N-4-Tert-Butyl Benzyl Haloperidol Chloride Suppresses Ca²⁺-dependent Egr-1 Expression and Subsequently Inhibits Vascular Smooth Muscle Cell Proliferation Induced by Angiotensin II

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Key Words

Vascular smooth muscle cell • Early growth response gene (Egr-1) • Platelet-derived growth factor (PDGF) • Calcium • N-4-Tert-Butyl benzyl haloperidol chloride (C₃) • G₁ cell-cycle regulatory proteins cyclin D1 and Cdk2 • Cell-cycle inhibitory proteins p21Cip1

Abstract

Background: N-4-Tert-Butyl benzyl haloperidol chloride (C₃) was a novel calcium antagonist synthesized in our laboratory. The present study is to explore the effect of C₃ on vascular smooth muscle cell proliferation and the mechanism involved. **Methods:** The effects of C₃ on Ang II-induced cytosolic free Ca²⁺ concentration change, VSMC proliferation, the key early growth response factor 1 (Egr-1) were evaluated by laser scanning confocal microscopy, microtiter tetrazolium (MTT) proliferation assay, flow cytometry analysis, Western blot and RT-PCR analysis, respectively. An extracellular Ca²⁺ chelator EGTA and antisense Egr-1 oligodeoxyribonucleotides (ODNs) were used to establish the relation between Ca²⁺-dependent Egr-1 expression induced by Ang II and VSMC proliferation. **Results:** C₃ attenuated the

Ang II-induced extracellular Ca²⁺ influx, inhibited VSMCs proliferation and arrested VSMCs in G₁-phase. C₃ also triggered a significant reduction in PDGF-A and cyclin D1, Cdk2 along with an overexpression of p21Cip1. Antisense Egr-1 ODNs inhibited VSMCs proliferation, which was related to G₁-phase arrest, due to inhibiting the expression of Egr-1 and C₃ inhibited the overexpression of Egr-1. **Conclusion:** Egr-1 may play a key role in Ang II-induced proliferation of VSMCs. C₃ inhibits vascular smooth muscle cell proliferation and the mechanism is involved with the inhibition of over-expression of Egr-1.

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Introduction

Vascular smooth muscle cell (VSMC) proliferation is a common pathognomonic feature of hypertension, atherosclerosis, restenosis and other cardiovascular diseases [1-3]. Inhibiting the proliferation of VSMCs is an important target in the treatment of cardiovascular diseases [4]. Ca²⁺ plays an important role as an intracellular messenger in physiological activities such as proliferation or apoptosis of cells [5]. The change in concentration of

intracellular Ca^{2+} is an initial factor that triggers signal transduction related to proliferation of cells [6].

Early growth response factor 1 (Egr-1) is one of the immediate-early genes and a member of the zinc finger family of transcription factors; it plays a significant role in cell proliferation [7-10]. Egr-1 is a nuclear protein with sequence-specific DNA binding activity and can induce the expression of multiple downstream target genes, including monocyte chemotactic protein 1 (MCP-1), platelet-derived growth factor (PDGF) A and B, G1 cell-cycle control proteins cyclin D1 and Cdk2, which promote cell growth and movement, and Cdk inhibitors p21Cip1 [9-13]. Egr-1 is believed to act as an intracellular "third messenger". It has a low basal level in normal vessel walls but is rapidly and transiently expressed in VSMCs and endothelial cells in response to arterial injury [9]. In injured vascular cells, Egr-1 has profound chemotactic and mitogenic effects, which may contribute to the structural remodeling that typically occurs in the pathogenesis of vascular diseases [9, 14]. Egr-1 expression is induced by Ca^{2+} stimulation, and its up-regulation is Ca^{2+} dependent [15]. Ca^{2+} , an intracellular "second messenger", transmits mitogenic signals to the nucleus, where Egr-1 is activated [16]. Since Egr-1 plays a key master regulatory role in multiple vascular pathological processes in structure remodeling [17, 18], blocking the Ca^{2+} -dependent overexpression of Egr-1 might be an important therapeutic strategy in inhibiting development of atherosclerosis, intimal thickening after arterial injury and restenosis after angioplasty.

