

蛋白激酶C在糖基化终末产物介导结肠平滑肌细胞内钙离子浓度中的作用

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■背景资料

糖尿病(diabetes mellitus, DM)胃肠动力障碍与胃肠自主神经、Cajal间质细胞及平滑肌细胞病变相关, 平滑肌病变在DM胃肠动力障碍中的研究已成为重点, 其中DM结肠平滑肌细胞内钙离子信号通路异常, 导致钙离子浓度异常, 是本病的病理基础之一。

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Advanced glycation end products inhibit intracellular calcium concentration in colon smooth muscle cells in a protein kinase C-dependent manner

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Abstract

AIM: To investigate the effect of advanced glycation end products (AGEs) on intracellular calcium concentration in isolated colonic smooth muscle cells and the possible mechanisms involved.

METHODS: Colonic smooth muscle cells were

isolated from normal adult rats, and immunofluorescence staining for α -actin was used to identify smooth muscle cells. The responsiveness of colonic smooth muscle cells to AGEs was measured by confocal laser scanning microscopy. Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was determined by Fluo3/AM based digital microfluorimetric measurement. Protein kinase C (PKC) activity was detected by PKC activity assay. PKC inhibitor chelerythrine was used to examine the role of PKC in AGEs-mediated inhibition of $[\text{Ca}^{2+}]_i$ in colonic smooth muscle cells.

RESULTS: Colonic smooth muscle cells were successfully isolated from normal rats and identified by immunofluorescence staining. AGEs inhibited $[\text{Ca}^{2+}]_i$ in a concentration-dependent manner. AGEs at a concentration of 50 or 100 $\mu\text{g/mL}$ significantly inhibited the mean $[\text{Ca}^{2+}]_i$ compared with the control group ($56.7\% \pm 3.6\%$, $78.6\% \pm 5\%$ vs $99.6\% \pm 3.1\%$, $P < 0.05$, $P < 0.01$). PKC activity increased in SMCs treated with 50 $\mu\text{g/mL}$ or 100 $\mu\text{g/mL}$ of AGEs compared with the control group. Pretreatment with chelerythrine ($1 \mu\text{mol/L}$) reduced AGEs-mediated inhibition of $[\text{Ca}^{2+}]_i$ ($70.7\% \pm 3.7\%$ vs $87.1\% \pm 2.5\%$, $P < 0.05$).

CONCLUSION: AGEs inhibit $[\text{Ca}^{2+}]_i$ in colonic smooth muscle cells in a PKC-dependent manner.

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Key Words: Advanced glycation end products; Smooth muscle cell; Protein kinase C; Ca^{2+}

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摘要

目的: 探讨糖基化终末产物(advanced glycation end products, AGEs)对结肠平滑肌细胞内钙离子浓度的影响及可能机制。

方法: 酶解法分离培养SD大鼠结肠平滑肌细胞(smooth muscle cell, SMC), α -actin免疫荧光鉴定; 激光共聚焦显微镜检测(SMC)钙闪烁; 蛋白激酶C(protein kinase C, PKC)活性检测试剂盒检测细胞PKC活性。

结果: 细胞免疫荧光鉴定大鼠结肠SMC。不同浓度AGEs(50、100 $\mu\text{g/mL}$)刺激结肠SMC后, 与对照组比较, 钙离子浓度显著降低($56.7\% \pm 3.6\%$ 、 $78.6\% \pm 5\%$ vs $99.6\% \pm 3.1\%$, $P < 0.05$, $P < 0.01$), 50 $\mu\text{g/mL}$ 为体外最大有效浓度。与对照组相比, AGEs(50、100 $\mu\text{g/mL}$)升高细胞内PKC活性。PKC抑制剂chelerythrine可阻断AGEs介导的钙离子浓度降低($70.7\% \pm 3.7\%$ vs $87.1\% \pm 2.5\%$, $P < 0.05$)。

结论: AGEs可激活PKC通路、从而降低胞内钙离子的浓度, 最终抑制大鼠结肠平滑肌收缩。

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关键词: 糖基化终末产物; 结肠平滑肌细胞; 蛋白激酶C; 钙离子

