

·基础研究·

转录因子X盒结合蛋白1参与视网膜色素上皮抗氧化防御机制

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摘要:【目的】视网膜色素上皮(RPE)的氧化损伤在年龄相关性黄斑变性(ARMD)的发病机制中起着核心作用。本研究旨在探讨内质网应激及其重要的转录因子X盒结合蛋白1(XBP1)在丙烯醛引起的RPE氧化损伤中起到的作用,从而为阐明ARMD的发病机制提供思路。【方法】用75 μmol/L丙烯醛分别处理人视网膜色素上皮细胞系(ARPE-19)2~24 h,观察内质网应激蛋白葡萄糖调节蛋白78(GRP78)及XBP1的表达。用XBP1 siRNA转染细胞,下调XBP1蛋白水平,用Western blot检测抗氧化基因Nrf2、SOD2在蛋白水平的表达;用DCF染色观察细胞活性氧产物(ROS)的产生;用TUNEL染色观察细胞凋亡改变。细胞用XBP1 siRNA或对照siRNA预处理后,加入丙烯醛,检测RPE细胞的凋亡情况。7只XBP1^{flax/flax}小鼠,一眼视网膜下注射Cre腺病毒,对侧眼视网膜下注射GFP腺病毒作对照。1周后取眼球。其中3只小鼠用TRIzol提取RPE的RNA,用实时RT-PCR法,检测XBP1的下游基因ERdj4和p58IPK在RNA水平的表达。另4只小鼠眼球作视网膜冰冻切片,观察腺病毒在视网膜下腔的表达,用免疫荧光染色法检测XBP1以及抗氧化基因Nrf2和SOD2在RPE的表达。【结果】丙烯醛分别处理RPE细胞2 h和4 h,GRP78的表达明显增加。处理6 h XBP1蛋白激活。XBP1表达的下调伴有抗氧化基因Nrf2和SOD2表达的减少,细胞内ROS的增加,并诱发细胞的凋亡。敲低XBP1以后丙烯醛对细胞的毒性作用明显增加。通过视网膜下注射Cre腺病毒成功转染XBP1^{flax/flax}小鼠RPE。与对照组相比,转染Cre腺病毒的小鼠RPE内的XBP1蛋白表达明显减少,RPE内的ERdj4和p58IPK RNA水平显著下调;抗氧化基因Nrf2和SOD2表达明显减少。【结论】丙烯醛作用于RPE细胞,激活内质网应激以及转录因子XBP1。抑制XBP1引起RPE内抗氧化基因表达的减少,ROS的产生增加,并且增加丙烯醛的细胞毒性。XBP1参与RPE细胞内抗氧化应激防御机制。

关键词: 丙烯醛;年龄相关性黄斑变性;内质网应激;X盒结合蛋白1

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Role of X-box Binding Protein 1 in Acrolein-induced Oxidative Injury of Retinal Pigment Epithelium

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Abstract: 【Objective】The oxidative injury of retinal pigment epithelium (RPE) plays a key role in the pathogenesis of age-related macular degeneration (ARMD). This study is to investigate the effects of endoplasmic reticulum stress and the vital transcriptional

