

氢气对高氧暴露下肺泡II型上皮细胞损伤的保护作用机制研究

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摘要 该文探讨了氢气对高氧暴露下早产大鼠II型肺泡上皮细胞(type II alveolar epithelial cells, AECII)损伤的作用机制研究。将原代分离培养的早产鼠AECII分为4组: 空气组、高氧组、高氧+氢气组、高氧+氢气+PD98059组。空气组、高氧组分别置于氧浓度为21%的空气和95%的氧气中; 高氧+氢气组在高氧暴露前加入氢气; 高氧+氢气+PD98059组在高氧暴露前同时加入氢气和ERK1/2特异性抑制剂PD98059, 再置于氧浓度为95%的密闭氧仓中。各组均培养24 h后, CCK-8法检测细胞增殖能力; 流式细胞术检测细胞凋亡情况; Western blot检测ERK1/2、p-ERK1/2、Bax蛋白质水平; 荧光定量PCR检测Bax和caspase-3 mRNA水平。结果发现, 与空气组比较, 高氧组细胞凋亡率显著增加($P<0.01$), 细胞增殖能力、p-ERK1/2蛋白质水平明显降低($P<0.01$), Bax蛋白质与mRNA水平均明显升高($P<0.01$), caspase-3 mRNA水平明显升高($P<0.01$); 与高氧组比较, 高氧+氢气组细胞凋亡率显著减低($P<0.01$), 细胞增殖能力升高($P<0.01$), p-ERK1/2蛋白质水平明显升高($P<0.01$), Bax蛋白质与mRNA水平均降低($P<0.05$), caspase-3 mRNA水平降低($P<0.05$); 当加入ERK1/2抑制剂PD98059后, 氢气的保护作用被消除($P<0.05$)。氢气可通过激活MAPK-ERK1/2信号通路抑制凋亡相关基因的表达, 改善高氧导致AECII的凋亡和增殖受限, 促进细胞存活。

关键词 高氧; II型肺泡上皮细胞; 氢气; MAPK-ERK1/2信号通路

Protection Mechanism of Hydrogen Against Hyperoxia-Induced Injury in Type II Alveolar Epithelial Cells

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Abstract This work was aim to investigate the protection mechanism of hydrogen against hyperoxia-induced injury in type II alveolar epithelial cells (AECII). AECII were isolated and purified from premature rats and were divided into 4 groups: air group, hyperoxia group, hyperoxia+hydrogen group, hyperoxia+hydrogen+PD98059 group. Air group and hyperoxia group were exposed to 21% oxygen and 95% oxygen, respectively. Hydrogen was added to the hyperoxia+hydrogen group before hyperoxia exposure. Hydrogen and ERK1/2 specific inhibitor

收稿日期: 2017-07-28

接受日期: 2017-11-29

国家自然科学基金(批准号: 30973218)和重庆市科委基础与前沿研究计划(批准号: CSTC2013jcyjA10031)资助的课题

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Received: July 28, 2017

Accepted: November 29, 2017

This work was supported by the National Natural Science Foundation of China (Grant No.30973218) and the Project of Foundation and Frontier Research Program of Chongqing Science and Technology Commission (Grant No.CSTC2013jcyjA10031)

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网络出版时间: 2018-01-29 17:35:03

URL: <http://kns.cnki.net/kcms/detail/31.2035.Q.20180129.1734.022.html>

PD98059 were added to hyperoxia+hydrogen+PD98059 before hyperoxia exposure, and then placed into 95% oxygen for 24 h. The ability of cell proliferation was measured by CCK-8 colorimetric assay. The cell apoptosis was analyzed by flow cytometry. The protein levels of ERK1/2, p-ERK1/2, Bax were detected by Western blot. The mRNA levels of *Bax* and *caspase-3* were assessed by qPCR. Compared with air group, cell apoptosis rates significantly increased ($P<0.01$), cells proliferation significantly decreased ($P<0.01$), the mRNA levels of *Bax* and *caspase-3* significantly increased and p-ERK1/2 significantly decreased under hyperoxia exposure ($P<0.01$). While with hydrogen treatment, the ability of cells proliferation markedly increased ($P<0.01$), cell apoptosis rates, *Bax*, *caspase-3* mRNA levels notably decreased ($P<0.05$) and p-ERK1/2 protein levels increased ($P<0.05$). The effect of hydrogen was abolished after treatment with inhibitor PD98059 ($P<0.05$). Hydrogen can inhibit the expression of apoptosis related-genes via activating the MAPK-ERK1/2 signaling pathway to improve the proliferation restriction of AECII cells induced by hyperoxia, and promote the survival of cells.

