·基础研究·

内质网应激PERK途径在高糖诱导血管平滑肌细胞向成骨样细胞转化中的作用

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【摘 要】目的 研究高糖刺激SD大鼠胸主动脉血管平滑肌细胞（VSMCs）向成骨样细胞转分化中内质网应激蛋白激酶R样内质网激酶（PERK）途径的作用。**方法** 原代培养VSMCs，分为正常对照组（5mmol/L D-葡萄糖），甘露醇组（5mmol/L D-葡萄糖+25mmol/L甘露醇），高糖组（30mmol/L D-葡萄糖），高糖+PERK-小干扰RNA（siRNA）转染组（30mmol/L D-葡萄糖+PERK-siRNA转染），高糖+RNA干扰阴性对照组（30mmol/L D-葡萄糖+乱序RNA转染）。分别作用48和72h。逆转录-聚合酶链反应及Western blot分别从信使RNA（mRNA）和蛋白质水平检测不同时间点内质网应激相关分子GRP78、PERK、p-PERK、eIF2α、p-eIF2α、ATF4的改变，以及成骨样细胞标志性分子核心转录因子（Cbfα-1）、骨钙素以及平滑肌细胞标志性分子α-SMA的改变情况。应用碱性磷酸酶（ALP）活性检测试剂盒检测ALP的活性。**结果** 与正常对照组和甘露醇组比较，在mRNA水平，高糖组和高糖+RNA干扰阴性对照组PERK和eIF2α表达升高（$P < 0.05$），PERK-siRNA转染后其表达水平均降低（$P < 0.05$），在蛋白质水平，PERK、p-PERK、eIF2α、p-eIF2α表达升高（$P < 0.05$），PERK-siRNA干预后其表达均降低（$P < 0.05$）。高糖刺激VSMCs后ALP活性升高（$P < 0.05$），PERK-siRNA转染后其表达均降低（$P < 0.05$）。**结论** 内质网应激PERK途径在高糖诱导VSMCs由收缩表型向成骨样细胞表型转化中发挥作用，抑制PERK途径可以部分阻止这一转化过程。

【关键词】蛋白激酶R样内质网激酶；高糖；肌细胞，平滑肌；成骨样细胞；转化

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PERK pathway in transformation of smooth muscle cells to osteoblast-like cells induced by high glucose

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【Abstract】 Objective To investigate the role of endoplasmic reticulum stress protein kinase R-like endoplasmic reticulum kinase (PERK) pathway in the transdifferentiation of SD rat aortic vascular smooth muscle cells (VSMCs) to osteoblast-like cells by high glucose. **Methods** The primary cultured VSMCs from rat's aortic segments were divided into 5 groups, including normal control group (5mmol/L D-glucose), mannitol group (5mmol/L D-glucose plus 25mmol/L mannitol), high glucose group (30mmol/L D-glucose), high glucose + PERK-small interference RNA (siRNA) group (30mmol/L D-glucose plus PERK siRNA transfection), and high glucose + scramble RNA group (30mmol/L D-glucose plus scramble RNA transfection). At 48 and 72h after the corresponding treatment, the expression of endoplasmic reticulum stress markers, including GRP78, PERK, p-PERK, eukaryotic initiation factor (eIF)2α, and p-eIF2α, osteo inductive factors such as Cbfα-1, osteocalcin, and smooth muscle cells marker α-smooth muscle antibody (α-SMA) at messenger RNA (mRNA) and protein levels were detected by RT-PCR and Western blotting, respectively. The activity of alkaline phosphatase (ALP) was measured by ALP activity testing kit. **Results** When compared with the normal group and the mannitol group, high glucose in presence or absence of scramble RNA transfection resulted significant increases in the mRNA levels of PERK and eIF2α ($P < 0.05$), while PERK siRNA transfection decreased the expression ($P < 0.05$). High glucose also induced high protein expression of PERK, p-PERK, eIF2α, and p-eIF2α ($P < 0.05$), but PERK siRNA transfection decreased the expression ($P < 0.05$). The activity of ALP was enhanced by high glucose ($P < 0.05$), but partially blocked by PERK siRNA ($P < 0.05$). **Conclusion** Endoplasmic reticulum stress PERK pathway plays a role in the transdifferentiation of VSMCs from the contractile phenotype to osteoblast phenotype induced by high

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glucose, and the transformation process can partially blunted by suppressing the PERK pathway.

【Key words】 protein kinase R-like endoplasmic reticulum kinase (PERK); high glucose; myocytes, smooth muscle; osteoblast-like cells; transformation

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血管钙化在糖尿病和尿毒症患者中十分常见,与心肌梗死、血管顺应性下降、血管成形术时夹层形成有关,血管钙化也被认为是心血管疾病的重要危险因素之一[1,2]。钙化的血管对血管舒张剂抵抗,比正常血管更加僵硬,更容易导致血栓形成和动脉粥样硬化斑块破裂。最近的研究表明,血管钙化不是一个被动的、血管疾病终末期、退行性变的表现,是一个主动的受到精确调控的过程,和骨生成极为相似[3]。血管钙化是在多种刺激因素下血管平滑肌细胞(vascular smooth muscle cells, VSMCs)向成骨样细胞表型转化(transformation)的过程[4]。VSMCs向成骨样细胞转分化(transdifferentiation)后碱性磷酸酶(alkaline phosphatase,ALP)活性增加,基质小泡形成,表达骨形态发生蛋白(bone morphogenetic proteins,BMPs),例如BMP2,以及骨基质蛋白,如骨钙素(osteocalcin bone Gla protein,BGP)、骨连蛋白(osteonectin)、骨唾液酸蛋白(bone sialoprotein)[1]。高糖刺激可以引起VSMCs向成骨样细胞转分化,引起成骨样细胞标志性分子核心转染因子(Cbfα-1)和BGP表达升高,ALP活性增加[5]。高糖和葡萄糖代谢产物葡萄糖胺可以引起血管平滑肌细胞内质网应激,与动脉粥样硬化形成有关[6]。内质网应激可以引起未折叠蛋白反应(unfolded protein response,UPR),UPR包括IRE1α、XBP1和蛋白激酶R样内质网激酶(protein kinase R-like endoplasmic reticulum kinase,PERK)途径[7]。VSMCs向成骨样细胞表型转化的机制还未完全阐明,本实验旨在揭示内质网应激PERK途径是否在高糖诱导VSMCs向成骨样细胞转分化过程中发挥作用。

