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Inhibitory effect of trapidil on proliferation of cultured rat aortic smooth muscle cells induced by endothelin-1

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KEY WORDS trapidil; endothelin-1; thoracic aorta; cultured cells; vascular smooth muscle; flow cytometry; hyperplasia

AIM: To study the effect of trapidil (Tra) on endothelin-1-induced proliferation of cultured rat aortic vascular smooth muscle cells (VSMC). **METHODS**: The [^{3}H]TdR incorporation into DNA assay, the number of VSMC, and cell cycle distribution were measured. **RESULTS**: Pretreated with endothelin-1 100 nmol·L^{-1}, cell number, [^{3}H]TdR uptake, and cell mitogenic activity increased 134 % ± 23 %, 210 % ± 70 %, and 86 % ± 18 %, respectively. This proliferation was inhibited by Tra 5, 50, 500 μmol·L^{-1}. The inhibitory rates were 12 % – 48 %, 35 % – 54 % and 15 % – 47 %, respectively. Tra did not influence the proliferation of VSMC without endothelin-1 pretreatment. **CONCLUSION**: Tra antagonized the proliferation of VSMC induced by endothelin-1.

The proliferation of vascular smooth muscle cells (VSMC) is a critical event in the development of atherosclerosis and restenosis[1,2]. Several growth factors and cytokines play important roles in proliferation of VSMC. Endothelin-1, a vasoactive peptide derived from endothelial cells, exhibits potent vasoconstriction and VSMC proliferation effect[3].

Trapidil (Tra), a coronary vasodilator, inhibits the proliferation of glioma cells[4]. The aim of this study is to investigate the effects of Tra on endothelin-1 induced proliferation of VSMC.

MATERIALS AND METHODS

Cell culture[5] VSMC from the thoracic aorta of Wistar rats (clean, ♂, $n = 6$, 170 ± s 30 g, Experimental Animal Center of Beijing Medical University, Certificate № 01-3056, The Administrative Commission of Medical Experimental Animals of Beijing) were isolated by outgrowth of the explants method. Cells in the 4th – 10th passages were used.

[^{3}H] TdR incorporation into DNA[6] Cells were seeded at 1×10^8 cell·L^{-1} into 24 well plastic plates by trypsinization (Gibco), incubated for 24 h in RPMI-1640 medium (Gibco) with 10 % new-born calf serum (Gibco). Cell synchronization was initiated by serum-free RPMI-1640 for 24 h. Added endothelin-1 100 nmol·L^{-1} (Sigma) and/or different concentration of Tra 5, 50, or 500 μmol·L^{-1} (gift from Wuhan Second Pharmaceutical Factory, white powder, M_r 205.25, purity >98.5 %, dissolved in physiologic saline solution), after incubated for 16 h, [^{3}H]TdR 37 kBq (China Institute of Atomic Energy, Beijing) was added, and incubated for 8 h. The reaction was stopped by addition of cold PBS. Cells were harvested by the filter paper on Millipore, then treated with 10 % trichloroacetic acid. The filter papers were dried and placed in the scintillation solution (PPO/POPOP). The radioactivity of [^{3}H] was measured by a liquid scintillation counter (Beckman LS3801, USA).

Cell growth At passage, cells were seeded at 1×10^5 cells/well in 24-well plastic plates with trypsin, incubated in RPMI-1640 medium for 24 h, replaced by serum-free RPMI-1640 for 24 h, supplemented with endothelin-1 and/or Tra. After 48 h the number of VSMC was determined by a hemocytometer.