A growing body of evidence has indicated that angiotensin II (Ang II) can stimulate protein synthesis and induce cell proliferation in VSMCs, and Ca^{2+} signals are also critical for proliferation induced by Ang II in VSMCs [19]. $[\text{Ca}^{2+}]_i$ can be elevated by either release of Ca^{2+} from intracellular stores or influx of extracellular Ca^{2+} . The mechanisms whereby Ang II stimulates extracellular Ca^{2+} influx may involve stored-dependent or stored-independent calcium entry [20, 21]. Our laboratory synthesized a novel series of quaternary ammonium salt derivatives of haloperidol, with a structure completely different from that of the calcium channel blockers used clinically. The derivatives can antagonize myocardial ischemia/reperfusion (I/R) injury in rats by inhibiting Egr-1 overexpression [22, 23], which might be related to the inhibitory effect on the L-type calcium channel [24]. *N*-4-Tert-Butyl benzyl haloperidol chloride (C_3) (Fig. 1) was one of the derivatives (In another study, we had proved C_3 was also a novel potent calcium antagonist [data not shown here, see supplemental data]).

In this study, we aimed to observe the effects of C_3 on Ang II- induced elevation of $[\text{Ca}^{2+}]_i$ and proliferation of VSMCs. Our study clearly establishes a role for C_3 in limiting vascular VSMCs regrowth *in vitro* and reveals the cellular and molecular mechanism related to Ca^{2+} -dependent Egr-1 expression.

Materials and Methods

Animals

Male Sprague-Dawley rats weighing 180-200 g were supplied by the Animal Center, Shantou University Medical College, China. All animals were treated in compliance with The Guide for the Care of Use of Laboratory Animals by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and followed the rules of the National Animal Protection of China.

Rat VSMC culture

Isolation of VSMCs followed the method of Campbell et al. [25]. VSM cells were isolated from the thoracic aorta of 10- to 12-week-old SD rats. VSMc were suspended in growth medium (DMEM supplemented with 10% [v/v] fetal bovine serum [FBS; JRH Biosciences, Lenexa, KS], 2.2 g/L NaHCO_3 , 100 U/mL penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin) and seeded into collagen-coated 24-well plates. Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in air. Cells at passage 4 to 6 were used for all experiments. The identification of VSMCs was confirmed by their morphology and immunoreactivity with SMC α -action (Santa Cruz, CA). Rat VSMCs are prone to loose calcium channel expression in culture, sometimes. Calcium elevations in cells by depolarization with high K^+ medium were determined to prove that these cells used in our study continued to express calcium channels in relevant quantities.

Determination of $[\text{Ca}^{2+}]_i$ level by laser scanning confocal microscopy

Level of $[\text{Ca}^{2+}]_i$ in adherent cells was determined by means of laser scanning confocal microscopy (ACAS/ULTIMA312, Meridian Instruments) as previously described [24]. VSMCs were grown on 24-mm glass coverslips in 6-well plates in DMEM containing 10% FBS. After 24 h, the cells were washed twice with a HEPES-buffered physiological saline solution (HBSS) (in mmol/L: 130 NaCl, 2.5 KCl, 1.2 MgCl_2 , 10 HEPES, 10 glucose, and 2 CaCl_2 , pH adjusted to 7.4 with NaOH), then incubated in a solution supplemented with 20 $\mu\text{mol}/\text{L}$ Fluo 3-AM for 60 min in the dark at 37 °C. Then extracellular Fluo 3 was removed by washing with HBSS three times. The Fluo-3 dye was excited with a 488-nm wavelength argon laser beam, and the emission fluorescence was monitored at 530 nm. The change in $[\text{Ca}^{2+}]_i$ was represented by the ratio of fluorescence intensity (FI) after $(\text{FI}-\text{FI}_0)/\text{FI}_0$ to that (control, FI_0) before the addition of drugs $[(\text{FI}-\text{FI}_0)/\text{FI}_0]$. In this experiment, images were scanned every 2 s according to the response of cells after administering the drugs. The data were stored electronically for later analysis.

