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Effects of isorhynchophylline on angiotensin II-induced proliferation in rat vascular smooth muscle cells

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Abstract

Proliferation of vascular smooth muscle cells (VSMCs) is a crucial event in cardiovascular diseases. Isorhynchophylline, an alkaloid from a traditional Chinese medicine *Gambirplant*, has been used to treat cardiovascular diseases. The aim of this study was to investigate the effects of isorhynchophylline on angiotensin II (Ang II)-induced proliferation of rat VSMCs. VSMCs were isolated from rat artery and cultured for 14 days before experimentation. The effect of isorhynchophylline on Ang II-induced proliferation was evaluated by cell number, MTT assay and flow cytometry, and nitric oxide (NO) content and activity of NO synthase (NOS) were measured. The expression of proto-oncogene c-fos, osteopontin (OPN) and proliferating cell nuclear antigen (PCNA) mRNAs was measured by real-time RT-PCR. VSMC cultures were verified by morphology and immunostaining with α-smooth muscle actin. Isorhynchophylline (0.1–10.0 μM) was not toxic to VSMCs, but markedly decreased Ang II (1.0 μM)-enhanced cell number and MTT intensity, and blocked cell transition from G_0/G_1 to S phase. Furthermore, isorhynchophylline increased the NO content and NOS activity, and suppressed Ang II-induced over-expression of c-fos, OPN and PCNA. Thus, isorhynchophylline was effective against Ang-II induced cell proliferation, an effect that appears to be due, at least in part, to increased NO production, regulation of the cell cycle, and depressed expression of c-fos, OPN and PCNA related to VMSC proliferation.

Introduction

Proliferation of vascular smooth muscle cells (VSMCs) is a crucial event in the pathogenesis of hypertension, atherosclerosis and restenosis. (Ross 1993; Rutherford et al 1997). Angiotensin II (Ang II) acts not only as a vasoactive peptide, but also as a growth factor for VSMC proliferation (Daemen et al 1991; Okamoto et al 2004). In particular, Ang II has been shown to stimulate proliferative and hypertrophic growth of VSMCs via angiotensin type-I receptor binding (Schieffer et al 1996). Other investigators have emphasized the important roles of over-expression of proto-oncogene c-fos (Stepien et al 1998), osteopontin (OPN) (Wen et al 2002) and proliferating cell nuclear antigen (PCNA) (Tzeng et al 2007) in VSMC proliferation; modulation of these signalling molecules may prevent VSMC proliferation. Thus, inhibition of VSMC growth may contribute to the treatment of cardiovascular diseases.

Isorhynchophylline is an alkaloid from a traditional Chinese medicine *Gambirplant* (*Uncaria rhynchophylla* Miq Jacks), which is used to treat ailments of the cardiovascular and central nervous systems, such as hypertension and convulsions (Hsieh et al 1999), and to prevent platelet aggregation and thrombosis (Xie et al 2007). The major active pharmacological ingredients are reported to be indole alkaloids such as rhynchophylline, isorhynchophylline, hirsutine and corynantheine, which have been identified, isolated and purified in a number of laboratories (Yamanaka et al 1983; Zhang et al 1998). Isorhynchophylline is effective in lowering blood pressure, and this effect is thought to be mediated through vasodilatory action (Shi et al 2003). However, the effects of isorhynchophylline on VSMC proliferation as a mechanism of its beneficial effects have not been investigated.

In the present study we investigated the effects of isorhynchophylline on the proliferation of rat VSMC induced by Ang II in-vitro, and explored the possible mechanisms of the inhibitory action through cellular production of nitric oxide (NO) and regulation of the cell cycle. The expression of OPN is thought to promote adhesion and spreading of vascular cells, and is an indicator of VSMC proliferation (Liaw et al 1995); the expression of PCNA is a sensitive measure of cell proliferation (Lavezzi et al 2005). Thus, the expression of both genes was examined using real time RT-PCR.

