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Antiproliferative effects of *D*-polymannuronic sulfate on rat vascular smooth muscle cells and its related mechanisms[1]

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KEY WORDS *D*-polymannuronic sulfate; angiotensin Ⅱ; calcium; cell cycle; protein synthesis inhibitors; nitric oxide; vascular smooth muscle; cell culture

ABSTRACT

AIM: To investigate the inhibitory effects of *D*-polymannuronic sulfate (DPS) on the proliferation of rat vascular smooth muscle cells (VSMC) induced by angiotensin Ⅱ (Ang Ⅱ) and its related mechanisms. **METHODS**: The effects of DPS on Ang Ⅱ-induced proliferation of VSMC were evaluated by MTT assay. The intracellular free Ca^{2+} concentrations, protein contents, and cell cycle were analyzed by flow cytometry. **RESULTS**: DPS 0.001 - 100 mg/L blocked the cell cycle at the $G_0/G_1 \rightarrow S$ transit and prevented the cells from entering into the G_2/M phase, and its inhibitory effects on an increase in intracellular free Ca^{2+} concentrations and the protein synthesis of VSMC were also observed. Also, the suppressing actions of DPS on intracellular Ca^{2+} were completely blocked by *L*-NAME, a nitric oxide synthase inhibitor, indicating that the counteracting effects on a rise in intracellular free Ca^{2+} contents by DPS might be mediated by participation of NO. **CONCLUSION**: DPS exerted an inhibitory effect on Ang Ⅱ-induced proliferation of VSMC and its related mechanisms were considered to be related to its inhibition on the increment of intracellular Ca^{2+} concentrations, which subsequently suppressed the synthesis of DNA and protein of VSMC.

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INTRODUCTION

A number of growth factors have been identified to be proliferation-stimulating factors for VSMC *in vitro*, including angiotensin Ⅱ (Ang Ⅱ)[1]. Angiotensin Ⅱ has been documented to stimulate the proliferation of VSMC by enhancing extracellular Ca^{2+} influx or increasing intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) released from sarcoplasmic reticulum[2], which was subsequently accompanied by an increase in the synthesis of DNA and protein[3,4]. Previous work from our laboratory has demonstrated that *D*-polymannuronic sulfate (DPS), a kind of marine sulfated polysaccharides extracted from brown algae with specific means of fractionation and chemical modification, exerted antihypertensive effects through inhibiting proliferation of VSMC by increasing nitric oxide (NO) synthesis and decreasing Ang Ⅱ production both *in vivo* and *in vitro*[5,6]. Our findings, together with other evidence that Ang Ⅱ has been proved to participate in enhancing a rise in $[Ca^{2+}]_i$ and the synthesis of DNA and protein of the proliferated VSMC, prompted us to consider whether DPS might afford an inhibitory effect on the synthesis of DNA and protein in the proliferated VSMC.

This paper firstly dealt with the antiproliferative effects of DPS on VSMC and its related mechanisms such as intracellular free Ca^{2+} contents, cell cycle, and protein synthesis.

MATERIALS AND METHODS

Drug and reagents DPS ($M_r = 4000$) was provided by Marine Drugs and Food Institute, Ocean University of Qingdao. Medium 199 (M199) was purchased from Hyclone Company (Logan, UT, USA). Fetal calf serum (FCS) was obtained from Whittaker Bioproducts (Walkersville, MD, USA). Angiotensin Ⅱ, N^{W}-nitro-*L*-arginine methyl ester (*L*-NAME), propidium iodide

(PI), RNase, fluorescein isothiocyanate (FITC), Fluo-3, and acetocymethyl ester (AM) were purchased from Sigma Chemical Co (St Louis, MO, USA). All the other reagents were of analytical grade.

VSMC culture Rats were purchased from Shandong Experimental Animal Center (Grade Ⅱ, Certificate № 200001003). Rat medial aortic smooth muscle cells were isolated and cultured as described previously[7]. VSMC were used from the 5th to the 8th passage.

MTT assay for VSMC proliferation induced by Ang Ⅱ The cells at density of 1×10^4 cells per well were seeded into 96-well plates and incubated at 37 ℃ for 24 h. And then cells were exposed to DPS at the final concentrations ranging from 0.001 to 100 mg/L for another 24 h. After 24 h, Ang Ⅱ at the final concentration of 0.1 μmol/L was added to the cells with or without DPS except the control cells for another 24 h, respectively. The antiproliferative activities of DPS were evaluated by MTT assay[8]. The percentage of inhibition of cell growth was derived from the following formula: % inhibition = $(1 - A_{DPS+Ang\ II}/A_{Ang\ II}) \times 100$ %.

