Silence of ClC-3 chloride channel inhibits cell proliferation and the cell cycle via G₁/S phase arrest in rat basilar arterial smooth muscle cells

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Abstract. *Objectives:* Previously, we have found that the ClC-3 chloride channel is involved in endothelin-1 (ET-1)-induced rat aortic smooth muscle cell proliferation. The present study was to investigate the role of ClC-3 in cell cycle progression/distribution and the underlying mechanisms of proliferation. *Materials and methods:* Small interference RNA (siRNA) is used to silence ClC-3 expression. Cell proliferation, cell cycle distribution and protein expression were measured or detected with cell counting, bromodeoxyuridine (BrdU) incorporation, Western blot and flow cytometric assays respectively. *Results:* ET-1-induced rat basilar vascular smooth muscle cell (BASMC) proliferation was parallel to a significant increase in endogenous expression of ClC-3 protein. Silence of ClC-3 by siRNA inhibited expression of ClC-3 protein, prevented an increase in BrdU incorporation and cell number induced by ET-1. Silence of ClC-3 also caused cell cycle arrest in G₀/G₁ phase and prevented the cells' progression from G₁ to S phase. Knockdown of ClC-3 potently inhibited cyclin D1 and cyclin E expression and increased cyclin-dependent kinase inhibitors (CDKIs) p27^{KIP} and p21^{CIP} expression. Furthermore, ClC-3 knockdown significantly attenuated phosphorylation of Akt and glycogen synthase kinase-3 β (GSK-3 β) induced by ET-1. *Conclusion:* Silence of ClC-3 protein effectively suppressed phosphorylation of the Akt/GSK-3 β signal pathway, resulting in down-regulation of cyclin D1 and cyclin E, and up-regulation of p27^{KIP} and p21^{CIP}. In these BASMCs, integrated effects lead to cell cycle G₁/S arrest and inhibition of cell proliferation.

INTRODUCTION

Cell proliferation must at some time point lead to cell volume increase (Bortner & Cidlowski 2004; Lang *et al.* 2005). This evokes a regulatory volume decrease process through activation of various channels/transporters, including chloride (Cl⁻) and potassium (K⁺) channels, which induces effluxes of Cl⁻, K⁺ and H₂O, and returns the cell volume to normal size (Wondereg

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et al. 2001; Wang *et al.* 2002; Lang *et al.* 2005) and recent evidence suggests the emerging roles of the CIC-3 Cl⁻ channel in cell proliferation. Endothelin-1(ET-1) induced cell proliferation with an increase in endogenous CIC-3 protein expression in rat aortic pulmonary artery smooth muscle cells and human prostate cancer epithelial cells (Wang *et al.* 2002; Lemonnier *et al.* 2004; Dai *et al.* 2005). The antisense protein specific to CIC-3 Cl⁻ channels significantly inhibited cell proliferation (Wang *et al.* 2002); however, its roles in cell cycle progression and its underlying mechanisms still remain to be clarified.

Based on our previous findings on the roles of CIC-3 in cell proliferation, this study aimed to explore how silence of the CIC-3 channel by small interference RNA (siRNA) influenced basilar artery smooth muscle cells' (BASMCs) cell cycle progression/distribution, expression of which key proteins are responsible for regulating cell cycle progression and the underlying mechanisms of these processes. We found that here deficiency in the CIC-3 Cl⁻ channel inhibits cell proliferation through interference in the cell cycle G₁/S transition, which is related closely to the Akt/glycogen synthase kinase-3 β (GSK-3 β) signal transduction pathway.

MATERIALS AND METHODS

Cell culture

Male Sprague-Dawley rats were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All procedures complied with the standards for care and use of animal subjects as stated in the Guide for the Care and Use of Laboratory Animals (issued by the Ministry of Science and Technology of China, Beijing). Basilar arteries were isolated aseptically and placed in Krebs buffer as described previously (Shi *et al.* 2007). After fat and connective tissue were cleaned away, the artery strip was cut into ~0.5 mm pieces and then was plated in Dulbecco's modified essential medium (DMEM)/F-12 containing 20% foetal calf serum. BASMCs were cultured in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Cells were identified as smooth muscle cells by morphology and immunostaining with monoclonal antibody specific for smooth muscle α -actin (Xiao *et al.* 2002). Passages 8–14 BASMCs at 70–90% confluence was in 35-mm dishes or 96-well plates and growth was arrested by incubation in 0.2% calf serum/DMEM/F-12, 72 h before experiments.