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Materials and Methods

Reagents

Isorhynchophylline (purity ≥ 96% via HPLC) was provided by the Guangxi Institute of Traditional Chinese Medicine (Nanning, China). Ang II and MTT were purchased from Sigma-Aldrich (St Louis, MO, USA). Trypsin-EDTA and Dulbecco modified Eagle medium (DMEM) were purchased from Gibco BRL (New York, USA). Fetal bovine serum (FBS) was from Hanzhou Season's Co. (Hanzhou, China). Kits for the measurement of NO levels and NO synthase (NOS) activity were purchased from Nanjing Jiancheng Bio-engineering Institute (Nanjing, China). RNeasy Mini Kits and the primers of c-fos, OPN and PCNA were from Takara Biological Technology (Dalian, China). SYBR green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). Trizol was from Jingmei Biological Engineering Co. (Shenzhen, China).

Animals

The animal studies were approved by the Animal Ethics Committee of Zunyi Medical College. Male Sprague–Dawley rats (Grade SPF 2, Certificate no. scxk 20020003), 200–250 g, were purchased from the Animal Center of the Third Military Medical University (Chongqing, China). The rats were maintained in an air-conditioned animal facility at 23 ± 1°C, relative humidity 50 ± 2% and a 12 h light/dark cycle (light on 8:00 am–8:00 pm). Water and food were freely accessible. All animals were acclimatized to the facilities for 7 days before isolation of VSMCs.

Cell culture and identification

VSMCs were isolated from the thoracic aorta by enzymatic digestion (Battle et al 1994). VSMCs were grown in a 5% CO_2 atmosphere at 37°C in DMEM supplemented with 100 units mL^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10% FBS. Under these conditions, VSMCs were verified by staining with antibody against α-smooth muscle actin and by morphology. Experiments were performed with cells from passages 3–8.

Cell number

VSMCs were plated into 24-well plates at a density of 5×10^4 cells per well in DMEM supplemented with 10% FBS for 24 h. Cells were then rinsed with phosphate-buffered saline (PBS) and cultured in medium containing 2% FBS, with or without Ang II (1.0 μM) and isorhynchophylline at the required concentrations for an additional 24 h. The concentration of Ang II used to stimulate VSMC proliferation was selected according to the literature (Watanabe et al 2001) and from our preliminary studies. Cells were then trypsinized and counted under a light microscopy.

MTT assay

VSMCs at a density of 6×10^3 cells per well (in 96-well plates) were subjected to the same treatments as above, followed by addition of MTT at a final concentration of 0.5 mg mL^{-1} for 4 h at 37°C. The medium was then removed and reduced MTT (blue formazan product) was solublized by adding 100 μL DMSO to each well. After agitation of the plates for 15 min, the optical density of the solublized formazan product in each well was measured at 492 nm using an automated microplate reader.

Cell cycle regulation

Cell cycle regulation was determined by flow cytometry. Briefly, cells were seeded into 24-well plates at a density of 5×10^5 cells per well and allowed to attach overnight. Cells were treated as described above. VSMCs were then harvested by trypsin treatment, washed with cold PBS, and then stained with propidium iodide (PI) solution (50 mg mL^{-1} PI, 100 mg mL^{-1} RNase, and 0.1% Triton X-100 in PBS). The distribution of cell cycle phase in the stained cells was analysed by flow cytometry.

Measurement of NO content and NOS activity

Cells were cultured in DMEM supplemented with 2% FBS and various concentrations of isorhynchophylline and Ang II for 24 h. NO content was determined by measuring the level of nitrite accumulated in the supernatant using a colorimetric reaction with Griess reagent. Briefly, supernatants were collected and mixed with an equal volume of Griess reagent (0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% H_3PO_4). The mixture was incubated in the dark for 10 min at room temperature, and the absorbance at 540 nm measured using a microplate spectrophotometer. The concentration of nitrite in the samples was determined from a sodium nitrite standard curve.

The NOS activity of the Ang II-induced VSMCs was measured using a commercially available kit, according to the manufacturer's instructions. Formation of NO from L-arginine and oxygen is catalysed by NOS; the NO and nucleophilic material produce a coloured compound, the concentration of which is determined spectrophotometrically at 530 nm (Feelisch & Noack 1987).