Keywords hyperoxia; type II alveolar epithelial cells; hydrogen; MAPK-ERK1/2 signaling pathway

氧疗是临床上治疗低氧血症的重要手段,但长时间暴露于高浓度的氧会产生过量的活性氧类(reactive oxygen species, ROS),引起肺泡II型上皮细胞(type II alveolar epithelial cells, AECII)损伤和死亡,最终导致新生儿尤其是早产儿慢性肺疾病(chronic lung disease, CLD)或支气管肺发育不良(bronchopulmonary dysplasia, BPD)的发生^[1-3]。氢气(hydrogen, H₂)是自然界最小的气体分子,可自由扩散到细胞的任何部位。同传统抗氧化剂相比,氢气具有选择性的抗氧化作用,即能够选择性地清除ROS,而对发挥信号调节作用的ROS无明显作用^[4]。越来越多的研究表明,氢气在人类多种疾病^[5-7](包括多种因素导致的急慢性肺疾病^[8-11])中发挥着保护作用。本课题组在前期研究中发现,氢气可减轻高氧导致的AECII氧化损伤,减少凋亡,促进其增殖,发挥一定的保护作用^[12],但其作用机制尚不清楚。MAPK-ERK1/2(mitogen activated protein kinase-extracellular signal regulated kinase1/2)信号通路是促进细胞增殖的一条重要的细胞信号转导通路,可介导细胞生长、分化、凋亡等多种生理过程。研究表明,MAPK-ERK1/2通路可通过影响Bcl-2(B-cell lymphoma-2)家族成员的活化和表达调控内源性的凋亡途径,从而有助于细胞的稳定^[13]。因此,我们推测,氢气可能通过参与调节MAPK-ERK1/2信号通路的表达起到保护高氧对肺损伤的作用。

本研究通过观察氢气和ERK1/2信号分子特异性抑制剂PD98059干预对高氧致AECII增殖、凋亡以及Bax、caspase-3、ERK、p-ERK表达的影响,初

步探讨氢气是否通过调控MAPK-ERK1/2信号途径对高氧致AECII损伤发挥保护作用。

1 材料与方法

1.1 实验动物与主要试剂

SPF级孕19 d SD雌鼠24只,体质量240~260 g,购于重庆医科大学实验动物中心。胰蛋白酶、胎牛血清(FBS)、DMEM/F12培养基购于美国Gibco公司。I型胶原酶购于美国Sigma公司;CCK-8溶液购于日本同仁公司。Annexin V-FITC/PI凋亡试剂盒购于南京凯基生物科技发展有限公司。兔抗鼠ERK1/2、p-ERK1/2、Bax单克隆抗体购于美国Abcam公司。辣根过氧化物酶标记的羊抗兔二抗、蛋白质提取及BCA蛋白质定量检测试剂盒购于北京碧云天生物技术有限公司。TRIzol总RNA提取试剂盒、逆转录试剂盒、荧光定量PCR试剂盒购于日本TaKaRa公司。

1.2 早产大鼠AECII的分离、培养及鉴定

取孕19 d SD大鼠,经剖宫产取出胎鼠,分离、纯化、培养AECII,具体提取方法参考文献[14]。经透射电镜鉴定细胞和改良巴氏染色法证实细胞存活率及纯度均在90%以上后用于实验。该动物实验已通过重庆医科大学伦理委员会审核。

1.3 富氢培养基的制备

按照第二军医大学孙学军教授等^[15]申请的专利方法制作。取250 mL软袋生理盐水,抽空袋内气体和生理盐水,将含10%胎牛血清DMEM/F12完全培养基和H₂注入软袋中,0.4 MPa加压6 h,每周重新制备,4 °C保存。制作过程需无菌操作。采用亚甲蓝-

氧化还原滴定法检测完全培养基中氢气浓度。取含氢培养基5 mL, 然后用亚甲蓝胶体铂MB-Pt试剂滴定, 直至蓝色恰好不再继续消失作为浓度计算滴定法点。具体操作和计算方法参考文献[16]。