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factor X-box binding protein 1 (XBP1) in acrolein-induced oxidative damage of RPE. **【Methods】** RPE cells were treated with acrolein (75 $\mu\text{mol/L}$) for 2 ~ 24 h, expression of glucose regulated protein 78 (GRP78) and XBP1 was determined by Western blot analysis. After being transfected with XBP1 siRNA with 24 h, the expression of XBP1 was knocked-down in RPE cells. Protein level of Nrf2 and SOD2 was then determined by Western blot analysis and intracellular Reactive Oxygen Species (ROS) generation was determined by DCF staining. Acrolein was added for 8 h after being transfected with XBP1 siRNA or control siRNA for 24 h. Apoptosis was detected by TUNEL assay before and after the treatment. Subretinal injection of Cre or GFP adenovirus was performed in XBP1^{flax} mice. After 1 week the mice were sacrificed. Total RNA was extracted from mice eyecups using TRIzol and real-time RT-PCR was performed to determine the two XBP1 down-stream genes, ERdj4 and p58IPK. Cryosectioning and immunofluorescent staining were performed to look at the expression of XBP1, Nrf2 and SOD2 in mice RPE. **【Results】** Protein level of GRP78 was significantly un-regulated after exposure to acrolein for 2 and 4 h. XBP1 was activated after acrolein treatment for 6 h. Knock-down of XBP1 by siRNA down-regulates anti-oxidant genes expression and increased ROS generation in RPE cells. Loss of XBP1 exacerbates acrolein-induced cell apoptosis. XBP1 was knocked-down in the RPE of XBP1^{flax} mice after subretinal injection of Cre adenovirus. Decreased mRNA level of ERdj4 and p58IPK, and decreased Nrf2 and SOD2 expression were seen in the Cre-injected group. **【Conclusions】** Acrolein induces ER stress and activates XBP1 in RPE cells. Knock-down of XBP1 down-regulates anti-oxidant genes expression, increases ROS generation, and exacerbates acrolein-induced cell apoptosis. XBP1 plays a role in the anti-oxidant defense in the RPE cells.

Key words: acrolein; age-related macular degeneration; ER stress; X-box binding protein 1

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年龄相关性黄斑变性(age-related macular degeneration, ARMD)是一种常见的严重致盲眼病。患者常双眼先后或同时发病,临床上以视力进行性下降、视物变形和黄斑区变性为主要特征。ARMD是西方国家50岁以上老年人低视力和盲目的首要原因之一^[1]。在中国,ARMD已跃居我国60岁以上人群致盲眼病的第4位,其发病率在65岁以上人群中为6.98%,70岁以上高达17.6%,估计目前在中国至少有两千万人患ARMD。但目前ARMD的具体发病机制仍未阐明,因此在其防治方面仍十分棘手^[2]。已有研究结果提示,与年龄相关的抗氧化保护机制的减弱及过强的氧化应激在ARMD的发病中起着主要作用^[3]。视网膜色素上皮(retinal pigment epithelium, RPE)功能障碍和变性是导致光感受器细胞损害及ARMD系列临床病理变化的早期特征性改变^[4]。ARMD的早期,功能性RPE的死亡与过强的氧化应激有关。此外,氧化应激破坏RPE内质网氧化还原的内环境,激活内质网应激信号通路,加重氧化损伤,并可引发内质网介导的凋亡信号通路。内质网应激与氧化应激的相互作用是Alzheimer等神经变性疾病发病机制的研究热点,但在ARMD发病机制的研究方面报道较少。吸烟是目前ARMD发病机制中较确定的主要危险因素之一^[5]。研究表明,烟雾中含有高浓度的强氧化剂,可以特异性地作用于RPE,引起氧化损伤及细胞凋亡,但具体机制尚不清楚^[6]。X盒结合蛋白1(X-box binding

protein 1, XBP1)是内质网内主要的转录因子,也是内质网应激保护机制的主要协调因子^[7]。本研究探讨内质网应激和XBP1在烟雾的主要成分——丙烯醛引起的RPE氧化损伤和细胞死亡中所起到的作用,以及XBP1对细胞内抗氧化基因的调节作用,以期探讨ARMD的发病机制提供思路。

1 材料与方法

1.1 试剂

丙烯醛购自美国Sigma公司。MG132购自美国Millipore公司。Lipofectamine购自美国Invitrogen公司。Cre腺病毒^[8]为美国Oklahoma大学糖尿病研究中心Takahashi Yusuke博士馈赠。XBP1 siRNA购自美国Santa Cruz公司。TUNEL试剂盒购自Roche公司。一抗GRP78购自美国Cell Signaling Technology公司。一抗XBP1, Nrf2均购自于美国Santa Cruz公司。SOD2购自美国Assay Designs公司。 β -actin购自美国Abcam公司。