Real-time RT-PCR

Expression of c-fos, OPN and PCNA mRNA was determined by real-time RT-PCR. Total RNA was isolated using Trizol agent and purified with RNeasy Mini Kit. RNA was quantified spectrophotometrically at 260/280 nm, dissolved in diethyl-pyrocarbonate-treated water, and stored at −80°C. Total RNA was reverse-transcribed with MuLV reverse transcriptase and Oligo-dT primers (listed in Table 1). The SYBR green PCR Master Mix was used for real-time PCR analysis. The relative differences in expression between groups were expressed using cycle time (Ct) values. The Ct values of the genes of interest were first normalized with β-actin of the same sample, and then the relative difference between the control and each treatment group was calculated and expressed as a relative increase, setting the control at 100%. Assuming that the Ct value is reflective of the initial starting copy and that there is 100% efficiency, a difference of one cycle is equivalent to a 2-fold difference in starting copy.

Table 1 Primer sequences of β-actin, c-fos, osteopontin (OPN) and proliferating cell nuclear antigen (PCNA)

Gene	GeneBank access number	Forward primer	Reverse primer
β-actin	NM_031144	TGACAGGATGCAGAAGGAGA	TAGAGCCACCAATCCACACA
c-fos	NM_022197	GTCTTCCTTTGTCTTCACCTACCC	CCCTGCCTTCTCTGACTGCT
OPN	NM_012881	TCCTGCGGCAAGCATTCTC	TCAGCCAAGTGGCTACAGCATC
PCNA	NM_022381	GAGCTTGGCAATGGGAACATTA	TGAACTGGCTCATTCATCTCTATGG

Statistical analysis

Data are expressed as mean ± s.d. Data were analysed by one-way analysis of variance followed by multi-range tests, using the SPSS 11.5 for Windows statistical program (SPSS, Chicago, IL, USA). $P < 0.05$ was considered statistically significant.

Results

Effects of isorhynchophylline on Ang II-induced VMSC proliferation

Isolated rat VMSCs were cultured continuously for 14 days to allow the cells to reach confluence. VMSCs were verified by morphology and staining with antibody against α-smooth muscle actin of VSMCs (Figure 1). Cultures typically containing more than 95% VSMCs were used in the experiments. Isorhynchophylline treatments at concentrations used in this study did not affect cell morphology or cell growth (not shown).

Ang II (1.0 μM) significantly promoted the proliferation of VSMC, as evidenced by the cell number counting (Table 2) and the MTT intensity (Figure 2). Isorhynchophylline treatment significantly attenuated Ang II-induced cell proliferation in a concentration-dependent manner. The highest concentration tested (10 μM) abolished Ang-II induced VMSC proliferation.

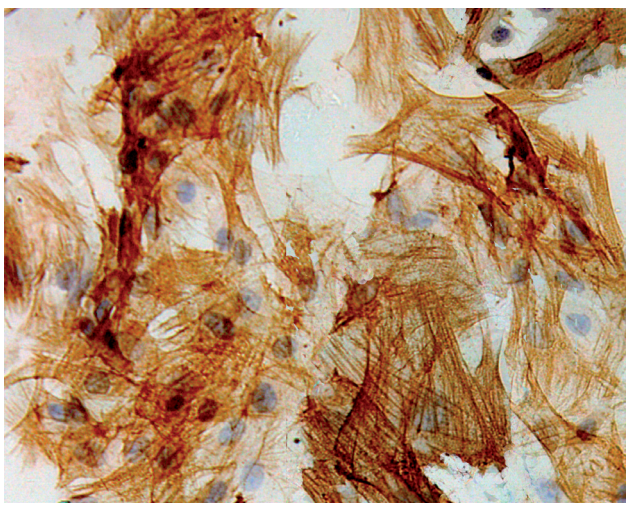

Figure 1 Identification of vascular smooth muscle cells by α-smooth muscle actin immunohischemistry staining.

Effect of isorhynchophylline on cell cycle regulation

The proportions of Ang II-induced VSMCs entering S phase and G_2/M phase were 33% and 15%, respectively, which were significantly higher than in the control group (Table 3). Isorhynchophylline (1.0 μM and 10.0 μM) significantly decreased the percentage of cells in S and G_2/M phases, and increased the percentage of cells in G_0/G_1 phase compared with the Ang II group.

Effects of isorhynchophylline on NO content and NOS activity

Ang II at the final concentration of 1.0 μM decreased NO release. However, the decreases in cellular nitrite were significantly recovered by isorhynchophylline treatment at concentrations of 1.0 μM and 10.0 μM (Figure 3A). Moreover, Ang II decreased the activity of NOS, which was reversed by the addition of isorhynchophylline at concentrations of 1.0 μM and 10.0 μM (Figure 3B).

