊抗兔(生产批号: 26)、羊抗鼠二抗(生产批号: 34)均购于美国Cell signaling公司; β-actin 抗体(生产批号: 4AL261918F)购于中国四正柏生物科技有限公司; 12% SDS-聚丙烯酰胺凝胶(生产批号: 024A1250)购于中国上海雅酶生物科技有限公司; BCA蛋白定量试剂盒(生产批号: TF268082), 快速增强化学发光(electro-chemi-luminescence, ECL)试剂盒(生产批号: 190115-36)均购于美国Pierce公司。

1.2 方法

1.2.1 *动物模型* 通过腹腔注射LPS(10 μg/kg)联合*D*-Gal(700 mg/kg)建立急性肝损伤模型, LPS/*D*-Gal均溶于生理盐水。32只实验小鼠分为四组, 每组8只。正常对照组: LPS/*D*-Gal(-)、AKG(-); 单独处理组: LPS/*D*-Gal(-)、AKG(+); 模型组: LPS/*D*-Gal(+)、AKG(-); 干预组: LPS/*D*-Gal(+)、AKG(+)。AKG(50 mg/kg)溶于生理盐水, 在注入LPS/*D*-Gal前0.5 h经腹腔注入小鼠。各组小鼠均自由饮食, 在注入LPS/*D*-Gal后6 h经腹腔注射1%戊巴比妥钠, 处死小鼠, 采集血浆和肝组织标本。

1.2.2 *转氨酶检测* 血液标本在4 °C、5 000 r/min条件下离心10 min, 取上层血浆按比例稀释, 根据转氨酶检测试剂盒说明书采用比色法进行检测。在波长505 nm处检测吸光度值, 根据标准曲线计算AST和

ALT的酶活力。

1.2.3 组织学分析 小鼠肝右叶固定于4%多聚甲醛, 石蜡包埋。肝组织切片用苏木精–伊红(hematoxylin and eosin, H&E)染色, 在光学显微镜(olympus, Japan)下观察肝组织病理学改变。Suzuki评分作为染色切片的评估标准[12]。

1.2.4 caspase活性检测 肝组织caspase-3、caspase-8和caspase-9活性检测根据caspase活性检测试剂盒说明书采用比色法进行检测。称取30 mg肝组织, 加入裂解液, 匀浆, 4 ℃、16 000 ×*g*离心15 min, 取上清液用于检测。Bradford检测蛋白含量, 每个样本设立样本孔和对照孔, 在405 nm处测出吸光度值。

1.2.5 细胞凋亡检测 4%多聚甲醛固定肝组织, 石蜡包埋, 肝组织切片, 根据TUNEL检测试剂盒说明书检测凋亡细胞。用二甲苯脱蜡 1 h; 依次用100%酒精、90%酒精、80%酒精、70%酒精进行脱水, PBS洗3次; 滴加蛋白酶K于37 ℃孵育30 min, PBS洗3次; 滴加含0.1 g枸盐酸钠的0.1% TristonX-100于4 ℃孵育4 min, PBS洗3次; 滴加H_2O_2室温孵育20 min, PBS洗3次; 滴加TUNEL反应液37 ℃孵育60 min, PBS洗3次; 滴加过氧化物酶(peroxidase, POD) 37 ℃孵育30 min, PBS洗3次; DAB显色; 苏木精复染, 烘干, 树脂盖片。在光学显微镜(Olympus, Japan)下观察凋亡细胞的数量。

1.2.6 免疫印迹 称取肝组织30 mg, 加入300 μL裂解液、6 μL磷酸化酶抑制剂、3 μL PMSF, 于4 ℃、12 000 ×*g*条件下离心15 min。收集上清液根据BCA法检测蛋白浓度, 计算上样体积。制备12% SDS-快速凝胶, SDS-PAGE电泳分离蛋白; 电转至硝酸纤维素膜, 5%脱脂奶粉室温封闭2 h; 一抗切割型caspase-3抗体(1:1 000)、β-actin抗体(1:1 000), 4 ℃孵育过夜。复温, TBST洗3次; 室温孵育二抗(羊抗兔1:2 000, 羊抗鼠(1:4 000) 2 h, TBST洗3次; 采用ECL发光显影, 使用Image J对条带进行灰度分析。