Gene	Forward Primer	Reverse primer	Amplicon (bp)
Egr-1	5'-GCAACACTTTGTGGCCTGAA-3'	5'-GAGTTGGGACTGGTAGGTGT-3'	512
GAPDH	5'-AGCAGTCCCGTACACTGGCAAAC-3'	5'-TCTGTGGTGATGTAAATGTCCTCT-3'	252

Table 1. Primers for PCR

To investigate angiotensin II (Ang II)-induced intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx, Ang II was added into the cultured cells in Ca^{2+} -containing or -free HBSS (2 mmol/L CaCl_2 was replaced with 1 mmol/L EGTA), then changes in fluorescence were recorded. In Ca^{2+} -free HBSS, when the fluorescence induced by Ang II returned to the basal level, 1 mmol/L Ca^{2+} was reintroduced into the medium. To investigate the effect of C_3 on Ang II-induced changes in fluorescence, VSMCs were incubated for 20 min with 1 $\mu\text{mol/L}$ C_3 before adding Ang II.

Preparation of Egr-1 oligodeoxyribonucleotides (ODNs)

Sense, antisense, and scrambled-sequence phosphorothioate Egr-1 ODNs of 15 base-pair length were commercially synthesized. The sequence of antisense Egr-1 ODN was 3'-TAC CGT CGC CGG TTC -5' as described previously [22]. For control experiments, sense (3'-GAA CCG GCG ACG GTA -5') and scrambled-sequence (3'-TCG TGC CGC TGC CAT -5') Egr-1 ODNs were used [26]. All phosphorothioate ODNs were commercially synthesized and purified by high-performance liquid chromatography (Takara Biotechnology, Dalian, China). FITC-labeled antisense ODN was used to check the cellular uptake of ODN.

Proliferation and survival assays

VSMCs were seeded into 96-well plates at 5×10^3 cells/well for 3-(4, 5-dimethylthiazol -2-yl) -2, 5-diphenyl tetrazolium bromide (MTT) assay. They were incubated for 12 h in DMEM supplemented with 10% FBS at 37 °C. After being deprived of serum for 24 h, cells were washed with phosphate-buffered saline (PBS). Cells were incubated with Ang II (1 $\mu\text{mol/L}$) for a further 24 h in DMEM containing 0.1% FBS in the absence (Ang II treatment alone) or presence of various concentrations of C_3 (0.1, 1, 10 $\mu\text{mol/L}$), or only vehicle (0.1% DMSO). In the antisense experiments, Cells were incubated with Ang II (1 $\mu\text{mol/L}$) for 24 h with or without the 24 pre-transfection of 0.4 $\mu\text{mol/L}$ ODNs or only with vehicle (0.1% DMSO), compared with control group. The MTT assay, as a measure of the number of viable cells, was as described previously [27]. The absorbance of the lysate at 562 and 630 nm was measured with an EL340 Microtiter Plate Reader (Bio-Tek Instruments, Winooski, VT). The difference between the values at 562 and 630 nm was used as an index of the number of viable cells. Lactate dehydrogenase (LDH) release was quantified with use of a CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI) according to the manufacturer's instructions.

Fig. 1. Chemical structure of *N*-4-Tert-Butyl benzyl haloperidol chloride (C_3).

Cell-Cycle Analysis

DNA content of cells underwent flow cytometry analysis as described [28], with minor modifications. In brief, after being deprived of serum for 24 h, cells were incubated with Ang II (1 $\mu\text{mol/L}$) for 24 h in DMEM containing 0.1% fetal calf serum in the absence (Ang II treatment alone) or presence of 0.1, 1, 10 $\mu\text{mol/L}$ C_3 , 1 mmol/L EGTA, or DMSO (0.1%). In the antisense experiments, Cells were incubated with Ang II (1 $\mu\text{mol/L}$) for 24 h with or without the 24 h pre-transfection of 0.4 $\mu\text{mol/L}$ ODNs or only with vehicle (0.1% DMSO), compared with control group. Then cells were collected by trypsinization, pelleted by centrifugation, and resuspended in 0.5 ml PBS. The same volume of 70% ethanol in PBS was added to the cell suspension and fixed for 30 min at 4 °C. After a washing with PBS, cells were resuspended in 1 ml of 2.5 mg/ml RNase for 30 min at room temperature in the dark. The pelleted cells were suspended in 0.5-1.0 ml of 50 mg/ml propidium iodide and analyzed by use of a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA). Cell-cycle distribution analysis involved use of Modifit cell-cycle analysis software (Becton Dickinson).

