前胡丙素对 Ang II 致离体血管平滑肌细胞肥厚及胞内钙、NO 含量和信号转导的影响

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摘要: 目的 前胡丙素(Pra-C)对血管紧张素 II(Ang II)致离体培养大鼠血管平滑肌细胞(SMCs)肥厚模型胞内游离钙浓度、NO 含量和信号转导的影响。方法 以 Ang II 刺激 SMCs 形成肥厚模型,用倒置显微镜测定 SMCs 面积;用 Fura-2/AM 测定单细胞内[Ca^{2+}]i,Griess 法测定 NO 含量;在 PMA 和 ST(PKC 激动剂及抑制剂)、PTX(Gi 蛋白敏感毒素)作用下观察 Pra-C 对 KCl 和 NE 所致胞内[Ca^{2+}]i浓度变化的影响。结果 Pra-C 组 SMCs 细胞面积较肥厚组减小 39.01 %,并接近正常细胞水平;NO 含量明显增加;胞内[Ca^{2+}]i对 KCl 和 NE 激动的反应明显低于肥厚组。PMA 使肥厚 SMCs [Ca^{2+}]i升高,而 ST 及 PTX 则使之降低,Pra-C 均使之恢复正常。结论 Pra-C 抑制 Ang II 致体外培养细胞 SMCs 肥厚,改善肥厚细胞因 PKC 和 Gi 蛋白的信号转导改变所致的[Ca^{2+}]i改变。

关键词: 前胡丙素; 血管平滑肌; 细胞内游离钙; 一氧化氮

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高血压时细胞内钙代谢异常及与药物作用的关系是目前研究的热点。细胞内信号转导异常导致钙代谢改变及细胞内钙浓度增高,而细胞的钙超载可进一步影响血管内皮和平滑肌细胞的功能[1~3]。因此,抗高血压药物降压的同时能否减轻细胞内钙超载及改善异常信号转导,对肥厚的形成和逆转及心功能恢复有重要影响。前胡丙素(praeruptorine C, Pra-C)是从中药白花前胡中提取的单体成分,本室多年研究证明其有钙拮抗作用[4]。本室曾报道前胡丙素可逆转肾型高血压(RHR)及自发型高血压(SHR)大鼠左室肥厚,防止缺血及肥厚心肌细胞内钙升高[5~9]。由于高血压的主要病理在血管,我们曾报道长期口服前胡丙素能防治 RHR 及 SHR 大鼠的血管肥厚,降低细胞内钙的升高,提高 NO 含量。本文在离体培养的大鼠血管平滑肌细胞(SMCs),用 Ang II 激动形成肥厚模型,观察 Pra-C 对肥厚细胞的影响及与蛋白激酶 C(protein kinare C, PKC)系统及 Gi 蛋白的关系。

材 料 与 方 法

药品与动物 前胡丙素(Pra-C)为白色粉末状,由南京中医药研究所植化室提供,以聚乙二醇 400 配成 2 %浓度,4 ℃避光保存。Ang II, M_{199}, ionomysin, staurosporine (ST), phorbol 12-myristate 13-acetate (PMA), pertussis toxin (PTX), Fura-2/AM 均购自 Sigma 公司。Fura-2/AM, 用 DMSO(Merck)配成 1 mmol·L^{-1},分装后置于 - 20 ℃保存。实验用 Spraque-Dawley (SD)大鼠,由江苏省实验动物中心提供。

SMCs 的分离培养 取体重(253 ±15) g 的健康 SD 大鼠,♀ ♂各半,在无菌条件下取出主动脉从弓至髂总动脉段,按文献[10]方法进行分离培养。用药勺轻轻刮去内皮,切割成 2 mm × 2 mm 组织块,将组织块贴入灭菌的 25 mL 培养瓶中,内膜面贴于瓶壁上,在 37 ℃,5 % CO_2 孵箱中培养 2 h 后加入含青霉素及链霉素和 10 %胎牛血清的 M_{199} 培养基,3 d 后换液,4 ~ 6 d 后可见动脉片周围细胞形成单层生长,呈明显的极向排列,"峰和谷"各半,将融合的细胞用 0.125 %胰蛋白酶进行消化,在倒置显微镜下见到细胞间连接疏松将要脱落时,加入含 10 %小牛血清的 M_{199} 培养基终止消化,将含细胞培养液稀释成 3.0×10^5·mL^{-1},继续分瓶培养传代,实验用细胞为 3 ~ 5 代,用电子显微镜确认为平滑肌细胞。

