·基础研究·

脂联素抑制内质网应激干预慢性间歇性缺氧所致肾损伤

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【摘 要】 **目的** 探讨脂联素(Ad)对慢性间歇性缺氧(CIH)所致肾损伤的干预作用及相关机制。**方法** 60只成年Wistar大鼠随机分成4组:正常对照(NC)组、NC+Ad组、CIH组和CIH+Ad组。其中CIH组和CIH+Ad组大鼠接受CIH处理4个月。其余2组接受正常空气处理,同时NC+Ad组和CIH+Ad组大鼠接受Ad(10 μg)治疗,每周2次,持续4个月。荧光显微镜下观察活性氧(ROS)的水平。TUNEL染色检测肾脏细胞凋亡情况。Western blotting检测各组大鼠肾脏组织中GRP78、CHOP、IRE1、PERK、pro-ATF6蛋白的表达,以反映各组大鼠内质网应激情况。采用SPSS 17.0软件进行统计学分析。计量资料用均数±标准差($\bar{x}\pm s$)表示,组间比较用t检验。**结果** 实验满4个月时,组间比较示各参数在NC与NC+Ad组间差异均无统计学意义(P均>0.05)。与NC组和NC+Ad组比较:(1)ROS水平在CIH组显著增高,而CIH+Ad组低于CIH组,但仍高于NC组和NC+Ad组(P均<0.05);(2)肾细胞凋亡率和反映凋亡的caspase-12和caspase-3蛋白水平在CIH组明显增加,CIH+Ad组较CIH组明显减少,但仍然高于NC组和NC+Ad组(P均<0.05);(3)CIH组肾脏组织的GRP78、CHOP、IRE1、PERK蛋白水平明显增加,在CIH+Ad组明显减少,但仍高于NC组和NC+Ad组(P均<0.05);pro-ATF6蛋白水平在CIH组明显降低,在CIH+Ad组有所增加,但仍然低于NC组和NC+Ad组(P均<0.05)。**结论** CIH可以通过激活ROS和ERS相关的细胞凋亡途径导致肾脏损伤,而补充外源性Ad后,可能通过抑制ROS,进而抑制ERS,保护肾脏细胞。

【关键词】 慢性间歇性缺氧;脂联素;活性氧;内质网应激
【中图分类号】 R322.3 【文献标志码】 A 【DOI】 10.11915/j.issn.1671-5403.2017.09.161

Adiponectin protects rats against chronic intermittent hypoxia-induced kidney injury through inhibiting endoplasmic reticulum stress

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【**Abstract**】 **Objective** To determine the intervention effects of adiponectin (Ad) on rat kidney injury induced by chronic intermittent hypoxia (CIH), and investigate the underlying mechanisms. **Methods** Sixty adult Wistar rats were randomly divided into 4 groups: normal control (NC group), normal control plus Ad supplement (NC+Ad group), CIH, and CIH plus Ad supplement (CIH+Ad group) groups. The rats in both CIH and CIH+Ad groups were submitted to a CIH environment for 4 months, while the rats in NC and NC+Ad groups were housed with the normal air for 4 months. In addition, the rats in NC+Ad and CIH+Ad groups were treated with an intravenous injection of Ad at a dosage of 10 μg per injection, twice a week, for 4 successive months. Fluorescence microscopy was used to observe the levels of reactive oxygen species (ROS). TUNEL staining was employed to detect the apoptosis of renal cells. Western blotting was adopted to measure the expression levels of GRP78, CHOP, IRE1, PERK and pro-ATF6 in renal tissues of rats in each group to indicate the stress of endoplasmic reticulum in rats. SPSS statistics 17.0 was used to analyze the data. The measurement data were expressed as mean±standard deviation ($\bar{x}\pm s$), and student's t test was used for the comparison between the groups. **Results** In the end of 4 months' treatment, no significant differences were found in all the parameters between the NC and NC+Ad groups (all $P>0.05$). Compared with the NC and NC+Ad groups, our findings were as follows: (1) ROS levels were significantly higher in the CIH group, while the levels in the CIH+Ad group was though lower than those in the CIH group, but higher than those in the NC and NC+Ad groups (all $P<0.05$); (2) the percentage of apoptotic renal cells and protein levels of caspase-12 and caspase-3 were all significantly higher in the CIH group, while in the CIH+Ad group, they were lower than in the CIH group, but