1 材料与方法

1.1 试剂

低糖DMEM培养液(Gibco),优质胎牛血清(PPA),D-Hanks液(北京索莱宝科技有限公司),胰蛋白酶、D-葡萄糖、D-甘露醇(Amresco公司),抗Cbfα-1抗体、抗p-PERK抗体、抗GRP78、抗BGP抗体购自(Santa Cruz公司),抗PERK抗体、抗eIF2α抗体、抗p-eIF2α抗体、抗ATF4抗体(CST公司),辣根过氧化物酶标记的羊抗兔、羊抗鼠二抗(北京中彬金桥生物科技有限公司),ALP活性检测试剂盒(南京建成生物公司),PCR引物由上海生工生物工程股份有限公司合成。其余试剂均为国产分析纯。

1.2 细胞培养和分组

植块法进行大鼠胸主动脉VSMCs培养,方法简述如下:健康雄性SD大鼠100g,由军事医学科学院实验动物中心提供。乙醚吸入麻醉,颈椎脱臼处死,无菌条件下迅速取出胸主动脉,立即移入有冰的含有无菌D-Hanks液的平皿中。剥离血管外的纤维脂肪组织,去除血管腔的血污,移入另一有冰的无菌D-Hanks液的平皿中,纵向剪开血管,用眼科弯镊钝头轻刮血管腔内膜面,分离血管中层平滑肌细胞,移入含有20%胎牛血清的DMEM培养液中,将中膜剪成1mm×1mm×1mm的组织块,将组织块转移入25cm^{2}塑料培养瓶瓶底,培养瓶预先用少许胎牛血清润湿包被,组织块摆置均匀,间距0.2～0.5cm翻转培养瓶使瓶底朝上,向培养瓶内加2～3ml含20%胎牛血清的DMEM培养液,拧松培养瓶盖,置于37℃,5%的CO_2培养箱中4～6h(如组织贴壁不牢可延长时间),待组织块与瓶底贴附后,再次翻转培养瓶,注意操作轻柔,使培养液轻轻漫过组织块,置于37℃,5%的CO_2培养箱,绝对静止培养5d,一般在5～7d见有细胞从组织块边缘长出,以后每3d换液1次。约2周组织块周围可爬满细胞,细胞达到80%融合时即可传代,0.125%胰酶(含EDTA)消化、传代,取4～8代细胞用作实验。培养的VSMCs传至第2代时,用α平滑肌抗体(α-smooth muscle antibody,α-SMA)作为标志分子进行免疫荧光染色鉴定。

实验前24h更换无血清的DMEM培养基,VSMCs随机分为5组:(1)正常对照组(5mmol/L D-葡萄糖),细胞常规培养48或72h至实验结束;(2)甘露醇组(5mmol/L D-葡萄糖+25mmol/L甘露醇),细胞培养48或72h至实验结束;(3)高糖组(30mmol/L D-葡萄糖),高糖培养基培养48或72h至实验结束;(4)高糖+PERK-小干扰RNA(siRNA)转染组(30mmol/L D-葡萄糖+PERK-siRNA转染),转染前换OpitMEM培养液,采用Lipo2000阳离子脂质体进行转染,转染4～6h后换高糖培养基培养48或72h至实验结束;(5)高糖+RNA干扰阴性对照组[30mmol/L D-葡萄糖+乱序(scramble)RNA转染],将PERK-siRNA换为乱序RNA,余同组(4)。

1.3 逆转录-聚合酶链反应(reverse transcription polymerase chain reaction,RT-PCR)

采用TRIzol-A+提取总RNA,Thermo NanoDrop 2000分光光度计测定RNA浓度,琼脂糖凝胶电泳显示清

晰的18S条带。按VigoScript第一链互补DNA（cDNA）合成试剂盒操作步骤将RNA逆转录成cDNA，进行PCR扩增，各目的基因上下游引物（P1，P2）见表1。PCR产物电泳图像采用Image–Pro Plus4.1图像分析软件分析平均吸光度值，以目的片段和18S平均吸光度值的比值表示目的基因信使RNA（mRNA）的相对水平。

1.4 Western blot

收取各组第48和72小时细胞，采用RIPA蛋白提取液提取平滑肌细胞总蛋白，BCA法蛋白定量后分装，–80℃保存。各组样品蛋白上样量为80μg，进行SDS-聚丙烯酰胺凝胶电泳（SDS-PAGE，相对分子质量＞90ku为10%分离胶，而＜90ku为12%分离胶）。电转移蛋白至硝酸纤维膜上，5%的牛血清白蛋白（BSA）封闭40min后分别加入抗GRP78、p-PERK、eIF2α、p-eIF2α、ATF4、Runx2、α-SMA多克隆抗体（均为1∶500）、抗PERK多克隆抗体（1∶1000）4℃孵育杂交过夜，用1×TBS-T洗膜后以相应辣根过氧化物酶标记的二抗孵育1h，以GAPDH（1∶1000）单克隆抗体重复上述实验，作为上样对照及内参照。抗原-抗体复合物用ECL法显示，暗室X线胶片曝光，采用Image-Pro Plus图像分析软件分析各蛋白条带的平均吸光度值，以目标蛋白吸光度值/β-actin吸光度值的比值反映目标蛋白相对水平。

1.5 ALP活性检测

ALP活性检测试剂盒测定ALP活性。各组细胞干预48和72h结束后，收集细胞用冷PBS洗3次，加入150μl/孔（24孔板）0.05%的TritonX-100，超声波破碎细胞10s，收集悬液，4℃12000×*g*离心15min，上清即为待测样本，蛋白用BCA法定量，各样本按照说明书分别加入反应试剂，紫外分光光度计测520nm处的吸光度值，将计算出的数值除以该样品的蛋白含量，得到各样本单位蛋白中ALP的活性。

