

Ripasudil对PDGF-BB诱导人肺动脉平滑肌细胞增殖和迁移的影响

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[摘要] 目的:研究Rho激酶抑制剂ripasudil对血小板源性生长因子(platelet-derived growth factor, PDGF)-BB诱导人肺动脉平滑肌细胞(human pulmonary arterial smooth cells, HPASMCs)增殖和迁移的影响及其相关机制。方法:培养HPASMCs,随机分为control组、PDGF-BB组、PDGF-BB+ripasudil组、ripasudil组。采用CCK-8法检测细胞活力;EdU掺入法检测HPASMCs增殖;Transwell实验检测HPASMCs迁移;Real-time PCR检测基质金属蛋白酶(matrix metalloproteinase, MMP)-2 mRNA表达;Western blot检测MMP-2蛋白表达以及肌球蛋白磷酸酶目标亚基1(myosin phosphatase target subunit 1, MYPT1)、细胞外调节蛋白激酶1/2(extracellular regulated protein kinases 1/2, ERK1/2)、p38激酶和蛋白激酶B(protein kinase B, PKB/Akt)的磷酸化。结果:与control组相比,ripasudil能显著抑制PDGF-BB诱导HPASMCs增殖及迁移($P < 0.01$),降低MMP-2 mRNA及蛋白的表达($P < 0.05$),下调MYPT1、ERK1/2、p38及Akt的磷酸化($P < 0.05$)。结论:Ripasudil抑制PDGF-BB诱导的HPASMCs增殖和迁移,可能与下调MYPT1、ERK1/2、p38及Akt的磷酸化有关。Ripasudil可能是治疗肺动脉高压的潜在药物。

[关键词] ripasudil;肺动脉高压;Rho激酶;Rho激酶抑制剂

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Effects of ripasudil on proliferation and migration of human pulmonary arterial smooth cells induced by platelet-derived growth factor-BB

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[Abstract] **Objective:** To investigate the effects of ripasudil on proliferation and migration of human pulmonary arterial smooth cells (HPASMCs) induced by platelet-derived growth factor (PDGF)-BB and the mechanisms underlying. **Methods:** Cultured HPASMCs were divided into four groups: the control group, the PDGF-BB-treated group, the PDGF-BB and ripasudil-treated group and the ripasudil-treated group. CCK-8 was applied to investigate cell viability and EdU assay was used to evaluate the proliferation of HPASMCs. Transwell assay was employed to examine cell migration. The expression of matrix metalloproteinase-2 (MMP-2) was determined by real-time PCR and Western blot. The levels of phosphorylated myosin phosphatase target subunit 1 (MYPT1), extracellular regulated protein kinases 1/2 (ERK1/2), p38, and protein kinase B (PKB/Akt) were detected by Western blot. **Results:** Compared with the control group, ripasudil blocked the proliferation and migration of HPASMCs challenged by PDGF-BB ($P < 0.01$). Ripasudil suppressed PDGF-BB-induced upregulation of MMP-2 ($P < 0.05$). Moreover, ripasudil inhibited PDGF-BB-induced phosphorylation of MYPT1, ERK1/2, p38, and Akt ($P < 0.05$). **Conclusion:** Ripasudil significantly inhibited PDGF-BB-induced proliferation and migration of HPASMCs, which might be attributed to the inhibition of MYPT1, ERK1/2, p38 and Akt. Ripasudil, a Rho kinase inhibitor, might be a potential therapeutic option in PAH.

[Key words] ripasudil; pulmonary arterial hypertension; Rho kinase; Rho kinase inhibitors