Western blot analysis of Egr-1, PDGF-A, cyclin D1, Cdk2, p21Cip1

Total protein extracts were prepared from cultured cells with cell lysis buffer containing a protease inhibitor cocktail (aprotinin, leupeptin, pepstatin A, and phenylmethanesulfonyl fluoride). The protein concentration was determined by use of the Bradford protein assay kit (Bio-Rad, Hercules, CA). An equal amount of total protein (100 μg) was subjected to SDS-PAGE (10%), followed by electrophoretic transfer to

Fig. 2. Effect of C_3 (1 μ M) on Ang II-induced intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx. VSMCs were pretreated with C_3 for 20 min before Ang II stimulation. (A) Ang II-induced Ca^{2+} response in Ca^{2+} -containing medium. Ang II (1 μ M) was added at the time point indicated in the figure. (B) VSMCs were stimulated with Ang II (1 μ M) in Ca^{2+} -free medium containing 1 mM EGTA. After a return to the basal level, 1 mmol/L Ca^{2+} was reintroduced into the medium. Values are means $\pm$ S.E.M (n=24). * $P < 0.01$ compared with Ang II alone treatment.

nitrocellulose membranes. Western blot was performed as described previously [22] to determine the Egr-1, cyclin D1, Cdk2, p21Cip1 and PDGF-A protein levels after the cells were incubated for 2 h (for Egr-1) and 24 h (for cyclin D1, Cdk2, p21Cip1 and PDGF-A). The following antibodies were used: antibodies against Egr-1 Cdk2, PDGF-A (Santa Cruz Biotechnology; 1:1000 at room temperature for 3 h), cyclin D1, p21Cip1 (Cell Signaling Technology; 1:1000 at room temperature for 3 h), and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich Inc. USA; 1:5000 at room temperature for 3 h). The blots were then washed with TBS-T and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:2000) at room temperature for 2 h. Visualization was by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) and exposure to autoradiographic film (Kodak, Rochester, NY). Blots were then reprobbed with GAPDH (1:5000, Sigma-Aldrich Inc. USA) to confirm equal loading of cell lysates.

RT-PCR

Egr-1 mRNA level was analyzed by semi-quantitative RT-PCR as in a previous study [23]. Total RNA was extracted from cultured cells using TRIzol reagent (Invitrogen, Carlsbad, CA). The sequences of forward and reverse primers for rat Egr-1 and GAPDH are in Table 1. The band relative densities of mRNA were analyzed by use of Fluorchem 8900 (Alpha Innotech, Miami, FL).

Statistical analysis

All data are expressed as mean $\pm$ S.E.M. Student's unpaired *t* test was used to compare differences between two groups and ANOVA for more than two groups. $P < 0.05$ was considered statistically significant.

Fig. 3. (A) Effect of Ang II on proliferation of VSMCs over time. Data were obtained after 48 h of incubation with Ang II (1 μ M) or DMSO control. (B) Effects of C_3 on LDH activity of cultured VSMCs. Results are mean $\pm$ S.E.M. of 5 independent experiments. * $P < 0.05$, ** $P < 0.01$ between Ang II and control from 12h to 48h.

Fig. 4. Effect of C_3 on proliferation of cultured VSMCs(A); on cell cycle progression of cultured VSMCs (A total of 10,000 nuclei were analyzed from each sample) (B); on cell-cycle regulatory molecules (C). Cells were incubated with Ang II (1 μ M) for 24 h in DMEM containing 0.1% FBS with or without C_3 , or only with vehicle (DMSO), compared with control group. Results are mean $\pm$ S.E.M. of 5 independent experiments. In Western blots, Values are mean $\pm$ S.E.M. of 3 separate experiments. [#], $P < 0.01$ compared with control. ^{*} $P < 0.05$, ^{**} $P < 0.01$ compared with Ang II alone treatment.