1.4 AECII高氧损伤模型的建立及实验分组

将分离提纯的AECII培养24 h后分为空气组、高氧组、高氧+氢气组和高氧+氢气+PD98059组(富氢培养基中预先加入ERK1/2抑制剂PD98059, 使其终浓度为20 $\mu\text{mol/L}$), 空气组置于37 $^{\circ}\text{C}$ 、5% CO_2 细胞培养箱中继续培养, 其余各组置于密闭氧仓, 通入含5% CO_2 和95% O_2 的混合气体后连同氧仓一同置于细胞培养箱, 各组细胞干预并培养24 h。

1.5 CCK-8检测细胞存活率

将原代分离的AECII接种于96孔板, 密度约为 1.5×10^4 /孔。按上述方法处理24 h后, 每孔加入10 μL CCK-8溶液和90 μL 的DMEM/12完全培养基混合液, 放入细胞培养箱继续培养4 h。酶标仪检测450 nm处各孔光密度(D)值。光密度值越大表明细胞增殖能力越强, 存活率越高。

1.6 流式细胞术检测细胞凋亡率

高氧处理细胞24 h后收集各组细胞。PBS洗涤细胞2次, 1 000 r/min离心5 min, 收集约 5×10^5 细胞沉淀后, 加入500 μL 染色缓冲液以及5 μL Annexin V-FITC, 并混匀。再加入5 μL PI染色液混匀, 室温避光15 min, 流式细胞仪检测细胞凋亡情况。Annexin V-FITC染色阳性和PI染色阴性表示早期凋亡, Annexin V和PI染色均为阳性表示晚期凋亡或坏死, Annexin V-FITC和PI染色均为阴性表示细胞存活。

1.7 Western blot检测Bax、ERK1/2、p-ERK1/2蛋白质水平

收集各组细胞, 采用蛋白质裂解液提取总蛋白, BCA蛋白质定量试剂盒测定蛋白质浓度。每个样本取40 μg 蛋白质上样, 以10% SDS-聚丙烯酰胺凝胶电泳、电压115 V电转75 min至聚偏氟乙烯(PVDF)膜, 用含5%脱脂奶粉的TBST封闭1 h。分别加入1:3 000稀释的Bax、ERK1/2、p-ERK1/2和GAPDH单克隆抗体, 4 $^{\circ}\text{C}$ 孵育过夜后用TBST清洗3次, 再加入1:4 000稀释的辣根过氧化物酶标记的二抗室温孵育90 min, TBST洗10 min, 洗3次。使用Millipore显影剂进行曝光检测, Quantity One 4.5.0软件分析各蛋白质条带密度, 以目的蛋白/相应内参得到各蛋白质的相对值进

行统计学分析。

1.8 qRT-PCR检测Bax、caspase-3 mRNA水平

采用TRIzol试剂提取AECII总RNA, 紫外线分光光度计测定RNA纯度和浓度。按照试剂盒说明书将提取的RNA逆转录为cDNA, 以Bax、caspase-3和GAPDH基因序列设计PCR引物, 在实时荧光定量PCR仪上进行扩增, 反应体系10 μL , 反应条件为: 95 $^{\circ}\text{C}$ 30 s, 95 $^{\circ}\text{C}$ 延伸5 s, 各基因以各自的最适温度退火30 s, 共39个循环。根据扩增曲线和溶解曲线, 在曲线上读出Ct值, 以GAPDH为内参, 用 $2^{-\Delta\Delta\text{Ct}}$ 法计算各基因mRNA的相对水平。各基因相应引物如下: Bax(正义链: 5'-GAA TTG GCG ATG AAC TGG AC-3'; 反义链: 5'-GCA AAG TAG AAA AGG GCA ACC-3'), caspase-3(正义链: 5'-GAC TGC GGT ATT GAG ACA GA-3'; 反义链: 5'-CGA GTG AGG ATG TGC ATG AA-3'), GAPDH(正义链: 5'-GCA AGT TCA ACG GCA CAG TC-3'; 反义链: 5'-TCA CCC CAT TTG ATG TTA GCG-3')。