1.2 细胞处理及转染

人RPE细胞系(ARPE-19)购自美国American Type Culture Collection公司。用DMEM/Ham's F12 50/50混合培养基培养。细胞达到80%~90%融合时,换成1%FBS培养基饥饿培养12~24 h后,加丙烯醛处理。细胞至50%融合时,用XBP1 siRNA或对照siRNA,与Lipofectamine 2000转染。

24 h后收细胞作免疫印迹分析或DCF染色。

1.3 免疫印迹实验

处理后的细胞加入含蛋白酶抑制剂的蛋白裂解液提取蛋白。用BCA试剂盒(美国Pierce Biotechnology公司)作蛋白定量。每泳道加入等量的25 mg蛋白样本,作SDS-PAGE凝胶电泳,随后电转膜。膜作常规封闭后,加入一抗4℃孵育过夜。一抗分别为:抗GRP78(1:5 000),抗XBP1(1:500),抗Nrf2(1:500),抗SOD2(1:4 000),抗 β -actin(1:5 000)。清洗后加入HRP结合的二抗孵育1 h。膜以增强化学发光底物(Thermo Fisher Scientific公司)显影。用Image Lab软件(Bio-Rad Laboratories)作条带的半定量分析。

1.4 细胞DCF染色

PE细胞转染成功后,加入10 μ mol/L DHDCF(美国Invitrogen公司),置于37℃温箱避光孵育45 min。用读板器做荧光素定量分析,激发光波长485 nm,发射光波长535 nm。

1.5 细胞TUNEL染色

RPE细胞转染成功后,用75 μ mol/L丙烯醛处理8 h。处理后的RPE细胞用40 g/L多聚甲醛溶液固定1 h,接着用含有0.1% Triton X-100的0.1%枸橼酸缓冲液打孔2 min,然后加入含有核苷酸和末端脱氧核糖核酸转移酶(TdT)的TUNEL反应混合液。阴性对照不含TdT酶。孵育完成后,用PBS冲洗3次,加入含DAPI的封片剂(美国Vector Laboratories公司),在荧光显微镜下观察及计数。

1.6 实验动物

动物饲养及实验操作遵守中山大学中山眼科中心动物实验伦理规定以及“眼科和视觉研究动物使用”的ARVO声明。将loxP位点靶向导入到XBP1基因内含子旁侧的外显子2,产生的转基因XBP1^{fllox/fllox}小鼠再与C57/BL6小鼠交配,5代后用于实验^[9]。饲养环境采用12 h光照-黑暗循环。

1.7 视网膜下注射

视网膜下注射采用美国World Precision Instruments公司的超微注射器完成。小鼠麻醉后,用1%环戊通散瞳,以31G针头在周边部透明角膜作穿刺口。将微泵注射器自带的35G钝性针头从穿刺口进入前房,经由瞳孔,绕过晶状体进入玻璃体,到达穿刺对侧的视网膜下腔后,注射1 μ L [含10⁹个病毒颗粒(viral particle, VP)]^[10]。1周后处死小鼠取眼球。

1.8 组织切片及免疫荧光染色

取小鼠眼杯作视网膜冰冻切片。免疫荧光染色所用一抗分别为抗Nrf2(1:100);抗SOD2(1:250),以及抗XBP1(1:100)。Cy3结合的二抗购自美国Jackson ImmunoResearch Laboratories公司。用DAPI作细胞核标记。

1.9 实时RT-PCR

用TRIzol试剂(美国Invitrogen公司)从小鼠眼杯中提取总RNA。使用Maxima First Strand cDNA合成试剂盒(含有oligo dT引物以及随机六聚体引物,美国Fermentas公司)合成cDNA。将SYBRH Green PCR Master Mix(美国Bio-Rad Laboratories)作定量实时RT-PCR分析。引物如下:ERdj4:正向GCTGGCTGATCACATTCTGCT,反向GCCGTCC AACATGCCACTA;p58IPK:正向TCCTGGTGGACC TGCAGTACG,反向CTGCGAGTAATTTCTTCCCC。目标基因的mRNA水平以18 s核糖核酸RNA作内参。