收稿日期: 2017-06-16; **修回日期**: 2017-07-15
基金项目: 国家自然科学基金(81470273);临床医学科技专项——公共卫生关键技术应用(BL2014083)
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still higher than in the NC and NC + Ad groups (all $P < 0.05$); (3) the protein levels of GRP78, CHOP, IRE1 and PERK in the renal tissues were all significantly higher in the CIH group, while in CIH + Ad group, the levels were lower than in the CIH group, but higher than in the NC and NC + Ad groups (all $P < 0.05$); the protein level of pro-ATF6 in the renal tissues was lower in the CIH group, while in the CIH + Ad group, the level was higher than in the CIH group, but lower than in the NC and NC + Ad groups (all $P < 0.05$). **Conclusion** CIH induces kidney injury through activation of ROS and ERS-associated cellular apoptotic pathway, while exogenous Ad supplement protects the renal cells by inhibiting ROS and then ERS.

【Key words】 chronic intermittent hypoxia; adiponectin; reactive oxygen species; endoplasmic reticulum stress

This work was supported by the National Natural Science Foundtion of China (81470273) *and Clininal Medical Science and Technology Project — Application of Key Technology of Public Health Care* (BL2014083).

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慢性间歇性缺氧(chronic intermittent hypoxia, CIH)是阻塞性睡眠呼吸暂停综合征(obstructive sleep apnea syndrome, OSAS)的重要特征。许多研究报道 CIH 和心血管损伤[1,2]、颏舌肌损伤、肝脏损伤[3]等有密切关系。最近研究结果显示,在没有糖尿病和高血压的重症 OSAS 患者中,慢性肾脏疾病的发病率较高[4]。Sim 等[5]发现,在早期肾病患者中,OSAS 发病率较高[5]。因此,我们推测 CIH 可能会导致肾损伤。

内质网应激(endoplasmic reticulum stress, ERS)是细胞为了应对过度刺激造成的内质网错误折叠及未折叠而产生的保护性反应。ERS 通过 IRE1、PERK、ATF6 这三条通路调节。当内质网发生应激时,三条通路被激活,过度的内质网应激可以导致细胞凋亡[6-8]。X-框结合蛋白 1(X-box binding protein 1)作为 ER 应激的重要价质,其有防止氧化应激的能力。同时,在动物和人的肾脏缺血再灌注过程中,ERS 也被发现发挥重要作用[9,10]。

脂联素(adiponectin, Ad)是一个重要的脂肪因子,在人体内起到重要的保护作用。Cammisotto 等[11]发现,在远端肾小管组织中,Ad 通过结合脂肪受体激活腺苷酸活化蛋白激酶(AMP-activated protein kinase, AMPK)而抑制糖原合成。另外,Ad 是尿蛋白的重要调控因子,它激活 AMPK 通路,减少肾小球足细胞中 Nox4 的生成,从而调节氧化应激[12]。然而,Ad 对 CIH 导致的肾脏损伤的保护作用目前并不清楚。本研究旨在观察 CIH 对肾脏的影响以及 Ad 的保护作用及相关机制。

1 材料与方法

1.1 实验动物

60 只成年 Wistar 大鼠(上海 Silake 公司)随机分为 4 组:正常对照(normal control, NC)组、NC + Ad 组、CIH 组和 CIH + Ad 组。依据参考文献[13]建立 CIH 大鼠模型。大鼠被饲养在设有空气控制器的特殊鼠笼里。2 min 为 1 个循环,第 1 min 时控制氧浓度从 21% 降低到 5% ~6%,持续 15 ~20 s;再接下来的 1 min,控制氧浓度恢复到正常水平(21%)。CIH 组和 CIH + Ad 组大鼠接受间歇性缺氧处理。其余组大鼠接受正常空气处理。同时 NC + Ad 组和 CIH + Ad 组大鼠接受 Ad(10 μg)尾静脉注射,每周 2 次。其余组大鼠接受生理盐水处理。间歇性低氧每天持续 8 h,共 4 个月。

1.2 活性氧反应染色

新鲜大鼠肾脏组织包埋于乙醇干冰中获得最佳切割温度,制成 8 μm 的切片。接下来将切片浸没在 10 μmol/L 的超氧化物阴离子荧光探针(dihydroethidium, DHE)溶液中,37℃孵育 30 min。在荧光显微镜下观察活性氧(reactive oxygen species, ROS)的水平。