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肺动脉高压(pulmonary arterial hypertension, PAH)是一类由各种因素引起的肺血管阻力进行性增高的肺循环疾病,预后差^[1]。过去20年,以前列腺素类、内皮素受体拮抗剂及5型磷酸二酯酶抑制剂为代表的靶向治疗使PAH的5年生存率提升至57%^[2]。近年来,Rho相关卷曲螺旋形成蛋白激酶(Rho associated coiled coil forming protein kinase, ROCK,Rho激酶)已成为治疗PAH的新型治疗靶点。Rho/ROCK通路、丝裂原活化蛋白激酶(mitogen-activated protein kinase,MAPK)通路、PI3K/Akt通路是调控细胞增殖和迁移的重要通路。Rho/ROCK通路中Rho激酶可被血小板源性生长因子(platelet derived growth factor,PDGF)-BB活化,激活下游底物肌球蛋白磷酸酶目标亚基1(myosin phosphatase target subunit 1,MYPT1)等,促进肺动脉平滑肌细胞(pulmonary arterial smooth cells,PASMCs)增殖及迁移^[3],参与肺血管重塑,促使PAH发生发展。PDGF-BB同时通过激活Rho/ROCK通路和MAPK通路促进血管平滑肌细胞产生基质金属蛋白酶(matrix metalloproteinases,MMPs),降解细胞外基质(extracellular matrix,ECM),促使细胞迁移^[4]。Rho激酶抑制剂fasudil(法舒地尔)、Y27632等已被证实具有显著的治疗PAH潜力,其中法舒地尔已进入临床研究阶段^[5]。目前Rho激酶抑制剂已有170余种^[6],但被用于研究治疗PAH的药物甚少,这限制了Rho激酶抑制剂降低肺动脉压力疗效的探究。法舒地尔通过气道给药治疗PAH减少了体循环压力降低等不良反应^[5],故而寻找疗效更佳的Rho激酶抑制剂意义重大。Ripasudil是近年新研发的Rho激酶抑制剂^[7],于2014年在日本被批准用于治疗青光眼,对Rho激酶靶点的选择性优于法舒地尔及Y27632^[8],可能具有治疗PAH潜力。目前,尚无有关ripasudil治疗PAH或作用相关肺血管细胞的研究。本研究探讨了ripasudil对PDGF-BB诱导的人肺动脉平滑肌细胞(human pulmonary arterial smooth cells,HPASMCs)增殖、迁移的影响及其相关机制。

1 材料和方法

1.1 材料

人肺血管平滑肌细胞(HPASMCs)、平滑肌细胞培养基、胎牛血清(ScienCell公司,美国),PDGF-BB(RD公司,美国),ripasudil(中国药科大学),CCK8试剂盒(同仁公司,日本),EdU细胞增殖检测试剂盒(广州锐博公司),结晶紫染液(上海碧云天生物技

术公司),RT-PCR试剂盒(TaKaRa公司,日本),MMP-2 PCR引物(上海捷瑞生物工程有限公司),GAPDH抗体(Proteintech公司,美国),p-MYPT1、MYPT1抗体(Bioworld公司,美国),MMP-2、p-Akt、Akt、p-ERK1/2、ERK1/2、p-p38、p38抗体(CST公司,美国)。

1.2 方法

1.2.1 细胞培养、分组

HPASMCs常规培养在含1%平滑肌细胞生长因子和2%胎牛血清的平滑肌细胞培养基中,置于37℃、含5%CO₂培养箱中。隔天换液,按1:3比例传代,6~10代细胞用于实验。

1.2.2 CCK8细胞活力检测

取对数生长期HPASMCs以 1×10^4 个/孔接种于96孔板。待细胞贴壁长至60%时,去除血清饥饿24h。将细胞分为6组:control组(空白培养基),PDGF-BB组(PDGF-BB 20 ng/mL),PDGF-BB+ripasudil组根据药物不同浓度共4组(ripasudil先于PDGF-BB 30 min加入,PDGF-BB 20 ng/mL,ripasudil分别为0.01、0.10、1.00、10.00 $\mu\text{mol/L}$);研究药物单独对细胞活力影响时,分为5组,control组,ripasudil组(剂量同前)。每组设5个复孔。各组细胞培养48h后,加10 μL /孔CCK8试剂,孵育3~4h后,以酶标仪检测样品在波长为450 nm时的吸光度。

1.2.3 EdU细胞增殖检测

待细胞生长至对数生长期后制成浓度为 2×10^4 个/mL无血清培养基细胞悬液,接种于细胞培养板片,每个孔接种500 μL 细胞悬液;贴壁生长至60%时,去除血清饥饿24h,弃去培养基,分为4组,control组(空白培养基),PDGF-BB组(20 ng/mL),PDGF-BB+ripasudil组(ripasudil先于PDGF-BB 30 min加入,PDGF-BB 20 ng/mL,ripasudil 10 $\mu\text{mol/L}$)及ripasudil组(10 $\mu\text{mol/L}$),作用48h后按EdU试剂盒说明书操作,荧光显微镜下观察并拍照,计算EdU的标记率。