1.10 统计学分析

定量资料用平均数 \pm 标准差来表示。两组定量资料对比时用非配对Student *t*检验。3组或3组以上对比时用one-way ANOVA分析。 $P < 0.05$ 为差异具有统计学意义。

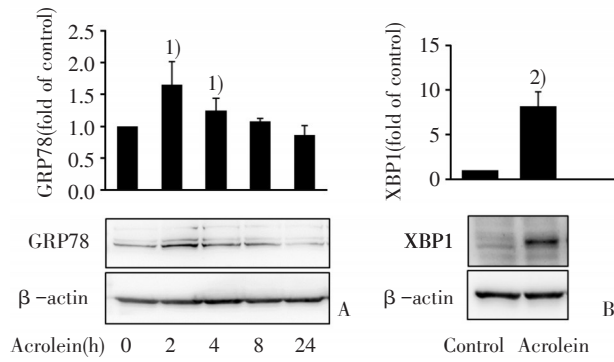
2 结果

2.1 丙烯醛激活RPE细胞的内质网应激

用75 μ mol/L丙烯醛分别处理人RPE细胞2、4、8或24 h,用免疫印迹法检测内质网应激标记蛋白——葡萄糖调节蛋白78(glucose regulated protein 78, GRP78)的表达。结果发现在处理后2、4 h,GRP78的表达均明显增加,分别为对照组的(1.65 \pm 0.36)倍($P < 0.05$),以及(1.25 \pm 0.19)倍($P < 0.05$;图1A)。用蛋白酶体抑制剂MG132预处理4 h,再以丙烯醛处理6 h,可以观察到RPE细胞XBP1表达的明显上调,为对照组的(8.20 \pm 1.59)倍($P < 0.01$;图1B)。

2.2 抑制XBP1的表达对RPE细胞的影响

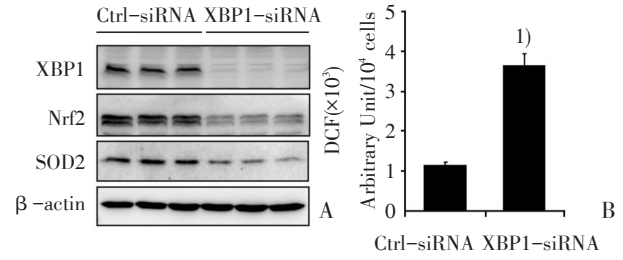
在体外用XBP1 siRNA或对照siRNA转染人RPE细胞24 h,可以观察到XBP1蛋白表达的明显下调,伴有抗氧化基因Nrf2和SOD2蛋白表达的明显减少(图2A)。细胞内DCF染色显著增加,是对照组的(3.70 \pm 0.20)倍($P < 0.01$;图2B)。



A: RPE cells were treated with acrolein (75 $\mu\text{mol/L}$). B: RPE cells were pretreated with proteasome inhibitor MG132 for 4 h, followed by acrolein treatment for 6 h. Data was expressed as mean \pm SD ($n = 3$). 1) $P < 0.05$ vs. acrolein 0 h, 2) $P < 0.01$ vs. control.