1.3 细胞凋亡的末端脱氧核苷酸转移介导 dUTP 缺口末端标记染色检测

肾脏组织经过多聚甲醛固定 48 h 后,脱水、浸蜡。制成 5 μm 的石蜡切片。切片脱蜡脱水,使用蛋白酶 K 室温下孵育 15 min。然后使用末端脱氧核苷酸转移介导 dUTP 缺口末端标记染色(terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling, TUNEL)反应液在 37℃ 下处理切片 60 min。使用 TUNEL-POD 处理 30 min 后,分别使用 3,3' - 二氨基联苯胺(3,3'-diaminobenzidine, DAB)和苏木素处理 1 min。最后脱水封片,在光学显微镜下观察。随机选择 5 张病理切片评价肾脏细胞凋亡情况。

1.4 Western blotting

为反映各组大鼠内激网应激情况,Western blotting 检测各组大鼠肾脏中 GRP78、CHOP、IRE1、PERK、pro-ATF6 蛋白的表达。取肾脏组织于裂解液(Thermo)中匀浆,10 000 × *g* 离心 5 min,取上清使用 BCA 法进行蛋白定量。取等量蛋白在含有十二烷基硫酸钠的聚丙烯酰胺凝胶中电泳,电转移至聚

偏氟乙烯膜上。将膜在封闭液中封闭 1 h,然后浸入一抗中 4℃过夜孵育。再与辣根过氧化物酶标记的二抗孵育。最后使用 ECL 显色,X 线放射自显影,扫描,拍片。

1.5 统计学处理

采用 SPSS 17.0 软件进行统计学分析。计量资料用均数 ± 标准差($\bar{x} \pm s$)表示,组间比较用 t 检验。$P<0.05$ 为差异具有统计学意义。

2 结 果

2.1 ROS 水平

与 NC 组和 NC + Ad 组比较,CIH 处理后,ROS 水平明显升高($P<0.05$)。而给予 Ad 处理后,ROS 水平明显下降,但仍然高于 NC 组和 NC + Ad 组($P<0.05$)。NC 组和 NC + Ad 组 ROS 水平差异无统计学意义($P>0.05$;图 1)。

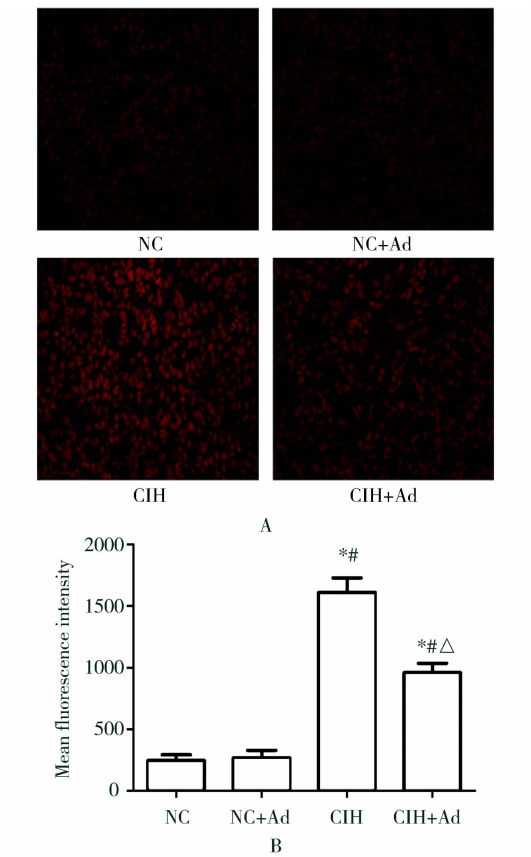


图 1 四组肾脏组织 ROS 水平

Figure 1 ROS levels of renal tissue in four groups

A: ROS fluorescence staining(×400); B: densitometric evaluation. $n=5$. Compared with NC group, $^{*}P<0.05$; compared with NC + Ad group, $^{\#}P<0.05$; compared with CIH group, $^{\triangle}P<0.05$

2.2 肾脏细胞凋亡

TUNEL 染色显示,与 NC 组(0.5671% ±0.1786%)和 NC + Ad 组(0.5207% ±0.1589%)相比,CIH 组(6.1510% ±0.2797%)大鼠肾脏细胞凋亡明显增加($P<0.05$)。而给予脂联素补充后,肾脏细胞凋亡明显减少(4.0590% ±0.2454%),但仍然高于 NC 组和 NC + Ad 组($P<0.05$)。NC 组和 NC + Ad 组之间差异无统计学意义($P>0.05$;图 2A,2B)。与NC 组和 NC + Ad 组相比,CIH 组和 CIH + Ad 组 caspase-12 和 caspase-3 蛋白水平(图 2C,2D)明显增加(均$P<0.05$)。而 CIH 组仍然高于 CIH + Ad 组($P<0.05$)。NC 组和 NC + Ad 组之间差异无统计学意义($P>0.05$)。