1.9 统计学方法

所有数据以 $\bar{x} \pm s$ 表示, 采用SPSS 17.0软件进行统计学分析, 各组间差异比较采用单因素方差分析, 其两两比较采用LSD法(方差齐), 方差不齐则采用Dunnett's T3检验。 $P < 0.05$ 为差异具有统计学意义。

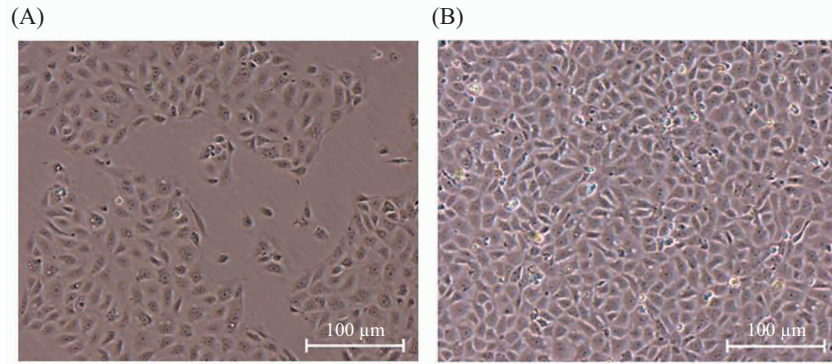
2 结果

2.1 AECII的形态学观察

倒置相差显微镜下观察发现, 体外培养24 h, AECII贴壁, 呈岛状生长, 细胞呈多边形或立方形, 透亮度高(图1A); 体外培养48 h, AECII处于对数生长期, 胞体进一步伸展, 细胞增殖明显, 细胞之间连接紧密(图1B)。

2.2 氢气和抑制剂PD98059对高氧导致AECII细胞增殖能力的影响

利用CCK-8实验检测高氧、氢气、PD98059对AECII生长的影响, 结果表明, 与空气组比较, 高氧暴露组AECII的增殖能力明显减弱, 细胞生长受到抑制, 差异具有统计学意义($P < 0.01$); 同高氧组相比, 加入氢气后, AECII的增殖能力明显升高($P < 0.01$); 当加入抑制剂PD98059, 氢气的这种保护作用被消除($P < 0.01$)(图2)。该结果表明, 氢气能够明显改善高氧导致的AECII增殖受限, 促进细胞存活, 而氢气的这种作用能被ERK1/2特异性抑制剂所阻滞。



A: 空气培养AECII 24 h; B: 空气培养AECII 48 h。

A: air culture 24 h of AECII; B: air culture 48 h of AECII.

图1 倒置相差显微镜下观察AECII形态学变化

Fig.1 Morphological observation of AECII under inverted phase contrast microscope

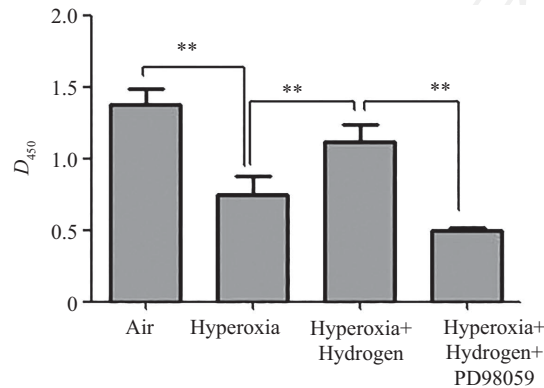
** $P < 0.01$.