Effects of isorhynchophylline on expression of c-fos, OPN and PCNA mRNA

Ang II significantly increased the expression of c-fos, OPN and PCNA by approximately 6-fold. However, isorhynchophylline (0.1–10.0 μM) significantly suppressed the over-expression of c-fos mRNA in a concentration-dependent manner. Similarly, isorhynchophylline (1.0 μM and 10.0 μM) markedly decreased the over-expression of OPN and PCNA mRNA (Figure 4).

Discussion

The proliferation and migration of VSMCs induced by various growth factors can lead to a variety of pathological processes, including atherosclerosis, hypertension and restenosis after balloon angioplasty (Ranganna et al 2000; Natarajan & Nadler 2003). It is well known that Ang II induces VSMC proliferation (Rudijanto 2007), and accelerated VSMC proliferation is a characteristic feature of the arteries of patients and animals with high blood pressure. Consequently, inhibition of VSMC proliferation represents a potentially important therapeutic strategy for the treatment of vascular diseases such as atherosclerosis and restenosis. In this paper, we demonstrate that isorhynchophylline significantly inhibited VSMC proliferation induced by Ang II, as evidenced by the cell number counting, MTT intensity and flow cytometry. Furthermore, isorhynchophylline prevented

Table 2 Effect of isorhynchophylline (isorhy) on proliferation of vascular smooth muscle cells

Groups	Cell number (1×10^4 mL^{-1})			
	12 h	24 h	48 h	72 h
Control	12.67 ± 1.28	13.58 ± 0.52	14.00 ± 0.75	15.08 ± 0.76
Ang II (1.0 μM)	14.66 ± 0.20	15.92 ± 0.38**	17.91 ± 0.52*	18.83 ± 0.37**
Ang II + Isorhy 0.1 μM	14.25 ± 0.25	15.58 ± 0.63	17.42 ± 0.39	18.33 ± 0.14
Ang II + Isorhy 1 μM	13.24 ± 0.90	14.41 ± 0.38##	16.50 ± 0.66#	17.50 ± 0.91
Ang II + Isorhy 10 μM	12.50 ± 0.25	13.66 ± 0.15##	14.58 ± 0.29##	15.65 ± 0.29##

Results are mean ± s.d. (n = 5). *$P < 0.05$; **$P < 0.01$ vs control group; #$P < 0.05$; ##$P < 0.01$ vs Ang II group.

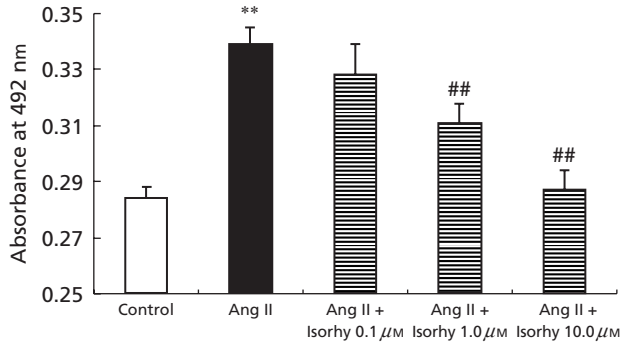

Figure 2 Effects of isorhynchophylline (Isorhy) on Ang II-induced proliferation of vascular smooth muscle cells, determined by MTT intensity. Results are mean ± s.d. (n = 6). **$P < 0.01$ vs control group ; ##$P < 0.01$ vs Ang II group.

Table 3 Effect of isorhynchophylline (Isorhy) on the cell cycle of vascular smooth muscle cells

	Cell cycle phase (%)		
	G_0/G_1 phase	S phase	G_2/M phase
Control	77.5 ± 1.31	17.3 ± 1.87	5.20 ± 1.53
Ang II (1.0 μM)	52.6 ± 2.52**	32.6 ± 1.36**	14.8 ± 2.40*
Ang II + Isorhy 0.1 μM	61.4 ± 2.15	27.8 ± 2.24	10.7 ± 1.94
Ang II + Isorhy 1 μM	70.3 ± 1.54##	22.0 ± 1.39##	7.60 ± 1.48#
Ang II + Isorhy 10 μM	76.3 ± 0.93##	17.9 ± 2.43##	5.70 ± 1.15#

Results are mean ± s.d. (n = 5). *$P < 0.05$; **$P < 0.01$ vs control group; #$P < 0.05$; ##$P < 0.01$ vs Ang II group.