1.6 统计学处理

所有实验重复3遍，计量资料用均数±标准差表示，采用SPSS13.0对实验数据进行统计分析，用单因素方差分析（one-way ANOVA）进行多组间比较，多个样本均数两两比较采用*q*检验。$P < 0.05$为差异有统计学意义。

2 结 果

2.1 VSMCs原代培养及其鉴定

VSMCs原代培养及其鉴定结果如图1所示。

2.2 PERK-siRNA在高糖诱导VSMCs向成骨样细胞转分化中的作用

2.2.1 各组VSMCs Cbfα-1，ALP，BGP，α-SMA mRNA的表达变化 PCR结果显示，高糖处理后，相对于正常对照组、甘露醇组和高糖+PERK-siRNA组，Cbfα-1、ALP、BGP mRNA表达升高，差异具有统计学意义（$P < 0.05$）；高糖+PERK-siRNA组相对于高糖组和高糖+乱序RNA组，Cbfα-1，ALP，BGP mRNA表达降低，差异具有统计学意义（$P < 0.05$；图2～图4）。各组间α-SMA mRNA表达差异均无统计学意义（$P > 0.05$；图5）。

表1 PCR反应目的片段引物
Table 1 Primer sequence for PCR

Gene	Primer sequence		T(℃)	Length(bp)
GRP78	P_1	TCT GGT TGG CGG ATC TAC TC	59.85	345
	P_2	TCT TTT GTC AGG GGT CGT TC	57.80	
PERK	P_1	AAG ATG GTA CAG TGG ACG GC	57.80	300
	P_2	CCG TGT TCC TGG TGA AAT CT	57.80	
eIF2α	P_1	GGT GCC TAT GAT GCC TTT AAG C	60.90	497
	P_2	GCT TTG GCT TCC ATT TCT TCT G	61.00	
ATF4	P_2	GCT TTG GCT TCC ATT TCT TCT G	59.09	202
	P_2	TTG GCG AGA GAA TCT GCC TT	59.38	
Cbfα-1	P_1	CGC CTC ACA AAC AAC CAC AG	59.80	258
	P_2	TCA CTG CAC TGA AGA GGC TG	59.80	
BGP	P_1	CAA CCC CAA TTG TGA CGA GC	59.80	181
	P_2	TGC CGT CCA TAC TTT CGA GG	59.80	
ALP	P_1	GGA CCC TGC CTT ACC AAC TC	61.90	270
	P_2	CGT CTT GGA GAG AGC CAC AA	59.80	
α-SMA	P_1	AAC TGG TAT TGT GCT GGA CTC TG	60.20	399
	P_2	CTG TTA TAG GTG GTT TCG TGG AT	58.40	
18S	P_1	CCA ACC CGG TCA GCC CCC TC	61.17	279
	P_2	TAC CTC CCC GGG TCG GGA GT	60.18	

PERK: protein kinase R-like endoplasmic reticulum kinase; eIF2α: eukaryotic initiation factor 2α; BGP: bone Gla protein; ALP: alkaline phosphatase; α-SMA: α-smooth muscle antibody; PCR: polymerase chain reaction

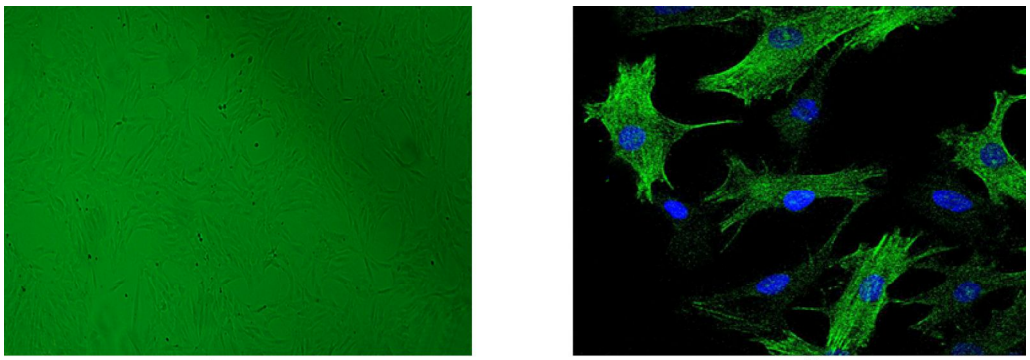


图1 血管平滑肌细胞原代培养及其鉴定

Figure 1 Primary culture and identification of vascular smooth muscle cells

α-SMA: α-smooth muscle antibody. A: cultured vascular smooth muscle cells (× 100); B: anti-α-SMA immunofluorescence staining (× 600)

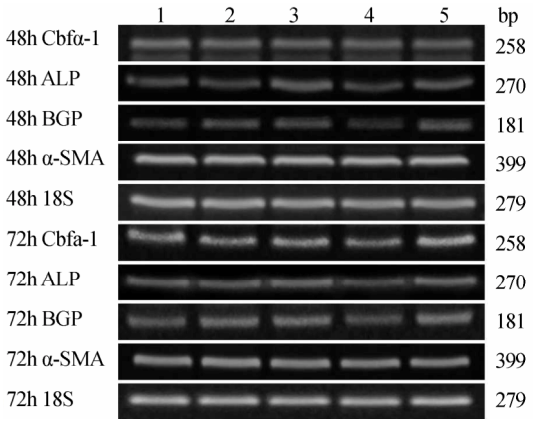


图2 各组细胞作用不同时间Cbfα-1，ALP，BGP和α-SMA mRNA的表达情况

Figure 2 Expression of Cbfα-1, ALP, BGP and α-SMA mRNA in five groups at different time points

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA group; ALP: alkaline phosphatase;BGP: bone Gla protein; α-SMA: α-smooth muscle antibody