图2 CCK-8检测氢气和抑制剂PD98059对AECII细胞增殖能力的影响

Fig.2 Effect of hydrogen and inhibitor PD98059 on the proliferation of AECII

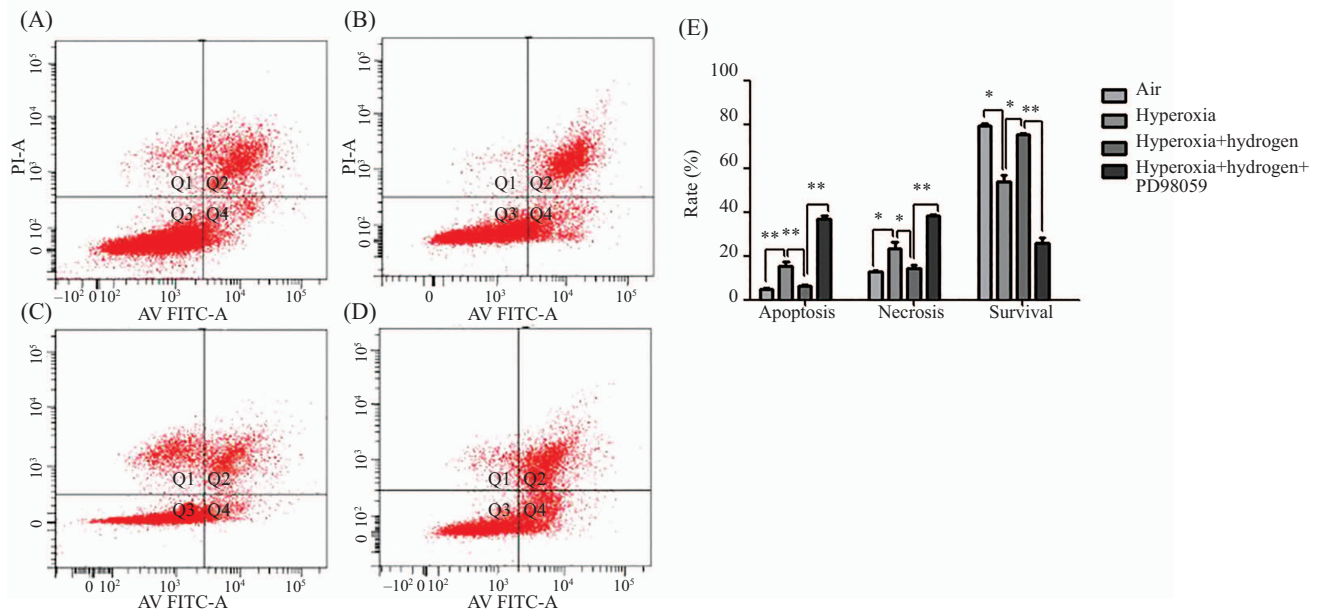
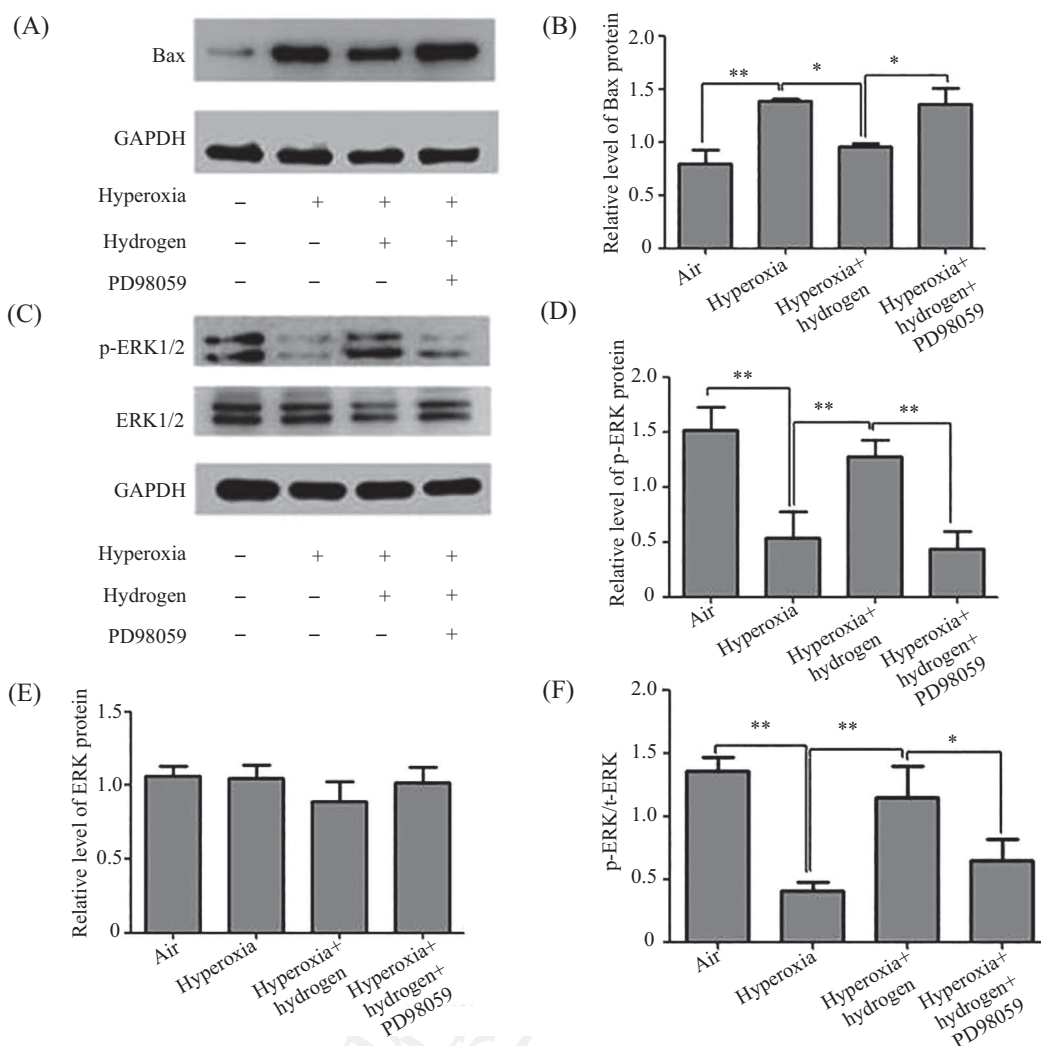
A: 空气组; B: 高氧组; C: 高氧+氢气组; D: 高氧+氢气+PD98059组; E: 定量分析各组细胞早期、晚期凋亡率和存活率。* $P < 0.05$, ** $P < 0.01$ 。A: air group; B: hyperoxia group; C: hyperoxia+hydrogen group; D: hyperoxia+hydrogen+PD98059; E: quantitative analysis of early and late apoptosis and cell survival. * $P < 0.05$, ** $P < 0.01$.

