

doi: 10.13241/j.cnki.pmb.2018.20.007

补骨脂酚通过激活 ROS 强化 TRAIL 的抗肝细胞癌 HepG2 细胞作用的机制研究*

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摘要 目的:探究补骨脂酚(Bakuchiol, Bak)对肿瘤坏死因子相关凋亡诱导配体(Tumor necrosis factor- related apoptosis-inducing ligand, TRAIL)抗 HepG2 细胞作用的影响及内在机制。**方法:**常规培养 HepG2 细胞, 给予梯度浓度的 Bak 处理, 检测细胞活力。联合应用 Bak 与 TRAIL 处理, 检测细胞活力。Western blot 检测 Bak 处理后氧化应激水平、死亡受体 4(Death Receptor 4, DR4)、DR5 的表达变化。联合应用 Bak 与 TRAIL 检测凋亡情况。进而引入 ROS 清除剂 NAC, 联合 NAC 处理后, 检测 ROS、DR4、DR5 以及凋亡情况。**结果:** Bak 剂量依赖地抑制了 HepG2 细胞的活力, 联合应用 Bak+TRAIL 对细胞活力的抑制作用优于单独用药。Bak 处理后氧化应激水平升高, 体现在 ROS 增加, GSH 水平下降; Western blot 检测发现 Bak 处理后 DR4、DR5 表达增加。联合应用 Bak+TRAIL 显著增加了细胞凋亡蛋白 Bax 的表达, 抑制了抗凋亡蛋白 Bcl2 的表达。引入 ROS 阻断剂 NAC 处理后, 与 Bak+TRAIL 组相比, ROS 水平下降, DR4、DR5 表达减少。凋亡检测发现 NAC 处理降低了 Bak+TRAIL 引起的细胞凋亡。**结论:** Bak 可以显著增强 TRAIL 引起的 HepG2 细胞凋亡, 该作用可能与 Bak 激活氧化应激而上调 DR4、DR5 表达有关。

关键词:肝细胞肝癌; 补骨脂酚; 肿瘤坏死因子相关凋亡诱导配体; 氧化应激; 死亡受体

中图分类号:R-33; R735.7 **文献标识码:**A **文章编号:**1673-6273(2018)20-3835-05

Bakuchiol Enhanced TRAIL Induced HepG2 Cell Apoptosis through Activating Oxidative Stress*

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ABSTRACT Objective: To investigate the roles of Bakuchiol (Bak) on HepG2 cell and the effects of co-treatment of Bak and Tumor necrosis factor- related apoptosis-inducing ligand (TRAIL) on HepG2 cells, as well as the inner mechanisms. **Methods:** Cells were treated with different concentrations of Bak or Bak+TRAIL and cell vitality was detected 24 h later. After Bak treatment, cell oxidative stress levels were examined through DCF-DA staining and ROS, GSH detection. Western blot analysis was conducted to detect the expressions of Death receptor 4 (DR4) and DR5. After Bak+TRAIL treatment, cell apoptosis status was detected. Furthermore, ROS scavenger NAC was used to inhibit oxidative stress and DR4, DR5, Bax expressions were detected. **Results:** Both Bak and TRAIL can reduce HepG2 cell vitality. Co-treatment of Bak and TRAIL further reduced the cell vitality. Bak treatment increased ROS production, DR expressions and reduced cellular GSH level. Co-treatment of Bak and TRAIL induced higher level of apoptosis compared with single-drug treatment. Compared with the Bak + TRAIL group, NAC treatment reduced ROS level and reduced DR expression, as well as the Bak+TRAIL induced apoptosis levels. **Conclusion:** Bak enhances TRAIL induced HepG2 cell apoptosis and tumor growth via activating cellular oxidative stress and up regulating DR4 and DR5 expressions.

