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基础研究 BASIC RESEARCH

NO体外对肠上皮细胞表达紧密连接蛋白Occludin的影响

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CONCLUSION: NO can directly kill intestinal epithelial cells. NO may affect protein and mRNA expression of the tight junction protein occludin in a dose-dependent manner.

Key Words: NO; Sin1; Caco-2 cell line; Tight junction protein occludin; Western blotting; Real time polymerase chain reaction

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■背景资料
国内报道NO对紧密连接影响甚少, 本文采用NO与Caco-2细胞共培养, 观察NO对Occludin的影响. 尽管许多国外学者在NO对紧密连接的影响上做了大量工作, 但NO影响紧密连接的机制还有待解决.

Effects of NO on the tight junction protein occludin in intestinal epithelial cells *in vitro*

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Abstract

AIM: To study the effects of NO on the intestinal mucosal barrier and on the tight junction protein occludin in intestinal epithelial cells *in vitro*.

METHODS: Colon cancer cell line (Caco-2 cells) was treated with Sin1, a NO donor, in a dose-dependent manner for 24 hours. The protein and total RNA of Caco-2 cells were extracted. Changes in occludin protein mRNA in Caco-2 cells stimulated by NO were determined by Western blotting and real-time quantitative pclymerase chain reaction, respectively.

RESULTS: The killing effect of NO on Caco-2 cells was dose-dependent. When treated with Sin1 at 125, 250, 500 and 1000 μmol/L doses, the levels of occludin protein (375 ± 0.5, 374 ± 0.8, 363 ± 0.3, 363 ± 0.7) and mRNA (0.689 ± 0.01, 0.578 ± 0.09, 0.554 ± 0.03, 0.619 ± 0.04) were significantly decreased compared with those in untreated Caco-2 cells (398 ± 0.7, 1, respectively, $P < 0.01$).

摘要

目的: 探讨一氧化氮(NO)对肠上皮细胞表达紧密连接蛋白Occludin的影响, 以研究NO对肠黏膜屏障的作用机制.

方法: 将NO的供体Sin1与肠上皮细胞株Caco-2共培养24 h, 采用MTT方法观察NO对肠上皮细胞的作用, 并分别提取细胞蛋白和总RNA, 采用免疫蛋白印迹(Western blot)蛋白半定量方法和实时定量聚合酶链式反应(RQ-PCR)方法检测不同NO浓度对Caco-2细胞表达紧密连接蛋白Occludin蛋白和mRNA表达的影响.

结果: 随着Sin1浓度升高(125, 250, 500和1000 μmol/L)NO对细胞的杀伤作用产生并逐渐增大, Occludin蛋白表达量和mRNA的相对表达量与无Sin1刺激时蛋白及mRNA的表达量相比明显降低(蛋白: 375±0.5, 374±0.8, 363±0.3, 363±0.7 *vs* 398±0.7; mRNA: 0.689±0.01, 0.578±0.09, 0.554±0.03, 0.619±0.04 *vs* 1, 均$P<0.01$).

结论: NO可直接损伤肠上皮细胞, 同时以剂量依赖形式在蛋白和分子水平影响紧密连接蛋白Occludin的表达.

关键词: 一氧化氮; Sin1; Caco-2细胞; 紧密连接蛋白Occludin; 免疫印迹; 实时定量聚合酶链式反应

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■创新盘点
NO是细胞内和细胞间的信使, 参与多种胃肠道的生理和病理过程, 本文采用NO的供体Sin1与Caco-2细胞共培养来观察NO体外对紧密蛋白的影响.

0 引言

Occludin是一种整体膜蛋白, 是紧密连接的重要组成部分, 对上皮细胞的紧密连接至关重要[1]. 紧密连接的建立和稳定是由生长因子、细胞因子和激素等严密调节的[2-3]. NO是细胞内和细胞间的信使, 广泛分布于消化道, 参与多种胃肠道的生理和病理过程, 如胃肠道运动、内脏的血流量调节、黏膜的保护以及炎症反应等, 是机体生理和病理生理过程中的一种重要化学介质. NO是否对紧密连接有影响, 国内报道甚少. 我们采用NO的供体Sin1与Caco-2细胞共培养, 观察NO对Occludin的影响.

1 材料和方法

1.1 材料 Caco-2细胞株(美国ATCC公司), NO供体-Sin1、抗人Occludin抗体和β-actin(Sigma公司), RNeas Mini kit, T7体外转录试剂盒和Real time quantitative PCR试剂盒(大连宝生物工程有限公司).