Measurements of intracellular free Ca^{2+} contents in VSMC by flow cytometry The cells were seeded into 6-well plates at a density of $2 \times 10^6 - 5 \times 10^6$/well and incubated at 37 ℃ for 24 h. Then the cells were arrested to growth by M199 containing 0.4 % FCS. After 48 h, the cells were pretreated with DPS at final concentration of 1 mg/L or in combination with *L*-NAME 0.1 mg/L for another 24 h. Then cells were exposed to Ang Ⅱ at the final concentration of 100 μmol/L and allowed to grow for further 48 h when cells were proliferated at a density over 1×10^7/L. Intracellular free Ca^{2+} contents were determined with calcium-sensitive fluorochrome Fluo-3. Fluo-3 was loaded into the cells with the acetoxymethyl ester. Stock solution of Fluo-3-AM (1 mmol/L in dry Me_2SO) was stored at −20 ℃. For loading, VSMC (1×10^7/L) were mixed with the Fluo-3-AM working solution at the final concentration of 2500 μmol/L and incubated for 30 min at 37 ℃. During incubation the cells were shaken every 10 min. After 30 min, the cells were washed in PBS solution for three times. The intracellular free $[Ca^{2+}]_i$ were determined by flow cytometry (Becton Dickinson, USA).

Determination of cell cycle and protein contents of VSMC The cells were treated as described previously. Briefly, VSMC were arrested to growth by M199 containing 0.4 % fetal calf serum. After 48 h, VSMC were exposed to 10 % M199 with or without DPS at the final concentration of 1 mg/L for another 24 h. Ang Ⅱ 0.1 μmol/L was added to the cells at designed groups. After 48 h, the cells were digested by a mixture of 0.25 % trypsin and 0.02 % edetic acid and centrifuged at 150 × *g* for 10 min, the pellets were washed in PBS solution twice, and then cells were dyed in a mixture of PI (50 mg/L) and RNase (3000 kU/L) at 4 ℃ for 30 min. The cells for protein contents determination were treated as described previously, and dyed with FITC at the final concentration of 0.05 mg/L. The cell cycle was determined and the protein contents were analyzed by flow cytometry (Becton Dickinson, USA), respectively.

Statistical analysis Data were expressed as $\bar{x} \pm s$ except for cell cycle expressed as percentage and analyzed with *t*-test. In all cases, differences were considered significant if $P < 0.05$.

RESULTS

Effects of DPS on Ang Ⅱ-induced proliferation of VSMC To examine the inhibitory effects of DPS on VSMC proliferation, cells were exposed to DPS at defined concentrations for 24 h, prior to addition of Ang Ⅱ at final concentration of 0.1 μmol/L. Addition of Ang Ⅱ promoted the proliferation of VSMC by 52 %, and the effects were attenuated by DPS at the final concentrations ranging from 0.001 to 100 mg/L, with maximal effect observed at concentration of 1 mg/L (Tab 1).

Tab 1. Effects of DPS at different concentrations on the proliferation of VSMC induced by Ang Ⅱ. $n = 7$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* **control.** $^eP < 0.05$, $^fP < 0.01$ *vs* **Ang Ⅱ.**

		Absorbance
Control		0.42 ± 0.04
Ang Ⅱ		0.940 ± 0.023[c]
DPS/mg·L^{-1}	0.001	0.88 ± 0.04[e]
	0.01	0.69 ± 0.04[f]
	0.1	0.58 ± 0.03[f]
	1	0.505 ± 0.008[f]
	10	0.76 ± 0.03[f]
	100	0.091 ± 0.018[e]

Effects of DPS on intracellular free Ca^{2+} contents in VSMC It is well-known that Ang Ⅱ is a strong stimulatory factor for increasing intracellular Ca^{2+} in VSMC. In our experiment, Ang Ⅱ at the final concentration of 0.1 μmol/L increased $[Ca^{2+}]_i$ to nearly two folds, compared with that of control. Addition of DPS

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1 mg/L to VSMC, which was an optimal antiproliferative concentration, decreased $[Ca^{2+}]_i$ by 47 %, indicating that DPS afforded an inhibitory effect on the increment of $[Ca^{2+}]_i$. In addition, the effect of DPS was reversed by the presence of *L*-NMAE at the final concentration of 0.1 mg/L (Fig 1).