核心提示: 本研究提示糖基化终末产物(advanced glycation end products, AGEs)可激活结肠平滑肌细胞内的蛋白激酶C(protein kinase C, PKC), PKC磷酸化下游底物丝/苏氨酸残基, 调节结肠平滑肌细胞内钙离子浓度, 参与细胞收缩等功能。

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0 引言

糖尿病胃肠动力障碍是糖尿病(diabetes mellitus, DM)的慢性并发症之一, 发生于75%的DM患者^[1,2]。糖基化终末产物(advanced glycation end products, AGEs)在DM患者血清及各种组织中均显著升高, 他与DM肾病、DM血管病变的研究颇多, 与DM胃肠动力障碍的研究很少^[3,4]。细胞外钙内流和内钙释放是平滑肌细胞(smooth muscle cell, SMC)收缩的决定因素; DM结肠

SMC内钙离子信号通路异常, 导致钙离子浓度异常^[5,6]。本文探讨AGEs是否影响结肠SMC内钙离子浓度及可能机制, 为DM胃肠道动力障碍的研究提供依据。

1 材料和方法

1.1 材料 清洁级SD大鼠, 体质量150-200 g, 由南京医科大学实验动物中心提供; DMEM培养基、胎牛血清、青链霉素混悬液、大豆胰蛋白酶抑制剂(Gibco, USA); α -actin抗体(Abgent, USA); AGEs(Merck Millipore, Germany); FITC标记山羊抗大鼠IgG二抗(Bioworld, USA); 蛋白激酶C(protein kinase C, PKC)检测试剂盒(Enzo, USA); PKC抑制剂chelerythrine、II型胶原酶(Sigma, USA); Fluo-3/AM(Invitrogen, USA)。

1.2 方法

1.2.1 结肠SMCs的分离及原代培养: SD大鼠断颈处死, 快速自肛门上2 cm取结肠10 cm左右, 用Hepes-Ringer缓冲液反复冲洗, 去除黏膜和浆膜层、剪碎平滑肌组织、加入消化液(0.1%的II型胶原酶和0.01%的大豆胰蛋白酶抑制剂)消化、离心, 含10%胎牛血清及DMEM培养液重悬细胞、过筛; 锥虫蓝染色确认细胞活力 $>90\%$, 于 $37\text{ }^{\circ}\text{C}$ 、 $95\%\text{O}_2$ 和 $5\%\text{CO}_2$ 条件下培养, SMCs长至致密单层时, 传代培养, 采用2代SMCs进行实验。

1.2.2 结肠SMCs的鉴定: 取对数生长期的SMCs, 胰蛋白酶消化、制成单细胞悬液、接种到放有载玻片的培养皿中、 CO_2 培养箱培养1-3 d, 待SMCs长至单层时, 吸去培养液, PBS冲洗、冰丙酮固定、PBS冲洗、BSA封闭30 min后吸去、加 α -actin一抗(1:100), 阴性对照不加一抗, $4\text{ }^{\circ}\text{C}$ 过夜, PBS冲洗; 加羊抗兔IgG二抗(1:500), 室温避光湿盒中孵育1 h, 冲洗、hoechst33258染核3 min、PBS冲洗、封片、镜下观察特异性荧光。

1.2.3 细胞内钙离子浓度的检测: 用DMSO溶解的Fluo-3/AM为钙荧光探针, SMCs接种于玻底皿中, PBS冲洗后, 加5 $\mu\text{mol/L}$ 的Fluo-3/AM, 置于 $37\text{ }^{\circ}\text{C}$ 、 $95\%\text{O}_2$ 和 $5\%\text{CO}_2$ 孵育箱40 min、PBS冲洗; 激光共聚焦显微镜激发光波488 nm, 发射光波为515 nm, 采样间歇为2 s, 记录单个SMC在给予AGEs前后荧光强度变化(荧光强度表示为F/F₀, F₀为初始荧光强度)。

1.2.4 PKC活性检测: 蛋白裂解液提取各组细胞蛋白, $4\text{ }^{\circ}\text{C}$ 、12000 r/min、5 min、取上清, BCA法测定蛋白总量, 加待检样品30 μL 于包被的反应孔中, $37\text{ }^{\circ}\text{C}$ 孵育2 h, 洗涤、加酶标抗体30 μL ,

■研究前沿

糖基化终末产物(advanced glycation end products, AGEs)在DM患者血清及各种组织中均显著升高, 他与DM肾病、DM血管病变的研究颇多。但AGEs与DM结肠平滑肌病变相关迄今为止尚未报道。

■ 相关报道

国内外研究报道DM胃肠动力障碍存在胃肠道平滑肌病变,且DM结肠平滑肌细胞内存在钙离子信号通路异常.

