

应激对大鼠结肠神经系统 nNOS 表达的影响

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Effect of stress on expression of neuronal nitric oxide synthase in colonic nervous system in rats

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Abstract

AIM: To investigate the effect of stress on the expression of neuronal nitric oxide synthase (nNOS) in colonic nervous system in rats.

METHODS: Thirty male SD rats were randomly divided into control group, stress group and NG-nitro-L-arginine methyl ester (L-NAME) group. The rat model of water immersion-restraint stress (WRS) was established. The expression of nNOS in colonic submucous plexus and myenteric plexus in the rats was examined by immunohistochemical staining and analyzed by computer image analysis system.

RESULTS: nNOS immune-positive substance was mostly expressed in the neurons of submucous plexus and myenteric plexus. In comparison with that in control group, the gray value of nNOS positive neurons in submucous plexus and myenteric plexus was significantly decreased ($P = 0.02$ or $P = 0.005$), and the density of nNOS positive neurons was increased markedly ($P = 0.04$ or $P = 0.01$) in stress group. Moreover, nNOS

expression in mucosal epithelial cells and lamina propria lymphocytes were also observed. In comparison with that in stress group, the gray value of nNOS positive neurons in submucous plexus and myenteric plexus was increased ($P = 0.04$), and the density of nNOS positive neurons was decreased ($P = 0.04$ or $P = 0.03$) in L-NAME group. nNOS expression was not significantly different between the rats of L-NAME and control group ($P > 0.05$).

CONCLUSION: WRS can increase the expression of nNOS in colonic nervous system in rats, which suggests nitric oxide (NO) may play an important role in WRS-induced function disorder of colon.

Key Words: Stress; Colonic nervous system; Neuronal nitric oxide synthase; Immunohistochemistry

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摘要

目的: 探讨应激对大鼠结肠神经系统nNOS表达的影响。

方法: SD大鼠30只随机分为对照组, 应激组和L-NAME组, 采用水浸-束缚应激(WRS)动物模型, 用免疫组织化学ABC法检测nNOS在大鼠结肠黏膜下神经丛和肌间神经丛的表达, 应用计算机图像分析系统对其表达进行定量分析。

结果: 与对照组比较, 应激组黏膜下神经丛和肌间神经丛的nNOS阳性神经元的灰度值明显减少($P = 0.02$ 或 $P = 0.005$), 阳性神经元细胞数的平均密度增加($P = 0.04$ 或 $P = 0.01$), 表达增强, 且在黏膜上皮细胞、固有层淋巴细胞也有nNOS表达。L-NAME组黏膜下神经丛和肌间神经丛的nNOS阳性神经元的灰度值较应激组增加($P = 0.04$), 平均密度下降($P = 0.04$ 或 $P = 0.03$), 表达减弱, 而与对照组比较均无明显差异($P > 0.05$)。

结论: 应激可引起大鼠结肠神经系统nNOS表达增强, 提示一氧化氮(NO)在应激所致的结肠功能失调中可能起重要作用。

关键词: 应激; 肠神经系统; nNOS; 免疫组化

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0 引言

肠道存在复杂的肠神经系统(enteric nervous system, ENS). ENS接受中枢神经系统的调控, 并通过各种神经介质调节肠道平滑肌的运动和腺体分泌, 维持其正常生理功能. 近年来发现应激参与肠道某些功能性疾病、自身免疫性疾病(如肠易激综合征, IBS)的发生、发展^[1-3]. 一氧化氮(nitric oxide, NO)是一种新型气体信号分子和神经递质, 可舒张胃肠平滑肌, 调节肠道血液循环, 参与细胞信号传递, 在肠道的生理及病理过程中具有重要的生物学功能^[4-6]. 有关应激对神经型一氧化氮合酶(nNOS)在大鼠肠神经系统表达的影响尚未见相关报道. 我们采用水浸-束缚应激(water immersion-restraint stress, WRS)动物模型^[7], 应用免疫组织化学方法, 旨在探讨应激对大鼠结肠神经系统nNOS表达的影响, 并为IBS动物模型的制作提供新的思路.

1 材料和方法

1.1 材料 左旋硝基精氨酸甲酯(NG-nitro-L-arginine methyl ester, L-NAME)购自美国Sigma公司, 使用前用生理盐水稀释(浓度为5 g/L); 兔抗鼠nNOS多克隆抗体(工作浓度为1:100)和免疫组织化学试剂盒(即用型SABC)均购自武汉博士德生物工程有限公司. 清洁级♂SD大鼠30只(浙江省实验动物中心提供), 体质量200-220 g, 随机分成对照组、应激组和L-NAME组, 每组10只. 实验前大鼠禁食24 h, 禁水1 h. L-NAME组大鼠在应激前30 min ip L-NAME 20 mg/kg, 对照组和应激组大鼠均于实验前30 min ip等体积生理盐水. 然后将应激组和L-NAME组大鼠束缚于鼠板上并垂直浸入20±1℃水浴中, 水面平大鼠胸骨剑突水平, 持续4 h. 对照组不施加应激.