1.2.4 Transwell迁移实验

待细胞生长至对数生长期后制成浓度为 4×10^5 个/mL无血清培养基细胞悬液,在24孔板底部分别加入600 μL 无血清培养基,Transwell小室加入100 μL 的细胞悬液,分组及各药物浓度同EdU实验,培养24h后,将上室未迁移的细胞擦去,以95%酒精固定,结晶紫染色,显微镜下随机取5个视野计数。

1.2.5 Real-time PCR

取对数生长期HPASMCs接种于6孔板,长至

70%时,饥饿24 h后,分组及药物浓度同EdU实验。药物作用48 h后,TRIzol法抽提各组细胞总RNA,以 β -actin为内参,按试剂盒说明进行RT-PCR。引物序列:MMP-2上游引物:5'-AACTACAACCTCTTC-CCTCGCAA-3',下游引物:5'-CAAAGGCATCATC-CACTGTCTCT-3'。 β -actin上游引物:5'-GGGAAAT-CGTGCGTGACATTAAGG-3',下游引物:5'-GCT-GATGATCTTGAGGCTGTTGTC-3'。

1.2.6 Western blot

取对数生长期的HPASMCs接种于6孔板,长至70%时,饥饿24 h,分组及药物浓度同EdU实验,作用48 h后,提取总蛋白,BCA测定蛋白含量,加入蛋白上样缓冲液后100℃煮10 min。SDS-PAGE进行电泳分离后,采用湿转膜法转至PVDF膜上,5%牛血清白蛋白(BSA)室温封闭2 h,加入一抗(MMP-2、GAPDH抗体稀释比例为1:1 000)4℃孵育过夜, TBST洗膜3次,每次10 min,加入二抗(抗体稀释比例为1:1 000),孵育1 h, TBST洗膜3次,每次10 min。加入发光反应液,凝胶电泳成像系统进行读片分析。以同样方法在PDGF-BB作用15 min后检测p-MYPT1、MYPT1、p-ERK1/2、ERK1/2、p-p38、p38、p-Akt、Akt蛋白表达水平。

1.3 统计学方法

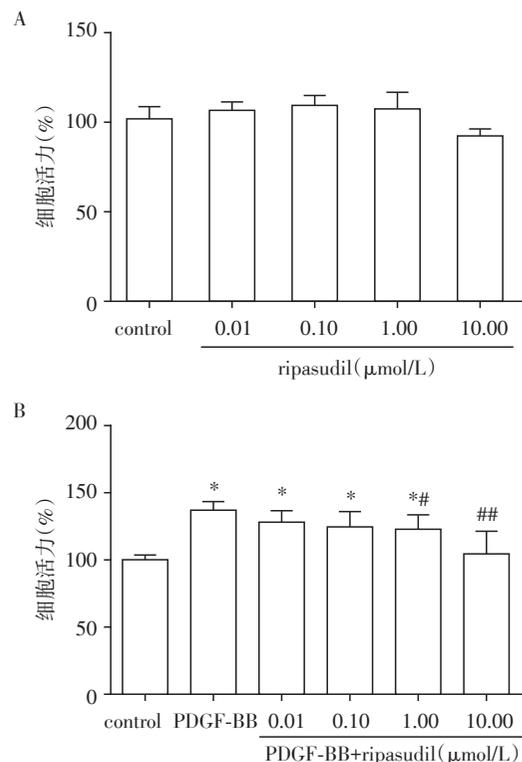
采用SPSS 22.0进行统计学分析,计量数据用均数 \pm 标准差($\bar{x} \pm s$)表示,多组间比较采用单因素方差分析,组间均数比较采用LSD法。实验重复3次或以上。 $P \leq 0.05$ 为差异有统计学意义。