图3 流式细胞术检测氢气和抑制剂PD98059对AECII细胞凋亡的影响

Fig.3 Effect of hydrogen and inhibitor PD98059 on the apoptosis of AECII detected by flow cytometry



A、C: Western blot检测各组Bax、ERK1/2、p-ERK1/2蛋白质水平; B: 半定量分析Bax蛋白质水平; D、F: 半定量分析p-ERK1/2蛋白质水平; E: 半定量分析ERK1/2蛋白质水平。* $P<0.05$, ** $P<0.01$ 。

A,C: the levels of Bax, ERK1/2, p-ERK1/2 proteins in each group by Western blot; B: semi-quantitative analysis of Bax, ERK1/2, p-ERK1/2 protein levels in each group; B: semi-quantitative analysis of Bax protein levels in each group; D,F: semi-quantitative analysis of phosphorylated ERK1/2 protein levels in each group; E: semi-quantitative analysis of ERK1/2 protein levels in each group. * $P<0.05$, ** $P<0.01$ 。

图4 各组AECII中Bax、ERK1/2、p-ERK1/2蛋白质水平

Fig.4 The protein levels of Bax, ERK1/2, p-ERK1/2 in each AECII group

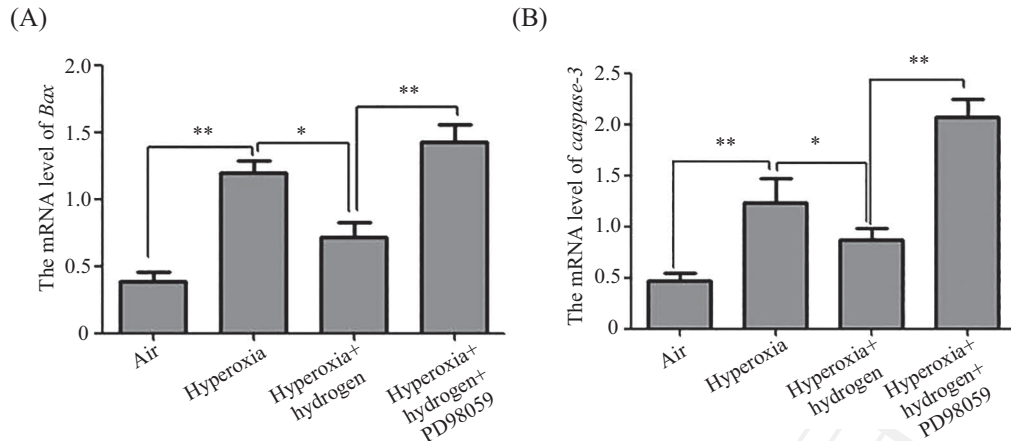
2.3 氢气和抑制剂PD98059对高氧导致AECII细胞凋亡的影响

利用Annexin V-FITC/PI双染法检测各组细胞的凋亡情况, 结果表明, 与空气组比较, 高氧组AECII早期和晚期凋亡率均升高, 存活率降低, 差异具有统计学意义($P<0.05$)。与高氧组比较, 高氧+氢气组细胞早期和晚期凋亡率呈现下降趋势, 存活率有所提高, 差异具有统计学意义($P<0.05$)。与高氧+氢气组比较, 高氧+氢气+PD98059组的早期和晚期凋亡率均明显升高, 存活率明显被抑制, 差异具有统计学意义($P<0.01$)(图3)。该结果表明, 氢气能够明显降低高氧导致的AECII的凋亡, 当加入ERK1/2特异性抑

制剂PD98059后氢气的这种保护作用被消除。

2.4 氢气和PD98059对Bax、ERK1/2、p-ERK1/2蛋白质水平的影响

Western blot检测各组相关蛋白质水平结果表明, 与空气组比较, 高氧组Bax蛋白质水平明显升高, 磷酸化ERK1/2蛋白质水平明显下降, 差异具有统计学意义($P<0.01$, 图4B和图4D)。与高氧组比较, 氢气组Bax蛋白质水平有所下降($P<0.05$), 磷酸化ERK1/2蛋白质水平升高($P<0.01$, 图4B和图4D)。当使用抑制剂PD98059干预后, 高氧+氢气+PD98059组同高氧+氢气组相比较, Bax蛋白质水平有所升高($P<0.05$, 图4B), 磷酸化ERK1/2蛋白



A: qRT-PCR检测基因*Bax* mRNA表达水平; B: qRT-PCR检测基因*caspase-3* mRNA表达水平。* $P < 0.05$, ** $P < 0.01$ 。

A: *Bax* mRNA expression detected by qRT-PCR; B: *caspase-3* mRNA expression detected by qRT-PCR. * $P < 0.05$, ** $P < 0.01$ 。

图5 各组AECII中*Bax*、*caspase-3* mRNA水平

Fig.5 The mRNA levels of *Bax*, *caspase-3* of AECII in each group

质水平呈明显下降趋势($P < 0.01$, 图4D)。各组总ERK1/2蛋白质水平无明显变化, 差异无统计学意义($P > 0.05$, 图4E)。

2.5 氢气和PD98059对*Bax*、*caspase-3* mRNA水平的影响

采用qRT-PCR检测各组*Bax*、*caspase-3* mRNA水平, 结果表明, 与空气组比较, 高氧暴露组*Bax*、*caspase-3* mRNA水平量明显升高($P < 0.01$, 图5)。高氧+氢气组同高氧组相比较, *Bax*、*caspase-3* mRNA表达水平有所下降($P < 0.05$, 图5)。与高氧+氢气组比较, 高氧+氢气+PD98059组*Bax*、*caspase-3* mRNA水平呈显著升高趋势, 差异具有统计学意义($P < 0.01$, 图5)。

3 讨论

氧化损伤是多数疾病的共同病理生理基础。氢气作为一种新型抗氧化物质, 关于氢气治疗疾病的研究在国际上逐渐成为热点。研究发现, 氢气在多脏器(心^[5]、肺^[10]、肠^[17]、眼^[18]、肾^[19]、脑^[20])损伤中均具有明显的治疗作用, 可通过中和羟自由基($\cdot\text{OH}$)、过氧亚硝基阴离子(ONOO^-)等多种氧化有毒产物而发挥抗氧化作用^[21-22]。从抗氧化出发, 氢气衍生出抗炎^[23-24]和减少细胞死亡等多种分子水平的作用机制。多项研究表明, 氢气可抑制*caspase-3*、*Bax*表达, 刺激*Bcl-2*、*Bcl-x1*表达发挥抗凋亡特性^[25-28]。本课题组前期研究亦探讨了氢气的抗氧化作用, 发现氢气能够明显改善高氧导致的AECII氧化损伤, 促进AECII在高氧环境下增殖, 降低AECII的凋亡^[12], 但对于氢