Key words: Hepatocellular carcinoma; Bakuchiol; Tumor necrosis factor- related apoptosis-inducing ligand; Oxidative stress; Death receptors

Chinese Library Classification(CLC): R-33; R735.7 **Document code:**A

Article ID:1673-6273(2018)20-3835-05

前言

肝细胞癌(Hepatocellular Carcinoma, HCC)是世界范围内

引起肿瘤相关死亡的第三大恶性肿瘤^[1,2]。目前,原发性肝癌的治疗是以手术为主的综合治疗,然而部分患者因为种种原因需要手术外的其他治疗手段,包括放化疗、生物治疗等,然而

* 基金项目:陕西省科学技术研究发展计划项目(2013K12-03-06)

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(收稿日期:2018-02-28 接受日期:2018-03-28)

治疗往往不尽人意。因此新药研发及联合用药成为目前的研究热点^[3,4]。

肿瘤坏死因子相关凋亡诱导配体 (Tumor necrosis factor-related apoptosis-inducing ligand, TRAIL) 是肿瘤坏死因子家族成员,具有诱导多种肿瘤细胞凋亡的作用,且对正常细胞毒性很小,是新近肿瘤治疗药物研发的一大突破^[5,6]。国内外已有研究证实 TRAIL 对 HCC 具有明确的抑制作用^[7,8]。死亡受体 4 (Death Receptor 4, DR4)、DR5 是 TRAIL 发挥促凋亡作用的受体^[9]。值得注意的是,外源干预上调 DR4、DR5 的表达能显著增强 TRAIL 的抗肿瘤作用^[10-12]。那么肝细胞癌中,能否通过调控 DR4、DR5 来强化 TRAIL 的促凋亡作用呢?

补骨脂酚 (Bakuchiol, Bak) 是中药补骨脂的活性成分,是一种异戊二烯基酚类化合物^[13]。既往研究证实 Bak 具有抗多种肿瘤的作用,氧化应激激活是介导 Bak 抗肿瘤作用机制之一^[14-16]。值得注意的是在其他肿瘤中 Bak 可以通过 ROS 强化 TRAIL 的抗肿瘤作用,但肝癌中作用及机制不清。本研究旨在探究补骨脂酚能否强化 TRAIL 的抗肝细胞肝癌的作用及具体机制。

1 材料和方法

1.1 材料

人肝细胞癌细胞系 HepG2 购自中科院上海细胞库。Bak、TRAIL、DCF-DA、NAC 购自 Sigma-Aldrich 公司 (美国)。抗 DR4、DR5、Bcl2、Bax、 β -actin 抗体购自 Cell Signaling Technology (美国)。CCK8 细胞活力检测试剂盒购自七海生物科技有限公司 (中国上海)。活性氧 (reactive oxygen species, ROS)、谷胱甘肽 GSH 检测试剂盒购自南京建成生物研究所 (中国南京)。蛋白定量试剂盒购自 ThermoFisher 公司 (美国)。Western blot 试剂购自索莱宝公司 (中国北京)。细胞培养耗材购自 Corning 公司 (美国); 胎牛血清、RPMI-1640 培养基购自 Hyclone 公司 (美国)。

1.2 方法

1.2.1 细胞培养 HepG2 细胞在完全培养基中常规培养。培养条件为 37℃、5% CO₂。细胞密度达到 80% 左右时进行相应处理。药物处理期间使用无血清培养基。

1.2.2 细胞活力检测 将对数期细胞接种到 96 孔板, 每孔

10000 个细胞。该部分实验分两部分。① 细胞贴壁后分为 4 组, 分别是 Control 组、10 μ M Bak 组、20 μ M Bak 组、40 μ M Bak 组, 给予相应处理 24 h。② 细胞贴壁后分为 4 组分别是 Control 组、20 μ M Bak 组、TRAIL 组、20 μ M Bak + TRAIL 组, 分别给予相应处理 24 h。24 h 后吸出培养基, 加入 CCK8 试剂与 RPMI-1640 培养基的工作液, 37℃、5% CO₂ 条件下继续培养 2 h, 于 450 nm 波长下检测吸光度, 即为其活力值。