Transfection of BASMCs with stealth siRNA

The sequence of the stealth siRNA duplex oligoribonucleotides against rat CIC-3 gene (GenBank Accession No. NM_053363) is 5'-UCACCAAGGAAACUGCAAGAAAGGC-3', and its corresponding complementary strand 5'-GCCUUUCUUGCAGUUCCUUGGUGA-3' (Invitrogen Life Technologies Inc., Carlsbad, CA, USA). The CIC-3 siRNA was transfected transiently with LipofectamineTM RNAiMAX according to the manufacturer's instructions and a negative stealth siRNA sequence was used as control. Briefly, siRNA and LipofectamineTM RNAiMAX were diluted in Opti-MEMI, respectively (Invitrogen Life Technologies Inc.). Then, the diluted siRNA duplex and the diluted LipofectamineTM RNAiMAX were combined for 20 min at room temperature to allow formation of transfection complexes. Complexes were then added to the cells while they were in the quiescent state and all were swirled gently to ensure uniform distribution. After incubation for 8 h at 37 °C, transfection mixture was removed and the cells were further incubated with normal growth conditions before use for experiments. The BLOCK-iT Fluorescent Oligo, siRNA-labelled fluorescein isothiocyanate (FITC; Invitrogen Life Technologies Inc.) was used for determination of siRNA transfection efficiency.

Cell counting and incorporation of bromodeoxyuridine

Cell proliferation was measured by counting and by bromodeoxyuridine (BrdU) incorporation as described previously (Xiao *et al.* 2002; Zhang *et al.* 2006), and according to the manufacturer's instructions (BrdU kits from Calbiochem, San Diego, CA, USA). After incubation, cells were mixed with 10 mmol/L BrdU for 18 h, and then the DNA was denatured by treatment with fixative/denaturing solution for 30 min, in order to enable anti-BrdU antibody binding to the incorporated BrdU. Subsequently, anti-BrdU monoclonal antibody was incubated for 1 h at room temperature. After washing, samples were treated with horseradish peroxidase-conjugated goat antimouse immunoglobulin G for 30 min. 100 mmol/L of colourless 3,3',5,5'-tetramethylbenzidine was then added as the substrate for horseradish peroxidase. The coloured reaction product was quantified by optical density determined at 450–540 nm, using a microplate reader.

Flow cytometry for cell cycle analysis

Cell cycle status was evaluated by flow cytometry as described previously (Zhang *et al.* 2006). Measurement of propidium iodide uptake was performed using fluorescence activated cell sorting. After appropriate treatment, BASMCs were collected in medium by centrifugation at 200 *g* for 5 min at 4 °C. Pellets were rinsed once with ice-cold phosphate-buffered saline (PBS) and were fixed in 70% ethanol for 24 h. Samples were then stained with staining buffer (PBS containing 50 µg/mL of propidium iodide, 10 µg/mL RNase A, 0.1% sodium citrate and 0.1 Triton X-100). DNA content was analysed by flow cytometry (EPICS XL, Beckman Coulter, Miami, FL, USA) to characterize population fraction in each phase of the cell cycle.