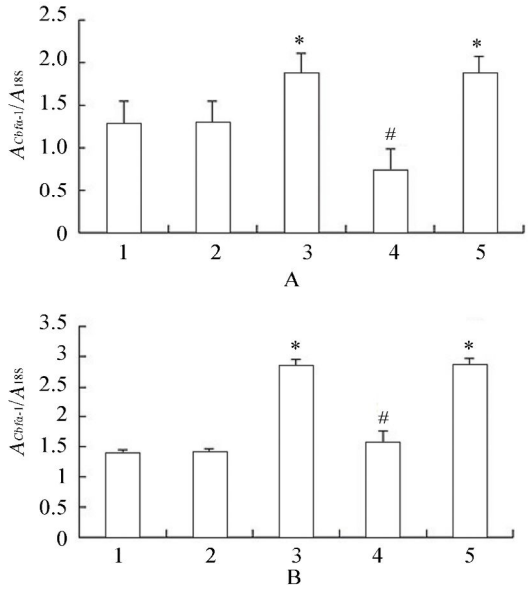


图3 各组细胞作用不同时间Cbfα-1 mRNA的表达情况

Figure 3 Expression of Cbfα-1 mRNA in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA group. A: 48h mRNA level; B: 72h mRNA level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

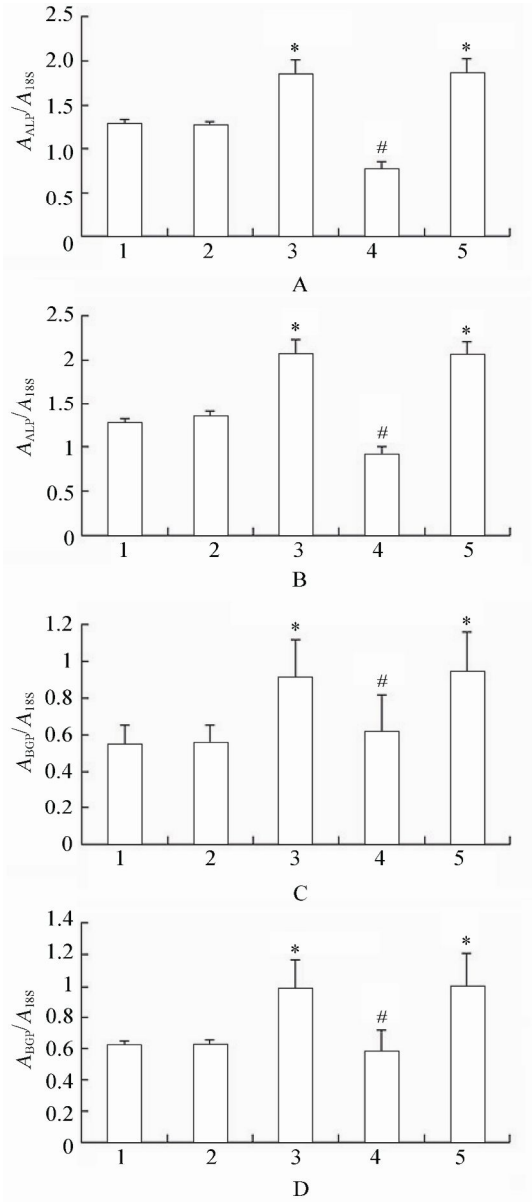


图4 各组细胞作用不同时间ALP mRNA和BGP mRNA的表达情况

Figure 4 Expression of ALP mRNA and BGP mRNA in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose+scramble RNA group. A: 48h ALP mRNA level; B: 72h ALP mRNA level; C: 48h BGP mRNA level; D: 72h BGP mRNA level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

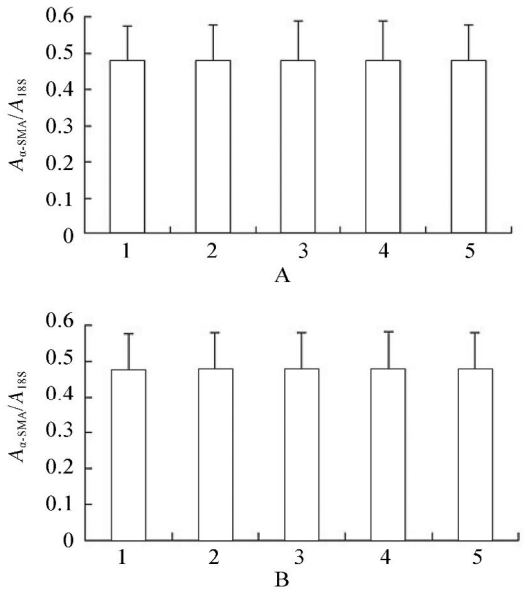


图5 各组细胞作用不同时间α-SMA mRNA的表达情况

Figure 5 Expression of α-SMA mRNA in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose+scramble RNA group; α-SMA: α-smooth muscle antibody

2.2.2 各组VSMCs Cbfα-1和α-SMA表达的变化 Western blot结果显示，高糖处理后，相对于正常对照组、甘露醇组和高糖 + PERK-siRNA组，Cbfα-1蛋白表达升高，且差异具有统计学意义（$P < 0.05$），高糖 + PERK-siRNA组相对于高糖组和高糖 + 乱序RNA组，Cbfα-1蛋白表达降低，且差异具有统计学意义（$P < 0.05$；图6，图7）。而α-SMA蛋白表达差异均无统计学意义（$P > 0.05$；图8）。

2.3 PERK-siRNA在高糖诱导引起VSMCs内质网应激

2.3.1 各组VSMCs GRP78，PERK，eIF2α，ATF4 mRNA的表达变化 PCR结果显示，高糖处理后，相对于正常对照组、甘露醇组和高糖 + PERK-siRNA组，GRP78，PERK，eIF2α和ATF4 mRNA表达升高，且差异具有统计学意义（$P < 0.05$）；高糖 + PERK-siRNA组相对于高糖组和高糖 + 乱序RNA组，GRP78，PERK，eIF2α和ATF4 mRNA表达降低，且差异具有统计学意义（$P < 0.05$；图9～图13）。

2.3.2 各组VSMCs GRP78，PERK，p-PERK，eIF2α，p-eIF2α，ATF4蛋白水平表达的变化 PCR结果显示，高糖处理后，相对于正常对照组、甘露醇组和高糖 + PERK-siRNA组，GRP78，PERK，p-PERK，eIF2α，p-eIF2α和ATF4蛋白表达升高，且差异具有统计学意义（$P < 0.05$）；高糖 + PERK-siRNA组相对于高糖组和高糖 + 乱序RNA组，GRP78，PERK，p-PERK，eIF2α，p-eIF2α和ATF4蛋白表达降低，且差异具有统计学意义（$P < 0.05$；图14～图16，图17～图20）。

