临床细胞生物学

利用FM4-64 FX标记大鼠血管平滑肌细胞的囊泡运输

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摘要 囊泡运输是大分子物质进入细胞的途径, 血管平滑肌细胞(vascular smooth muscle cells, VSMCs)与外界存在频繁的信息和物质交换, 该研究通过标识内吞囊泡来研究VSMCs的囊泡运输。体外培养大鼠胸主动脉VSMCs, 用血管紧张素II(angiotensin II, Ang II)刺激, 加入 FM4-64 FX短暂孵育后固定。通过免疫组化方法标记VSMCs血管紧张素II 1型受体(angiotensin II receptor type 1, AT1R), 检测内吞囊泡和AR1R转运之间的关系。受到Ang II的激活后, VSMC快速形成内吞囊泡, 将AT1R转运至胞质; 存在血管紧张素受体阻断剂(angiotensin receptor blocker, ARB)时, 内吞囊泡数量少, AT1R较少进入胞质。通过FM4-64 FX对胞内囊泡进行标识可以显示VSMCs的大分子物质运输, 可观察特定的分子在内吞囊泡上的分布和运输情况。

关键词 FM4-64 FX; 血管平滑肌细胞; 囊泡运输; 内吞

Using FM4-64 FX to Lable Transport Vesicles of Rat Vascular Smooth Muscle Cells

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Abstract This study aims to investigate vascular smooth muscle cells (VSMCs) vesicle transport by endocytic vesicles labeling. Rat VSMCs from the thoracic aortic were cultivated *in vitro*. VSMCs were stimulated with angiotensin II (Ang II) and incubated with FM4-64 FX shortly. Then VSMCs were fixed with paraformaldehyde and marked with angiotensin II type 1 receptor (AT1R) antibody by immunohistochemistry. With Ang II stimulation, VSMCs rapid formed endocytic vesicles and with it, AT1R was transported into the cytoplasm. Presence of an angiotensin receptor blocker (ARB) inhibited the number of endocytic vesicles formation and less AT1R entered the cytoplasm. Macromolecules transport of VSMCs can be illustrated by labeling the intracellular vesicles with FM4-64 FX dye. With this method, early endocytosis of VSMCs when the external environment changed can be investigated.

Keywords FM4-64 FX; vascular smooth muscle cells (VSMCs); vesicle transport; endocytosis

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FM4-64是一种亲脂性的苯乙烯染料, 对细胞无毒性, 在水性溶液中基本不显荧光。FM4-64 FX(FX: fixable)则是进行细胞囊泡染色后, 可用甲醛进行固定的产品, 便于加做免疫组化检测。它可插入到真核细胞的细胞膜磷脂双分子膜的外层; 当细胞膜内陷形成内吞囊泡后, 与细胞膜外层结合的FM4-64就被包裹在囊泡内, 染料从而获得明显的荧光性, 故可用于标记活细胞囊泡[1-2]。其最大激发/最大发射波长约为515/640 nm(发出红色荧光), 被研究者用于观察囊泡形态和动力学, 研究胞吞、胞吐过程和筛选酵母细胞突变体等。在荧光显微镜下, 可以动态观测囊泡的形成与运输。该染料常用于观察神经细胞的囊泡运输[3-5], 而未见用于了解血管平滑肌细胞的物质运输上。

本研究中, 我们利用血管紧张素II(angiotensin II, Ang II)刺激VSMCs, 用FM4-64 FX标记囊泡后用多聚甲醛固定细胞, 再通过免疫组织化学标记血管紧张素II 1型受体(angiotensin II receptor type 1, AT1R), 检测受到Ang II刺激后的VSMCs的内吞囊泡和AT1R转运之间的关系。