1.2 方法 大鼠用10 g/L戊巴比妥钠(30 mg/kg)颈后皮下注射麻醉. 剖腹后在距回肠末端3 cm处剪取长度为1.0 cm的结肠标本. 切开肠管后用生理盐水漂洗, 放入40 g/L多聚甲醛溶液固定, 常规石蜡包埋后制作4 μm切片, 用于HE染色和免疫组织化学分析. 免疫组织化学采用SABC法, 主要步骤包括: 石蜡切片脱蜡、梯度酒精脱水; 30 mL/L H₂O₂甲醇液室温孵育20 min灭活内源性过氧化物酶; 枸橼酸缓冲液(0.01 mol/L, pH 6.0)中92-100℃抗原修复20 min, 冷却; 30 mL/L正常山羊血清37℃孵育20 min; 一抗工作液(兔抗鼠nNOS)冰箱内4℃孵育24 h; 生物素标记的羊抗鼠IgG 37℃孵育20 min; 链霉卵白素37℃孵育20 min; SABC室温孵育20 min; DAB显色, 黄色或棕黄色产物为阳性标记;

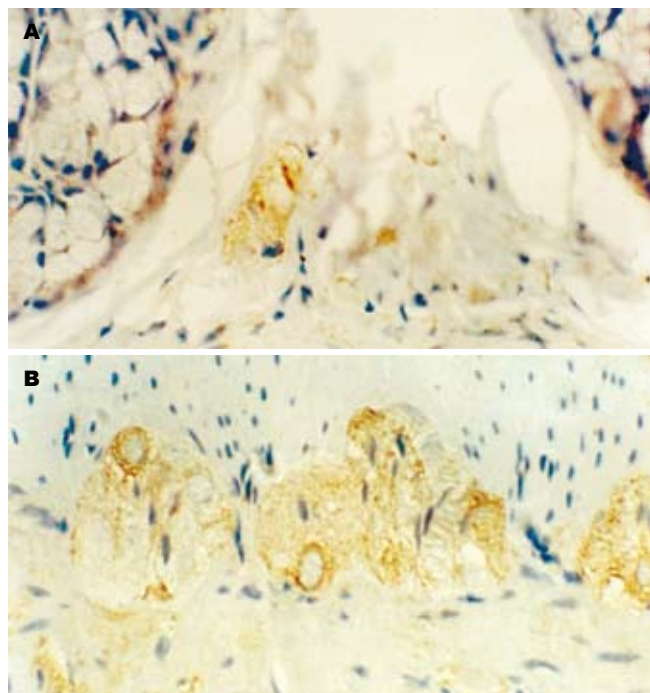


图1 对照组大鼠结肠神经系统nNOS表达(×400). A: 黏膜下神经丛; B: 肌间神经丛.

苏木素复染; 常规脱水、透明、封片; 光镜下观察. 以0.01 mol/L PBS(pH 7.4)代替一抗作为阴性对照. 每张切片随机连续选取不重叠的5个高倍视野(×400), 计数每个高倍视野内黏膜下神经丛和肌间神经丛的阳性神经元数, 取其平均值分别代表两种神经丛内阳性神经元的平均密度(阳性神经元细胞数/高倍视野); 并用Nikon Act-1图像分析仪和Image-Pro Plus 4.5图像分析软件测量阳性神经元的灰度值, 取平均值代表阳性神经元的灰度值以反映nNOS蛋白的相对含量.

统计学处理 所有数据均以均数±标准差(mean±SD)表示, 运用SPSS 10.0统计软件进行t检验, P<0.05为有统计学意义.

2 结果

2.1 病理组织学变化 光镜下对照组、应激组和L-NAME组大鼠结肠组织结构完整, 均未见明显损伤性改变.

2.2 结肠nNOS的表达 对照组大鼠nNOS免疫阳性产物主要见于结肠黏膜下神经丛和肌间神经丛的神经元, 定位于细胞质(图1). 应激组nNOS免疫阳性产物除在上述结构表达外, 黏膜上皮细胞、固有层淋巴细胞也有表达(图2). L-NAME组nNOS免疫阳性产物主要见于结肠黏膜下神经丛和肌间神经丛的神经元(图3).