Results

Effect of C_3 on Ang II-induced intracellular Ca^{2+} mobilization and extracellular Ca^{2+} influx

In Ca^{2+} -containing HBSS, Ang II caused a rapid peak increase in $[Ca^{2+}]_i$ level within 10 s, then a decrease to a sustained plateau at 180 s after Ang II adding, as seen on laser scanning microscopy; the basal $[Ca^{2+}]_i$ in VSMCs was not affected by C_3 (data not shown). In the presence of extracellular Ca^{2+} , 1 μ mol/L C_3 inhibited the Ang II-induced plateau level of $[Ca^{2+}]_i$ by 63.02%, without affecting the peak level (Fig. 2A). In Ca^{2+} -free medium containing 1 mmol/L EGTA, Ang II was also able to elicit an peak increase in $[Ca^{2+}]_i$, although to a lesser extent (Fig. 2B). Furthermore, in the absence of extracellular Ca^{2+} , the Ang II-induced peak level of $[Ca^{2+}]_i$ was not inhibited by C_3 . When the $[Ca^{2+}]_i$ level induced by Ang II returned to the basal level, the level of $[Ca^{2+}]_i$ could be re-elicited to another sustained plateau by 1 mmol/l Ca^{2+} at 100 s after Ca^{2+} adding. C_3 inhibited this sustained plateau by 56.16%.

Effect of C_3 on Ang II-induced cell proliferation and cell cycle progression

MTT assay revealed that Ang II induced proliferation of VSMCs in a time-dependent manner as compared with control treatment (Fig. 3). Various concentrations of C_3 (0.1, 1, 10 μ mol/L) inhibited the proliferation of VSMCs stimulated by Ang II for 24 h, by 16.02%, 25.91%, and 31.12% respectively, compared with the Ang II treatment alone (Fig. 4A). At the dose of 10 μ mol/L, C_3 showed the maximum effect (E_{max}) (Fig. 4A). The IC_{50} value was about 1 μ mol/L according to Hill equation: $E = E_{max} \times C^b /$

Fig. 5. Effect of antisense (AS), sense(S) and scrambled-sequence (SC) Egr-1 ODN on proliferation of cultured VSMCs (A); on cell cycle progression (A total of 10,000 nuclei were analyzed from each sample) of cultured VSMCs (B); on Egr-1 and PDGF-A protein level in cultured VSMCs (C). Cells were incubated with Ang II (1 μ M) for 24 h or 2 h (in western blot for Egr-1) with or without the 24 pre-transfection of ODNs, compared with control group. Results are mean $\pm$ S.E.M. of 5 independent experiments. #, $p < 0.01$ compared with control. *, $p < 0.01$ compared with Ang II treatment alone.

($C^b + IC_{50}^b$). To determine whether the growth inhibitory effect of C_3 was specific to the cell cycle phase, flow cytometry of VSMCs stimulated by Ang II for 24 h revealed that treatment with 0.1-, 1-, and 10 μ mol/L C_3 induced accumulation of cells in the G_1 phase, to 76.42%, 79.54% and 84.52%, respectively; accumulation with Ang II treatment alone was 63.71% ($P < 0.01$; Fig. 4B). Concomitantly, the proportion of cells in the S phase was remarkably decreased, by 12.37%, 8.92%, and 5.34% with 0.1, 1, and 10 μ mol/L C_3 , respectively; the proportion in the S phase with Ang II treatment alone was 20.34% ($P < 0.01$).

Whether treatment of C_3 causes apoptosis or necrosis in reducing the proliferation rate was investigated by LDH release assay. Cells treated with C_3 or the control did not significantly differ in mean LDH activity (Fig. 3B). Hence, cytotoxicity did not contribute to reducing the proliferation rate, and the reduction appears to be caused at least in part by decreased proliferation of cells.