1 材料与方法

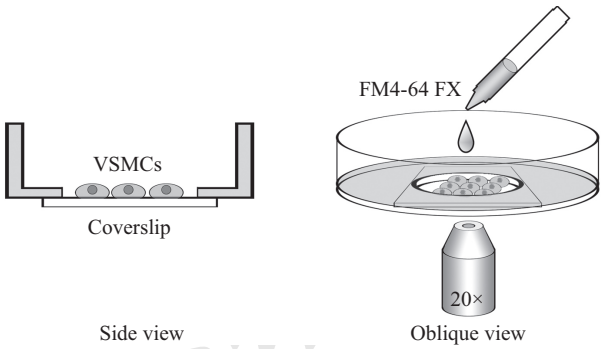
1.1 实验动物

本研究所涉及的动物实验符合NIH实验动物指南(NIH No.85-23, 1996年修订), 并通过泸州医学院实验动物中心许可。选择8周龄雄性Sprague-Dawler(SD)大鼠(体重150~200 g), 购于泸州医学院实验动物中心。

1.2 VSMCs原代及传代培养

处死大鼠, 取出新鲜胸主动脉。纵向剪开血管, 剥去外膜, 将内膜面置于0.2% I型胶原酶中孵育20 min, 轻轻吹打10 min, 使内皮细胞脱落, 弃去内皮细胞。用虹膜剪将血管中膜剪碎并均匀铺贴在培养瓶底部, 用含有10%胎牛血清的DMEM培养基(Gibco)培养5~7 d。当原代细胞生长到融合状态时, 通过免疫荧光标记SMC特有的α-SM-actin(SMA), 经鉴定VSMCs阳性率>95%后进行传代(图1)。使用2~7代VSMCs进行实验。

选用底部为盖玻片的培养皿, 用鼠尾胶原包被培养皿底部, 待其在超净台下风干后进行紫外线照射灭菌。将密度为1×10^5/mL的VSMCs接种于培养皿, 待细胞铺满后用于实验。该培养皿可置于倒置荧光显微镜及激光共聚焦显微镜下直接观察(图1)。



用鼠尾胶原包被培养皿底部载玻片并风干, 接种1×10^5/mL VSMCs, 待细胞贴壁后开始实验。培养皿可置于倒置显微镜或激光共聚焦显微镜上, 再用FM4-64 FX染色并实时观察。

The glass slide at the bottom of the culture plate was coated with rat tail collagen and air-dried. VSMCs in 1×10^5/mL density were planted to confluency. The culture plate could be placed on a laser scanning confocal microscope with a FM4-64 FX dyeing for real-time observation.

图1 实时观测FM4-46 FX染色血管平滑肌细胞(VSMCs)

Fig.1 Real-time observation of FM4-46 FX stained vascular smooth muscle cells

1.3 活细胞囊泡染色

实验前, 用无血清DMEM同步化VSMCs 12 h, 使细胞周期处于同步。吸去培养基, 补充新鲜DMEM, 将培养皿置于荧光显微镜下。

在暗处用双蒸水将粉末状FM4-64 FX稀释为1‰的溶液。将1 μL溶液加入培养皿中, 用640 nm(红色)滤光片立即观察胞膜结构。在成像实验中, 每1 μL溶液加入体积为1 mL的培养基, FM4-64 FX终浓度为1×10^{-6} mol/L。

将1×10^{-6} mmol/L的血管紧张素II(Ang II)加入到FM4-64 FX稀释溶液中, 再将此溶液加到培养皿中, 在同等条件下观察VSMCs的囊泡形成情况。

1.4 免疫组化标记ATIR, 与内吞囊泡荧光共定位

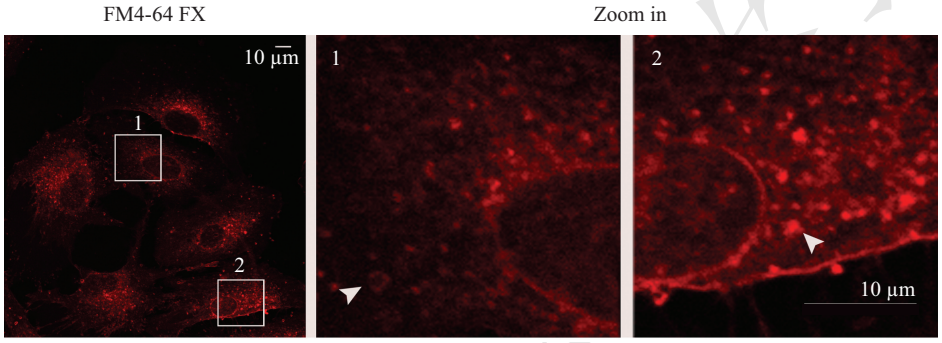
FM4-64 FX在紫外线下的激发光为640 nm(以红色表示)。用4%多聚甲醛在5 °C环境下固定VSMCs 8 h, 3‰ Triton X-100短暂破膜。稀释的兔抗鼠AT1R抗体(Sigma)在5 °C环境下孵育过夜。洗去一抗后, 用DyLight 488标记的抗兔IgG(Sigma; 激发光为525 nm, 以绿色表示)在暗盒里室温孵育2 h。洗去荧光二抗, 用640 nm和525 nm滤光片观察、拍摄, 用Image-Pro Plus分析图像。我们通过测量细胞内总的荧光强度来比较囊泡形成的相对数量。每个样品随机选取10个视野, 每个视野下测量9个细胞。