1.2.3 DCF-DA 法检测细胞 ROS 水平 将细胞接种至 96 孔板, 细胞贴壁后给予相应处理, 24 h 后弃去培养基, PBS 洗涤 3 次, 加入 10 μ L MDCFH-DA 溶液, 37℃、5% CO₂ 条件下继续培养 30 min, PBS 洗涤 3 次, 于 488 nm 激发波长和 605 nm 观测波长进行观察并采集照片。用 image pro plus 检测荧光强度。

1.2.4 ROS、GSH 检测 提取细胞培养上清或细胞, 严格参照生产商提供的说明书进行操作。

1.2.5 Western blot 检测 细胞处理完毕后, 收集细胞并提取总蛋白。进行蛋白定量后取 30 μ g 总蛋白进行 SDS-PAGE 凝胶电泳。然后将蛋白转移至 PVDF 膜上并用脱脂牛奶封闭。用相应抗体孵育 (DR4、DR5、Bcl2、Bax 按照 1:1000 稀释; β -actin 按照 1:2000 稀释) 于 4℃ 过夜。TBST 洗涤后用相应二抗孵育, 最后用 Bio-Rad 照相系统采集照片并分析。

1.3 统计学分析

所涉及实验均重复 6 次。所有计量资料数据均采用平均值 $\pm$ 标准误 (Mean $\pm$ SEM) 表示。用 SPSS18.0 统计软件进行统计学分析, 计量资料组间比较采用单因素方差分析, $p < 0.05$ 为有统计学差异。

2 结果

2.1 Bak 及 TRAIL 对 HepG2 细胞活力的影响

如图所示, 梯度浓度的 Bak (10 μ M、20 μ M、40 μ M) 处理 24h 后 HepG2 细胞活力呈剂量依赖性下降, 分别下降到对照组的 (96.3 $\pm$ 5.4)%、(72.8 $\pm$ 4.8)%、(44.6 $\pm$ 4.1)% ($P < 0.05$)。联合应用 Bak 与 TRAIL 对 HepG2 细胞的抑制作用更强, 细胞活力分别下降到对照组的 (71.6 $\pm$ 4.3)%、(67.4 $\pm$ 4.9)%、(36.8 $\pm$ 3.9)% ($P < 0.05$), 提示 Bak 处理强化了 TRAIL 的杀伤作用 (如图 1)。

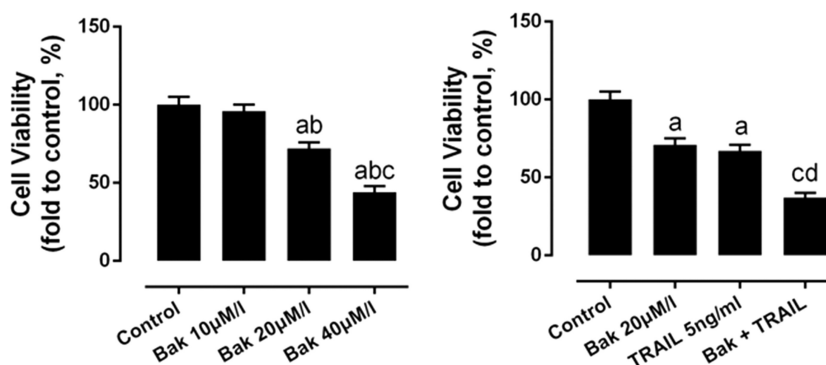


图 1 Bak 及 TRAIL 对 HepG2 细胞活力的影响

Fig. 1 Effects of Bak treatment and TRAIL on HepG2 cell vitality

Note: Data are expressed as $\bar{x} \pm$ SEM, $n=6$. ^a $P < 0.05$, compared with group Control; ^b $P < 0.05$, compared with 10 μ M Bak group; ^c $P < 0.05$, compared with 20 μ M Bak group; ^d $P < 0.05$, compared with TRAIL group. Bak: Bakuchiol