Western blot analysis

Western blot analysis was performed as described previously (Wang *et al.* 2002). Briefly, rat BASMCs were rinsed with ice-cold PBS and lysis buffer: Tris-HCl 50 mmol/L, NaCl 150 mmol/L, NaN₃ 0.02%, Nonidet P-40 1%, sodium dodecyl sulfate 0.1%, sodium deoxycholate 0.5% and 1% protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA). Protein was separated by using 8% SDS-PAGE and was transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp., Bedford, MA, USA). Membranes were blocked at room temperature for 2 h in PBST (in mmol/L: NaCl 130, KCl 2.5, Na₂HPO₄ 10, KH₂PO₄ 1.5, 0.1% Tween-20, and 5% bovine serum albumin, pH 7.4), incubated 1 h at room temperature initially with primary antibodies, and then with the appropriate secondary peroxidase-conjugated antibodies (HRP-linked antirabbit or antimouse secondary antibody and HRP-linked antibiotin antibody, 1 h at room temperature). Blots were developed using a chemiluminescence system (Cell Signalling Technology Inc., Beverly, MA, USA) and then were visualized by exposure to Kodak X-ray film. Density of target bands was accurately determined using a computer-aided 1-D gel analysis system. In this study, anti-CIC-3 antibody was purchased from Alomone Laboratories (Jerusalem, Israel) and Santa Cruz Biotechnology (Santa Cruz, CA, USA), antibodies against β -actin, cyclin D1, p27^{KIP}, total GSK3 β , phospho-GSK3 β (Ser9), total Akt, phospho-Akt (Ser473 and Thr308), secondary peroxidase-conjugated antibodies (HRP-linked) were from Cell Signalling Technology Inc. Cyclin-dependent kinase-2 (CDK2) was from BD Transduction Laboratories (San Diego, CA, USA), and cyclin E, p21CIP1 were from Santa Cruz Biotechnology.

Statistical analysis

All data are represented as means $\pm$ SEM. Statistical significance was tested by Student's *t*-test or one-way analysis of variance followed by Duncan's multiple range tests. *P* < 0.05 was considered significant.

Figure 1. Effects of CIC-3 siRNA transfection on endogenous CIC-3 protein expression in BASMCs. (a) Representative image of fluorescence in BASMCs (200 $\times$). Stealth siRNA labelled with FITC was transfected to BASMCs, and fluorescence was detected in these cells after 6 h incubation (transfection), while there was no fluorescence detectable in untransfected (control) cells, indicating where siRNA had been successfully transfected. (b) Western blot results show that 40 nmol/L CIC-3 siRNA decreased significantly endogenous CIC-3 expression, but transfection of LipofectamineTM RNAiMAX (lipo) and negative siRNA control (negative siRNA) did not significantly change CIC-3 expression ($n = 5$; ‡ $P < 0.01$ versus control).

RESULTS

Effect of CIC-3 siRNA on endogenous CIC-3 protein expression

Here in BASMCs, transfection efficiency was detected by uptake of siRNA-labelled FITC. As shown in Fig. 1a, under resting conditions fluorescence of the cells was negligible, while fluorescence was greatly increased in those treated with siRNA; this confirmed siRNA uptake by cells.

Endogenous expression of CIC-3 protein was decreased by $72.5 \pm 6.4\%$ after treatment with 40 nM CIC-3 siRNA for 48 h ($P < 0.01$, $n = 5$). Lipofectamine and negative siRNA transfection had no significant effects on CIC-3 expression (Fig. 1b). Expression of CIC-3 protein increased over 2-fold in the rat BASMCs after 48-h ET-1 (10 nM) treatment. CIC-3 siRNA at 40 nM suppressed ET-1-induced CIC-3 protein expression by $66.4 \pm 4.2\%$ (Fig. 2a).

Effect of CIC-3 silence on cell proliferation

The effects of CIC-3 knockdown on cell proliferation were determined by cell count and BrdU incorporation assay. Compared to control, 10 nM ET-1 led to 1.6 ± 0.2 -fold and 1.8 ± 0.3 -fold

Figure 2. Effects of CIC-3 knockdown on ET-1-dependent CIC-3 protein expression and cell proliferation. (a) Densitometric analysis shows stealth siRNA inhibits CIC-3 protein expression induced by 10 nmol/L ET-1, while transfections of LipofectamineTM RNAiMAX and negative siRNA control had no significant effect on CIC-3 protein expression ($n = 5$; * $P < 0.01$ versus control; ** $P < 0.01$ versus ET-1). Representative Western blots for CIC-3 protein expression are shown in the bottom. (b) Increase in cell growth induced by 10 nmol/L ET-1 was determined by counting the number of cells, and was significantly inhibited by incubation of CIC-3 siRNA for 48 h, but not by transfection of LipofectamineTM RNAiMAX and negative siRNA. (c) The effects of CIC-3 siRNA on cell proliferation induced by ET-1 were further determined by assaying BrdU incorporation. Incubation of CIC-3 siRNA for 48 h also significantly decreased BrdU incorporation ($n = 5$; ‡ $P < 0.01$ versus control; * $P < 0.01$ versus ET-1). These results show that the inhibitory effect of CIC-3 siRNA on ET-1 induced CIC-3 protein expression matches that of cell proliferation.