1.2 方法 参照文献[4]培养Caco-2细胞, 即应用含有200 mL/L胎牛血清, 10 g/L非必需氨基酸, 10 g/L谷氨酰胺, 1 g/L的丙酮酸钠, 青霉素-链霉素双抗液和用$NaHCO_3$调pH值的DMEM培养液将Caco-2细胞在37℃, 50 mL/L CO_2条件下进行培养, 每7 d按1∶2传代, 传代后7 d细胞生长达融合状态, 每次实验分为对照组(Sin1非添加组)和实验组(Sin1添加组), 实验时取5组非同代7 d生长达融合的细胞, 添加1000 μmol/L, 500 μmol/L, 250 μmol/L, 125 μmol/L, 0 μmol/L Sin1继续培养24 h后并收集Caco-2细胞提取总蛋白和总RNA, -130℃保存以待做Western blot和Real-time PCR使用.

1.2.1 MTT检测Sin1对Caco-2杀伤 取同代细胞接种于96孔板上, 待细胞生长达融合状态时用不同浓度Sin1处理24 h后每孔加入MTT 20 μL(5 g/L), 37℃孵箱孵育4 h, 用二甲亚砜终止反应, 492 nm波长酶标仪检测.

1.2.2 Western blot检测Sin1对Caco-2表达Occludin蛋白影响 6孔板培养细胞达融合状态后, 加入Sin1(浓度为1000 μmol/L, 500 μmol/L, 250 μmol/L, 125 μmol/L, 0 μmol/L)培养24 h, 加入蛋白裂解液, 提取蛋白, 分装, -130℃保存待用; 用紫外分光度计进行蛋白浓度测定, 然后经SDS-聚丙烯酰胺凝胶电泳(SDS-PAGE)、转膜、染膜, 观察结果并照相, 用天能GIS凝胶图象处理系统进行分析.

1.2.3 用Real-time PCR检测Caco-2细胞Occludin mRNA表达 取5组非同代7 d生长达融合状态的细胞, 添加1000 μmol/L, 500 μmol/L, 250 μmol/L, 125 μmol/L, 0 μmol/L Sin1在CO_2孵箱中继续培养24 h, 用大连Takara公司提供的试剂提取RNA, 提取的RNA利用凝胶电泳定量. 在体外将每个样本RNA 100 ng转录为cDNA, 然后进行定量PCR反应. Occludin的引物为: Occludin–F5'-AAGAGTTGACAGTCCCATGGCATAC-3', Occludin-R5'-ATCCACAGGCGAAGTTAATGGAAG-3'; GAPDH的引物: GAPDH-F5'- GCACCGTCAAGGCTGAGAAC-3', GAPDH-R5'-ATGGTGGTGAAGACGCCAGT-3'. 将构建的RNA标准品分别梯度稀释(10^{10}, 10^9, 10^8, 10^7, 10^6 copies/L)作为模板进行Real-time PCR反应, 分别制作目的基因occludin和管家基因GAPDH的标准曲线. 同时使用提取RNA样品在标准曲线上分别进行定量, PCR反应条件为95℃ 10 s, 然后95℃ 5 s和60℃ 20 s循环45次, 最后经60℃ 1 min和95℃ 5 s. 按下列公式进行相对表达量分析: Occludin mRNA的相对表达量 = occludin基因拷贝数/GAPDH基因拷贝数, 校正结果以0 μmol/L为1, 其余组与之比较.

2 结果

2.1 MTT检测结果 Sin1浓度为250 μmol/L, 500 μmol/L, 1000 μmol/L时对细胞有杀伤作用, 125 μmol/L无损害作用(图1).

2.2 NO对Caco-2细胞表达Occludin蛋白的影响 利用Western blot方法进行蛋白的半定量分析. Occludin的分子量为65 kDa, 结果表明在65 kDa位置有明显条带, 加入Sin1后蛋白表达量明显下降, Sin1浓度为125 μmol/L, 250 μmol/L, 500 μmol/L, 1000 μmol/L时Occludin蛋白表达量分别为375±0.5, 374±0.8, 363±0.3, 363±0.7, 与无Sin1刺激Caco-2的Occludin蛋白表达量398±0.7相比明显降低(P<0.01, 图2).

2.3 NO对Caco-2细胞Occludin mRNA相对表达量的影响 应检测的mRNA以0 μmol/L Sin1时Occludin mRNA的相对表达量设为1, 通过管家基因GAPDH的校正得出Sin1浓度为125 μmol/L, 250 μmol/L, 500 μmol/L, 1000 μmol/L时Occludin mRNA的相对表达量分别为0.689±0.01, 0.578±0.09, 0.554±0.03, 0.619±0.04, Sin1浓度为125 μmol/L, 250 μmol/L, 500 μmol/L, 1000 μmol/L刺激Caco-2表达Occludin的mRNA相对含量与无Sin1刺激Caco-2表达Occludin的mRNA相对含量相比明显下降(P<0.01), 在500 μmol/L达最低, 1000 μmol/L轻微升高接近125 μmol/L(图3).