2.2 Bak 处理对 ROS 水平的影响

为进一步明确 Bak 强化 TRAIL 杀伤效果的具体机制,我们检测了细胞 ROS 水平。DCF-DA 荧光染色发现,20 μ M、40 μ M Bak 处理显著增强了其 ROS 水平,ROS 荧光强度分别升高到 5245 ± 254 和 6774 ± 336 荧光单位($P < 0.05$)。ROS 检测试剂盒检测 ROS 结果与荧光染色一致,20 μ M、40 μ M Bak 处理

后 ROS 水平升高到对照组的 $(187.2 \pm 11.4)\%$ 和 $(254.3 \pm 16.2)\%$ ($P < 0.05$)。此外,我们检测了细胞还原型 GSH 水平,发现 20 μ M、40 μ M Bak 处理后还原型 GSH 水平下降到对照组的 $(75.6 \pm 4.9)\%$ 和 $(48.7 \pm 4.1)\%$ ($P < 0.05$),提示氧化应激激活(图 2)。

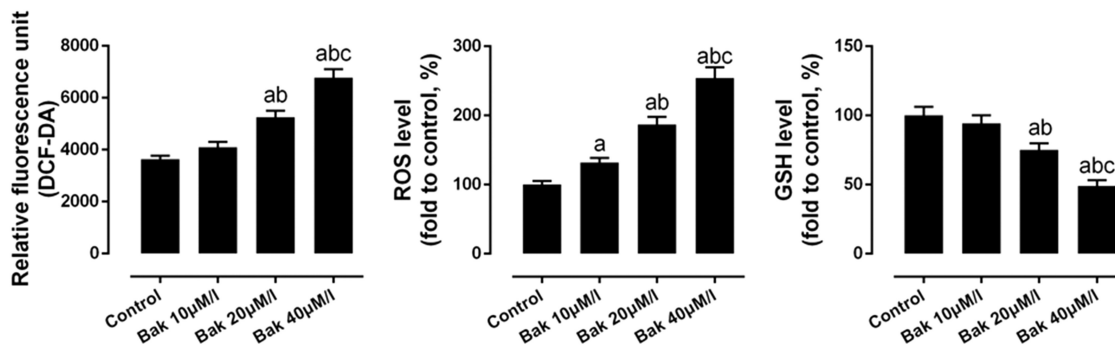


图 2Bak 处理对 ROS 水平的影响

Fig. 2 Effects of Bak treatment on cell ROS level

Note: Data are expressed as $\bar{x} \pm \text{SEM}$, $n=6$. ^a $P < 0.05$, compared with group Control; ^b $P < 0.05$, compared with 10 μ M Bak group; ^c $P < 0.05$, compared with 20 μ M Bak group. Bak: Bakuchiol

2.3 Bak 处理对 TRAIL 受体 DR4、DR5 表达的影响

既往研究证实,Bak 可通过激活氧化应激诱导 DR4 与 DR5 的表达。本实验中通过 Western blot 法检测了 Bak 处理后

的 HepG2 细胞,发现 DR4 与 DR5 的表达均有一定程度的上调($P < 0.05$)(图 3)。

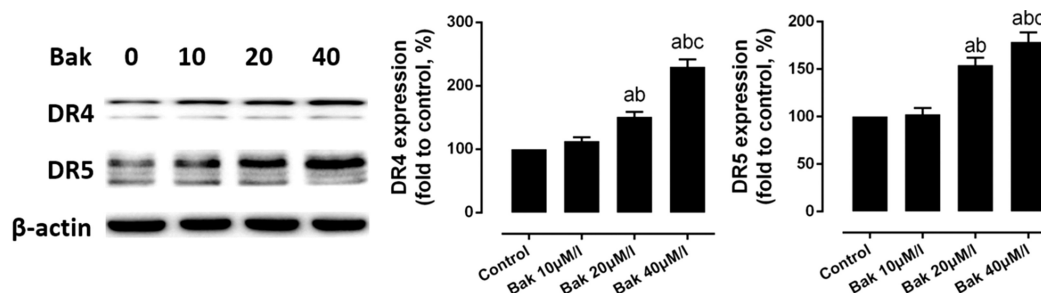


图 3 Bak 处理对 TRAIL 受体 DR4、DR5 表达的影响

Fig. 3 Effects of Bak treatment on DR4 and DR5 expression

Note: Data are expressed as $\bar{x} \pm \text{SEM}$, $n=6$. ^a $P < 0.05$, compared with group Control; ^b $P < 0.05$, compared with 10 μ M Bak group; ^c $P < 0.05$, compared with 20 μ M Bak group. Bak: Bakuchiol