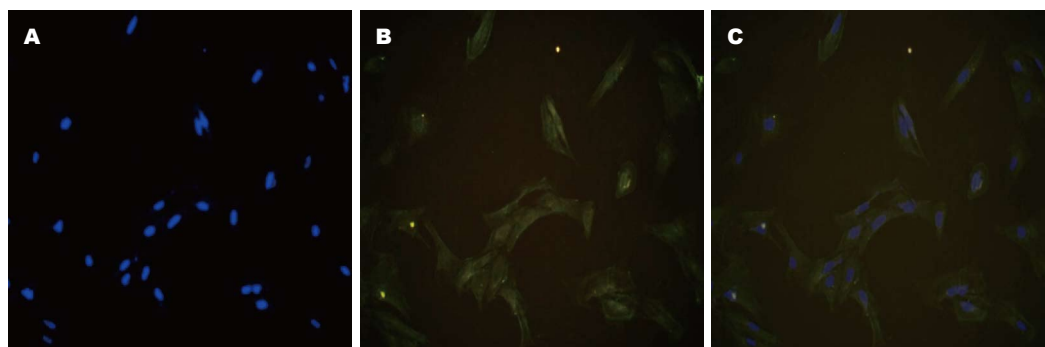


图1 大鼠结肠SMC鉴定(细胞免疫荧光×200). A: Hoechst33258染色细胞核(蓝色); B: α -actin染色(绿色); C: 融合.