1.2.7 统计学分析 所有计量资料均按均数±标准差($\bar{x}\pm s$)表示, 统计学意义采用GraphPad Prism 5.0软件进行分析。多组间统计学差异使用单因素方差分析(One-Way ANOVA), 两组间差异使Turkey检验进行比较。$P<0.05$表示具有统计学差异。

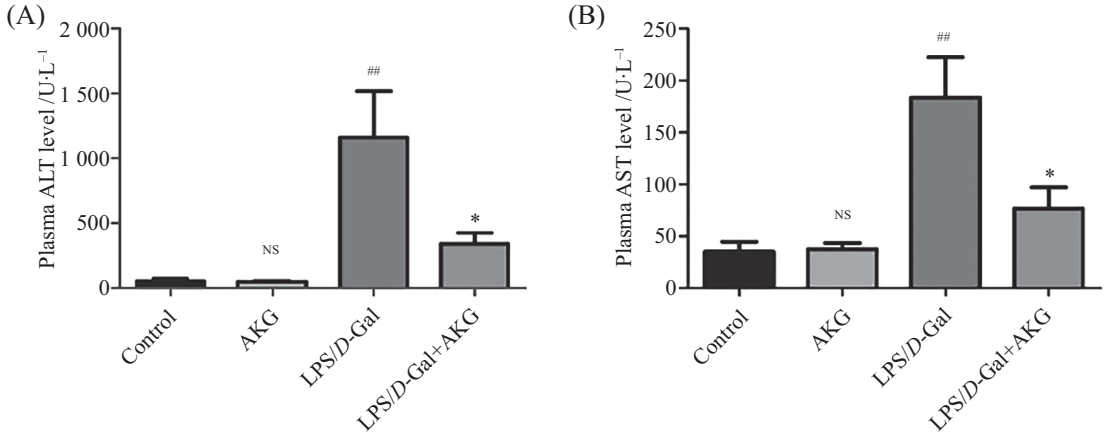
2 结果与分析

2.1 AKG降低LPS/*D*-Gal诱导的小鼠急性肝损伤转氨酶水平

血浆中转氨酶水平是反应肝损伤的常用指标[13], 我们检测了小鼠血浆中AST和ALT的水平以反映肝损伤程度。结果显示: AKG单独处理组与正常对照组无差异($P>0.05$), LPS/*D*-Gal模型组AST和ALT明显高于正常对照组($P<0.01$); 而AKG处理组血浆AST和ALT明显低于模型组($P<0.05$)(图1)。这一结果表明, AKG处理可降低模型小鼠肝损伤程度。