Ang II 致 SMCs 肥厚及细胞大小测定 在 5 mL 培养瓶中,用含 0.5 %胎牛血清的 M_{199} 培养基培养 48 h,使 SMCs 与 G_0/G_1 同步后进行实验。于 24 孔培养板中,将细胞调成每孔 3×10^4,分为正常(细胞)

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组:加等量溶剂;Ang II 组:只加 Ang II 10 nmol·L^{-1};Pra-C + Ang II 组:加 Pra-C 10 μmol·L^{-1}和 Ang II 10 nmol·L^{-1}。培养 4 d 后对各组细胞进行计数并测量大小,将待测细胞注入自制细胞池中,池底载玻片中部打一圆孔,底部用乳胶粘一盖玻片。在倒置显微镜闭路电视系统下用 MICC 软件测定细胞长、宽,并计算面积。每次测定 10 个视野内的全部细胞。

NO 的测定 参照微盘测定法及蛋白校正法[11]进行亚硝酸测定。使 SMCs 同步于 G_0/G_1 期,于 24 孔培养板中,将细胞调成每孔 3×10^4 个,加 Griess 试剂与亚硝酸盐反应,于紫外分光光度计 550 nm 测其吸光度的改变,作出亚硝酸浓度和蛋白含量的标准曲线,计算培养液中 NO 含量。

SMCs 单细胞内[Ca^{2+}]i的测定 参照文献[12]将同步化后的 SMCs 细胞,用胰蛋白酶消化,用培养基调细胞浓度为每孔 5×10^8·L^{-1},加终浓度为 3 μmol·L^{-1}的 Fura-2/AM,37 ℃水浴中孵育 30 min,用 Hanks 液冲洗 5 次,以防荧光干扰。于 AR-CM-MIC 阳离子测定系统(SPEX,美国)中测定单细胞内钙,用软件 DM3000 进行分析。

统计学处理 所有实验数据均以 $\bar{x} \pm s$ 表示,组间及组内前后差异用 t 检验。

结 果

1 Ang II 诱导的 SMCs 肥厚模型制备和前胡丙素对细胞肥厚的作用

SMCs 经 Ang II 10 nmol·L^{-1}刺激后,细胞数目无明显增加,但其大小变化明显,表明本实验所用浓度的 Ang II 使 SMCs 只肥厚而不增生。Pra-C 10 μmol·L^{-1}与 Ang II 共同孵育,细胞面积明显小于 Ang II 组($P < 0.001$),表明 Pra-C 可以防止 SMCs 肥厚,见表 1。

Table 1 Effect of Pra-C on the amount and area of isolated and cultured rat hypertrophied smooth muscle cells (HSMCs) induced by angiotensin II (Ang II) *in vitro*

Group	Dose	Amount / cells	Length / μm	Width / μm	Area / μm^2
Normal	-	5.1 ±0.3	14.5 ±2.4	2.46 ±0.28	36 ±3
Pra-C + Ang II	10 μmol·L^{-1} +10 nmol·L^{-1}	5.20 ±0.26	15.7 ±1.4	2.60 ±0.28	41 ±4
Ang II	10 nmol·L^{-1}	5.24 ±0.25	20.1 ±1.1***	3.33 ±0.25***	67 ±4***

HSMCs (hypertrophied smooth muscle cells) were induced by angiotensin II (Ang II) 10 nmol·L^{-1} for 4 d; cell area = length × width; Pra-C 10 μmol·L^{-1} was incubated with HSMCs for 30 min. $n = 12$, $\bar{x} \pm s$. *** $P < 0.001$ *vs* Pra-C + Ang II group

2 前胡丙素对 Ang II 致肥厚 SMCs 产生 NO 的影响

肥厚细胞产生 NO 的能力明显降低,降低原水平 40 %,前胡丙素组细胞产生 NO 的能力明显提高,接近对照组,说明 Pra-C 不仅能防止 SMCs 肥厚、还能恢复细胞产生 NO 的能力,见表 2。