2.3 内质网应激

与 NC 组和 NC + Ad 组相比,CIH 组大鼠肾脏组织的 GRP78 和 CHOP 蛋白水平明显增加($P<0.05$;图 3),而 CIH 组大鼠给予脂联素补充后,GRP78 和 CHOP 蛋白水平明显减少,但仍然高于 NC 组和NC + Ad组($P<0.05$)。NC 和 NC + Ad 组间差异无统计意义($P>0.05$)。与 NC 组和 NC + Ad 组相比,CIH 组大鼠肾脏组织的 IRE1(图 4)和 PERK(图 5)通路蛋白水平明显增加(P 均 <0.05),给予脂联素补充后,IRE1 和 PERK 通路蛋白水平明显减少,但仍然高于 NC 组和 NC + Ad 组($P<0.05$;图 6)。CIH 组的 pro-ATF6 蛋白表达水平低于 NC 组、NC + Ad 组和CIH + Ad组($P<0.05$),而 CIH 组 pro-ATF6 蛋白表达水平则低于 NC 组和 NC + Ad 组,三条内质网应激通路蛋白水平在 NC 组和 NC + Ad组间差异无统计学意义($P>0.05$)。

3 讨 论

OSAS 是一种慢性全身性疾病。Fleischmann 等[14]发现睡眠呼吸障碍患者高发慢性肾脏病 3 期。Kawakami 等[9]研究也发现在睡眠呼吸障碍的病人中慢性肾脏病相对比较高。因此我们推测 OSAS 可能在慢性肾脏病的病程发展中起重要作用。在本研究中,我们发现长期的 CIH 虽然没有导致肾脏功能的损伤,但是肾脏细胞凋亡明显增加。经过 CIH 处理,肾脏凋亡细胞及细胞凋亡生物标志物 caspase-3 蛋白水平升高,这可能是 CIH 造成肾脏损害的机制之一。而给予脂联素后,细胞凋亡明显减少。因此,我们发现 Ad 对 CIH 导致的肾脏损伤具有保护功能。为了评估 Ad 在肾脏细胞凋亡时起保护作用的机制,我们研究了 ERS 的蛋白水平变化。在本实验中,ERS 的两种生物标志物 GRP78 和 CHOP 水平均明显升高,由此证实 CIH 激活了 ERS。尽管 ERS 开始时是一种保护性反应,但是越来越多证据表明,过度的 ERS 会导致细胞凋亡[6,7,15-17]。