($P < 0.01$, $n = 5$) increments in cell number (Fig. 2b) and BrdU incorporation, respectively (Fig. 2c). CIC-3 siRNA at 40 nM could produce $82.6 \pm 6.4\%$ and $79 \pm 5.7\%$ inhibition of ET-1-induced cell growth and BrdU incorporation, respectively, while treatments of lipofectamine and negative siRNA had no significant on ET-1-induced cell growth and BrdU incorporation, excluding the non-specificity of the siRNA and/or transfection reagent.

Effect of CIC-3 silence on cell cycle progression

As shown in Fig. 3, serum-starved BASMCs in the control group were mostly in the G₀/G₁ (81.1%) and S phases (14.9%). This changed to a decrease from 81.1% to 62.6%, and an increase from 14.9% to 32.7% in S phase after treatment with ET-1 ($P < 0.01$, $n = 5$), indicating that ET-1 promotes cell cycle progression and accelerates cells' entrance into S phase. CIC-3

Figure 3. Flow cytometric analysis shows ET-1 accelerated entrance into G₁ and arrested entrance from G₁ to S phase. (a) Representative images of cell cycle analysis. (b) Statistical analysis shows the percent distribution of cells in G₀/G₁, S, G₂/M stages of the cell cycle ($n = 5$; ‡ $P < 0.01$ versus control; * $P < 0.01$ versus ET-1).

knockdown inhibited the effect of ET-1 on cell cycle progression with a decrease in cell population in S phase from 32.7% to 17.0% and an increase in cell population in G₀/G₁ from 62.6% to 78.9% (Fig. 3). These results suggest that deficiency in CIC-3 arrests the cell cycle in G₀/G₁ phase by preventing entrance into S phase in BASMCs.

Effect of CIC-3 silence on expression of cyclins and cyclin-dependent kinase inhibitors

G₁/S transition is governed by the balance of cyclins and cyclin-dependent kinase inhibitors (CDKIs) (Braun-Dullaeus *et al.* 1998). To explore the molecular mechanisms by which CIC-3 knockdown arrests G₁/S transition, we analysed the proteins regulating cell cycle progression in these cell phases, including cyclin D1, cyclin E, CDK2, p21^{CIP} and p27^{KIP}. As shown in Fig. 4, ET-1 increased expressions of cyclin D1 and cyclin E to 2.2 ± 0.2 -fold and 1.6 ± 0.1 -fold and

Figure 4. Effects of CIC-3 knockdown by incubation of 40 nM CIC-3 siRNA for 48 h, on protein expression of cyclin E, cyclin D1 and cyclin-dependent kinase inhibitors (CDKIs) p21 and p27. (a) Representative Western blot images of cyclins and CDKIs. (b and c) Densitometric analysis of the effects of CIC-3 knockdown on expression of cyclin D1 and cyclin E (b), and p21 and p27 (c), respectively. Expression of CDK2 was not changed by CIC-3 knockdown. (a) control; (b) ET-1; (c) ET-1 + LipofectamineTM RNAiMAX; (d) ET-1 + negative siRNA; (e) ET-1 + CIC-3 siRNA ($n = 5$; $\dagger P < 0.05$, $\ddagger P < 0.01$ versus control; $*P < 0.01$ versus ET-1).

decreased expressions of p21^{CIP} and p27^{KIP} by $70.9 \pm 6.4\%$ and $61.6 \pm 9.1\%$, respectively ($P < 0.01$, $n = 5$). Alterations induced by ET-1 were completely reversed by CIC-3 knockdown although no significant change of CDK2 was observed after CIC-3 knockdown.