图1 用丙烯醛处理RPE细胞后的免疫印迹结果

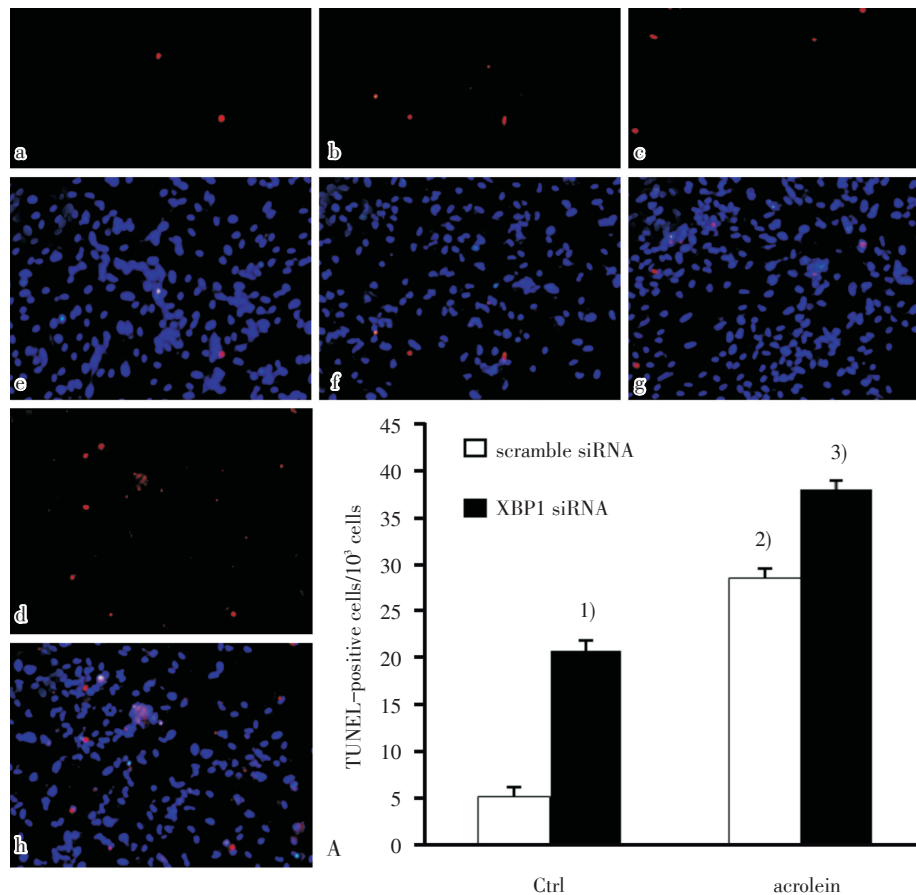
Fig.1 The Western blot results in RPE cells after acrolein treatment



Human RPE cells were transfected with XBP1 siRNA or control siRNA (scramble siRNA) for 24 h. A: Protein levels of XBP1, Nrf2 and SOD2 were determined by Western blot analysis. B: Intracellular ROS generation was determined by DCF staining. The fluorescence density was quantified by using a fluorescence plate reader with wavelength of 485/535 nm (mean \pm SD, $n = 3$). 1) $P < 0.01$ vs. Ctrl-siRNA.

图2 RPE细胞内敲除XBP1后的免疫印迹及DCF染色结果

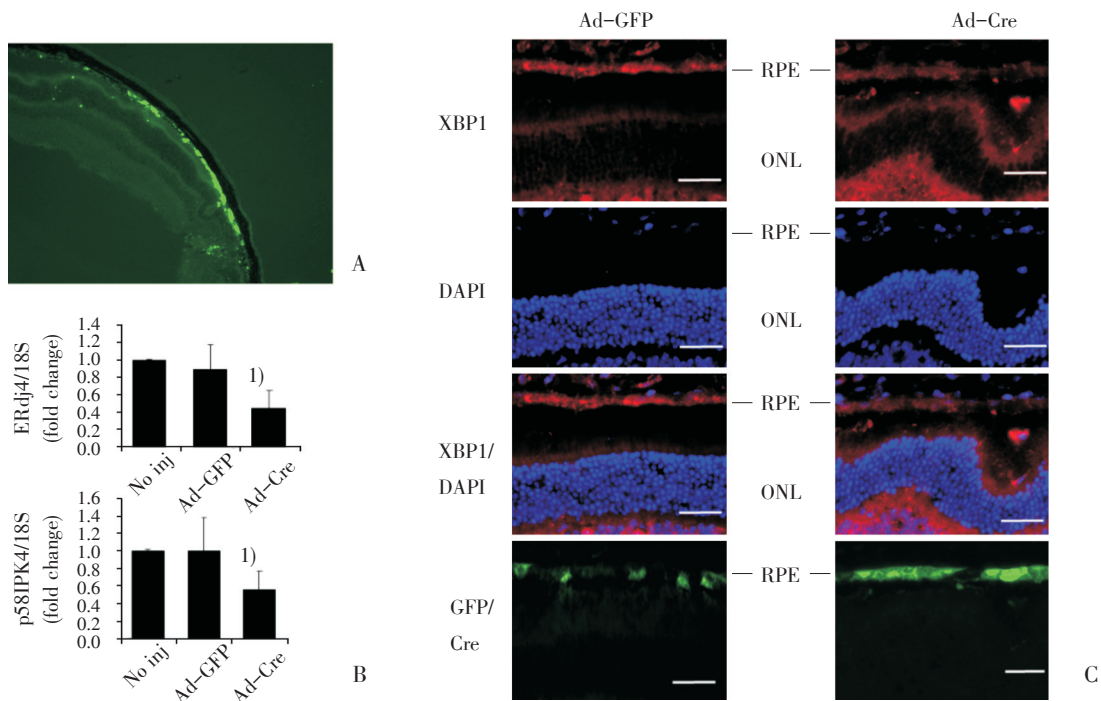
Fig.2 The Western blot and DCF staining results in RPE cells after knock-down of XBP1