2.3 图像分析结果 结肠黏膜下神经丛nNOS阳性神经元灰度值和平均密度的变化: 应激组nNOS阳性神经元灰度值较对照组明显减少(P<0.05), 阳性神经元的平均密度高于对照组(P<0.05), 即nNOS表达增强. 与应激组相比, L-NAME组黏膜下神经丛的nNOS阳性神经元

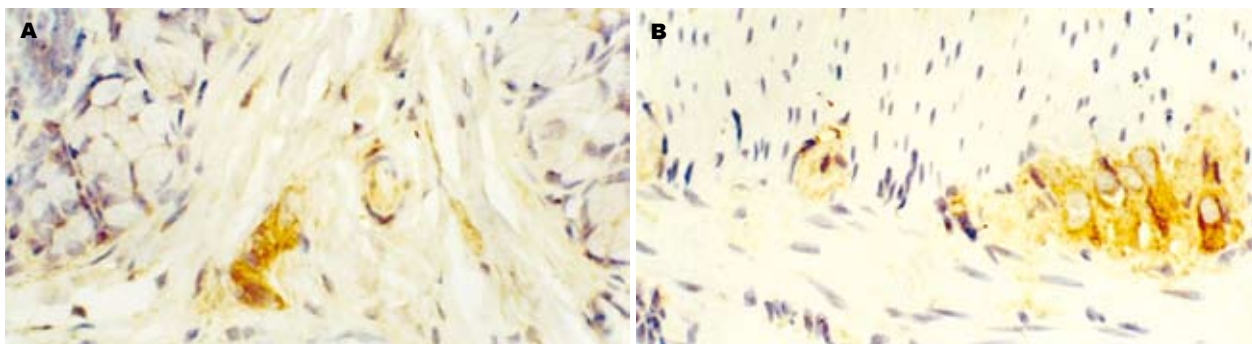


图2 应激组大鼠结肠神经系统nNOS表达($\times 400$). A: 黏膜下神经丛; B: 肌间神经丛.

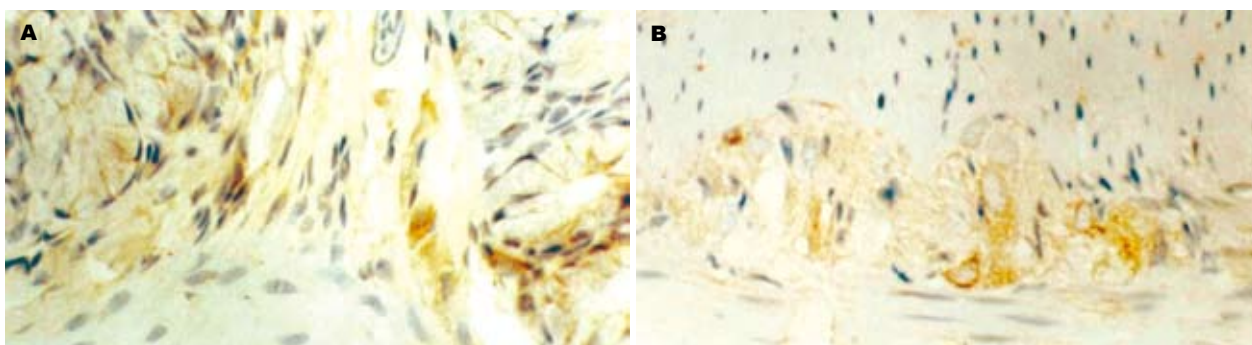


图3 L-NAME组大鼠结肠神经系统nNOS表达($\times 400$). A: 黏膜下神经丛; B: 肌间神经丛.

灰度值增加($P < 0.05$), 平均密度降低($P < 0.05$), 即nNOS表达减弱. 结肠肌间神经丛nNOS阳性神经元灰度值和平均密度的变化: 应激组肌间神经丛的nNOS阳性神经元灰度值较对照组显著减少($P < 0.01$), 平均密度高于对照组($P < 0.05$), 即nNOS表达明显增强. 与应激组相比, L-NAME组肌间神经丛的nNOS阳性神经元灰度值增加($P < 0.05$), 平均密度降低($P < 0.05$), 即nNOS表达明显减弱(表1).

表1 大鼠结肠黏膜下神经丛和肌间神经丛nNOS阳性神经元的灰度值和平均密度 ($n = 10$, mean \pm SD)

分组	黏膜下神经丛		肌间神经丛	
	灰度值	平均密度	灰度值	平均密度
对照组	178.18 \pm 3.29	1.56 \pm 0.34	173.96 \pm 4.12	7.12 \pm 1.56
应激组	174.34 \pm 4.84 ^a	2.08 \pm 0.59 ^a	166.36 \pm 5.25 ^b	8.92 \pm 1.97 ^a
L-NAME组	178.05 \pm 5.07 ^c	1.64 \pm 0.55 ^c	172.12 \pm 4.13 ^c	7.18 \pm 1.15 ^c

^a $P < 0.05$, ^b $P < 0.01$ vs 对照组; ^c $P < 0.05$ vs 应激组.