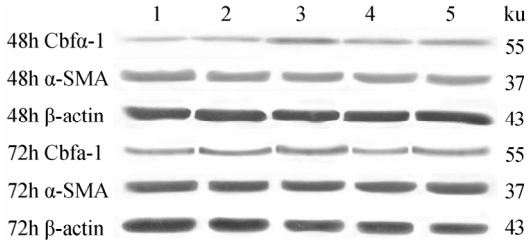


图6 各组细胞作用不同时间Cbfα-1和α-SMA蛋白的表达情况

Figure 6 Expression of Cbfα-1 and α-SMA protein in five groups at different time points

1: normal control group; 2: mannitol group; 3: high glucose group; HG+PERK- siRNA: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA; α-SMA: α-smooth muscle antibody

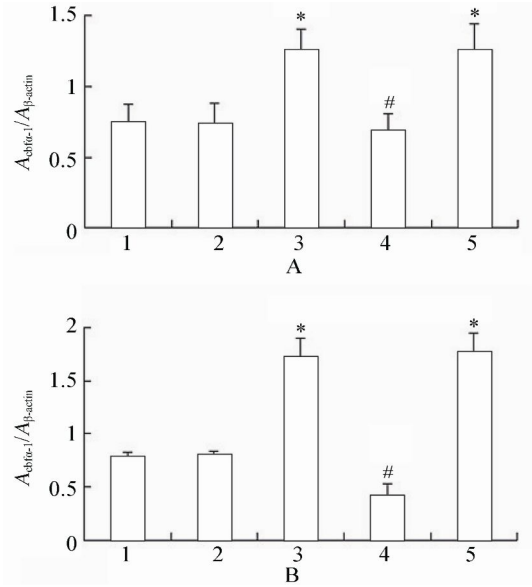


图7 各组细胞作用不同时间Cbfα-1蛋白的表达情况

Figure 7 Expression of Cbfα-1 protein in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+ PERK-siRNA group; 5: high glucose+scramble RNA group. A: 48h protein level; B: 72h protein level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

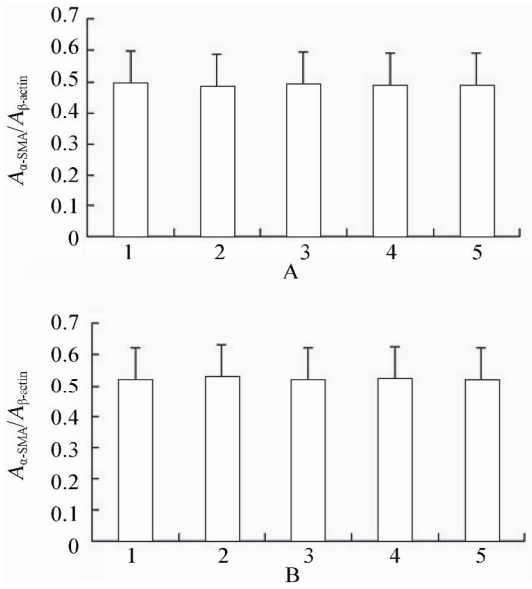


图8 各组细胞作用不同时间α-SMA蛋白的表达情况

Figure 8 Expression of α-SMA protein in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose+scramble RNA group; α-SMA: α-smooth muscle antibody. A: 48h protein level; B: 72h protein level

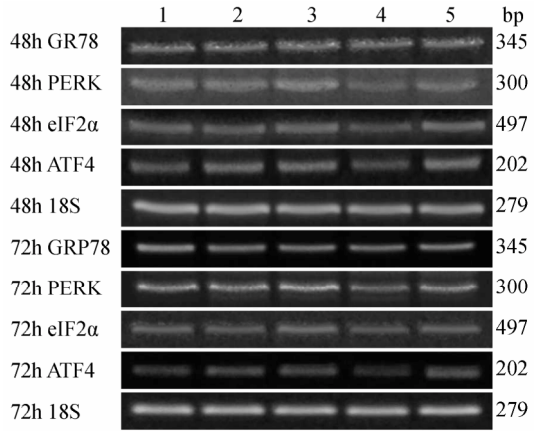


图9 各组细胞作用不同时间GRP78，PERK，eIF2α和ATF4 mRNA的表达情况

Figure 9 Expression of GRP78, PERK, eIF2α and ATF4 mRNA in five groups at different time points

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA group; PERK: protein kinase R-like endoplasmic reticulum kinase; α-SMA: α-smooth muscle antibody

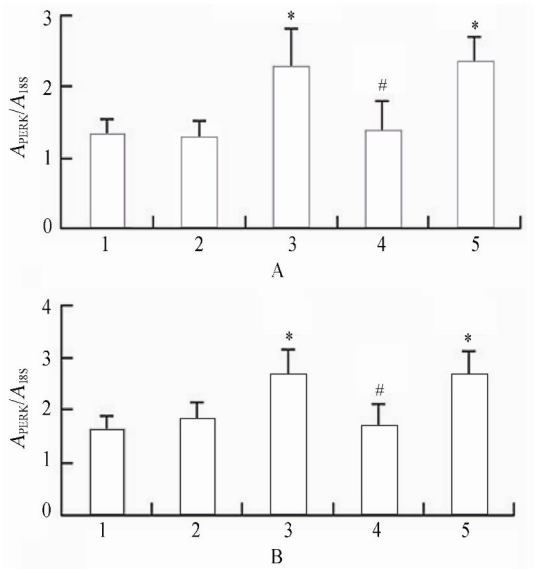


图10 各组细胞作用不同时间PERK mRNA的表达情况

Figure 10 Expression of PERK mRNA in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+Scramble RNA group; PERK: protein kinase R-like endoplasmic reticulum kinase. A: 48h mRNA level; B: 72h mRNA level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