1.5　ARB对VSMCs囊泡形成的影响

在VSMC的培养液中先加入1×10^{-6} mol/L的血管紧张素受体阻断剂(angiotensin receptor blocker, ARB)厄贝沙坦(Irbesartan), 孵育1 h, 再加入Ang II、FM4-64 FX, 继而用免疫组化的方法标记VSMCs的AT1R; 检测内吞囊泡形成和AT1R的分布情况。

1.6　统计学分析

实验结果用均数±标准差(mean±S.D.)表示, 用单因素方差分析(One-Way ANOVA)和最小显著差异法(Fisher's least significant difference, LSD)比较两组间的差异。$P<0.05$为有显著性差异, $P<0.01$为有极显著性差异。

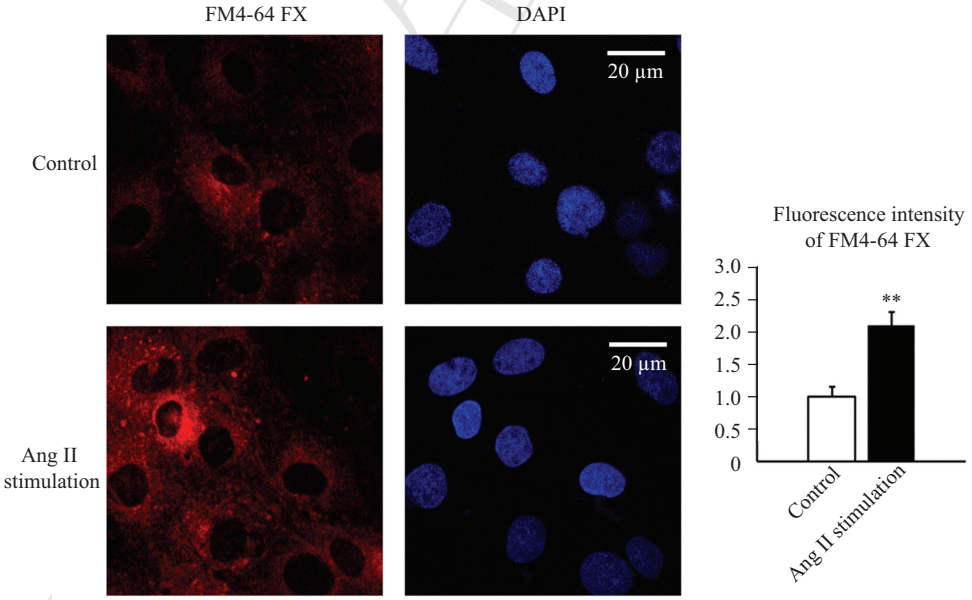


用1‰ FM4-64 FX对VSMCs进行囊泡染色并实时观察。通过对图片的局部放大可观察到细胞内的囊泡。FM4-64 FX溶液孵育细胞30 s, 箭头所示为囊泡。

The vesicles of VSMCs were stained with 1‰ FM4-64 FX for real-time observation. The intracellular vesicles could be observed by the enlarged image. The cells were incubated with FM4-64 FX solution for 30 s. Arrows indicated vesicles.