2 结果

2.1 Ripasudil对PDGF-BB诱导HPASMCs细胞活力及细胞增殖的影响

CCK8实验结果显示,ripasudil在10 μ mol/L及以下浓度对细胞活力的影响与control组相比,差异无统计学意义($P > 0.05$,图1A),表明10 μ mol/L ripasudil对HPASMCs无细胞毒性。与control组对比,PDGF-BB显著增强HPASMCs活力($P < 0.01$,图1B)。PDGF-BB+ripasudil组中1 μ mol/L对细胞活力的影响与PDGF-BB组相比,差异有统计学意义($P < 0.05$),在10 μ mol/L时明显抑制PDGF-BB诱导的细胞活力增强($P < 0.01$),且与control组无统计学差异($P > 0.05$),提示ripasudil能浓度依赖性地抑制PDGF-BB诱导的细胞活力增强并在10 μ mol/L可以逆转。故后续研究采用10 μ mol/L浓度开展。EdU实验结果显示,PDGF-BB组细胞增殖数与control组

相比差异有统计学意义($P < 0.01$,图2)。PDGF-BB+ripasudil组细胞增殖数与PDGF-BB组相比,差异有统计学意义($P < 0.01$),与control组相比差异无统计学意义($P > 0.05$)。ripasudil单独作用与control组相比,对HPASMCs增殖无影响($P > 0.05$)。以上结果证实PDGF-BB显著促进HPASMCs增殖,并提示ripasudil在10 μ mol/L浓度时能逆转PDGF-BB诱导的HPASMCs增殖。

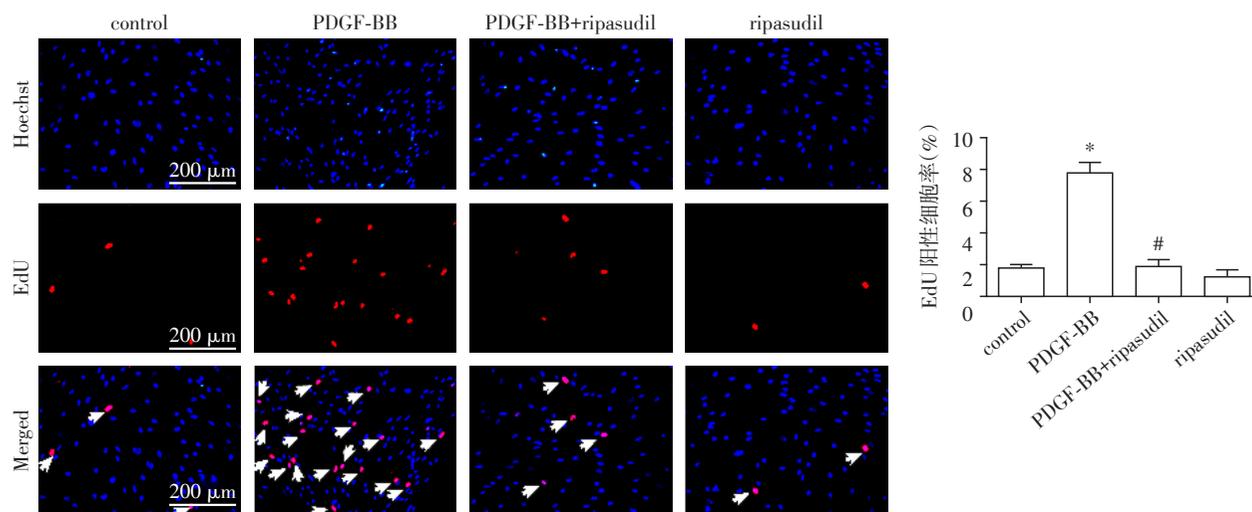


A: CCK8检测药物毒性($n=4$); B: CCK8检测细胞活力,与control组比较,* $P < 0.01$;与PDGF-BB组比较,* $P < 0.05$,# $P < 0.01$ ($n=6$)。

图1 Ripasudil对PDGF-BB诱导的HPASMCs活力的影响
Figure 1 Effects of ripasudil on the viability of HPASMCs challenged by PDGF-BB