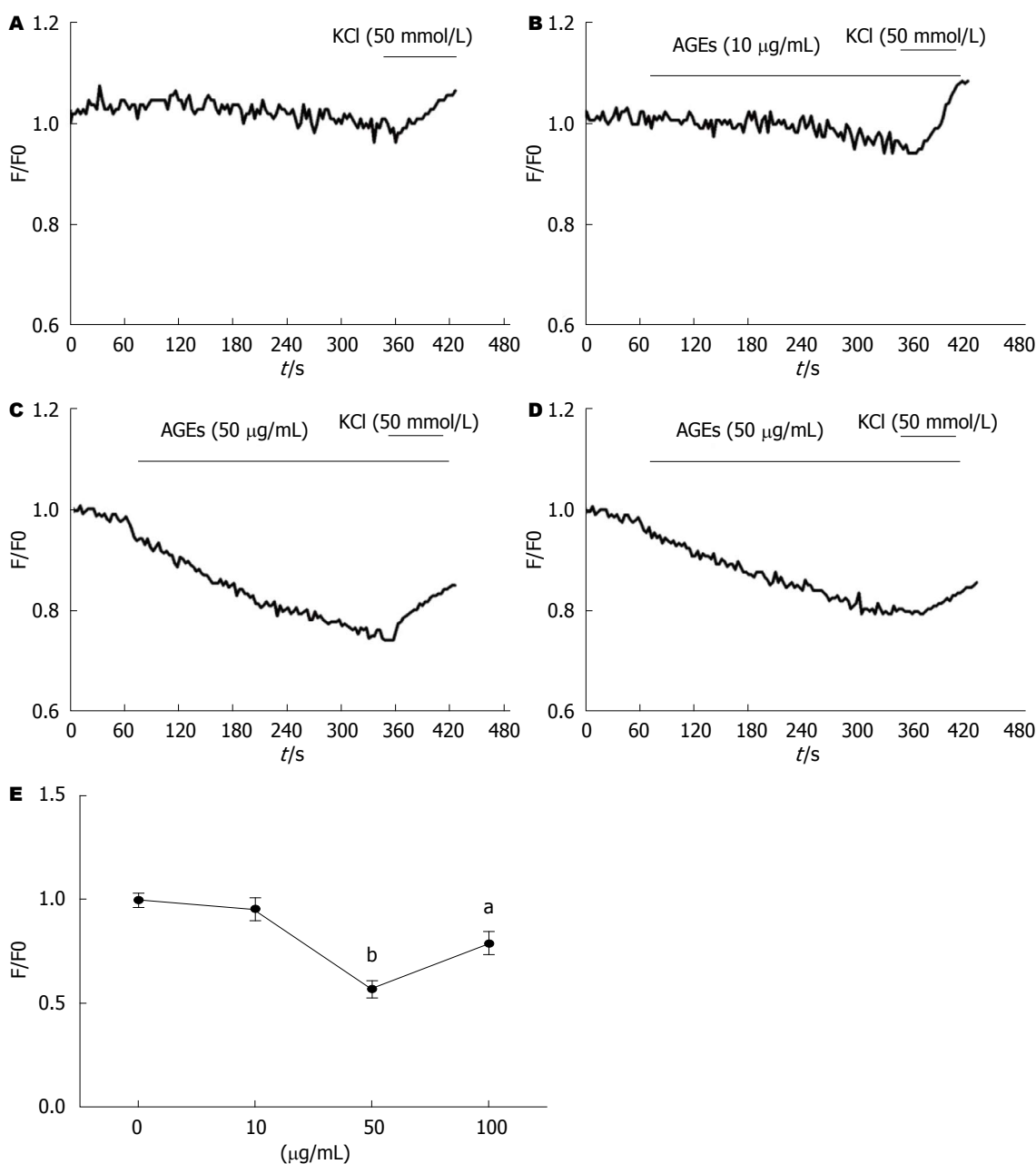


图2 Fluo-3/AM负载大鼠结肠SMC钙离子, 给予不同浓度AGEs荧光强度的变化. A: 对照组; B: 10 μ g/mL AGEs; C: 50 μ g/mL AGEs; D: 100 μ g/mL AGEs; E: 不同浓度AGEs介导结肠SMC荧光强度, 用F/F0表示, F: 实时荧光强度; F0: 基础荧光强度. ^a $P < 0.05$, ^b $P < 0.01$ vs 对照组.

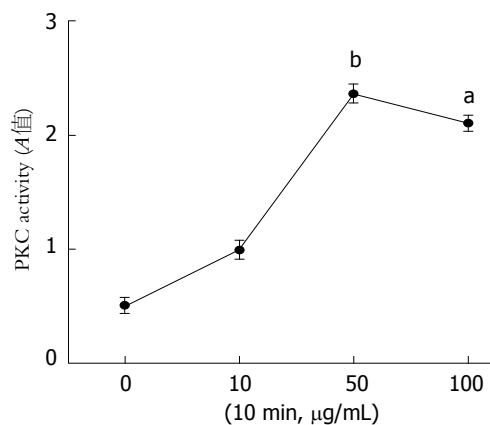


图3 不同浓度AGEs对结肠SMC中PKC活性的影响. AGEs在50 $\mu\text{g/mL}$ 和100 $\mu\text{g/mL}$ 时, 升高细胞内PKC活性. ^a $P<0.05$, ^b $P<0.01$ vs 对照组.

室温孵育2 h, 洗涤、加显色液30 μL 、显色30-45 min、终止反应, 在450 nm波长读板.

统计学处理 采用SPSS17.0软件包分析, 非参数检验之K-S检验进行数据正态性分析, 各组数据均呈正态分布, 以 $\text{mean} \pm \text{SD}$ 表示, 各组间比较采用单因素方差分析和成组 t 检验, $P<0.05$ 为差异有统计学意义.

2 结果

2.1 结肠SMCs鉴定(免疫荧光) 细胞核Hoechst染色呈蓝色, 大部分细胞 α -actin荧光反应阳性, 细胞呈梭形(胞质见红色荧光)(图1).

2.2 不同浓度AGEs对结肠SMCs中钙离子浓度的影响 与对照组相比, AGEs在50和100 $\mu\text{g/mL}$ 时, 降低Fluo-3/AM负载的钙离子荧光强度($56.7\% \pm 3.6\%$ 、 $78.6\% \pm 5\%$ vs $99.6\% \pm 3.1\%$, $P<0.05$, $P<0.01$), 说明AGEs可降低SMCs内钙离子浓度; AGEs在10 $\mu\text{g/mL}$ 时, 与对照组的差异无统计学意义; 实验结束后加入50 mmol/L氯化钾, 钙离子显著升高表示细胞活性良好(图2).