2.2 AKG减轻LPS/*D*-Gal诱导的小鼠急性肝组织病理学改变

在肝组织病理切片H&E染色后发现, LPS/

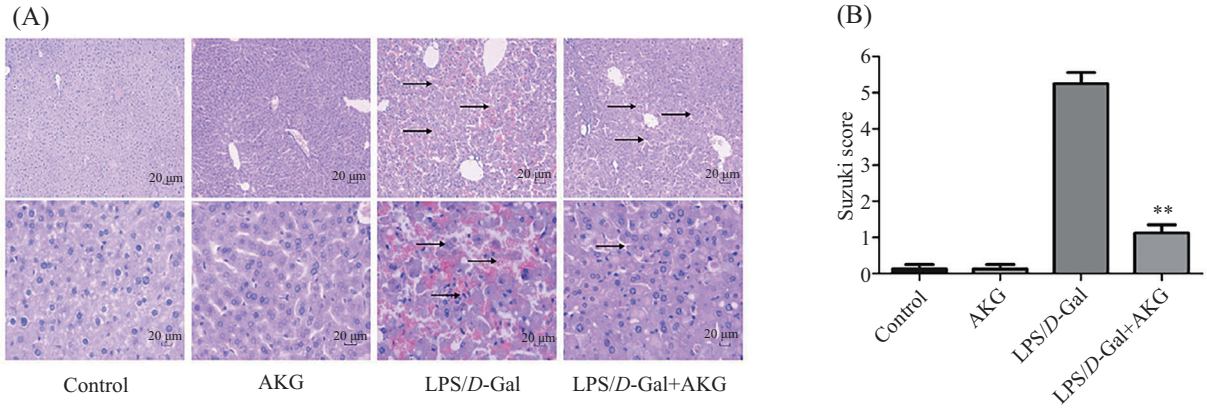


A: AKG可降低血浆ALT水平。B: AKG可降低血浆中AST水平。Control: 正常对照组; AKG: AKG单独处理组; LPS/*D*-Gal: LPS/*D*-Gal单独处理组; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG处理组。n=8, $^{NS}P>0.05$, 与正常对照组相比; $^{\#\#}P<0.01$, 与正常对照组相比; $^{*}P<0.05$, 与LPS/*D*-Gal组相比。

A: AKG reduces ALT in plasma. B: AKG reduces AST in plasma. Control: control group; AKG: AKG group; LPS/*D*-Gal: LPS/*D*-Gal group; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG group. n=8, $^{NS}P>0.05$ *vs* control group, $^{\#\#}P<0.01$ *vs* control group, $^{*}P<0.05$ *vs* LPS/*D*-Gal group.

图1 AKG降低LPS/*D*-Gal诱导的小鼠急性肝损伤转氨酶水平

Fig.1 AKG reduced LPS/*D*-Gal-induced acute liver injury in mice serum transaminase levels



A: Control组肝组织形态结构未见异常, 未见明显肝窦出血及肝细胞坏死; AKG组, 肝组织形态结构未见异常, 未见明显肝窦出血及肝细胞坏死; LPS/*D*-Gal组箭头所示小鼠肝组织肝窦出血及炎症细胞浸润明显; LPS/*D*-Gal+AKG组箭头所示小鼠肝组织肝窦出血及炎症细胞浸润明显减轻。肝组织切片用苏木精–伊红染色, 选取每组有代表性的肝切片。B: Suzuki评分。Control, 正常对照组; AKG, AKG单独处理组; LPS/*D*-Gal, LPS/*D*-Gal单独处理组; LPS/*D*-Gal+AKG, LPS/*D*-Gal+AKG处理组; n=8, $^{**}P$<0.01, 与LPS/*D*-Gal组相比。

A: Control, the morphology and structure of liver tissue showing normal tissue with on evidence of abnormalities, and no obvious hepatic sinusoid hemorrhage and hepatocyte necrosis were found; AKG, the morphology and structure of liver tissue showing normal tissue with on evidence of abnormalities, and no obvious hepatic sinusoid hemorrhage and hepatocyte necrosis were found; LPS/*D*-Gal, the arrows showed obvious hepatic sinusoid hemorrhage and inflammatory cell infiltration in the liver tissue of mice; LPS/*D*-Gal+AKG, the hepatic sinusoid hemorrhage and inflammatory cell infiltration in the liver tissue of mice shown by arrows were significantly reduced. Liver tissue sections were stained with hematoxylin-eosin, and the representative liver sections of each group were selected. B: Suzuki score. Control, control group; AKG, AKG group; LPS/*D*-Gal, LPS/*D*-Gal group; LPS/*D*-Gal+AKG, LPS/*D*-Gal+AKG group; n=8, $^{**}P$<0.01 *vs* LPS/*D*-Gal group.

图2 AKG减轻 LPS/*D*-Gal诱导的小鼠急性肝组织病理学改变

Fig.2 AKG attenuates LPS/*D*-Gal-induced acute liver histopathological changes in mice

D-Gal模型组有大量的肝细胞死亡, 并存在红细胞淤积在肝小叶中, 大量肝索结构被破坏的情况; 而在AKG处理之后肝细胞死亡、肝索结构破坏和红细胞淤积情况明显减轻(图2); 这一结果也表明, AKG处理能减轻LPS/*D*-Gal诱导的急性肝组织损伤。