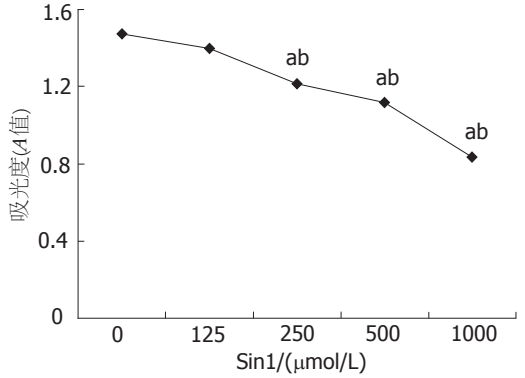

图 1 Sin1对细胞的杀伤作用. $^{a}P<0.05$ *vs* 125 μmol/L, $^{b}P<0.01$ *vs* 0 μmol/L.

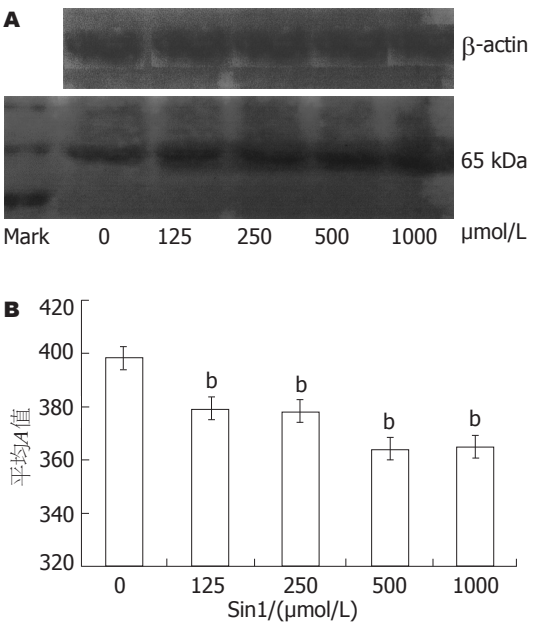

图 2 Occludin蛋白表达量随Sin1浓度升高明显下降. $^{b}P<0.01$ *vs* 0 μmol/L.

3 讨论

单层Caco-2细胞类似正常人肠上皮细胞, 在形态学上与人体肠上皮细胞相同, 并可分泌与人体相同的酶类、转化因子等[5], 此细胞来源于人体结肠癌细胞株, 在生长至融合后有柱状突起, 类似于小肠微绒毛. 向培养液一侧形成刷状缘并有分化良好的紧密连接, 是目前很好的体外肠屏障模型[6], 特别是在紧密连接蛋白表达研究中得到广泛应用[7-9]. 紧密连接是上皮屏障的一种重要的因子, 对相邻细胞的相互连接中起重要的作用. 他维持着细胞顶部和嗜碱性部位的分离, 是上皮渗漏的调节屏障[10]. 作为转膜蛋白的Occludin是紧密连接的重要组成部分, 他是分子质量为65 kDa的跨膜蛋白. NO由3型一氧化氮合酶(NO synthase, NOS)催化L-精氨酸合成, 他是

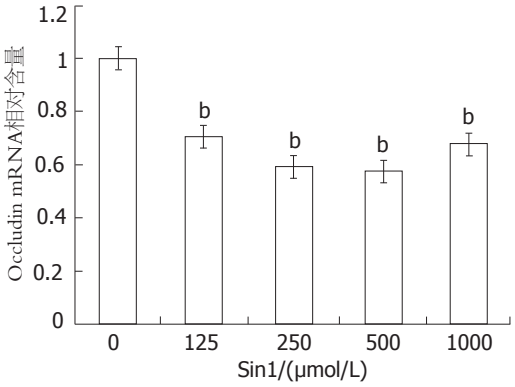

图 3 Occludin mRNA相对含量. $^{b}P<0.01$ *vs* 0 μmol/L.