Effect of C_3 on Cell-Cycle Regulatory Molecules and PDGF-A expression

The C_3 -induced G_1 arrest was further confirmed by examining the cellular levels of the G_1 cell-cycle control proteins cyclin D1, Cdk2 and Cdk inhibitors P21Cip1 in VSMCs. Western blot analysis confirmed that treatment with 0.1, 1, 10 μ mol/L C_3 downregulated the expression of cyclin D1 and Cdk2 proteins induced by Ang II (Fig. 4C). C_3 also could substantially inhibit PDGF-A expression, induced by Ang II in VSMCs (Fig. 6B). In addition, the expression of p21Cip1 attenuated by Ang II was substantially upregulated, stimulated by C_3 after 24 h (Fig. 4C). Based on these findings, C_3 is likely to block the cell-cycle progression through G_0/G_1 to S phase.

Extracellular Ca^{2+} is involved in Ang II-induced Egr-1 upregulation

To verify the role of the extracellular Ca^{2+} influx in the activation of Egr-1 induced by Ang II, VSMCs were incubated in Ca^{2+} -containing or -free HBSS (with 1 mmol/

Fig. 6. Effect of extracellular Ca^{2+} on Egr-1 protein in Ca^{2+} -containing or Ca^{2+} -free medium (A); Effect of C_3 on Egr-1, PDGF-A protein level (B) and Egr-1 mRNA level (C) in cultured VSMCs. Cells were incubated with Ang II (1 μM) for 24 h (for PDGF-A) or 2 h (for Egr-1). Values are mean $\pm$ S.E.M of 3 (for A), or 5 (for B,C) separate experiments. #, $P < 0.01$ compared with Control, *, $p < 0.01$ compared with Ca^{2+} -free EGTA (A) or Ang II treatment alone (B,C).

L EGTA) in the presence or absence of Ang II. Ang II-increased expression of Egr-1 was inhibited by extracellular Ca^{2+} chelation by 44.78%, and in the absence of Ang II, Egr-1 expression did not differ in Ca^{2+} -containing or -free HBSS (Fig. 6A).

Antisense Egr-1 ODNs reduced the Ang II-induced expression of Egr-1 and inhibited Ang II-induced cell proliferation and cell cycle progression

To confirm the cause-effect relation between Egr-1 expression and VSMC proliferation, we revealed FITC-labeled antisense Egr-1 ODN mainly in the nuclei of cultured VSMCs, which indicates the successful uptake of antisense Egr-1 ODN. Ang II caused a time-dependent induction of Egr-1 protein, beginning at 1 h, peaking at 2 h, and declining thereafter (data not shown). Antisense Egr-1 ODNs (0.4 $\mu\text{mol/L}$) inhibited the proliferation of VSMCs by 34.31% , compared with Ang II treatment alone, and increased the proportion of cells in the G_1 phase,

to 86.81%, whereas with Ang II treatment alone, the proportion was 63.74 ($P < 0.01$) (Fig. 5A,B). Furthermore, antisense Egr-1 ODNs inhibited the expression of PDGF-A and Egr-1 as compared with Ang II treatment alone (Fig. 5C). Treatment with vehicle, sense and scrambled-sequence Egr-1 ODNs did not produce different results.

C_3 reduced the expression of Egr-1 induced by Ang II

C_3 could inhibit VSMC proliferation and Cell-Cycle Regulatory Molecules, but to elucidate the relation between C_3 and Egr-1 expression, we used western blot analysis to investigate the effect of C_3 on Egr-1 protein expression induced by Ang II in VSMCs and the level of Egr-1 mRNA by RT-PCR. On stimulation with Ang II (1 $\mu\text{mol/L}$), the level of Egr-1 protein and mRNA increased at 1 h, peaked at 2 h, and returned nearly to baseline by 3 h (data not shown). Egr-1 protein and mRNA overexpression at 2 h could be inhibited by C_3 (0.1, 1, 10 $\mu\text{mol/L}$) (Fig. 6B,C).