Table 2 Effect of Pra-C on nitric oxide (NO) content in isolated and cultured rat hypertrophied smooth muscle cells (HSMCs) induced by angiotensin II (Ang II) *in vitro*

Group	Dose	NO/nmol·mg^{-1}(protein)·d^{-1}
Normal	-	6.0 ±0.5
Pra-C + Ang II	10 μmol·L^{-1} +10 nmol·L^{-1}	5.7 ±0.6
Ang II	10 nmol·L^{-1}	3.6 ±0.4***

HSMCs (hypertrophied smooth muscle cells) were induced by angiotensin II (Ang II) 10 nmol·L^{-1} for 4 d; Pra-C 10 μmol·L^{-1} was incubated with HSMCs for 30 min; NO content was measured by Griess method. $n = 10$, $\bar{x} \pm s$. *** $P < 0.001$ *vs* Pra-C + Ang II group

3 前胡丙素对 Ang II 致肥厚 SMCs [Ca^{2+}]i稳态的影响

分别用电压依赖性和受体操纵性激动剂 KCl 60 mmol·L^{-1}和 NE 10 μmol·L^{-1}使 Ang II 致肥厚 SMCs 的细胞内[Ca^{2+}]i比正常细胞的增加幅度分别高出 100 %和 145 %,Pra-C 组仅增加了 13 %和 19 %,均与正常接近。可见 Pra-C 在肥厚 SMCs 对 KCl 和 NE 两种激动剂诱导的[Ca^{2+}]i异常升高均有明显抑制作用,见表 3。

4 前胡丙素对 PMA,ST 及 PTX 致[Ca^{2+}]i升高的影响

将正常细胞及已制备好的肥厚平滑肌细胞(HSMCs)分为 3 组。正常细胞组:加等量溶剂;Pra-C 组:用 Ang II 制备好的肥厚细胞加 Pra-C 10 μmol·L^{-1}共同孵育 30 min;肥厚组:用 Ang II 制备好的肥厚细胞。PMA 0.1 μmol·L^{-1},PTX 200 ng·L^{-1}测定前 2 h 加入,ST 10 nmol·L^{-1}测定前 5 min 加入。以上试剂均指终浓度。对 PKC 系统激动剂 PMA 作

用后,KCl 和 NE 分别使 HSMCs 的[Ca^{2+}]i较正常组增加了 110.9 nmol·L^{-1}和 232.5 nmol·L^{-1}。与表 3 中未用 PMA 的 HSMCs [Ca^{2+}]i比较,KCl 和 NE 分别使之增高 32.1 nmol·L^{-1}和 34.3 nmol·L^{-1}。提示 PKC 转导通路激活,可显著增加已肥厚的血管平滑肌细胞内钙。PMA 的阻断剂 ST 可以阻滞此通路的信号转导。HSMCs 在 ST 作用后,再用 KCl 和 NE 激动,其胞内[Ca^{2+}]i较表 3 中未用 ST 者分别减低了 64.7 nmol·L^{-1}和 104.2 nmol·L^{-1},说明 PKC 信号转导受抑制时可以显著减轻肥厚平滑肌的胞内[Ca^{2+}]i。百日咳毒素(PTX)系作用于 G 蛋白中 Gi,Gi 为抑制性蛋白,在 PTX 作用后,HSMCs 的[Ca^{2+}]i,在 KCl 和 NE 激动下较表 3 中未用 PTX 者分别减低了 56.8 nmol·L^{-1}和 52.5 nmol·L^{-1}。可见,与 Pra-C 共同孵育后,对 PKC 信号系统的激动剂、抑制剂及 Gi 的影响,无论用 KCl 或 NE 激动均与正常细胞反应一致,说明 Pra-C 防止 Ang II 致细胞肥厚作用与细胞内钙及 PKC 和 Gi 有关,见表 4。

Table 3 Effect of Pra-C on [Ca^{2+}]i concentration alteration stimulated by KCl and NE in isolated and cultured rat hypertrophied smooth muscle cells (HSMCs) induced by angiotensin II (Ang II)