2.3 AKG抑制caspase的激活

在LPS/*D*-Gal暴露后6 h获取肝组织样本, 检测了caspase-3、caspase-8及caspase-9的活性。结果显示, AKG单独处理组小鼠与正常对照组小鼠中3种caspase酶活性无差异(P>0.05), LPS/*D*-Gal组小鼠肝组织caspase-3、caspase-8及caspase-9活性均明显高于正常对照组(P<0.01); AKG处理组中肝组织caspase-3、caspase-8及caspase-9蛋白酶活性均明显低于模型组(P<0.01)(图3)。我们也采用Western blot检测了肝组织中切割型caspase-3蛋白含量, 结果显示, AKG单独处理组与正常对照组无差异(P>0.05); LPS/*D*-Gal模型组切割型caspase-3明显高于正常对照组(P<0.01); AKG处理组肝内切割型caspase-3蛋白明显低于LPS/*D*-Gal模型组(P<0.01)(图4)。结果表明, AKG干预可以抑制LPS/*D*-Gal诱导的caspase-3的激活。

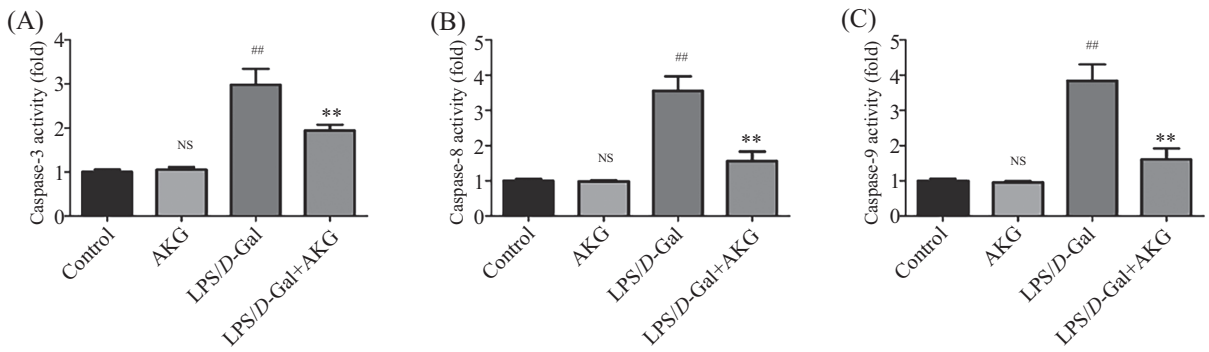
2.4 AKG抑制LPS/*D*-Gal诱导的肝细胞凋亡

TUNEL染色可将凋亡细胞的细胞核染成棕黄色, 伴随细胞皱缩、染色质凝集和凋亡小体的形成等典型的形态学改变。结果显示, 正常对照组及AKG单独处理组之间未见棕黄色TUNEL(+)细胞, LPS/*D*-Gal模型组可见大量胞核被染成棕黄色的凋亡细胞。而AKG处理组TUNEL(+)细胞显著少于LPS/*D*-Gal模型组(图5)。结果表明: AKG可抑制LPS/*D*-Gal诱导的肝细胞凋亡。

3 讨论

AKG具有多种生理功能, 包括参与三羧酸循环、调节肾小管酸碱平衡等[14]。近年来的研究还发现, AKG可在缺血再灌注诱导的急性肾损伤[15]、LPS诱导的急性肺损伤[16]等动物模型中发挥保护作用。在本研究中, 我们发现, AKG能显著减轻LPS/*D*-Gal诱导的急性肝损伤, 表现为AKG可抑制血浆转氨酶升高并改善组织病理学异常。本研究结果提示, AKG在急性肝损伤中可能具有一定的应用前景。

大量的肝细胞凋亡是LPS/*D*-Gal诱导的急性肝损伤的重要病理特征, 本研究发现, AKG处理后肝组织内TUNEL(+)凋亡细胞数目明显减少, 提示AKG在LPS/*D*-Gal诱导的急性肝损伤模型中的保护效应可能与其抗凋亡效应有关。与我们的结果相一致,