图2　VSMCs的囊泡染色

Fig.2　Vesicles staining of VSMCs

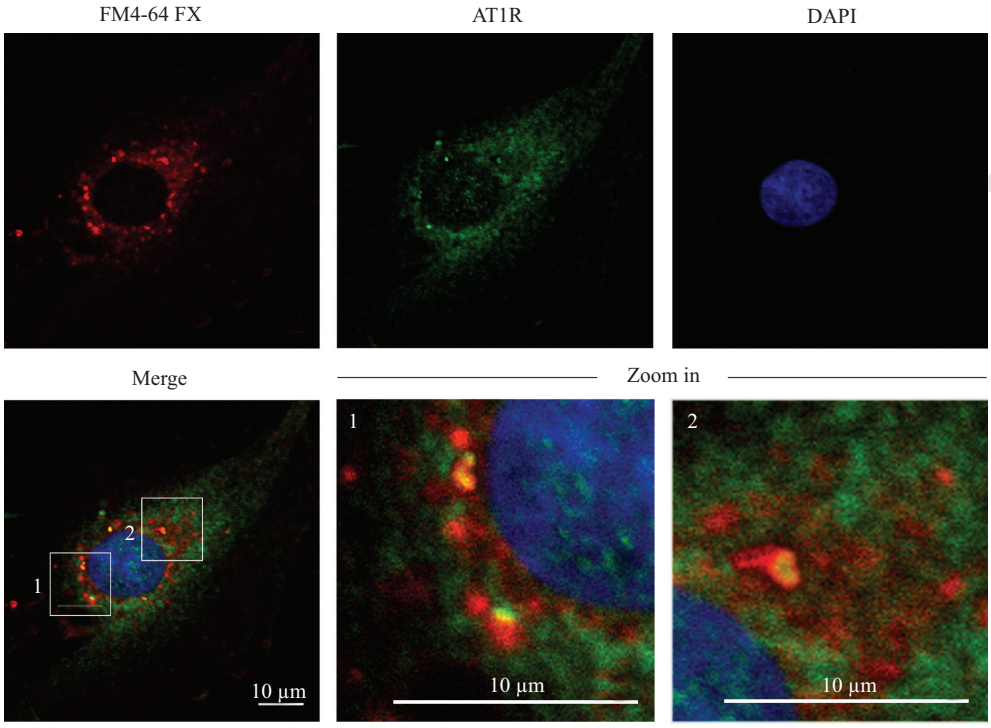


Ang II刺激组: 将Ang II加到FM4-64 FX稀释液中, 再将混合溶液加入VSMCs培养基。刺激组细胞内囊泡的荧光强度在短时间内达到高峰, 且最大荧光值较对照组高。对照组FM4-64 FX与细胞孵育30 s, Ang II刺激组孵育时间13 s; 图片的曝光时间均为100 ms; 实验重复4次; $^{**}P<0.01$, 与对照组相比较。

Ang II stimulation group: Ang II was added into to the FM4-64 FX dilution, and then the mixed solution was added into VSMCs culture medium. Intracellular vesicle fluorescence intensity of Ang II stimulation group reached to peak in a short time and the maximum fluorescence value was higher than that of the control group. Cells of the control group were incubated with FM4-64 FX solution for 30 s and cells of the Ang II stimulation group were incubated for 13 s. Image exposure time of the control group and Ang II stimulation group were both 100 ms. The experiment was repeated four times. $^{**}P<0.01$ *vs* control group.

图3　对照组和Ang II刺激组的细胞内囊泡形成情况

Fig.3　The intracellular vesicle formation of the control group and the Ang II stimulation group