2.4 各组VSMCs ALP活性检测

ALP检测结果显示，高糖处理后，相对于正常对照组、甘露醇组和高糖 + PERK-siRNA组，ALP活性差异具有统计学意义（$P < 0.05$）；高糖 + 乱序RNA相对于正常对照组、甘露醇组和高糖 + PERK-siRNA组，ALP活性差异具有统计学意义（$P < 0.05$；表2）。

3 讨 论

血管钙化是钙盐沉积在血管壁的一个主动的过程，和骨形成有许多相似之处。VSMCs发生表型转换在血管钙化中发挥重要作用，VSMCs的成骨样细胞表型一经形成，钙化促进因子Cbfα-1就会在血管平滑肌表达升高，Cbfα-1是成骨细胞标志性分子，其出现也标志着血管钙化的起始步骤[8]。血管钙化也和抑制血管钙化的因子减少有关，这些因子包括：

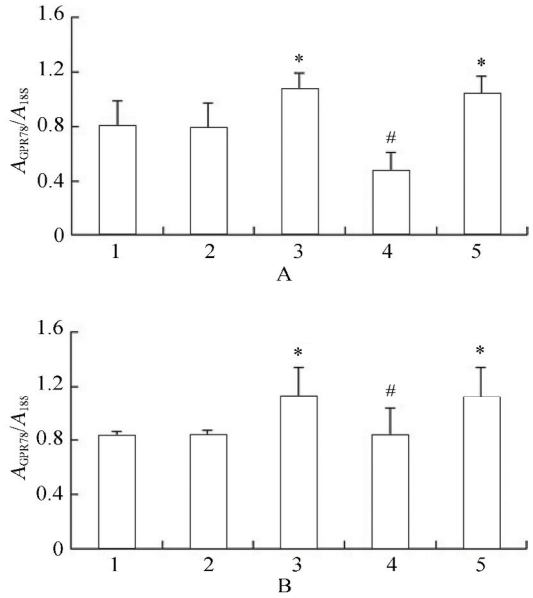


图11 各组细胞作用不同时间GRP78 mRNA的表达情况

Figure 11 Expression of GRP78 mRNA in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA group. A: 48h mRNA level; B: 72h mRNA level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

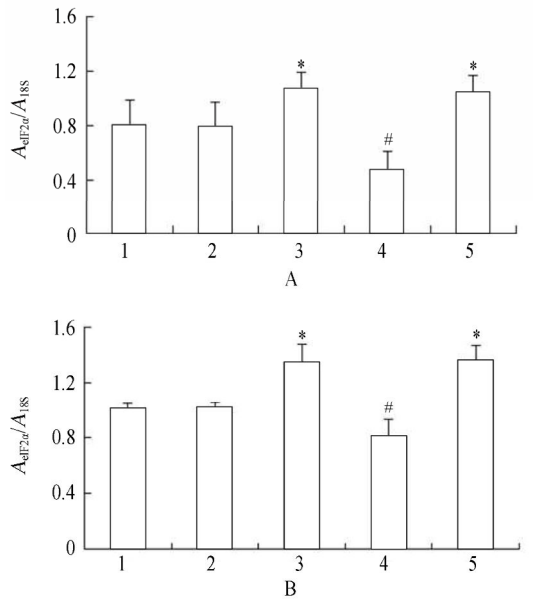


图12 各组细胞作用不同时间eIF2α mRNA的表达情况

Table 12 Expression of eIF2α mRNA in five groups at different time points (n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose + Scramble RNA group; α-SMA: α-smooth muscle antibody; eIF2α: eukaryotic initiation factor 2α. A: 48h mRNA level; B: 72h mRNA level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

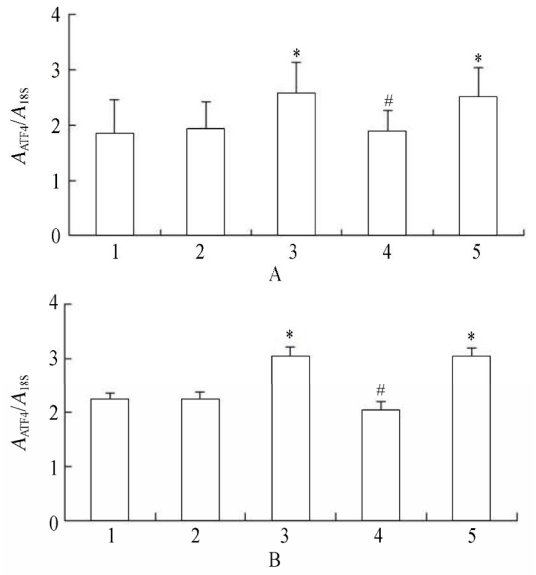


图13 各组细胞作用不同时间ATF4 mRNA的表达情况

Table 13 Expression of ATF4 mRNA in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose + scramble RNA group. A: 48h mRNA level; B: 72h mRNA level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

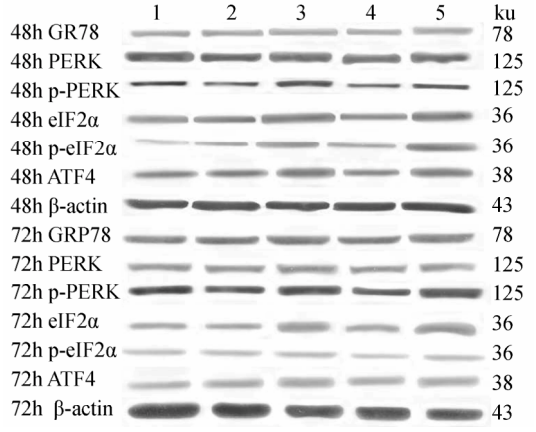


图14 各组细胞作用不同时间GRP78，PERK，p-PERK，eIF2α，p-eIF2α和ATF4蛋白的表达情况

Figure 14 Expression of GRP78, PERK, p-PERK, eIF2α, p-eIF2α and ATF4 protein in five groups at different time points

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA group; PERK: protein kinase R-like endoplasmic reticulum kinase; α-SMA: α-smooth muscle antibody