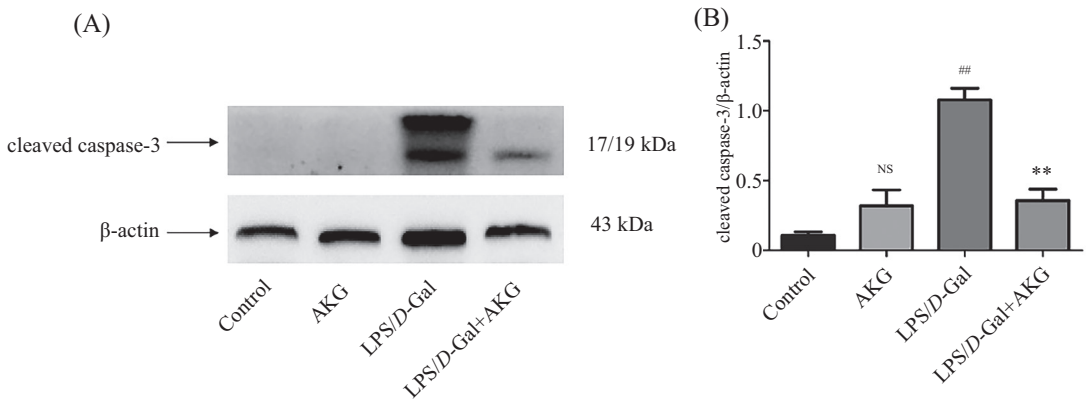


A: AKG降低肝组织caspase-3活性。B: AKG降低肝组织caspase-8活性。C: AKG降低肝组织caspase-9活性。Control: 正常对照组; AKG: AKG单独处理组; LPS/*D*-Gal: LPS/*D*-Gal单独处理组; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG处理组。n=8, ^{NS}P>0.05, 与正常对照组相比; $^{\#\#}P$<0.01, 与正常对照组相比; $^{**}P$<0.01, 与LPS/*D*-Gal组相比。

A: AKG reduces the activity of hepatic caspase-3. B: AKG reduces the activity of hepatic caspase-8. C: AKG reduces the activity of hepatic caspase-9. Control: control group; AKG: AKG group; LPS/*D*-Gal: LPS/*D*-Gal group; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG group. n=8; ^{NS}P>0.05 *vs* control group; $^{\#\#}P$<0.01 *vs* control group; $^{**}P$<0.01 *vs* LPS/*D*-Gal group.

图3 AKG抑制caspase的激活

Fig.3 AKG inhibits the activation of caspase



A: 用免疫印迹法检测肝组织中切割型caspase-3水平。切割型caspase-3和β-actin条带用箭头表示。B: 切割型caspase-3与β-actin相对含量。Control: 正常对照组; AKG: AKG单独处理组; LPS/*D*-Gal: LPS/*D*-Gal单独处理组; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG处理组。n=4, ^{NS}P>0.05, 与正常对照组相比; $^{\#\#}P$<0.01, 与正常对照组相比; $^{**}P$<0.01, 与LPS/*D*-Gal组相比。

A: the levels of cleaved caspase-3 in the liver were determined by immunoblot analysis. The bands of cleaved caspase-3 and β-actin are indicated by arrows. B: relative content of cleaved caspase-3 and β-actin. Control: control group; AKG: AKG group; LPS/*D*-Gal: LPS/*D*-Gal group; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG group. n=4; ^{NS}P>0.05 *vs* control group; $^{\#\#}P$<0.01 *vs* control group; $^{**}P$<0.01 *vs* LPS/*D*-Gal group.