2.3 不同浓度AGEs对结肠SMCs中PKC活性的影响 与对照组相比, AGEs在50 $\mu\text{g/mL}$ 和100 $\mu\text{g/mL}$ 时, 升高细胞内PKC活性(2.3600 ± 0.0723 、 2.1060 ± 0.0625 vs 0.5123 ± 0.0614 , $P<0.05$, $P<0.01$), 而AGEs在10 $\mu\text{g/mL}$ 时, 与对照组差异无统计学意义(图3).

2.4 PKC抑制剂chelerythrine阻断AGEs介导的钙离子浓度降低 预先给予PKC抑制剂chelerythrine(1 $\mu\text{mol/L}$)可显著升高Fluo-3/AM负载的钙离子荧光强度, 说明细胞内钙离子浓度明显增加; 再加入AGEs后荧光强度下降, 但下降较溶剂

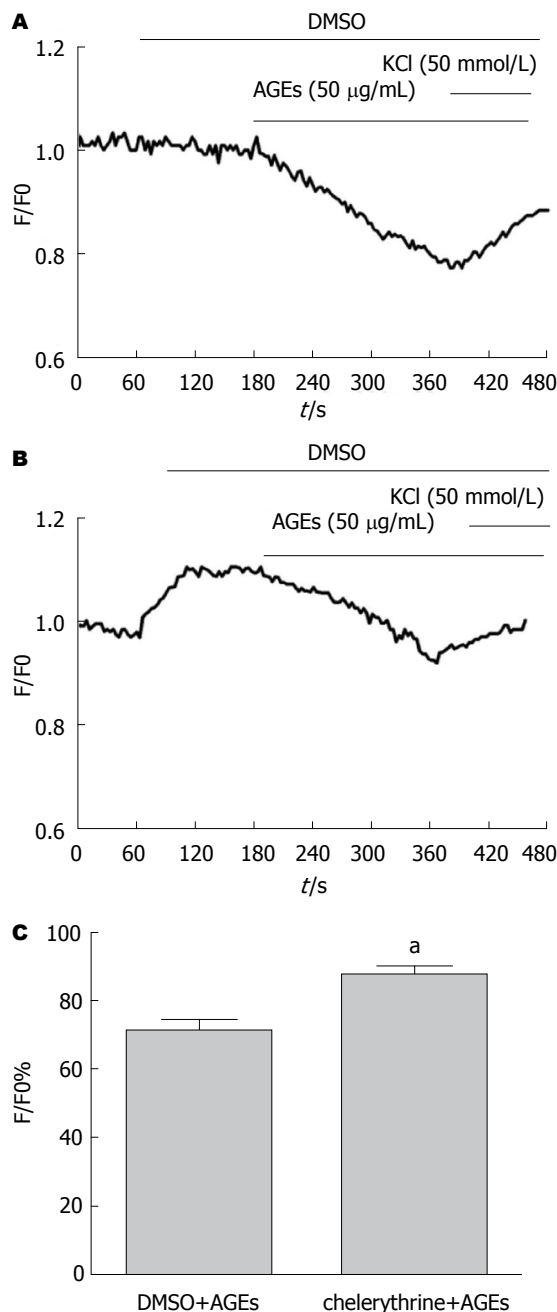


图4 PKC抑制剂chelerythrine阻断AGEs介导的钙离子浓度降低. A: 溶剂对照组; B: PKC抑制剂chelerythrine组; C: PKC抑制剂chelerythrine阻断AGEs介导的钙离子浓度降低, 用F/F0%表示, F: 实时荧光强度, F0: 基础荧光强度. ^a $P<0.05$ vs 溶剂对照组. AGEs: 糖基化终末产物.

对照组(DMSO, 1 μL)明显减少. 提示PKC抑制剂chelerythrine阻断AGEs介导的钙离子浓度降低($70.7\% \pm 3.7\%$ vs $87.1\% \pm 2.5\%$, $P<0.05$)(图4).