Human RPE cells were transfected with XBP1 siRNA (XBP1i) or scramble siRNA (Ctrl) as control for 24 h, transfected cells were then exposed to acrolein for 8 h, apoptosis was detected by TUNEL assay. A: Representative pictures of TUNEL. Red: TUNEL staining of apoptotic cells; blue: nuclear staining with DAPI. Magnification: 100 \times . (a, e) Ctrl; (b, f) XBP1i; (c, g) Ctrl + acrolein; (d, h) XBP1i + acrolein. B: Quantification of TUNEL-positive cells. 1) $P < 0.01$ vs. scramble siRNA, 2) $P < 0.01$ vs. scramble siRNA, 3) $P < 0.05$ vs. scramble siRNA + acrolein.

图3 RPE细胞敲除XBP1后暴露于丙烯醛的TUNEL染色结果

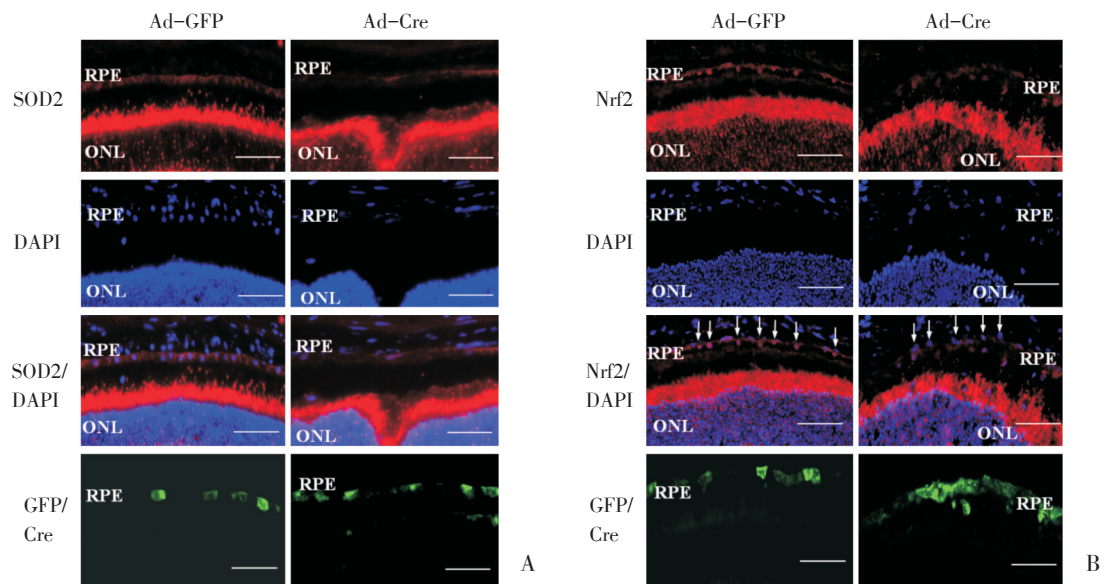
Fig.3 RPE apoptosis induced by acrolein after knock-down of XBP1