Cell cycle distribution analysis[7] Seeded at 1×10^8 cell·L^{-1} in 24 well plastic plates, cells were incubated in RPMI-1640 medium for 24 h, replaced by serum-free RPMI-1640 for 24 h, supplemented with endothelin-1 and/or Tra. After 24 h cells were isolated by 25 % trypsin, washed and fixed in 70 % ice-cold ethanol solution for 30 min, spun and

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washed twice in PBS, resuspended in PBS 0.5 mL. and RNase 150 U (Sigma) was added. After incubation at 37 ℃ for 30 min, 1.5 mL of propidium iodide (PI) 50 mg·L^{-1} (Sigma) was added and incubated for 40 min. Using an FACS440 instrument (Becton Dickinson, USA), PI fluorescence of DNA was measured. For fluorescence-activated cell sorting, single-parameter frequency histograms were generated. With the help of computer, cell cycle stages were estimated and the mitotical activity was calculated [Mitotical activity = $(S + G_1 + M)/(G_0 + S + G_2 + M)$].

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared with t test.

RESULTS

[^{3}H]TdR uptake in VSMC Compared with control group, endothelin-1 100 nmol·L^{-1} stimulated [^{3}H]TdR incorporation into DNA ($P < 0.01$), which was inhibited by Tra 5, 50, and 500 μmol·L^{-1} ($P < 0.05 - 0.01$). Not pretreated with endothelin-1, [^{3}H]TdR uptake in the VSMC were not affected by Tra ($P > 0.05$) (Tab 1).

Tab 1. Effects of Tra on [^{3}H]TdR uptake and cell unmber in cultured rat aortic smooth muscle cells. $n = 5$ rats, $\bar{x} \pm s$. $^cP < 0.01$ *vs* control group. $^eP < 0.05$, $^fP < 0.01$ *vs* endothelin-1 group.

Tra (μmol·L^{-1})	Endothelin-1 (nmol·L^{-1})	[^{3}H]TdR uptake (Bq)	Cell number (×10^8/L)
0	0	860 ± 110	4.2 ± 0.6
50	0	840 ± 150	4.2 ± 0.8
500		860 ± 110	4.0 ± 0.6
0	100	2 600 ± 360^c	10.0 ± 0.6^c
5	100	1 730 ± 610^e	8.8 ± 0.8^e
50	100	1 640 ± 230^f	7.5 ± 0.5^f
500	100	1 170 ± 130^f	5.2 ± 1.0^f

VSMC growth Endothelin-1 increased VSMC number ($P < 0.01$). Tra 5, 50, and 500 μmol·L^{-1} all showed inhibition on the proliferation of VSMC induced by endothelin-1 ($P < 0.05 - 0.01$) compared with ET group. The VSMC numbers were not affected by Tra (50 and 500 μmol·L^{-1}) without endothelin-1 pretreatment ($P > 0.05$) (Tab 1).

Cell cycle of VSMC Compared with the control group, endothelin-1 increased the ratio of S and G_2 + M phases of cell cycle. The ratio of VSMC treated with Tra on the S phase and the mitotical activity active stage was obviously decreased in a concentration-dependent manner ($P < 0.01$). Tra had no effect on the cell cycle in VSMC which was not pretreated with endothelin-1 ($P > 0.05$) (Tab 2).

DISCUSSION

Endothelin-1, a potent vasoconstrictor, significantly induces VSMC proliferation. Our study shows endothelin-1 induced augment of DNA synthesis in VSMC ([^{3}H]TdR incorporation and percentage distribution of S phase in cell cycle represent) and the cell growth (cell counting and mitotical activity represent), which is consistent with the reported paper[3]. Tra has a concentration-dependent inhibiting effect on augment of DNA synthesis in VSMC and on the cell growth of induced by endothelin-1. But Tra has no effect on the above parameters when it was used alone. The results showed that VSMC proliferation induced by endothelin-1 was inhibited by Tra, whereas the normal VSMC growth was not affected by Tra. It suggested that Tra exerted a cytostatic effect but not a cytotoxic effect on cell. This is consistent with Kuratsu's

Tab 2. Effects of Tra on % distribution of cell cycle in cultured rat aortic smooth muscle cells. $n = 5$ rats, $\bar{x} \pm s$. Mitotically active stage = $S + G_2 + M$. $^cP < 0.01$ *vs* control group. $^eP < 0.05$, $^fP < 0.01$ *vs* endothelin-1 group.