2.4 联合应用 Bak 与 TRAIL 对细胞凋亡的影响

将细胞分为 4 组,分别为 Control 组、Bak 组、TRAIL 组和 Bak+TRAIL 组。给予相应处理后检测凋亡抑制蛋白 Bcl2 以及促凋亡蛋白 Bax 的水平,发现 Bak 与 TRAIL 单独与联合用药都下调了 Bcl2 表达,上调了 Bax 表达(与对照组相比, $P < 0.05$),提示凋亡水平上升,而联合用药凋亡更为显著(分别与 Bak 和 TRAIL 组相比, $P < 0.05$)(图 4)。

2.5 阻断 ROS 对 Bak+TRAIL 联合用药的影响

为进一步明确 ROS 是否介导了 Bak 强化 TRAIL 效能这一作用,我们给予 ROS 清除剂 NAC 共处理。细胞分为 Control 组、Bak+TRAIL 组和 NAC+Bak+TRAIL 组。与 Bak+TRAIL 组相比,NAC+Bak+TRAIL 组细胞活力升高,DR4、DR5 表达下调,凋亡相关蛋白 Bax 表达下调($P < 0.05$),提示 NAC 处理弱化了 Bak+TRAIL 的作用,可能是通过抑制 DR4、DR5 表达来实

现的(图 5)。

3 讨论

近年来,中药活性成分的抗肿瘤作用研究是肿瘤治疗领域的研究热点。Bak 是中药补骨脂的活性成分,已有多项研究证实其对多种肿瘤有明确的抑制作用。Kim 等发现,Bak 可以直接调控造血细胞激酶(Hck)、B 淋巴细胞激酶(Blk)、和 p38 丝裂原激活蛋白激酶(p38MAPK)抑制皮肤癌的增殖^[14]。Park 等人发现,在 TRAIL 耐受的结肠癌细胞系中,Bak 可以通过上调死亡受体的表达来增强细胞对 TRAIL 的敏感性^[17]。值得注意的是,该过程可能是通过激活细胞 c-Jun 氨基末端激酶(JNK)通路和氧化应激来实现的^[17]。Li 等在乳腺癌中的研究发现,Bak 可能通过诱发乳腺癌细胞 S 期停滞,促进细胞凋亡来抑制其增殖^[15]。另一项乳腺癌研究发现,Bak 可以抑制乳腺癌肿瘤干细胞