一种多效性生物气体分子, 可参与调节血管舒张、突触传递、巨噬细胞的杀伤活性和免疫反应等, 是机体生理和病理生理过程中的一种重要化学介质[11]. 内生的NO能通过调节小肠的运动而调节肠道营养的运转[12], 他是胃肠道主要的非肾上腺素能-非胆碱能的神经递质[13], 他参与紧密连结蛋白包括Occludin的表达[14], 调节紧密连结的关和开[15], 他是紧密连结的重要调节因子[16]. 此外NO还是肠道黏膜防御的重要因子[17], 他调节黏膜免疫细胞的活性; 减少白细胞与内皮的相互黏附; 并调整黏膜血流; 减少上皮的渗漏; 刺激黏液的产生和碳酸氢盐的分泌[18-19]; 他还通过细胞毒特性进行黏膜防御[20], 由实验证实长期少量NO注射可减少实验性结肠炎症并加速其治愈[21]. 尽管在正常情况下NO对黏膜有防御作用, 但有研究显示NO对组织损伤起作用[22], 更有研究证明NOS抑制剂减弱结肠损伤和炎症反应[23-25], 此外NO还通过抑制CAMP依赖的CFTR(一种上皮细胞离子通道和氯分泌调节蛋白)抑制氯的分泌[26], 他能使细胞间紧密连接变得松弛而导致肠黏膜通透性增高, 能直接作用于肠黏膜, 导致高通透和细菌易位[27], 而且通过形成过氧化硝酸盐来损伤肠黏膜上皮[28]. 在体外NO作用于肠上皮细胞可导致钠、钾ATP酶抑制[29].

Sin1是NO的供体, 他可产生NO和等量的超氧化物, 用Sin1与细胞共培养24 h可引起大约50%细胞死亡[30]. 我们用Sin1刺激Caco-2, 观察NO对肠上皮表达Occludin的影响. MTT结果显示Sin1随浓度的加大对Caco-2细胞有杀伤作用, 这证明NO可直接作用于肠上皮细胞, 引起肠上皮细胞的损伤. NO的生物学作用除作为生物信使外, 还具有细胞毒作用, NO可作用于巯基使能量代谢或与抗氧化有关的酶失活, 并可直接

■应用要点

本文分别利用Western blot方法和Real-time PCR方法得出加入Sin1后Occludin蛋白和mRNA表达都明显减少, 从而得出NO在分子和蛋白水平影响紧密连结蛋白Occludin的表达, 并可直接杀伤场上皮细胞, 损伤肠黏膜屏障.

■同行评价
本文研究得出NO可直接损伤肠上皮细胞，同时以剂量依赖形式在蛋白和分子水平影响紧密连接蛋白Occludin的表达，方法成熟，设计合理，有一定的可读性.

损伤细胞DNA. 国外有学者利用NO另一供体—DETA-NONOate处理Caco-2发现, NO从分子和蛋白水平降低了紧密连接蛋白的表达[31-32]. 我们利用Western blot方法进行蛋白半定量检测发现加入Sin1后Occludin蛋白表达下降, 应用Real-time PCR检测加入Sin1后Caco-2表达Occludin mRNA水平得出加入Sin1后Occludin mRNA表达也明显减少, 这与国外学者报道相符. 由此得出NO在分子和蛋白水平影响紧密连接蛋白Occludin的表达, 并可直接杀伤肠上皮细胞, 损伤肠黏膜机械屏障.

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• 消息 •

全国消化及消化内镜诊断与治疗进展学术研讨会征文启事

本刊讯 为提高我国消化内镜诊疗技术的整体水平, 《中华消化内镜杂志》编辑部拟于2007-08在新疆乌鲁木齐市召开“全国消化及消化内镜诊断与治疗进展学术研讨会”, 邀请消化和消化内镜专家作有关专题学术报告. 会议将出论文汇编, 并授予继续教育Ⅰ类学分, 《中华消化内镜杂志》将择优刊登应征论文.

1 征文内容

征文内容包括消化系统疾病的内镜(食管镜、胃镜、十二指肠镜、小肠镜、大肠镜、肠道镜、腹腔镜、超声内镜等)诊疗技术; 内镜外科的临床应用及进展; 食管、胃、肠、肝胆、胰腺疾病的基础研究、临床诊治及其进展(炎症、溃疡、出血、肿瘤、异物等); 消化系统疾病的中医、中西医结合治疗及其进展; 消化内镜消毒及护理技术, 消化系统疾病的急诊护理.

2 征文要求

应征文章按《中华消化内镜杂志》稿约要求撰写打印, 并寄3000字以内全文及500字以内的论文摘要各一份; 已投《中华消化内镜杂志》尚未发表的稿件, 请注明稿号. 应征文章经单位推荐盖公章后, 寄南京市紫竹林3号《中华消化内镜杂志》编辑部卜小乐、赵在文同志收. 邮编: 210003. 信封左下脚注“征文”字样, 同时汇寄审稿费10元. 请自留底稿, 恕不退稿. 有关会议的具体事项另行通知. 联系电话: 025-83472831, 86086091.