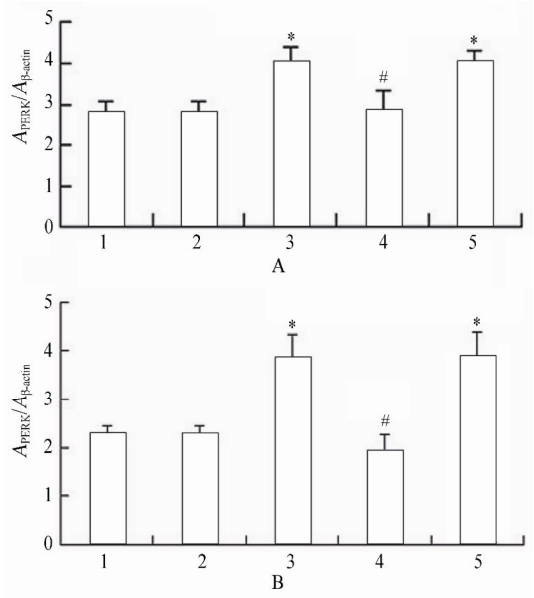


图15 各组细胞作用不同时间PERK蛋白的表达情况

Figure 15 Expression of PERK protein in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA group; PERK: protein kinase R-like endoplasmic reticulum kinase. A: 48h protein level; B: 72h protein level. Compared with 1,2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

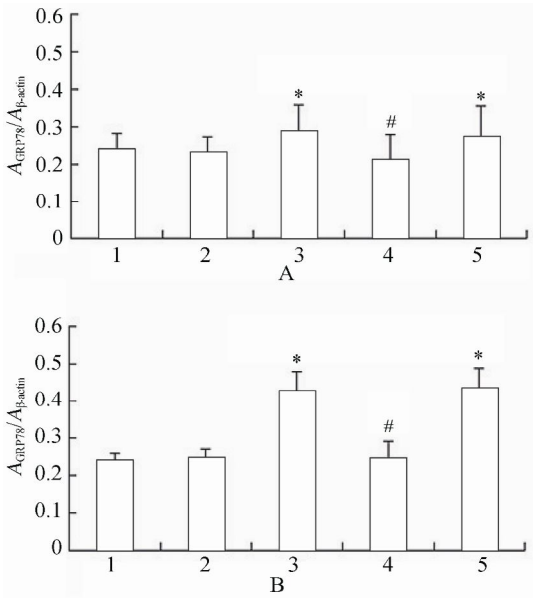


图16 各组细胞作用不同时间GRP78蛋白的表达情况

Figure 16 Expression of GRP78 protein in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose+PERK-siRNA group; 5: high glucose+scramble RNA group. A: 48h protein level; B: 72h protein level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

无机焦磷酸盐、基质Gla蛋白、骨桥蛋白、骨保护素、胎球蛋白、Smad6等[8]。

许多刺激因素可以导致VSMCs转分化，如高糖、糖基化终末产物、高磷、碱性成纤维细胞生长因子、硬脂酸盐、氧化应激、氧化低密度脂蛋白、维生素D等[1,9−11]。本研究中发现高糖刺激可以通过内质网应激PERK途径使VSMCs向成骨样细胞转分化，VSMCs表达成骨样细胞标志物Cbfα-1，BGP，ALP升高，内质网应激PERK通路激活。内质网应激PERK分子在发育过程中扮演重要角色，其功能缺失突变在人和小鼠会导致新生个体发育缺陷，如糖尿病、生长迟滞和多部位骨骼发育异常[12,13]。体外研究表明，内质网应激PERK途径在骨组织和骨细胞发育成熟中起重要作用[14]。

在体外培养和一些心血管病理生理过程中，VSMCs会经历表型转化。它们与其他肌细胞不同，是一种非终末分化细胞[14]。VSMCs是由间胚

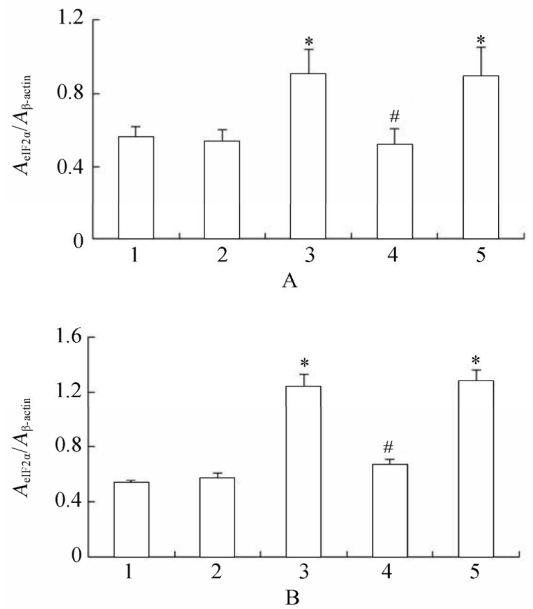


图17 各组细胞作用不同时间eIF2α蛋白的表达情况

Table 17 Expression of eIF2α protein in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose + scramble RNA group. A: 48h protein level; B: 72h protein level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

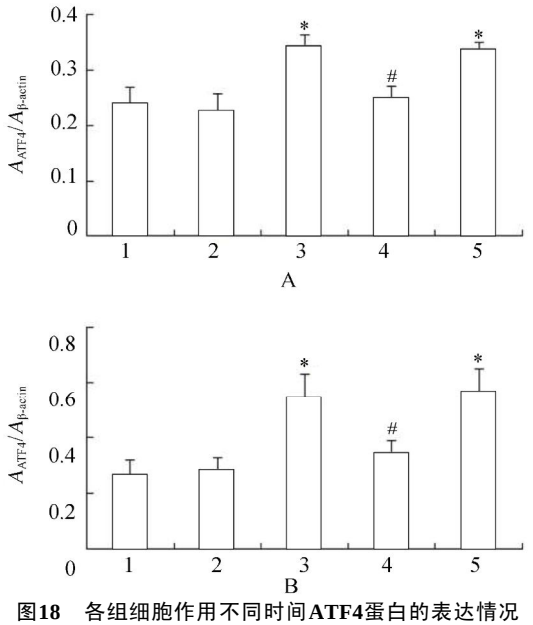


图18 各组细胞作用不同时间ATF4蛋白的表达情况

Table 18 Expression of ATF4 protein in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose + scramble RNA group. A: 48 protein level; B: 72 protein level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