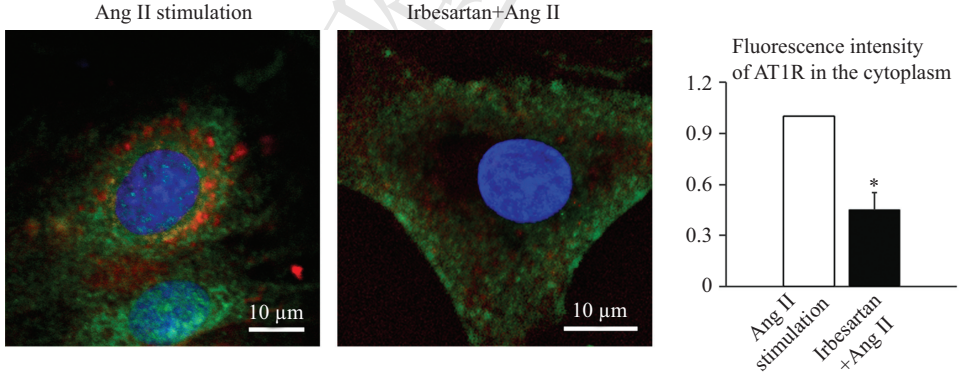


通过图像的局部放大, 可观察到红色和绿色信号叠加后的橘黄色, 提示内吞囊泡和AT1R存在共定位。FM4-64 Fx与细胞孵育13 s, 内吞囊泡标记为红色; AT1R通过荧光抗体标记为绿色; 细胞核用DAPI标记为蓝色。实验重复4次。

By locally enlarged the image, red and green signals superimposed to orange and indicated the colocalization of endocytic vesicles and AT1R. Cells were incubated with FM4-64 FX for 13 s. Endocytic vesicles were marked in red, AT1R was labeled by fluorescent green antibody and nuclei were labeled with DAPI in blue. The experiment was repeated four times.

图4 内吞囊泡和血管紧张素II 1型受体(AT1R)免疫荧光共定位

Fig.4 Immunofluorescence colocalization of endocytic vesicles and the angiotensin II type 1 receptor (AT1R)



Ang II刺激组: 细胞内有明显囊泡形成, 最大荧光强度高于对照组; AT1R和转运囊泡有共定位(橘黄色信号)。厄贝沙坦拮抗组: 囊泡荧光强度低, AT1R在细胞边缘上较为明显, 细胞质中较少。FM4-64 FX与细胞孵育13 s, 内吞囊泡通过染色标记为红色; AT1R通过荧光抗体标记为绿色; 细胞核用 DAPI 标记为蓝色。对照组和Ang II刺激组图片的曝光时间均为100 ms; 测量细胞质中的AT1R荧光强度, 10×40视野下计数10个细胞; 实验重复4次。*P<0.05, 与Ang II刺激组相比较。

Ang II stimulation group: significant intracellular vesicle formed and the maximum fluorescence intensity was higher than that in the control group; AT1R and transport vesicles had colocalization (orange signal). Irbesartan antagonist group: the vesicle fluorescence intensity was low and AT1R uniformly distributed on the cell surface. Cells of both groups were incubated with FM4-64 FX for 13 s. Endocytic vesicles were marked in red, AT1R was labeled by fluorescent green antibody and nuclei were labeled with DAPI in blue. The exposure time of the control group and Ang II stimulation group were both 100 ms. The fluorescence intensity of AT1R in the cytoplasm was measured by counting 10 cells in the 10×40 vision. The experiment was repeated 4 times. *P<0.05 compared to Ang II stimulation group.

图5 Ang II刺激组和厄贝沙坦拮抗组的细胞内囊泡染色及AT1R分布情况

Fig.5 Intracellular vesicles staining and AT1R distribution in Ang II stimulation group and irbesartan antagonist group

2　结果

2.1　FM4-64 FX清晰标示VSMCs内吞囊泡, 细胞经过甲醛固定后囊泡仍可见

将培养皿置于荧光显微镜下, 调整好焦距(40×), 将1‰的FM4-64 FX加入培养液中, 用640 nm的滤光片观察, 细胞间隙有微弱的荧光出现; 短时间内较明亮的荧光出现在细胞内。细胞内荧光即为被FM4-64 FX标记的内吞囊泡。

FM4-64用于标记活细胞内囊泡, 若需要固定细胞以便进一步研究, 则需要用FM4-64 FX。用FM4-64 FX对细胞内囊泡进行标记后, 吸去培养液, 用4%冰多聚甲醛在暗盒中固定细胞30 min。在荧光显微镜下仍可见清晰的细胞内荧光信号(图2)。

2.2　Ang II促进VSMCs内吞囊泡的形成

接受Ang II刺激后, VSMCs内吞囊泡形成速度和量明显上升。通过记录细胞内荧光出现的时间发现, 在没有Ang II刺激时, 细胞内荧光强度约在30 s达到顶峰, 而在Ang II的刺激下, 细胞内荧光强度在13 s即达到顶峰。在细胞内囊泡结构的荧光强度达到顶峰后, 通过 Image-Pro Plus软件计算对照组和Ang II刺激的细胞形成的囊泡的荧光强度, 发现经过Ang II刺激的VSMCs所形成的囊泡数量较未经Ang II刺激的平滑肌细胞高2.1倍(图3)。