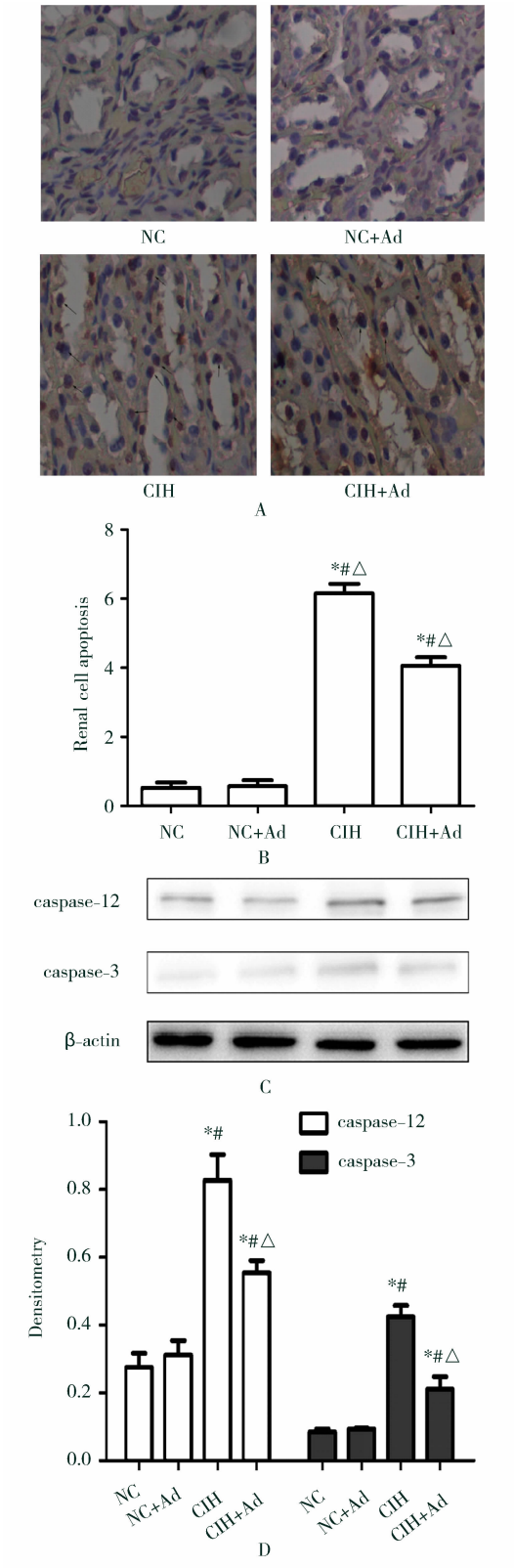


图 2 四组肾脏细胞凋亡情况检测

Figure 2 Renal cell apoptosis in four groups

A: TUNEL staining (× 400); B: densitometric evaluation; C: Western blotting; D: densitometric evaluation. $n = 5$. Compared with NC group, $^{*}P < 0.05$; compared with NC + Ad group, $^{\#}P < 0.05$; compared with CIH group, $^{\triangle}P < 0.05$

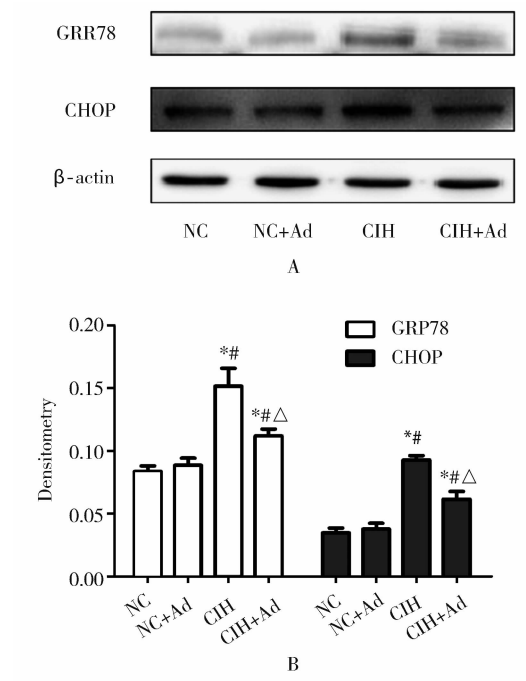


图 3 四组肾脏组织 GRP78 和 CHOP 蛋白表达水平

Figure 3 Expression of GRP78 and CHOP of renal tissue in four groups

A: Western blotting; B: densitometry evaluation. $n = 3$. Compared with NC group, $^{*}P < 0.05$; compared with NC + Ad group, $^{\#}P < 0.05$; compared with CIH group, $^{\triangle}P < 0.05$

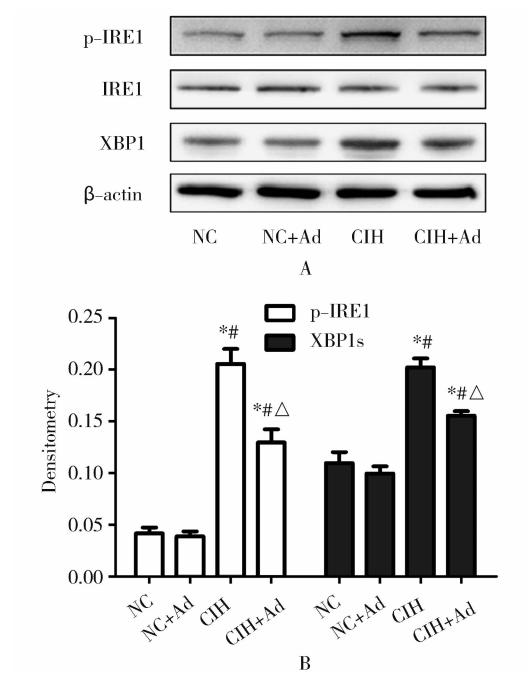


图 4 四组肾脏组织 IRE1 通路蛋白表达水平

Figure 4 Expression of IRE1 pathway proteins of renal tissue in four groups

A: Western blotting; B: densitometry evaluation. $n = 3$. Compared with NC group, $^{*}P < 0.05$; compared with NC + Ad group, $^{\#}P < 0.05$; compared with CIH group, $^{\triangle}P < 0.05$