2.2 Ripasudil对PDGF-BB诱导HPASMCs迁移能力的影响

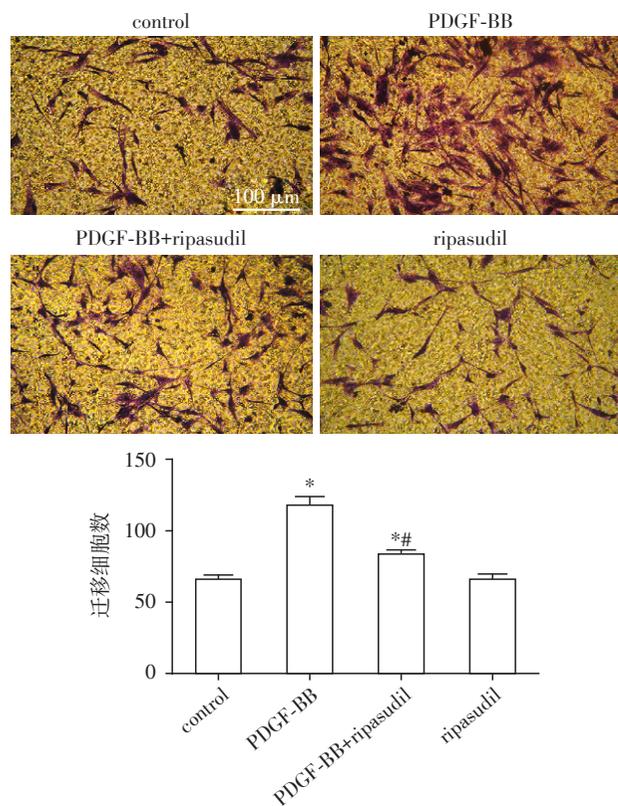
Transwell细胞迁移实验显示PDGF-BB组细胞迁移数与control组相比有统计学差异($P < 0.01$,图3),说明PDGF-BB能显著促进HPASMCs迁移。PDGF-BB+ripasudil组细胞迁移数分别与PDGF-BB组、control组相比有统计学差异($P < 0.01$)。ripasudil组与control组相比差异无统计学意义($P > 0.05$)。这表明ripasudil可以明显抑制PDGF-BB诱导的HPASMCs迁移,但未逆转,并其单独作用对细胞迁移无影响。



EdU实验检测细胞增殖($\times 200$),与control组比较,* $P < 0.01$;与PDGF-BB组比较,* $P < 0.01$ ($n=4$)。

图2 Ripasudil对PDGF-BB诱导的HPASMCs增殖的影响

Figure 2 Effects of ripasudil on the proliferation of HPASMCs challenged by PDGF-BB



Transwell实验检测细胞迁移($\times 100$),与control组比较,* $P < 0.01$;与PDGF-BB组比较,** $P < 0.01$ ($n=3$)。

图3 Ripasudil对PDGF-BB诱导的HPASMCs迁移的影响

Figure 3 Effects of ripasudil on the migration of HPASMCs challenged by PDGF-BB

2.3 Ripasudil对PDGF-BB诱导HPASMCs的MMP-2表达的影响

与control组相比,在PDGF-BB作用后,MMP-2

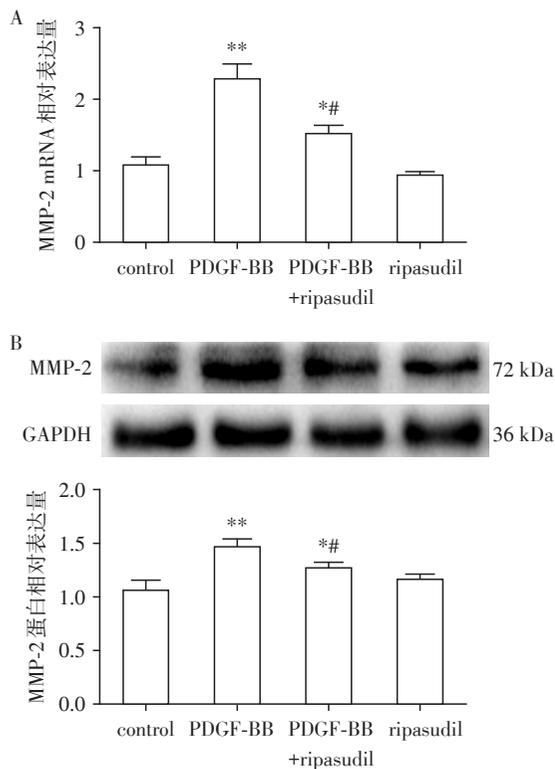
mRNA($P < 0.01$,图4A)及蛋白($P < 0.01$,图4B)表达有统计学差异。PDGF-BB+riipasudil组MMP-2表达与PDGF-BB组及control组相比有统计学差异($P < 0.05$)。结果表明PDGF-BB明显提高HPASMCs中MMP-2的表达,这可被riipasudil抑制,但未逆转。