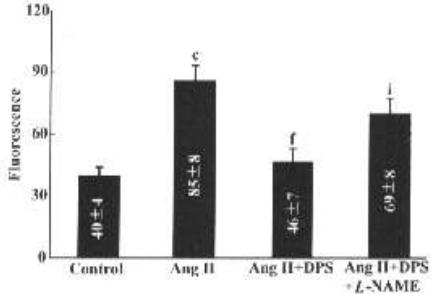

Fig 1. Effects of DPS on intracellular Ca^{2+} concentrations. Ang Ⅱ: 0.1 μmol/L; DPS: 1 mg/L; *L*-NAME 0.1 mg/L. $n=5$. $\bar{x} \pm s$. $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* Ang Ⅱ. $^iP<0.01$ *vs* Ang Ⅱ + DPS.

Effects of DPS on cell cycle of VSMC Flow cytometry analysis indicated that the Ang Ⅱ-treated VSMC entered into S phase and G_2/M phase by 38 % and 16 %, respectively, whereas the DPS-treated VSMC only by 30 % and 8 %. Most of DPS-treated VSMC were blocked at the cell cycle of G_0/G_1 phase (Tab 2).

Tab 2. Effects of DPS on cell cycle in VSMC. $\bar{x} \pm s$. $n=5$. $^cP<0.01$ *vs* control. $^eP<0.05$, $^fP<0.01$ *vs* Ang Ⅱ.

Group	Cell cycle (%)		
	G_0/G_1 phase	S phase	G_2/M phase
Control	72.2±2.9	25.0±1.7	3.0±1.6
Ang Ⅱ	46±4^c	38.1±2.5^c	16.0±2.7^c
DPS + Ang Ⅱ	59±5^f	30.0±2.9^e	8.0±1.9^f

Effects of DPS on protein contents of VSMC The cells exposed to Ang Ⅱ (0.1 μmol/L) for 24 h were observed to increase their protein synthesis by 77 %, compared with that of control. Pretreatment with DPS to the Ang Ⅱ-treated VSMC markedly decreased the synthesis of VSMC protein by 30 %, suggesting that DPS was also a potent protein synthesis inhibitor. Furthermore, the inhibitory effect of DPS on the protein synthesis of VSMC illustrated that DPS blunted cells to enter into G_2/M phase (Fig 2).

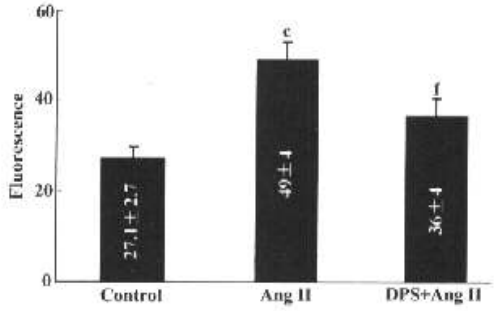

Fig 2. Effects of DPS on protein synthesis of VSMC. Ang Ⅱ: 0.1 μmol/L; DPS: 1 mg/L. $n=5$. $\bar{x} \pm s$. $^cP<0.01$ *vs* control. $^fP<0.01$ *vs* Ang Ⅱ.

DISCUSSION

DPS was found to inhibit proliferation of VSMC by suppressing a rise in intracellular Ca^{2+} concentrations, and followed by blocking cell cycle at the $G_0/G_1 \rightarrow S$ and preventing the cells from entering into the G_2/M phase.

Of many factors those are capable of stimulating VSMC proliferation, Ang Ⅱ has been the focus of considerable interests. It has been reported that addition of Ang Ⅱ to the cultured VSMC for 24 h was discovered to exert proliferation-promoting activity[3]. While DPS showed inhibitory effects on Ang Ⅱ-induced proliferation of VSMC.

Recently, accumulating evidence has demonstrated that cellular Ca^{2+} metabolism is an important determinant parameter of proliferation and migration in numerous cells types including VSMC. Cell cycle transition, for example, can be associated with dramatic changes in intracellular Ca^{2+} concentrations[9]. Moreover, proliferation of VSMC is thought to be mediated by locally produced growth factors such as Ang Ⅱ[4]. Although the mechanisms by which Ang Ⅱ stimulates VSMC proliferation has not been elucidated yet, many studies have shown that Ang Ⅱ increases the synthesis of DNA and protein in VSMC by elevating intracellular Ca^{2+} concentrations. As a matter of fact, Ang Ⅱ has been found not only to mediate extracellular Ca^{2+} influx, but also to stimulate $InsP_3$ formation in cultured rat smooth muscle cells, which generates cytoplasmic Ca^{2+} signals by activating Ca^{2+} release from Ca^{2+}-sequestering target organelles, including the sarcoplasmic reticulum[10]. Ang Ⅱ, in our experiment, was found to stimulate a rise in $[Ca^{2+}]_i$ as well as the synthesis of DNA and protein, which were completely reversed by DPS. This notion gave us a

good understanding of the precise mechanisms underlying antiproliferative effect of DPS for VSMC.