3 讨论

NO是近年来发现的一种小分子气体信号分子和重要的神经递质. 内源性NO以L-精氨酸(L-Arg)为底物, 在NADPH、 O_2 和原卟啉IX等因子辅助下, 由一氧化氮合酶(NOS)作用下产生. NO具有高度脂溶性, 合成后以扩散方式到达靶细胞, 与细胞中可溶性鸟苷酸环化酶(SGC)结合, 通过改变SGC的空间构型提高酶活性, 使细胞内环磷酸鸟苷(cGMP)生成增多, 激活依赖cGMP蛋

白激酶的钙泵, 从而参与细胞间信息传递^[8,9]. NOS是NO合成过程中唯一的限速酶, 其活性依赖于NADPH、 Ca^{2+} 和钙调蛋白, 可被左旋精氨酸结构类似物(如L-NAME)竞争性抑制^[10,11]. NOS包括三种亚型: 神经型NOS(neuronal NOS, nNOS)、内皮型NOS(endothelial NOS, eNOS)和诱导型NOS(inducible NOS, iNOS). 前两者合称为结构型NOS(constitutive NOS, cNOS). 酶细胞化学方法研究发现^[12-14], NOS广泛分布于哺乳动物的肠道组织, 但此方法不能区分cNOS与iNOS. 我们采用免疫组化方法显示, nNOS主要分布于大鼠结肠黏膜下神经丛和肌间神经丛神经元, 定位于细胞质. 上述结果为揭示nNOS在大鼠肠道中的分布规律提供了形态学资料, 并进一步证实NO是结肠重要的信号分子和神经递质.

结肠的神经支配较为复杂, 其运动形式也比小肠复杂且多变^[15,16]. 结肠肌间神经丛包括初级、二级及三级神经丛. 初级神经丛与二级神经丛相连形成网络, 其神经纤维穿入环行肌, 并直接支配环行肌的运动, 而纵行肌则由三级肌间丛支配. 肌间神经丛部分神经纤维伸至肠黏膜后形成感觉末梢. 而黏膜下神经丛的神经元不仅支配肠黏膜和黏膜肌层, 有些神经元还伸至肌间神经丛. 因此, 我们推测, 在生理状态下, 黏膜下神经丛内NO主要与结肠黏膜肌的收缩、腺体分泌和黏膜感觉有关, 而肌间神经丛NO主要参与了对结肠环行肌运动的调节.

研究表明, 应激通过神经体液调节, 常导致胃肠

功能失调, 表现为胃肠运动、分泌和消化转运时间的改变^[17-19]。然而, 应激引起或加重结肠功能紊乱的发病机制尚不清楚。有关NO与胃肠动力学的研究显示, 胃肠组织均可在cNOS催化下产生NO, 参与对肠道平滑肌收缩的非胆碱能非肾上腺素能(nonadrenergic noncholinergic, NANC)神经调节: NO含量减少时, 胃肠运动加快; 反之, 胃肠运动减慢^[20]。本研究显示, 水浸-束缚应激后黏膜下神经丛和肌间神经丛内nNOS阳性神经元的表达增强, 而预先用NOS抑制剂L-NAME处理可逆转nNOS的表达结果。我们推测, 水浸-束缚应激状态下, 大鼠结肠黏膜下神经丛和肌间神经丛的神经元nNOS表达增强, 由nNOS催化L-Arg生成的NO含量增加, 扩散至周围靶细胞后, 不仅抑制平滑肌的收缩, 导致结肠运动减弱, 而且抑制了结肠黏膜肌的收缩、腺体分泌和黏膜感觉。

不同的应激往往产生不同的胃肠运动效应^[21-25]。激怒、恐惧、痛性刺激和高强度训练可使健康人的胃排空延迟, 结肠运动增强; 动物经束缚制动、电刺激和强迫游泳等急性应激后, 也可产生胃肠排空延迟。近年来研究发现应激可诱发、加重IBS的症状^[26-28], 导致结肠动力增强(腹泻型IBS)或减弱(便秘型IBS), 但至今尚无一种成熟的IBS动物模型, 因此有关IBS发病机制的基础研究较少。本研究结果显示WRS后肠神经系统nNOS表达增强, 提示应激状态下抑制性神经递质NO产生增加, 参与了对肠道平滑肌活动的抑制, 但未引起结肠组织学变化。因此, 我们认为WRS可作为研究IBS肠道动力异常的较为理想的动物模型。

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