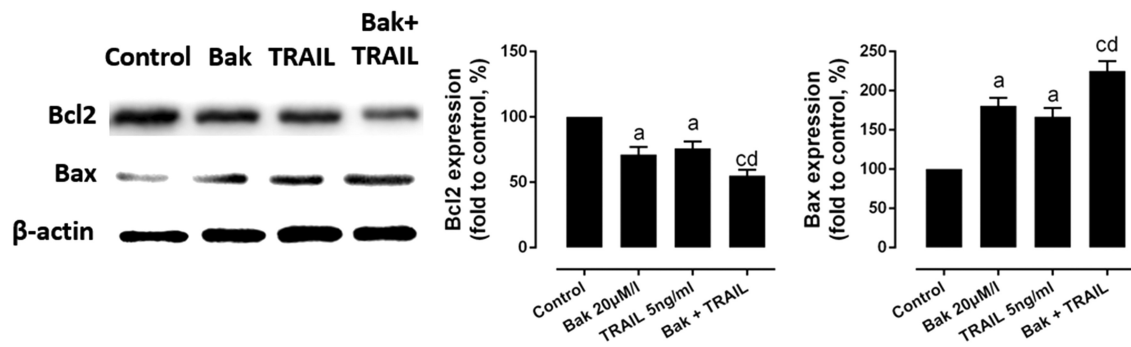


图4 联合应用 Bak 与 TRAIL 对细胞凋亡的影响

Fig. 4 Effects of Bak and TRAIL on cell Bcl2 and Bax expression

Note: Data are expressed as $\bar{x} \pm \text{SEM}$, $n=6$. ^a $P < 0.05$, compared with group Control; ^b $P < 0.05$, compared with 20 μM Bak group; ^c $P < 0.05$, compared with TRAIL 5 ng/ml group. Bak: Bakuchiol

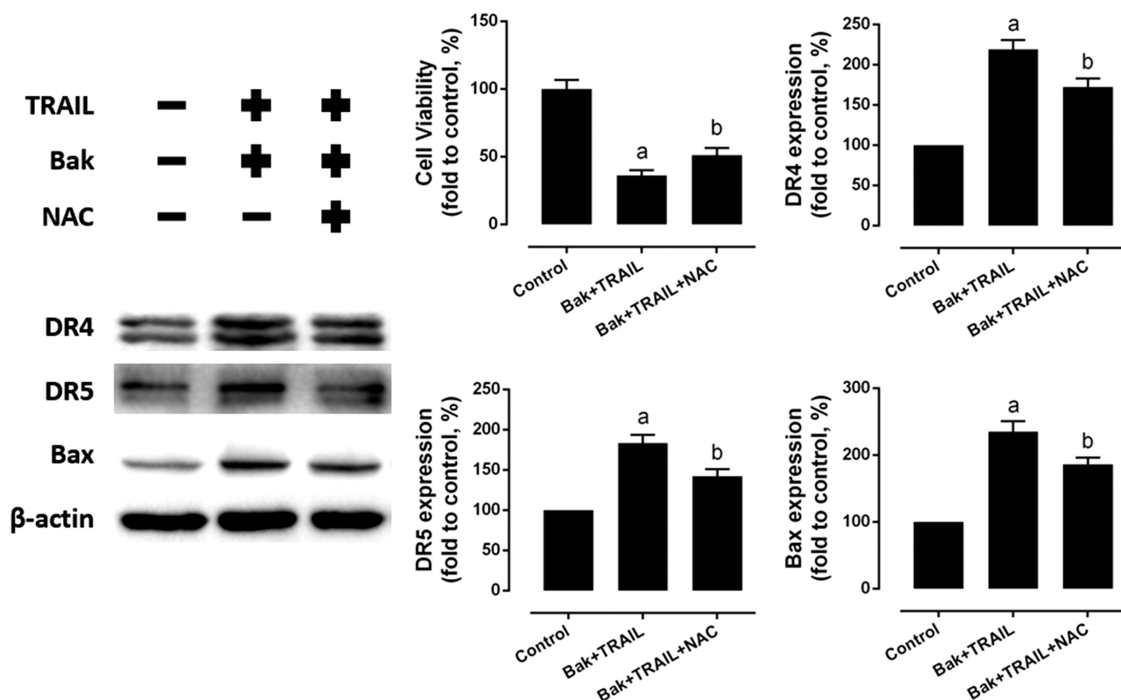


图5 阻断 ROS 逆转了 Bak+TRAIL 上调 DR 受体增强细胞凋亡的效应

Fig. 5 ROS inhibition attenuated Bak+TRAIL induced DRs increase cell apoptosis

Note: Data are expressed as $\bar{x} \pm \text{SEM}$, $n=6$. ^a $P < 0.05$, compared with group Control; ^b $P < 0.05$, compared with Bak + TRAIL group. Bak: Bakuchiol

增殖、转移,该过程可能与 Bak 上调 CK18 表达,下调 Notch3、FASN、TGFBR1 以及 ACVR1B 有关^[18]。此外,Lin 等发现,Bak 可以将胃癌细胞系 SGC-7901 抑制在 Sub-G1 期,同时诱发细胞凋亡^[16]。然而肝癌中 Bak 的作用特别是作用机制尚无明确研究。我们首先确认了 Bak 对肝细胞癌的直接作用。Bak 处理显著抑制了 HepG2 细胞的增殖。