Group	Dose	[Ca^{2+}]i/nmol·L^{-1}	
		KCl/60 mmol·L^{-1}	NE/10 μmol·L^{-1}
Normal	-	171 ±5	174 ±9
Pra-C + Ang II	10 μmol·L^{-1} +10 nmol·L^{-1}	192.3 ±2.9	208 ±8
Ang II	10 nmol·L^{-1}	341 ±17***	425 ±14***

HSMCs (hypertrophied smooth muscle cells) were induced by angiotensin II (Ang II) 10 nmol·L^{-1} for 4 d; [Ca^{2+}]i was measured with Fura-2/AM; KCl (60 mmol·L^{-1}) or NE (10 μmol·L^{-1}) were added 5 min before [Ca^{2+}]i measurement *in vitro*; Pra-C 10 μmol·L^{-1} was incubated with HSMCs for 30 min. $n=8$, $\bar{x}\pm s$. *** $P<0.001$ *vs* Pra-C + Ang II group

Table 4 Effect of Pra-C on [Ca^{2+}]i concentration alteration stimulated by KCl and NE when incubated with PMA, ST and PTX in isolated and cultured rat hypertrophied smooth muscle cells (HSMCs)

Group	Dose	Activator	[Ca^{2+}]i / nmol·L^{-1}		
			ST/10 nmol·L^{-1}	PMA/0.1 μmol·L^{-1}	PTX/200 ng·L^{-1}
Normal	-	KCl	170 ±10	263 ±14	171 ±9
		NE	173 ±9	227 ±21	188.0 ±0.9
Pra-C + Ang II	10 μmol·L^{-1} +10 nmol·L^{-1}	KCl	163 ±15	277 ±14	172 ±9
		NE	173 ±14	264 ±34	182 ±7
Ang II	10 nmol·L^{-1}	KCl	277 ±14***	373 ±15***	284 ±10***
		NE	321 ±19***	460 ±17***	372 ±9***

HSMCs (hypertrophied smooth muscle cells) were induced by angiotensin II (Ang II) 10 nmol·L^{-1} for 4 d; [Ca^{2+}]i was measured with Fura-2/AM; PMA (phorbol 12-myristate 13-acetate) 0.1 μmol·L^{-1}, PTX (pertussis toxin) 200 ng·L^{-1} were incubated 2 h before [Ca^{2+}]i measurement; ST (staurosporine) 10 nmol·L^{-1} was incubated 5 min before [Ca^{2+}]i measurement; KCl 60 mmol·L^{-1} or NE 10 μmol·L^{-1} were given 5 min before [Ca^{2+}]i measurement. $n=8$, $\bar{x}\pm s$. *** $P<0.001$ *vs* Pra-C + Ang II group

讨 论

高血压的形成与心血管局部血管紧张素 II(Ang II)诱发的血管肥厚有关。文献报道[13,14],与对 SHR 表现为增生不同,Ang II 在某种浓度下可使体外大鼠血管平滑肌细胞表现为只肥厚而不增生。本研究用 Ang II 诱导离体培养血管平滑肌细胞形成肥厚并证实细胞无明显增生,表现出与整体 RHR 及 SHR 相似的病理生理反应,如细胞体积增大、细胞内[Ca^{2+}]i显著升高,对 KCl 及 NE 反应更敏感,NO 含量降低。结果表明 Pra-C 与 Ang II 致肥厚 SMCs 共同孵育,可防止细胞肥厚、阻滞平滑肌细胞内钙升高,使对 KCl 与 NE 的反应正常化,NO 含量与正常细胞相近。说明 Pra-C 能防止血管肥厚、降低血管平滑肌细胞内[Ca^{2+}]i以及恢复血管对电压依赖性及受体操纵性钙通道激动剂的异常反应。

胞内[Ca^{2+}]i升高是 Ang II 诱导细胞肥厚信息传递的中心环节[15~17]。心肌细胞和 VSMC 膜上存在特异性 Ang II 受体,Ang II 通过活化 AT 受体激活细胞膜磷脂酶系统,生成 IP_3 和 DG,后两者使细胞内 Ca^{2+} 浓度升高及 PKC 活化。而 PKC 活力增高一方面可导致细胞核酸及蛋白合成增加、促进肥厚发生发展,另一方面对细胞内许多酶产生磷酸化修饰作用,参与细胞内基因调控,进一步影响细胞功能。因此,本文 Pra-C 防止血管肥厚作用与其通过钙拮抗作用改善钙代谢异常有重要关系。G 蛋白又称鸟