Discussion

Proliferation of VSMC is a crucial event in the development of vascular diseases. Over the past several years, remarkable progress has been achieved with the introduction of two kinds of drug, rapamycin (Sirolimus) and paclitaxel (Taxol) that were approved by American Food and Drug Administration [26, 29]. However, the scope of clinically proven anti-proliferation agents is extremely limited, and additional new compounds are needed. In our group, we synthesized one haloperidol-derivative (F2). F2 had vasodilator effect and can antagonize myocardial ischemia/reperfusion (I/R) injuries, which were related to the inhibitory effect on Egr-1 up-expression [22, 23]. In order to find the more efficient compound, we synthesized a series of haloperidol derivative. In the screening of vasodilator effect of haloperidol derivative, C3 showed the most potent calcium channel antagonism and was selected for the study of antiproliferation activity on VSMCs.

Ca^{2+} is considered to be an intracellular signal implicated in proliferation as well as vasoconstriction [30]. The influx of extra cellular Ca^{2+} is a mechanism able to sustain long lasting intracellular Ca^{2+} elevations: this signal, activated by many growth factors and mitogens in normal and pathological cell, is linked to DNA transcription and duplication, finally leading to cell proliferation [31, 32]. So Ca^{2+} influx is a key signal in the control of cell proliferation and Ca^{2+} entry regulation can be a therapeutic approach for the control of cell proliferation [33]. We had provided the profound evidence that C_3 inhibited the influx of extracellular Ca^{2+} , which was taken as crucial signal leading to cell proliferation, induced by Ang II. The role of ACE and Ang II in neointimal thickening has been firmly established by animal studies. Ang II is involved in the remodeling of large and resistance arteries during hypertension and the inflammatory and tissue response in atherosclerosis [34]. Ang II has been shown to stimulate proliferative and hypertrophic growth in VSMCs by enhancing cytosolic $[\text{Ca}^{2+}]_i$ level [19, 35]. Ang II causes a rapid peak increase in $[\text{Ca}^{2+}]_i$ level by mobilizing Ca^{2+} from intracellular storage, followed by a decrease to a sustained plateau expression phase, which is induced by Ca^{2+} influx from the extracellular space across the cell membrane through the calcium channel [36]. The mechanisms whereby angiotensin II stimulates extracellular Ca^{2+} influx may involve voltage-operated Ca^{2+} channels (VOCC), receptor-gated Ca^{2+} channels, et al. The best-characterized Ca^{2+} entry pathway is VOCC [37]. Recently, there is considerable evidence that store-

operated Ca^{2+} channels (SOCs) in SMCs also play an important role in cell proliferation [20, 21]. The antiproliferative effects of several kinds of Ca^{2+} modulators such as dihydropyridines, thapsigargin, and 2-aminoethoxydiphenyl borate (2-APB) on VSMCs have been demonstrated recently. Dihydropyridine derivatives, such as amlodipine, nicardipine, and nimodipine inhibited A431 cell growth with IC_{50} values of 20-30 $\mu\text{mol/L}$. This is related to the direct or indirect modulation on thapsigargin-sensitive Ca^{2+} stores or/and store-operated Ca^{2+} entry, other than L-type Ca^{2+} channels [38]. Many studies also indicates the antiproliferative effect of 2-APB, with relevance to the store-operated Ca^{2+} entry, and its effect depended on the cell types [39]. In addition, mitochondria has the potential to regulate intracellular Ca^{2+} signaling and control cell growth [40, 41]. No doubt, the different target positions are relevance to the distinct antiproliferative effects of Ca^{2+} modulators. Our study indicated, C_3 inhibited VSMCs growth potently with IC_{50} values of 1 $\mu\text{mol/L}$. The comparison of C_3 and other Ca^{2+} modulators will be made by us on the same cell, in the same condition in the future. In this study, C_3 pretreatment attenuated the sustained Ca^{2+} entry response induced by Ang II, without affecting the initial Ca^{2+} mobilization response in Ca^{2+} -containing medium. Its target might be the Ca^{2+} channels on the cell membrane. Further studies about the direct or indirect effects of C_3 on SOC or/and VOCC are needed to clarify the detailed mechanisms for C_3 to attenuate angiotensin II-induced elevation of $[\text{Ca}^{2+}]_i$.