Ang II-induced reduction in cellular NO production and attenuated the overexpressions of c-fos, OPN and PCNA mRNA. These results clearly demonstrate that isorhynchophylline is beneficial against vascular proliferative disorders associated with overproduction of Ang II.

Isorhynchophylline isolated from different herbs has many biological actions. For example, isorhynchophylline isolated from hooks and stems of *U. sinensis* (Oliv.) showed strong effects on Ca^{2+} influx and protected against glutamate-induced neuronal cerebellar granule cell death (Shimada et al 1999). Isorhynchophylline isolated from *U. tomentosa* exerted a beneficial effect on scopolamine-induced memory impairment in mice (Mohamed et al 2000), and that isolated from *U. genus* had anti-hypertension effects in spontaneously hypertensive rats, probably through actions on the *N*-methyl-D-aspartate

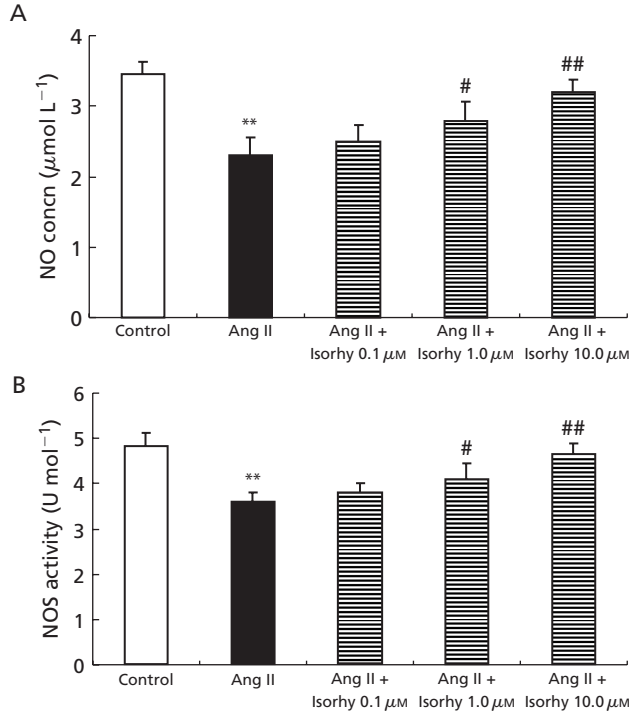

Figure 3 Effects of isorhynchophylline (Isorhy) on the content of NO (A) and the activity of NOS (B). Results are mean ± s.d. (n = 6). **$P < 0.01$ vs control group; #$P < 0.05$; ##$P < 0.01$ vs Ang II group.

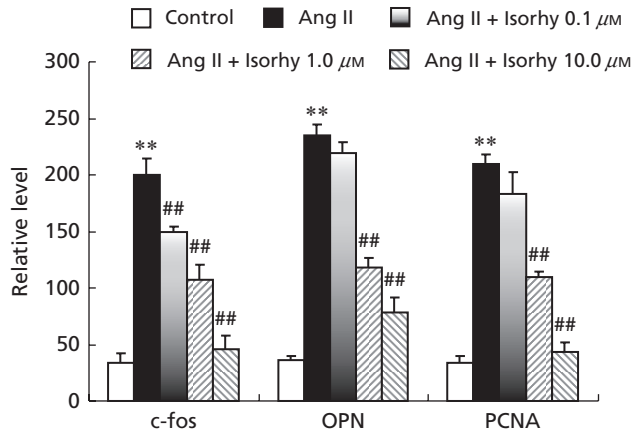

Figure 4 Effects of isorhynchophylline (Isorhy) on expression of c-fos, OPN and PCNA mRNA of VSMCs. Results are mean ± s.d. (n = 6). **$P < 0.01$ vs control group; ##$P < 0.01$ vs Ang II group.

receptor (Watanabe et al 2003). Isorhynchophylline may also exert beneficial effects on brain function through serotonin 5-HT_{2A} receptors in mice (Matsumoto et al 2005). Isorhynchophylline isolated from *U. rhynchophylla* (Miquel) has been shown to act via the inhibition of multiple calcium pathways and NO pathways to produce vasodilatation in rat aortic rings (Zhang et al 2004).