3 讨论

本实验证实: AGEs可以影响细胞内钙离子浓度, 其通过活化PKC, 来降低细胞内钙离子浓度; 而PKC抑制剂chelerythrine则可阻断AGEs降低细

■创新盘点

文本首次采用激光共聚焦的方法观测AGEs对结肠平滑肌细胞内钙离子浓度的影响及可能机制, 为DM胃肠道动力障碍研究提供依据.

■应用要点

本研究提示AGEs抑制结肠平滑肌细胞内钙离子浓度,可能是DM胃肠动力障碍的相关机制,为临床治疗DM胃肠动力障碍提供治疗靶点。

胞内钙离子浓度的作用。提示PKC参与了AGEs抑制大鼠结肠SMCs钙信号的作用,减少钙离子释放^[7,8]。

细胞外钙内流和内钙释放是SMCs收缩的决定因素,经典的钙致敏平滑肌收缩机制是:钙离子与钙调蛋白结合,再结合并激活肌球蛋白轻链激酶(myosin light chain kinase, MLCK),激活的MLCK磷酸化肌球蛋白轻链(myosin light chain, MLC),促进肌动蛋白和肌球蛋白之间的横桥周期、导致平滑肌收缩。而肌球蛋白轻链磷酸酶(myosin light chain phosphatase, MLCP)可使肌球蛋白轻链去磷酸化、导致平滑肌松弛^[9,10]。

研究发现PKC有多种亚型,其中 α 、 β 、 γ 等亚型主要分布于胃肠SMC上,PKC可被DAG、 Ca^{2+} 或磷脂激活,参与调节细胞收缩、细胞增殖、代谢和凋亡等多种过程^[11-13]。生理条件下,磷脂酶C(phospholipase C, PLC)激活,水解4,5-二磷酸磷脂酰肌醇(4,5-phosphatidylinositol biphosphate, PIP2)产生三磷酸肌醇(inositol triphosphate, InsP3)和甘油二酯(diacylglycerides, DAG),InsP3与内质网上InsP3R结合、引起内质网钙离子释放,细胞内钙离子浓度升高;同时PKC活化负反馈调节钙离子的释放^[14-16],PKC这种保护性负反馈机制一方面源于细胞内钙过度释放、导致内质网自身应急信号的激活,另一方面可防止细胞内钙过度释放引起能量浪费和细胞损伤^[17,18];该生理过程决定着多种生物学效应,如钙依赖性酶和通道的激活,细胞收缩等^[19-21]。另有文献报道,其他一些蛋白激酶如PKG、PKA也能负反馈调节细胞内钙离子浓度,发挥相应的生物学功能^[22-24]。

文献报道AGEs在DM患者血清及组织中比正常人明显升高,他可通过氧化应激、糖基化修饰某些蛋白在DM并发症中起重要作用^[25,26]。本实验的新颖点:从DM结肠SMCs病变入手,探讨AGEs是否通过SMCs内钙离子途径、最终影响SMCs收缩的。遗憾的是:AGEs作用受体及信号通路尚不明确(尤其是胃肠平滑肌AGEs的作用受体不明),因此没有特异性受体拮抗剂^[27-29],不能直接证明AGEs降低SMCs内钙离子浓度的信号通路。另外,调节SMCs内钙离子的机制很多,AGEs是否影响细胞外钙离子内流?是作用于胞膜上T型钙通道或者L型钙通道还是抑制内质网上InsP3R3或ryanodine受体释放钙离子^[30]?仍有待研究证实。最后,SMCs内钙离子浓度的检测还

可以结合流式细胞术及膜片钳技术多重证实。

总之,AGEs可以降低结肠SMC内钙离子浓度从而抑制收缩。AGEs激活PKC降低细胞内钙离子浓度,是细胞内钙离子降低的重要因素。DM患者结肠动力障碍与患者血清及组织中AGEs增多相关,AGEs对结肠平滑肌是否有直接抑制作用,以及AGEs降低结肠SMCs内钙离子的其他机制,仍待研究。

4 参考文献

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同行评价

本文有一定的学术价值, 研究论点新颖, 为探索DM胃肠动力障碍、平滑肌病变提供了新思路。

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