TRAIL 是新型抗肿瘤药物,其作用机制为与肿瘤细胞表面的死亡受体结合,诱导肿瘤细胞发生凋亡^[19]。目前联合应用其他药物强化 TRAIL 的肿瘤杀伤作用逐渐引起人们的关注^[20,21]。有文献证实,可以通过药物联用的方式,强化 TRAIL 的杀伤作用,该作用主要是通过上调 TRAIL 受体 DR4、DR5 等的表达来实现的^[17]。Lim 等研究发现,MDL-12330A 可以通过 CHOP 依赖的方式上调 DR5 的表达,进而增强 TRAIL 的杀伤作用。Chen 等也证实,芹黄素可以通过 p53 依赖的方式上调 DR5 的表达,进而增强 TRAIL 对非小细胞肺癌的杀伤作用。然而肝细

胞癌中 TRAIL 与 Bak 联合应用作用如何,以及内在调控机制尚不明确。因此本研究在探究了 Bak 对肝细胞癌直接作用的基础上,探究与 TRAIL 联用的效果。

氧化应激是指体内氧化与抗氧化作用失衡,倾向于氧化。微观上看,氧化应激本质上是细胞线粒体功能受损,功能紊乱,释放出大量氧自由基,引起细胞氧化应激相关凋亡的过程^[22]。氧化应激在肿瘤药物治疗的过程中发挥重要作用。研究表明,多种中药提取物杀伤肿瘤细胞是通过激活氧化应激来实现的^[23,24]。席夫碱可以通过激活氧化应激,诱导化疗耐药的肿瘤细胞发生凋亡^[25]。Blanquer 等研究发现,黄腐酚可以调控乳腺癌 MCF-7 细胞内氧化应激水平并影响其增殖、转移能力^[26]。那么在 Bak 抗肝细胞癌作用中,氧化应激发挥何种作用呢? 我们研究发现,ROS 指标提示 Bak 剂量依赖地激活了 HepG2 细胞的氧化应激。

有研究证实,氧化应激的激活可以上调死亡受体 DR4、

DR5 的表达,从而敏化 TRAIL 的抗肿瘤作用^[27-30]。Ma 等研究发现,毒胡萝卜素可以通过激活内质网应激和氧化应激,上调 CHOP 和 DR5 的表达,增强 TRAIL 的杀伤作用^[27]。Kim 等研究发现紫花前胡素可以通过激活氧化应激和内质网应激,增强非小细胞肺癌细胞对 TRAIL 处理的敏感性^[28]。Tochigi 等研究发现,在 TRAIL 耐药的黑色素瘤细胞中,双氧水可以激活细胞氧化自由基的生成,强化 TRAIL 的杀伤作用^[31]。

本研究证实 Bak 处理后 TRAIL 受体 DR4、DR5 表达上调,提示 Bak 处理可能增强 TRAIL 对 HepG2 细胞的杀伤作用。因此我们进一步检测了联合应用 TRAIL 及 Bak 的抗 HepG2 的作用,发现联合用药效果优于单独用药。为进一步明确氧化应激在该过程的具体作用,我们在给药的同时,给予 ROS 清除剂 NAC 处理抑制细胞内氧化应激,发现 NAC 处理后逆转可 Bak+TRAIL 引起的细胞增殖抑制,western blot 结果显示,NAC 处理抑制了 DR4、DR5 表达的上调,同时也下调了凋亡指标 Bax 的表达。以上结果提示 Bak 联合 TRAIL 有更为显著的抗 HepG2 细胞的作用,该作用可能是通过激活 ROS 促进 DR4、DR5 的表达来实现的。本研究首次明确了 Bak 与 TRAIL 联合用药的抗肝细胞癌的作用及可能机制,为进一步相关研究及临床应用提供了理论参考。

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