层分化发育而来，对于血管的收缩和舒张有着十分重要的作用。Cbfα-1又称为Runx2，是成骨细胞发育分化的掌控因子，在骨形成中起关键作用，其在血管平滑肌细胞中表达升高被认为是向成骨样细胞分化的明确的早期标志[15]。Cbfα-1控制着其下游与成骨细胞发育分化有关的蛋白分子，如BGP、骨桥蛋白（osteopontin）和Ⅰ型胶原[16]。

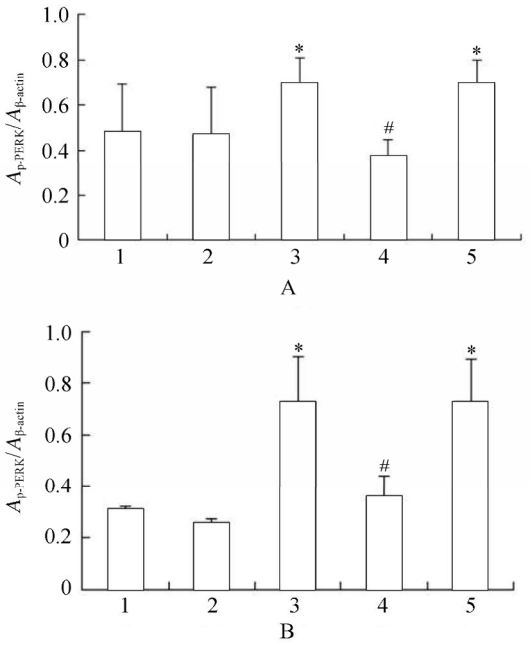


图19 各组细胞作用不同时间p-PERK蛋白的表达情况

Table 19 Expression of p-PERK protein in five groups at different time points (n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose + Scramble RNA group. A: 48h protein level; B: 72h protein level. Compared with, 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

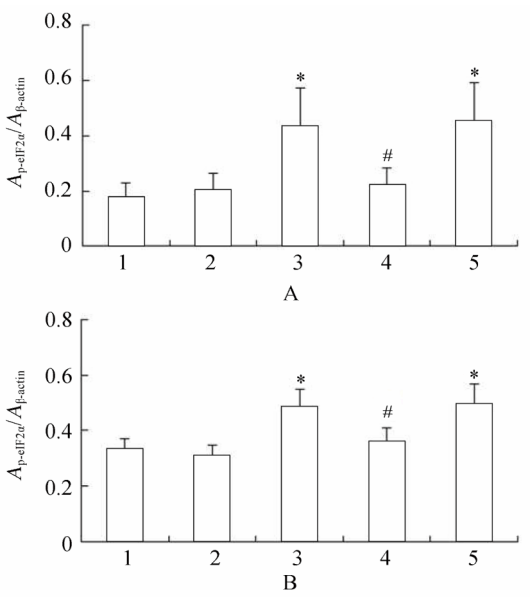


图20 各组细胞作用不同时间p-eIF2α蛋白的表达情况

Table 20 Expression of p-eIF2α protein in five groups at different time points(n = 3)

1: normal control group; 2: mannitol group; 3: high glucose group; 4: high glucose + PERK-siRNA group; 5: high glucose + scramble RNA group; p-eIF2α: p-eukaryotic initiation factar 2α. A: 48h protein level; B: 72h protein level. Compared with 1, 2 and 4, $^{*}P < 0.05$; compared with 3 and 5, $^{\#}P < 0.05$

本实验中我们用RNA干扰（RNA intenference，RNAi）技术干扰PERK的表达，成功地使其在高糖环境刺激下mRNA和蛋白质水平表达降低，其激活形式——磷酸化的PERK也降低，同时其下游eIF2α在mRNA和蛋白水平也降低，磷酸化的eIF2α表达也下降，成骨样细胞的标志物Cbfα-1和BGP表达下降，

表2 各组细胞作用不同时间ALP活性检测

Table 2 The activity test of ALP in five groups at different time points (n = 3, U/g protein, $\bar{x} \pm s$)

Group	48h	72h
CON	72.00 ± 3.62	75.00 ± 3.35
Mannitol	70.00 ± 3.91	72.00 ± 3.78
HG	99.00 ± 6.23*	113.00 ± 5.79*
HG + PERK-siRNA	69.00 ± 4.34#	83.00 ± 4.45#
HG + scramble-RNA	99.00 ± 6.25*	110.00 ± 5.92*

CON: normal control group; Mannitol: mannitol group; HG: high glucose group; HG + PERK-siRNA: high glucose + PERK-siRNA group; HG + scramble RNA: high glucose + scramble RNA group. Compared with CON, Mannitol, HG + PERKsiRNA groups, $^{*}P < 0.05$; compared with HG, HG + scramble RNA groups, $^{\#}P < 0.05$

ALP mRNA表达也下降。VSMCs在高糖刺激下，PERK-siRNA干预后其ALP活性下降。作为阴性对照的乱序RNA处理组同高糖组相比未见明显差异。甘露醇组和正常对照组相比，未引起内质网应激PERK途径表达改变，也未见成骨样细胞标志表达变化。

血管钙化的确切机制目前仍不十分清楚，但迄今认为其不是一个被动的钙盐沉积的过程，而是一个主动的、和骨形成有许多形似之处的可调控的过程。VSMCs向成骨样细胞转分化这一细胞学行为在血管钙化的发生中发挥重要作用。血管钙化的关键问题是VSMCs由收缩表型向成骨样细胞表型转化，而细胞表型转化是细胞生物学的重大科学问题[17]。本实验利用RNAi技术敲低PERK，成功阐明了内质网应激PERK途径在高糖诱导VSMCs向成骨样细胞转分化过程中发挥作用，并且干预PERK可以抑制这一转分化的细胞生物学过程，有助于对血管钙化这一病理生理现象进行干预，可以作为血管钙化早期预防的靶点。

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