A: Visualization of the GFP fluorescence of Cre adenovirus in mice RPE after subretinal injection. B: Significant decreased mRNA level of two XBP1 downstream genes, ERdj4 and p58IPK, could be seen in the Cre virus injected group (Ad-Cre) compared to the GFP (Ad-GFP) and no-injection group (No inj) (mean±SD, n=3), 1) P<0.05 vs. No inj. C: Immunostaining of XBP1 (red) in retinal cryosections from Cre virus injected (Ad-Cre) and GFP virus injected mice (Ad-GFP) (Scale bars: 50 μm). GFP fluorescence revealed the infection of adenovirus Cre or GFP in the RPE layer. Blue: nuclear staining with DAPI. RPE, retinal pigment epithelium; ONL, outer nuclear layer.

图4 用腺病毒转染敲低小鼠RPE细胞内XBP1的表达

Fig.4 Knock-down of XBP1 in the mice RPE by subretinal injection of adenovirus



Retina sections were stained with SOD2 (A) or Nrf2 (B) antibody and a secondary Cy3-labeled antibody. DAPI was stained to show the location of cell nuclei. GFP fluorescence revealed the infection of adenovirus Cre or GFP in the RPE layer. A: The merged images showed marked decreased expression of SOD2 in the RPE of Cre-injected mouse (Ad-Cre). B: The merged images showed marked decreased expression of Nrf2 in the RPE nuclei (white arrow) in the Cre-injected mouse (Ad-Cre). RPE, retinal pigment epithelium; ONL, outer nuclear layer. Scale bars: 50 μm.

图5 敲除XBP1后,小鼠RPE内抗氧化基因的表达

Fig.5 Expression of anti-oxidant genes in mice RPE after loss of XBP1

以 XBP1 siRNA 或对照 siRNA 转染成功后,用丙烯醛处理 RPE 细胞 8 h,用 TUNEL 染色检测凋亡的细胞,发现转染 XBP1 siRNA 后 RPE 凋亡细胞数为 $(20.8 \pm 2.5)/10^3$ 个,明显多于对照 siRNA 组 $[(5.1 \pm 2.5)/10^3$ 个] $(P < 0.01)$ 。对照 siRNA 组用丙烯醛处理后细胞凋亡明显增加 $[(28.5 \pm 4.9)/10^3$ 个] $(P < 0.01)$ 。如用 XBP1 siRNA 预处理,丙烯醛引起的死亡细胞数明显多于对照 siRNA 组 $[(38.0 \pm 5.7)/10^3$ 个], $P < 0.05$ (图 3)。

2.3 敲除 XBP1^{flox/flox} 小鼠 RPE 内 XBP1 基因的影响

7 只 XBP1^{flox/flox} 小鼠,一眼视网膜下注射 Cre 腺病毒,对侧眼视网膜下注射 GFP 腺病毒作对照。1 周后处死小鼠。其中 4 只小鼠取眼球作视网膜冰冻切片,可以观察到 RPE 层有腺病毒的绿色荧光,提示转染成功(图 4A)。Cre 腺病毒组 RPE 内 XBP1 的免疫荧光染色明显弱于对照组(图 4C)。其中 3 只小鼠处死后提取 RPE 内的 RNA,分析 XBP1 的两个下游分子,ERdj4 和 p58IPK 在 RNA 水平的表达。结果发现 ERdj4 和 p58IPK 在 RNA 水平的表达明显降低($P < 0.05$),提示 XBP1 在 RPE 的表达被成功敲低(图 4B)。RPE 细胞内的 Nrf2 及 SOD2 的表达明显降低(图 5 A、B)。