2.3　通过荧光共定位, 可观察AT1R通过内吞囊泡进入胞质

通过标记囊泡的FM4-64 FX(红色)和标记AT1R的荧光二抗(绿色)进行共定位, 发现脱离细胞膜表面进入胞质有红色和绿色荧光共存的现象, 说明AT1R和和配体结合后通过内吞囊泡进入细胞质(图4)。

2.4　ARB抑制受Ang II介导的内吞囊泡形成

在VSMCs培养液中加入1×10^{-6} mmol/L厄贝沙坦孵育1 h后, 加入Ang II、FM4-64 FX进行囊泡染色。结果发现, VSMCs的内吞囊泡形成减少; 免疫组织化学染色提示, AT1R在细胞膜上分布较为明显, 细胞质中较少。和未加入厄贝沙坦的对照组相比, 加入厄贝沙坦后VSMCs内吞囊泡减少, 免疫组织化学染色可见AT1R在细胞质内没有典型的颗粒状荧光(图5)。

3　讨论

VSMCs是组成血管壁和提供机械支持的主要细胞成分, 与外界环境之间存在密切的信息与物质交换[6-8]。细胞外环境如生化、力学等改变将引起VSMCs产生生物学适应, 包括增殖、凋亡水平和迁移能力的变化, 导致高血压、动脉粥样硬化、血管成形术后再狭窄等情况发生[9-14]。因此, 研究VSMCs与外界的信息和物质交流是十分必要的。

囊泡运输是包括蛋白质在内的大分子进入细胞的方式[15], 这种有膜包被的小型泡状结构像集装箱一样将待运输的分子包裹起来, 送到目的地释放[16-17], 货物送达后, 磷脂双分子层则重新回到细胞膜[18-19]。我们的实验结果提示, Ang II刺激VSMCs后, 由于配体受体复合物需转运进入胞质, VSMCs形成的内吞囊泡数量明显增多, 且速度加快。可以通过对内吞囊泡的标记, 观察到VSMCs在受到Ang II刺激后囊泡运输的情况。

Ca^{2+}促进囊泡释放[20-21], 正常情况下, 受到外界刺激后, 囊泡形成和释放速度极快, 为实验条件下观测造成一些困难。我们采用不含Ca^{2+}的培养液, 使囊泡释放的速度变慢, 从而对于动态观察囊泡运输过程有帮助。实验中我们观察到, 接受Ang II刺激后, 平滑肌细胞在2~3 s开始出现内吞囊泡, 10 s达到高峰。

Ang II是重要的血管活性物质, 它不但促进VSMCs收缩, 对其增殖和迁移也具有作用[22-23]。Ang II与细胞膜表面的Ang II受体(主要为1型受体, 即AT1R)结合, 配体–受体复合物通过内吞囊泡进入细胞内。在细胞质中, Ang II和AT1R解离, Ang II产生生理学作用, 如通过激活磷脂酶C(phospholipase C, PLC)升高胞质内Ca^{2+}离子浓度, 进而激活一系列细胞反应。AT1R则由于被快速磷酸化和内吞, 暂时失去对Ang II的敏感性, 一部分AT1R将被运输到溶酶体而被降解, 一部分则被送回细胞膜表面而再利用[24-25]。通过我们的实验可直接观测到AT1R随内吞囊泡进入细胞内的情况。

如上所述, VSMCs通过囊泡运输与外界进行物质和信息交换。通过FM4-64标记内吞囊泡, 再通过免疫荧光标记研究的分子, 可以观测被运输的分子囊泡转运过程。本研究所采用的内吞囊泡的标记方法, 能够为了解VSMCs在外环境变化(血流运动、血液成份变化、与邻近细胞的相互交流)时的初期反应打开一扇窗口, 对于其他细胞外大分子物质的运输以及细胞膜表面蛋白受体的转运的研究可能也有同等的价值。

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