2.4 Ripasudil对PDGF-BB诱导HPASMCs的MYPT1、ERK1/2、p38及Akt磷酸化水平影响

与control组相比,PDGF-BB作用15 min后p-MYPT1、p-ERK1/2、p-p38及p-Akt磷酸化水平明显增加,有统计学差异($P < 0.05$,图5)。与PDGF-BB组相比,PDGF-BB+riipasudil组p-MYPT1、p-ERK1/2、p-p38及p-Akt磷酸化水平明显下调,差异有统计学意义($P < 0.05$)。与control组相比,PDGF-BB+riipasudil组p-MYPT1磷酸化水平差异无统计学意义($P > 0.05$),p-ERK1/2、p-p38及p-Akt磷酸化水平差异有统计学意义($P < 0.05$)。这提示riipasudil能逆转PDGF-BB诱导的p-MYPT1上调,完全抑制了PDGF-BB诱导的Rho激酶活性的增高,并显著抑制PDGF-BB诱导的p-ERK1/2、p-p38及p-Akt上调,部分抑制了ERK1/2、p38及Akt通路的激活。

3 讨论

PAH是以肺血管阻力进行性增高为特征、最终导致右心衰竭或死亡的一组疾病。肺血管重构在PAH形成过程中起重要作用,主要包括肺血管细胞增殖、凋亡失调以及细胞迁移增强。肌球蛋白轻链(myosin light chain,MLC)磷酸化后能促进肌动蛋白-肌球蛋白交联桥形成,参与细胞增殖、迁移及收缩

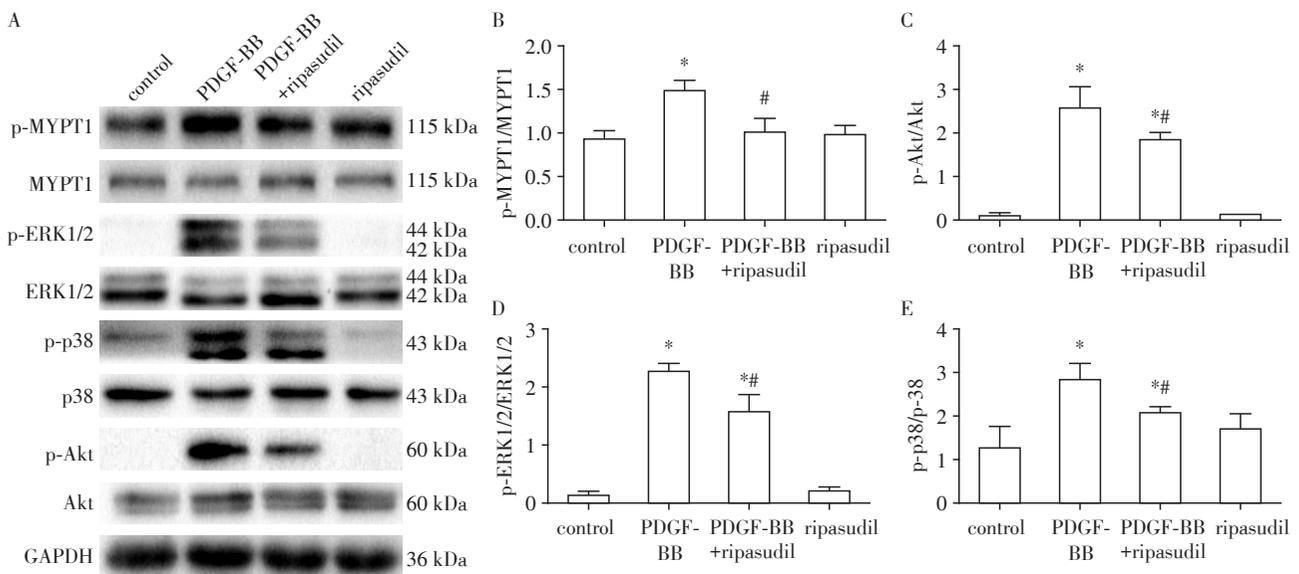


A: RT-PCR 检测 MMP-2 mRNA。B: Western blot 检测 MMP-2 蛋白量。与 control 组比较, * $P < 0.05$, ** $P < 0.01$; 与 PDGF-BB 组比较, # $P < 0.05$ ($n=3$)。