C_3 is a novel agent acting as a calcium-entry inhibitor. In this study, we found that VSMC proliferation induced by Ang II was inhibited by C_3 . In addition, the inhibition of VSMC growth by C_3 was related with a G_1 -phase arrest at multiple points within the machinery governing the G_1/S transition. The expression of G_1 control proteins cyclin D1 and Cdk2 was down-regulated and associated with profound increased p21Cip1 and p27Kip1 protein levels. Cyclin D1 is a critical regulator involved in cell-cycle progression through the G_1 phase into the S phase, thereby contributing to cell proliferation. In combination with cyclin E, Cdk2 is necessary for the G_1 -to-S-phase transition. Cdk2 activity is also necessary for entry into mitosis because it activates mitotic cyclin-cdc2 kinase activity [12]. We found that C_3 inhibited the expression of cyclin D1 and Cdk2, further indicating the effects of C_3 on the proliferation inhibition. The subsequent decrease in cyclin D1 may cooperate with the early induction of p21Cip1 to arrest cells in the G_1 phase and thereby further contribute to C_3 -induced growth inhibition.

Ca^{2+} , as a second messenger, transmits mitogenic signals to the nucleus. Potential nuclear targets include the transcription factors ELK-1, Egr-1 and c-Fos, which are known to regulate cellular growth, differentiation, and migration [13, 42]. Egr-1 plays the most important role. Egr-1 expression is induced by Ca^{2+} stimulation, and its up-regulation is Ca^{2+} dependent [16]. Here we used EGTA to confirm that extracellular Ca^{2+} is involved in the Ang II-induced Egr-1 expression. Thus, change in influx of extracellular calcium regulated Egr-1 expression.

The Egr family includes Egr-1, Egr-2, Egr-3, and Egr-4. The family members share a highly homologous DNA binding domain that recognizes an identical DNA response element, although their flanking regions are much less conserved. Among the family members, the best characterized is Egr-1. Egr-1 not only is a crucial regulator of cell growth, differentiation, but also can be activated by a variety of stimuli. Egr-1 expression can be greatly induced on balloon dilatation of the carotid artery or denudation of the arterial endothelium [12]. Egr-1 protein is biologically active. In injured vascular cells, Egr-1 displays chemotactic and mitogenic effects [12, 15]. Antisense ODNs or DNA enzymes targeting Egr-1 inhibited VSMC proliferation after mechanical injury *in vitro* [43]. In this study, overexpression of Egr-1 in concomitance with high level of PDGF-A, an important downstream growth factor of Egr-1, and cells proliferation, cells cycle progression also proved the Egr-1 biological activity. Antisense Egr-1 ODNs reduced the VSMCs proliferation induced by Ang II, arrested VSMCs in G_1 -phase, and inhibited the expression of PDGF-A.

It confirmed the downregulation of Egr-1 transcriptional activity following incubation with antisense ODNs. Thus, Egr-1 could be a potential therapeutic target to inhibit proliferation and lesion formation.

Since Ang II enhanced cytosolic $[\text{Ca}^{2+}]_i$ level, and then induced rapid activation of Ca^{2+} -dependent Egr-1 expression, we further investigated the effect of C_3 on the expression of Egr-1. C_3 potentially inhibited the upregulation of Egr-1 with Ang II-induced increased level of $[\text{Ca}^{2+}]_i$ in VSMCs. Thus, C_3 , a novel calcium antagonist, inhibits the proliferation and retards the progression of the cell cycle in VSMCs. Its mechanism might be related to the inhibition of the influx of extracellular Ca^{2+} and subsequent inhibition of Ca^{2+} -dependent Egr-1 overexpression. However, the existence of another pathway or whether C_3 has direct effects on Egr-1 expression or proliferation needs further study.

Our findings imply that inhibition of VSMC proliferation via a Ca^{2+} -dependent Egr-1 expression pathway could be a potential therapeutic approach to inhibit lesion formation, and Ca^{2+} influx is a key signal in the control of cell proliferation. In addition, our study provides a basis for further studies of novel calcium channel blockers that reverse vascular remodeling and atherosclerosis induced by hypertension.

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