Cell cycle phase	G_1	S	G_2 + M	S + G_2 + M
Control	61.3 ± 2.8	25.7 ± 2.3	13.0 ± 1.9	38.7 ± 2.8
Tra (50 μmol·L^{-1})	61.0 ± 1.8	24.6 ± 1.1	14.4 ± 1.6	39.0 ± 1.8
Tra (500 μmol·L^{-1})	60.6 ± 1.8	25.6 ± 2.8	13.8 ± 1.8	39.4 ± 1.8
Endothelin-1 (100 μmol·L^{-1})	28 ± 5^c	52 ± 4^c	20 ± 3^e	72 ± 5^c
Endothelin-1 + Tra (5 μmol·L^{-1})	38.9 ± 4.0^f	45.2 ± 2.3^e	15.9 ± 3.1	61.1 ± 4.0^c
Endothelin-1 + Tra (50 μmol·L^{-1})	44.5 ± 2.2^f	39.2 ± 1.4^f	16.4 ± 2.2	55.5 ± 2.2^f
Endothelin-1 + Tra (500 μmol·L^{-1})	61.7 ± 2.8^f	23.6 ± 1.4^f	14.8 ± 2.2^e	38.3 ± 2.8^f

report[4].

The antiproliferation effect activity of Tra might be related to the $[Ca^{2+}]_i$, metabolism of arachidonic acid, and cell cycle. Many studies have suggested the importance of $[Ca^{2+}]_i$ on cell proliferation. Endothelin-1 increased the $[Ca^{2+}]_i$, caused a rapid and transient increase in c-*fos* and c-*myc* mRNA level and stimulated DNA synthesis in VSMC[8]. Calcium antagonists had inhibited proliferation of VSMC and had antiatherosclerotic effect. Tra was capable of inhibiting the inward calcium current in mouse neuroblast/glioma hybrid cells[9]. Whether Tra could decrease $[Ca^{2+}]_i$ induced by endothelin-1 in VSMC, further study is needed.

VSMC proliferation was related with peroxide levels and imbalance of the PGI_2/TXA_2 ratio. Endothelin-1 potently stimulates the formation of TXA_2 and caused a 5.5 fold increase above basal TXB_2 level after 34 min of incubation[10]. Addition of Tra to the human umbilical veins perfused *in vitro* resulted in a significantly stimulation of PGI_2 production and increased the PGI_2/TXA_2 ratio.

It is well known that cell proliferation is related to cell cycle. In the moment, we have studied the effect of Tra on expression and kinase activities of some key regulatory enzymes (the result will be shown in other paper).

The fact that Tra can inhibit VSMC proliferation provides a new approach to anti-atherosclerotic and anti-restenosis therapy and makes a foundation for further study on the exact mechanism of this antiproliferation effect.

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曲匹地尔阻抑内皮素-1诱导的培养大鼠主动脉平滑肌细胞增生

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关键词 曲匹地尔；内皮素-1；胸主动脉；培养的细胞；血管平滑肌；流动血细胞记数；增生

目的：观察曲匹地尔（Tra）对内皮素-1 诱导的血管平滑肌细胞（VSMC）增生的影响．**方法**：测定 VSMC 数目，$[^3H]$TdR 参入细胞 DNA 的放射活性及 VSMC 的细胞周期分布．**结果**：内皮素-1 100 $nmol \cdot L^{-1}$处理大鼠 VSMC 后，细胞数，$[^3H]$TdR 摄取及细胞分裂增殖活性等分别增加 134 % ± 23 %，212 % ± 71 %和 86 % ± 18 %，Tra 5，50，500 $\mu mol \cdot L^{-1}$对上述参数的抑制率分别为 12 % - 48 %，35 % - 54 %和 15 % - 47 %．Tra 50 $\mu mol \cdot L^{-1}$和 500 $\mu mol \cdot L^{-1}$对未经内皮素处理的细胞的生长无明显作用．**结论**：Tra 对内皮素-1 诱导的培养大鼠 VSMC 的增生具有阻抑作用．