苷酸调节蛋白 ,在受体产生跨膜信号转导中起重要作用 ,被誉为“分子开关”或“细胞开关”。本研究用 PKC 激动剂 PMA 使肥厚平滑肌细胞[Ca^{2+}]i显著增加 ,PKC 抑制剂 ST 及 G-蛋白族中的抑制性蛋白 Gi ,则可降低肥厚平滑肌细胞内[Ca^{2+}]i的升高 ,在与 Pra-C 共同孵育时能改善信号转导改变所致胞内[Ca^{2+}]i异常 ,使其对电压依赖性及受体操纵性钙通道激动剂异常增高反应减轻 ,接近正常 ,说明 Pra-C 不仅具有钙拮抗剂的作用 ,而且对细胞信号转导亦产生影响 ,改善钙代谢异常 ,从而对高血压心肌、血管肥厚发挥治疗作用。

NO 作为调节心血管、神经、免疫的主要细胞信使 ,可以激活鸟苷酸环化酶而发挥重要的信息传递作用 ,文献报道 ,内源性和外源性 NO 皆可明显抑制 Ang II 诱导的动脉平滑肌细胞及高血压性心肌肥厚[18,19]。NO 抑制细胞增殖和血管重构、调节血管平滑肌张力、保护血管内皮等生理病理作用 ,有利于扩张血管、降低血压和改善血管肥厚。本文研究表明 ,Pra-C 可使 Ang II 致肥厚 SMCs NO 含量与正常细胞相近 ,表明其降低 Ang II 致肥厚作用与提高肥厚 SMCs NO 产量有关 ,是其降低血管肥厚的重要机制。

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EFFECTS OF PRAERUPTORIN C ON CELL HYPERTROPHY, INTRACELLULAR [Ca^{2+}]i, NITRIC OXIDE AND SIGNAL TRANSDUCTION IN ISOLATED HYPERTROPHIED RAT SMOOTH MUSCLE CELLS INDUCED BY ANGIOTENSIN II

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ABSTRACT: **AIM** To investigate the effects of praeruptorin C (Pra-C) on smooth muscle cell (SMC) hypertrophy, intracellular calcium ([Ca^{2+}]i), nitric oxide (NO) content and influence on cellular signal transduction in isolated cultured rat smooth muscle cell (SMC). **METHODS** Hypertrophied smooth muscle cells (HSMCs) were induced by angiotensin II (Ang II), cell area was measured under inverted microscope. Nitric oxide (NO) concentration was measured using Griess method. [Ca^{2+}]i was measured using Fura-2/AM. The responses to [Ca^{2+}]i elevation stimulated by KCl (60 mmol·L^{-1} or norepinephrine (10 μmmol·L^{-1}) were observed by incubation with phorbol 12-myristate 13-acetate (PMA), staurosporine (ST), the agonist and inhibitor of protein kinase C (PKC), and pertussis toxin (PTX), the sensitive toxin of Gi. **RESULTS** The cell area of SMCs were decreased by 39.01 % ($P < 0.001$) and NO content of SMCs were significantly increased in Pra-C + Ang II group. In presence of 60 mmol·L^{-1} KCl or 10 μmol·L^{-1} NE, [Ca^{2+}]i of SMCs in Pra-C + Ang II group was significantly decreased than that of Ang II group ($P < 0.001$) and closed to the normal group. Incubation of SMCs with PMA, ST and PTX, [Ca^{2+}]i of SMCs in Ang II group was increased by PMA and decreased by ST and PTX, but that of Pra-C + Ang II group was similar to the normal group. **CONCLUSION** These findings suggest that Pra-C can reduce vascular hypertrophy in isolated rat HSMCs, and this is associated with improvment of SMCs [Ca^{2+}]i level, NO content and cellular signal transdution of PKC and Gi.

KEY WORDS: praeruptorin C; vascular smooth muscle; [Ca^{2+}]i; nitric oxide