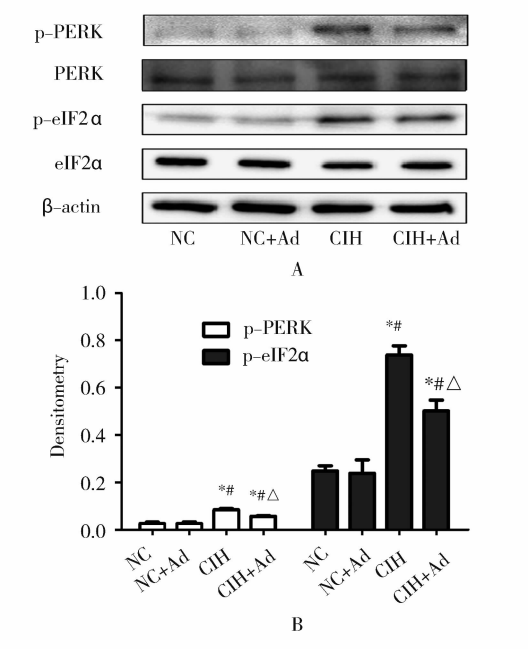


图5 四组肾脏组织 PERK 通路蛋白表达水平

Figure 5 Expression of PERK pathway proteins of renal tissue in four groups

A: Western blotting; B: densitometry evaluation. $n=3$. Compared with NC group, $^{*}P<0.05$; compared with NC + Ad group, $^{\#}P<0.05$; compared with CIH group, $^{\triangle}P<0.05$

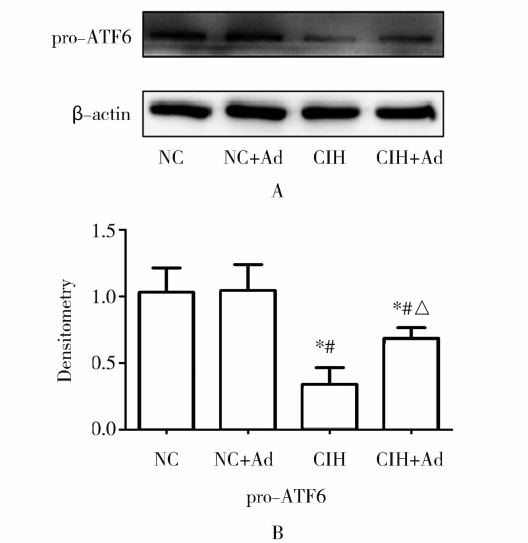


图6 四组肾脏组织 pro-ATF6 蛋白表达水平

Figure 6 Expression of pro-ATF6 protein of renal tissue in four groups

A: Western blotting; B: densitometry evaluation. $n=3$. Compared with NC group, $^{*}P<0.05$; compared with NC + Ad group, $^{\#}P<0.05$; compared with CIH group, $^{\triangle}P<0.05$

ERS 有 3 条通路:IRE1、PERK、ATF6。任何一条通路活化最终都能激活 CHOP 基因表达[18-20],最终导致细胞凋亡[21]。在本研究中,CIH 组三条通路均被激活,IRE1、PERK 蛋白水平升高,而 pro-ATF6 蛋白水平降低,CHOP 的蛋白水平亦明显升高。另外,caspase-12 是 ERS 相关细胞凋亡的特异性分子[22]。CIH 组的 caspase-12 蛋白水平明显上升。本研究结果提示,ERS 会导致肾脏细胞凋亡;给予 Ad 处理后,IRE1、PERK、CHOP、caspase-12 蛋白水平降低,而 pro-ATF6 蛋白水平升高。最终发现,脂联素通过抑制 ERS 起到保护肾脏的作用。

目前,很多研究已证实 CIH 可以使 ROS 水平升高[23,24]。本研究也发现 CIH 组的 ROS 蛋白水平明显升高。另外,越来越多的证据表明,ROS 可引起 ERS[25,26]。本研究亦发现 ROS 是 CIH 引起内质网应激的一个重要中介物,给予 Ad 治疗的 CIH 组,ROS 的水平明显降低,从而证实抑制 ROS 生成或许是 Ad 能够保护肾脏的重要机制。

综上所述,本研究发现脂联素可通过抑制 CIH 导致的 ROS,进而抑制 ERS 相关的肾脏细胞凋亡。

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（编辑：兆瑞臻）

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