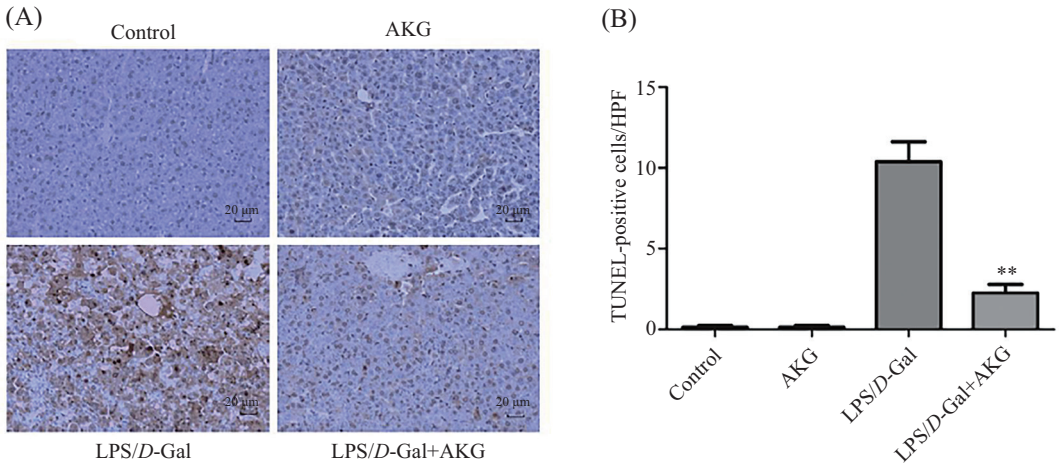
图4 AKG抑制cleaved caspase-3的产生

Fig.4 AKG suppressed the production of cleaved caspase-3

置于含有或不含有AKG和H_2O_2的杜尔贝科氏改良培养基中, AKG可降低猪肠上皮细胞(J2)中caspase-3的活性、抑制细胞凋亡, 从而保护肠道细胞免受H_2O_2引起的损伤[9]。此外, 在人肺腺癌细胞H1975中, AKG处理后减少低氧诱导的细胞凋亡[17]。由此可见, AKG对急性肝损伤的保护效应可能与其抗凋亡效应有关。

caspase级联激活是诱导细胞凋亡的主要信号通路。研究表明, caspase-3、caspase-8以及caspase-9在急性肝损伤中发挥重要作用[18-20]。在细胞凋亡过程中, caspase-8被裂解活化, 活化的caspase-8进一步激活促凋亡蛋白, 使大量细胞色素c释放到细胞质, 募集caspase-9酶原, 将募集的caspase-9酶原催化分解为活性分子, 从而活化caspase-3凋亡蛋白，进一步促进细胞凋亡的发生[21]。本研究中, AKG可抑制LPS/*D*-Gal诱导的caspase-3, caspase-8及caspase-9的活化, 进一步表明AKG通过抑制细胞凋亡而在LPS/*D*-Gal诱导的肝损伤中发挥保护作用。

目前, 关于AKG抗凋亡分子机制和信号通路并不十分清楚。在骨肉瘤细胞MHM和SJSA1中的



A: TUENL检测法检测肝细胞凋亡情况, TUNEL阳性细胞染色为深棕色。B: TUNEL阳性细胞计数。Control: 正常对照组; AKG: AKG单独处理组; LPS/*D*-Gal: LPS/*D*-Gal单独处理组; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG处理组。n=8; **P<0.01, 与LPS/*D*-Gal组相比。

A: the apoptosis of hepatocytes was detected by TUENL, and the TUNEL positive cells were stained dark brown. B: the count of TUNEL positive cells. Control: control group; AKG: AKG group; LPS/*D*-Gal: LPS/*D*-Gal group; LPS/*D*-Gal+AKG: LPS/*D*-Gal+AKG group. n=8; **P<0.01 *vs* LPS/*D*-Gal group.

图5 AKG抑制LPS/*D*-Gal诱导的肝细胞凋亡

Fig.5 AKG inhibits hepatocyte apoptosis induced by LPS/*D*-Gal

研究表明, AKG可通过促进自噬来减少p53激活剂Nutlin-3a诱导的细胞凋亡[22]; 在H_2O_2诱导的肠上皮细胞损伤中, AKG增加抗凋亡因子Bcl-2与促凋亡因子Bax的比例、稳定线粒体膜电位(mitochondrial membrane potential, MMP)减少细胞色素c的释放, 从而发挥抗凋亡保护效应[9]。本研究虽发现, AKG可抑制LPS/*D*-Gal诱导的肝细胞凋亡, 但相关分子机制仍有待进一步的深入研究。

综上所述, 本研究发现AKG干预可在LPS/*D*-Gal诱导的急性肝损伤中发挥保护效应, 这一效应可能与其抑制LPS/*D*-Gal诱导的肝细胞凋亡有关。虽然AKG抗凋亡的下游分子机制有待进一步研究, 但本研究结果初步提示, AKG可能在急性肝损伤中具有开发应用前景。

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