NO has been documented to afford an inhibitory action on extracellular Ca^{2+} influx by blocking voltage-gated Ca^{2+} channels or intracellular Ca^{2+} released from the sarcoplasmic reticulum through inhibiting the formation of $InsP_3$ in rat VSMC[11]. And other evidence indicated that *L*-NAME, a nitric oxide synthase inhibitor, blunted the suppressing effects afforded by NO on $[Ca^{2+}]_i$ in VSMC[12]. Previous work from our laboratory has illustrated that DPS inhibited the proliferation of VSMC by enhancing NO synthesis at its antiproliferative concentrations in VSMC[6]. In this study, DPS was found to decrease the intracellular Ca^{2+} contents at the optimal concentration of stimulating NO synthesis, which was attenuated by *L*-NAME. These findings suggested that the counteracting actions on a rise in $[Ca^{2+}]_i$ by DPS might be mediated by participation of NO.

As far as the impact of Ang Ⅱ on synthesis of DNA and protein was concerned, there existed different evidence. Geisterfer *et al* noted that the increase in DNA synthesis in response to Ang Ⅱ was much smaller than that in protein synthesis[13]. On the contrary, the observations by Campbell-Boswell and Robertson demonstrated similar response of synthesis of DNA and protein to Ang Ⅱ in VSMC, which were consistent with our data[14]. The discrepancy, we inferred, might be due to difference in VSMC species or serum used or other un-elucidated reasons.

In conclusion, the reversal of overload of intracellular Ca^{2+} and subsequently the inhibition on synthesis of DNA and protein might be a key to understand the antiproliferative mechanisms exerted by DPS. However, the detailed mechanisms by which DPS decreased the concentrations of Ca^{2+} as well as the synthesis of DNA and protein in VSMC require to be further investigated.

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D-硫酸多糖抗血管平滑肌细胞增殖作用及其机制[1]

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关键词 *D*-硫酸多糖；血管紧张素Ⅱ；钙；细胞周期；蛋白质合成抑制剂；一氧化氮；血管平滑肌；细胞培养

目的：探讨硫酸多糖(DPS)抗增殖作用及其机制. **方法**：用MTT法评价DPS抗大鼠主动脉平滑肌细胞(VSMC)增殖作用. 用流式细胞仪观察DPS对

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VSMC 胞浆内游离 Ca^{2+} 的含量、细胞周期和蛋白质含量的影响. **结果**: DPS 在 0.001 – 100 mg/L 浓度下, 对 Ang Ⅱ 诱导的 VSMC 增殖均有明显抑制作用, 最佳抑制浓度为 1 mg/L. 流式细胞术分析结果表明, DPS 在抗增殖的浓度(1 mg/L)下能抑制 VSMC 从 G_0/G_1 到 S 期的转换, 阻止 VSMC 进入 G_2/M 期; 减少 VSMC 蛋白质的合成. DPS 能明显抑制胞浆内游离 Ca^{2+} 浓度的升高, 此作用可被一氧化氮合酶抑制剂(*L*-NAME)所阻断, 提示一氧化氮可能介导了 DPS 降低胞浆内游离 Ca^{2+} 浓度的作用. **结论**: 硫酸多糖 DPS 可拮抗 Ang Ⅱ 诱导的 VSMC 增殖, 其作用机理可能与降低胞浆内游离 Ca^{2+} 浓度, 抑制 VSMC 的 DNA 及蛋白质合成有关.

(责任编辑 朱倩蓉)

Correction

Preventive effects of ginsenosides on osteopenia of rats induced by ovariectomy (2001; 22: 428 – 434). On page 429, right column, line 6, MAR/BV should be MAR/BS; page 431, Fig 1, the scale should be 1 mm not 1 μm; page 432, in Tab 3, "Parameters perimeter" should be "Label perimeter". The editors and authors are sorry for the mistakes.