3 讨论

吸烟是目前比较确定的 ARMD 的主要危险因素之一。烟雾与 RPE 损伤及 ARMD 进行性的 RPE 丧失密切相关^[11]。丙烯醛是烟雾中的主要成分,也是其中毒性最大的非饱和醛类,同时也是脂质过氧化的产物。已有研究表明,丙烯醛参与了细胞内多个生化反应过程,包括转录因子激活、基因表达的调节,以及细胞的死亡。丙烯醛在体外培养的 RPE 可引起明显的氧化应激和线粒体损伤,而内质网应激信号通路和 XBP1 在其中所起的作用未见报道^[12]。本研究结果提示,内质网转录因子 XBP1 在丙烯醛引起的 RPE 氧化损伤中起着重要作用。丙烯醛作用于 RPE,激活内质网应激,上调 XBP1 的表达。抑制 XBP1 的表达,RPE 内的抗氧化基因表达减少,细胞 ROS 增多,死亡增加,丙烯醛引起的细胞损伤更重。

XBP1 是包含 bZIP(basic leucine zipper)功能区的转录因子,是调节磷脂合成和内质网生物合

成的一个重要基因,也是重要的细胞生存因子。以往研究表明,缺乏 XBP1 的浆细胞无法完成细胞分化及行使正常功能^[13];敲除 XBP1 的小鼠肝组织和心肌组织不能正常发育^[14];敲除 XBP1 导致肠道 Paneth 细胞凋亡^[9]。然而另外有报道提示,主动脉内皮细胞 XBP1 的持续激活可以抑制细胞的正常功能并导致细胞凋亡^[15]。这些迥异的结果提示 XBP1 在不同类型的细胞,功能不同,其具体效应可能是通过调节参与细胞防御和凋亡的一系列基因起作用。本研究结果提示,XBP1 可以保护 RPE,对抗丙烯醛引起的细胞氧化损伤。敲除 XBP1 后,细胞内 DCF 染色增多,提示 ROS 产生增多,伴有细胞凋亡增加。XBP1 在 RPE 的具体作用机制尚不清楚。本研究提示,XBP1 对抗氧化基因 Nrf2 及 SOD2 可能存在一定的调节。在 ARPE-19 细胞敲除 XBP1 后,Nrf2 及 SOD2 的蛋白水平明显降低。在 XBP1^{flox} 小鼠通过视网膜下注射 Cre 病毒敲除 XBP1 后,可以观察到 Nrf2 和 SOD2 表达的明显下调,进一步证实了这一点。此外,研究提示 XBP1 激活后可以诱导一系列的非折叠蛋白反应基因,其中包括一些 ER 保护基因,例如 ERdj4 和 p58IPK。这些基因在氧化应激的情况下起着维持细胞活性的重要作用^[7]。p58IPK 在肿瘤细胞和神经元均有抗凋亡作用^[16-17]。

氧化应激在 ARMD 的发生和发展中起着重要作用^[3]。RPE 具有一套内源性的抗氧化防御系统,由一系列抗氧化基因组成,包括 SOD1、SOD2、Nrf2、过氧化氢酶等等。Imamura 等报道敲除 SOD1 基因的小鼠出现了 ARMD 的改变,例如玻璃膜疣、Bruch's 膜增厚、RPE 空泡化,以及视网膜 IS/OS 的破坏等^[18]。Justilien 等^[19]在小鼠视网膜下注射腺病毒,敲除 RPE 内的 SOD2 基因后,发现 RPE 出现显著的氧化损伤,并导致视网膜光感受器细胞的进行性死亡。Liu 等^[20]报道敲除 XBP1 的小鼠成纤维细胞抗氧化基因,包括过氧化氢酶、SOD1 等的表达降低,细胞更易受到过氧化氢的氧化损伤。本研究在体外和体内实验中发现,敲除 XBP1 后,RPE 的 Nrf2 和 SOD2 的表达均降低。XBP1 在 RPE 内调控抗氧化基因的具体机制尚未明。RPE 的抗氧化防御系统涉及复杂的作用机制,有多种转录因子和多条信号传导通路参与。XBP1 的具体调控机制有待进一步的研究。

综上所述,本研究结果提示内质网应激及转

录因子XBP1在丙烯醛引起的RPE氧化损伤中起着重要作用。RPE中的XBP1缺失足以引起细胞的氧化损伤和细胞死亡。XBP1参与RPE的抗氧化应激保护机制。本研究有望为从增强RPE及视网膜细胞内防御系统角度、早期预防和治疗ARMD方面带来新的思路。

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