气的抗凋亡作用及调控AECII增殖、凋亡的具体作用机制并未做深入研究。同时, 关于氢气对AECII的调控机制在国内外少有报道。所以, 本研究采用原代分离培养的II型肺泡上皮细胞建立高氧肺损伤模型, 更好地模拟体内环境, 从凋亡相关途径方面探讨氢气对高氧肺损伤的影响及其潜在的分子机制。研究结果发现, 高氧环境下AECII增殖受到明显抑制, 促凋亡蛋白*Bax*表达明显升高, qPCR结果也显示, 凋亡基因*Bax*和*caspase-3* mRNA水平明显升高, 导致细胞凋亡。当加入氢气干预后发现, 氢气能够明显抑制细胞凋亡、促进细胞增殖, 同时, 促凋亡蛋白*Bax*及凋亡基因*Bax*和*caspase-3* mRNA水平表达明显下降。这表明, 氢气能够通过改善高氧导致的AECII增殖受限而发挥细胞保护效应, 而这种保护作用可能与抑制凋亡基因*Bax*和*caspase-3*的表达有关。同时, 本研究还发现, 同空气组相比, 高氧组磷酸化ERK1/2蛋白质水平降低, 氢气能使磷酸化ERK1/2蛋白质水平较高氧组有所升高, 而总的ERK1/2蛋白质表达水平在各组未见明显差异。该结果表明, ERK1/2蛋白质的激活在氢气对高氧肺损伤修复中发挥着重要作用。于是, 我们推测, 氢气可能是通过MAPK-ERK1/2信号途径调控凋亡基因*Bax*和*caspase-3*的表达起作用的。

为进一步验证氢气发挥效应是通过MAPK-ERK1/2信号通路进行调控的, 本研究通过加入MAPK-ERK1/2信号通路特异性抑制剂PD98059后, 对各组MAPK-ERK1/2信号通路的活化情况作进一步检测。ERK1/2是一种丝/苏氨酸蛋白激酶, 具有

苏氨酸和丝氨酸双重磷酸化能力, 主要受丝裂源刺激物激活, 导致丝氨酸、苏氨酸双位点磷酸化, 随后引起下游一系列转录因子的活化, 最终发挥调节细胞增殖和分化的作用。Chen等^[29]发现, 在经皮冠状动脉成形术后注射富氢生理盐水可失活Ras-ERK1/2-MEK1/2和Akt通路, 抑制血管平滑肌细胞增殖, 阻止新生内膜增生, 防止血管再狭窄。Wang等^[30]研究表明, 激活ERK在脑缺血再灌注损伤中具有神经保护作用。本研究结果发现, 高氧能抑制AECII内ERK1/2的磷酸化, 氢气在高氧条件下则能促进ERK1/2磷酸化, 激活ERK1/2信号分子, 当使用ERK1/2信号分子特异性阻滞剂后, Bax和caspase-3的表达明显升高, p-ERK1/2被明显抑制, 此结果同Wang等^[30]研究结果相一致。本研究证明了氢气可通过促进信号转导蛋白ERK1/2的磷酸化对凋亡蛋白Bax和caspase-3进行调节, 从而发挥其抗凋亡作用。因此, 本研究证明, 在高氧暴露前使用氢气干预后, p-ERK1/2表达的上调导致细胞内凋亡基因表达水平的改变是促进AECII细胞增殖的主要分子机制之一, 有助于高氧导致肺泡上皮损伤的修复。

本研究室长期致力于高氧状态下II型肺泡上皮细胞的相关机制研究, 通过对AECII分离培养的不断摸索、反复改良, 最终具备成熟的原代AECII的分离培养技术并得到认可。研究室沿用此技术培养AECII做的相关研究已成功发表多篇研究成果^[31-33]。结合实验室以往发表的文章沿用此方法得到的AECII能够满足体外进一步实验的需求。

综上, 氢气可通过激活MAPK-ERK1/2信号通路, 下调凋亡基因的表达, 改善高氧导致的AECII增殖受限和凋亡, 促进细胞存活, 减轻高氧肺损伤, 有助于维持肺泡结构的完整性和功能的稳定性。该研究为氢气的临床应用提供了理论基础及新的作用靶点。

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