NO is an important endogenous vasodilator, the synthesis of which from L-arginine is catalysed by NOS. It is well known that decreased NO production plays an important role in the development of hypertension and atherosclerosis (Kunieda et al 2008). L-arginine–NO signalling in VSMCs is an important autocrine action of NO, in addition to the paracrine role of NO generated in the endothelium (Buchwalow et al 2004). The mechanism of NO-mediated inhibition of VSMC proliferation may involve the delay of cell cycle progression or the induction of apoptosis (Sarkar et al 1997; Tanner et al 2000). Ang II is known to antagonize the effects of NO on arterial cell proliferation (Kato et al 1996). The NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) inhibited isorhynchophylline-induced vaso-relaxing effects in isolated rat aortic rings (Zhang et al 2004). In this study, we found that VSMC proliferation was inhibited by isorhynchophylline, simultaneously accompanied by increased NO levels and NOS activity. Thus, increased NO production could be one mechanism for the antiproliferative effects of isorhynchophylline in VSMCs.

Aberrant proto-oncogene expression is related not only to tumorigenesis, but also to the pathogenesis of hypertension and atherosclerosis. Activiation of proto-oncogenes is an important mechanism for cell proliferation and cell hypertrophy (Naftilan et al 1989). Through a diversity of signal transduction pathways, Ang II induces the expression of c-fos mRNA and other early growth response genes associated with the proliferative response in VMSCs (Taubman et al 1989; Berk & Corson 1997). In this study, isorhynchophylline decreased expression of c-fos mRNA induced by Ang II in VSMCs, indicating that the inhibition of VSMC proliferation may be related to decreased expression of c-fos.

PCNA, a protein synthesized early in G_1 and S phases of the cell cycle, functions in cell progression, DNA replication and DNA repair. It is a very sensitive measure of cell proliferation, including VSMC proliferation (Lavezzi et al 2005). VSMC proliferation and PCNA expression are critical events in the development and progression of atherosclerosis. In this study, Ang II markedly promoted cell cycle progression in proliferative VSMCs, decreasing the percentage of cells in G_0/G_1 phase, and increasing the percentage of cells in S and G_2/M phases, together with increased expression of PCNA mRNA. Isorhynchophylline significantly blocked cell cycle progression in Ang II-treated VSMCs by decreasing the percentage of cells in S and G_2/M phases and increasing cells in G_0/G_1 phase. Isorhynchophylline also suppressed the expression of PCNA mRNA. These results suggest that the antiproliferative effect of isorhynchophylline may be partially mediated through decreasing PCNA mRNA expression and suppressing cell cycle progression.

OPN is a secreted acidic phosphoprotein that regulates a variety of cellular processes. Increased expression of OPN is associated with vascular injury. OPN is elevated in human atherosclerotic plaques (Giachelli et al 1993). OPN also promotes adhesion and spreading of vascular cells, and is a potent chemotactic factor for VSMCs (Liaw et al 1995). Over-expression of OPN is thought to play a central role in the induction of proliferative VSMC phenotypes (Parrish & Ramos 1997). In this study, Ang II-induced VSMC proliferation was associated with over-expression of OPN mRNA, which was significantly attenuated by isorhynchophylline. These inhibitory effects would potentially contribute to the antiproliferation effects of isorhynchophylline in cultured VSMCs.

Many pathways have been shown to be involved in Ang II-induced VSMC proliferation, including NO pathways (Buchwalow et al 2004), calcium signalling pathways (Trion et al 2008), and mitogen-activated protein kinase signalling pathways (Kanda et al 2001). All these pathways could play an integrated role in regulating VSMC proliferation, and could be involved in the pharmacological effects of isorhynchophylline.

Conclusion

Our study shows that isorhynchophylline is effective against Ang II-induced VSMC proliferation, an effect that appears to be due, at least in part, to increased NO production, regulation of the cell cycle, and depressed expression of c-fos, OPN and PCNA mRNA related to VMSC proliferation. Thus, isorhynchophylline may be useful against Ang II-induced vascular disorders.

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