图4 Ripasudil 对 PDGF-BB 诱导的 HPASMCs MMP-2 表达的影响

Figure 4 Effects of ripasudil on the expression of MMP-2 of HPASMCs challenged by PDGF-BB

等生物学过程。肌球蛋白轻链磷酸酶(myosin light chain phosphatase, MLCP)活化后可使MLC去磷酸化。Rho激酶活化后通过磷酸化MYPT-1,可使MLCP失活,从而上调MLC的磷酸化水平。Rho激酶抑制剂可通过与ATP竞争Rho激酶上ATP结合位点,抑制Rho激酶活化。目前研究的Rho激酶抑制剂主要有法舒地尔、Y27632、氮杂吡啶-1等,在多种肺动脉高压动物模型上治疗效果显著,已成为治疗PAH的新途径。法舒地尔对ROCK1与ROCK2半数抑制浓度(IC₅₀)为290 nmol/L与350 nmol/L。Y27632对ROCK1与ROCK2的IC₅₀为110 nmol/L与170 nmol/L。Ripasudil是新型Rho激酶抑制剂,为法舒地尔的衍生物,对ROCK1与ROCK2的IC₅₀分别为51 nmol/L与19 nmol/L,比法舒地尔与Y27632对Rho激酶具有更高的选择性^[9]。用于治疗眼高压时,在改变小梁网细胞形态、破坏肌动蛋白束及增加施莱姆氏管内皮细胞通透性等方面,同浓度ripasudil效果明显优于法舒地尔及Y27632,且低浓度ripasudil与相对较高浓度法舒地尔及Y27632作用效果相似^[8]。在人视网膜微血管内皮细胞上,30 μmol/L ripasudil抑制了血管内皮生长因子诱导的细胞增殖、迁移及MYPT-1的磷酸化,效果优于同浓度法舒地尔。同时在高压氧诱导的视网膜病变小鼠上也证实0.4% ripasudil明显抑制MYPT-1的磷酸化,而同浓度法舒地尔无抑制作用^[10]。故而ripasudil对Rho激酶的抑制作用



与 control 组比较, * $P < 0.05$; 与 PDGF-BB 组比较, # $P < 0.05$ ($n=3$)。

图5 Ripasudil 对 PDGF-BB 诱导的 HPASMCs 的 p-MYPT1、p-ERK1/2、p-p38 及 p-Akt 磷酸化水平的影响

Figure 5 Effects of ripasudil on the expression of p-MYPT1, p-ERK1/2, p-p38 and p-Akt of HPASMCs challenged by PDGF-BB

优于法舒地尔及Y27632,在抑制细胞增殖及迁移方面具有更大潜力。本研究也证实了ripasudil明显抑制了PDGF-BB诱导的HPASMCs增殖及迁移,具有治疗PAH潜力。

真核细胞G1期到S期的过程由细胞周期依赖激酶(CDKs)调控。CDKs抑制因子包括p21^{Waf1/Cip1}、p27^{Kip1}。在小鼠胚胎成纤维细胞中,Rho激酶可通过ERK通路上调细胞周期蛋白(cyclin)D1与p21^{Cip1},通过激活LIM激酶2(LIM kinase 2, LIMK2)上调cyclin A,并下调p27^{Kip1},该作用不依赖ERK通路^[11]。Y27632作用后可降低LIMK磷酸化、cyclin A、D1及p21,上调p27^{Kip1},进一步证实Rho激酶对细胞增殖的调控。MAPK通路是促进细胞增殖的经典通路,包括ERK1/2、p38等蛋白激酶,可被PDGF-BB激活,促进肺血管重构,在PAH发展过程中起重要作用^[12]。p38及其下游激活转录因子2(activating transcription factor 2, ATF-2)激活后也可调控cyclin A2、D1^[13]。虽然ERK同样可以激活ATF-2,但cyclin A的表达不依赖于ERK通路,说明ERK与p38对ATF-2的激活有差异。PI3K/Akt通路同样在促进细胞增殖中发挥重要作用,参与了PDGF-BB诱导的PASMCs增殖^[14],可通过多种途径调节细胞周期,包括磷酸化p21,阻碍其与CDKs结合,下调p27^{Kip1},上调cyclin D1,并抑制cyclin D和E的降解。有研究显示cyclin A可以调节Akt^[15],但暂无关于Akt通路是否介导调节cyclin A的研究。以上说明各通路之间对细胞增殖相关蛋白的调控并不完全重叠,可能有其特有的下游调控蛋白。研究显示Y27632作用后可以逆转缺氧引起的肺动脉内皮细胞的活力增加与增殖,下调细胞周期蛋白A、D1^[16]。本研究中,ripasudil虽然部分抑制了ERK1/2、p38及Akt通路,但可逆转Rho激酶介导的MYPT1磷酸化升高,故其可能是通过抑制Rho激酶下游特有的细胞增殖关键蛋白从而逆转PDGF-BB诱导的细胞增殖,且不被ERK、p38及Akt通路所介导。这也证明了细胞增殖过程是多个通路共同作用的结果。

MMPs可由PASMCs产生,降解ECM,促进细胞迁移,在肺血管重构中起重要作用^[17]。在大鼠主动脉平滑肌细胞上,MMP非选择性抑制剂可以阻断PDGF-BB诱导的迁移^[4],这表明MMPs是细胞迁移的关键。MMP-2是MMPs家族中重要成员,可由Rho/ROCK、ERK1/2、p38及Akt通路介导所产生^[4,18]。在纤维肉瘤细胞上,Akt、ERK1/2、p38抑制剂分别作用后,都可以逆转细胞侵袭及迁移^[18],这显示3条通路

在细胞迁移上都起关键作用。本研究中在Rho激酶活性被ripasudil下调至正常后,MMP-2表达与control组相比仍升高,这可能是通过ERK1/2、p38及Akt通路的部分激活促进其表达,进而促进细胞迁移。这表明Rho/ROCK通路是介导MMP-2产生及细胞迁移中的通路之一,其中Rho激酶活性升高完全抑制后,并不能完全逆转MMP-2表达升高及细胞迁移,但这也需要Rho激酶基因敲除等更精确的实验进行证实。

信号通路存在广泛的“crosstalk”。在血管平滑肌细胞中,经PDGF-BB作用,Rho激酶敲除后能下调ERK磷酸化,Rho激酶上调能促进ERK磷酸化,这显示Rho激酶是ERK通路上游之一^[19]。在不同细胞中,Rho/ROCK通路与PI3K/Akt通路之间关系各异^[20]。ROCK1缺失后使肥大细胞中Akt及ERK1/2磷酸化水平下调,表明Akt及ERK1/2可能是ROCK1下游之一^[21]。但在Hela细胞中,PI3K/Akt的下游雷帕霉素靶蛋白(mTOR)可能参与调节RhoA^[22]。溶血磷酸酯通过活化Rho激酶,进而激活p38,可促进人脐静脉内皮细胞表达白介素-8及单核细胞趋化因子-1^[23]。本研究发现,ripasudil可同时抑制MYPT1、ERK1/2、p38、Akt的磷酸化,其中对于ERK1/2、p38及Akt通路的抑制是否是通过抑制Rho激酶活性介导,需要研究进一步证实。

本研究结果显示,新型Rho激酶抑制剂ripasudil可明显抑制PDGF-BB诱导的人肺动脉平滑肌细胞的增殖及迁移,下调MMP2表达,降低MYPT1、Akt、ERK1/2、p38的磷酸化水平,其作用机制可能是通过抑制Rho/ROCK、ERK1/2、p38及Akt信号通路。今后将在动物模型上证实其治疗效果并与法舒地尔等药物进行比较。作为Rho激酶抑制剂,ripasudil有望成为治疗PAH的新型药物。

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