Effect of CIC-3 silence on Akt/GSK-3 β signal pathway

Phosphorylation of Akt and its downstream GSK-3 β has been documented as regulatory for cell cycle G₁/S progression in many cells, through control of cyclin degradation (Hixon *et al.* 2000; Stabile *et al.* 2003; Sedding *et al.* 2005; Heron-Milhavet *et al.* 2006). We investigated how deficiency in CIC-3 affects the activation of the pathways. Our results have demonstrated that the time-course response of phosphorylation of Akt (Ser473) to ET-1 was significantly impaired

Figure 5. Effects of CIC-3 knockdown by incubation of 40 nM CIC-3 siRNA for 48 h, on phosphorylation of Akt and GSK-3 β . (a) Representative images of Western blots show that loss of CIC-3 channel affects the time course response to ET-1 after CIC-3 was knocked down by siRNA, and ET-1 was added to observe phosphorylation of Akt (Ser473) at 10-min, 1-h and 12-h time point ($n = 5$). (b) Representative images of Western blots to show that ET-1 increased phosphorylation of Thr308 and its downstream target, GSK-3 β , at Ser9, but failed to increase this phosphorylation when CIC-3 was knocked down. However, deficiency of CIC-3 did not significantly alter protein levels of Akt and GSK-3 β . (c) Densitometric analysis of the effects of CIC-3 knockdown on inactivation of Akt/GSK-3 β signal transduction pathway. (a) control; (b) ET-1; (c) ET-1 + LipofectamineTM RNAiMAX; (d) ET-1 + negative siRNA; (e) ET-1 + CIC-3 siRNA; ($n = 6$; $^{\ddagger}P < 0.01$ versus control; $^*P < 0.01$ versus ET-1).

after 10-min and 1-h ET-1 treatment, owing to loss of CIC-3 (Fig. 5a). Moreover, deficiency in CIC-3 failed to increase the phosphorylation of Akt at Thr308 and GSK-3 β at Ser9 evoked by ET-1 (Fig. 5b). However, loss of CIC-3 did not alter total Akt and GSK-3 β activity.

DISCUSSION

Recent studies have found that the volume-regulated Cl⁻ channel (VRCC) current varies in different cell cycle phase, and Cl⁻ channel blockers suppress cell proliferation and arrested cells in G₀/G₁ phase with inhibition of Cl⁻ current (Duan *et al.* 1997; Shen *et al.* 2000; Chen *et al.* 2002, 2007). These data indicate that the VRCC plays an essential role in cell cycle progression and cell proliferation through regulation of cell volume. Although the molecular nature of VRCC has yet to be conclusively identified, our recent study on A10 vascular smooth muscle

cells (VSMCs) strongly supported that CIC-3 is the molecular component involved in activation or regulation of VRCC (Zhou *et al.* 2005; Guan *et al.* 2006). Given its potential importance for cell volume regulation in cell proliferation, its role attracts particular interest. In the present study, we found that ET-1-induced cell proliferation was accompanied by an increase in CIC-3 expression; siRNA of CIC-3 not only inhibited ET-1-induced expression of CIC-3 protein, but also prevented BrdU incorporation and cell proliferation. Similar correlation between the increase in CIC-3 expression and stimulation of mitogens has also been found in prostate cancer epithelial cells and pulmonary VSMCs (Lemonnier *et al.* 2004; Dai *et al.* 2005). These results provide compelling evidence that CIC-3 plays an important role in regulation of cell proliferation.

The BASMC proliferation is governed by passage through the cell cycle, and cell cycle progression is a tightly controlled event positively regulated by cyclins and CDKs, and negatively by CDKIs and tumour suppressor genes (Woo & Poon 2003). In this study, we found that knockdown of CIC-3 inhibited entrance from G₀/G₁ to S phase and arrested the cell cycle at the G₁/S checkpoint. To further clarify the mechanisms by which CIC-3 knockdown induced G₁/S arrest, we analysed expression of proteins responsible for the G₁/S checkpoint. The results show that loss of CIC-3 almost abolished ET-1-induced expression of cyclin D1 and cyclin E, but not the expression of CDK2. In addition, CIC-3 knockdown also reversed the suppressive effects of ET-1 on two CDKIs, p21^{CIP} and p27^{KIP}, which are widely reported to restrict G₁/S transition in the cell cycle by inhibiting several cyclin-CDK complexes (Sedding *et al.* 2003; Barchiesi *et al.* 2006). Together, our results demonstrate that silencing the CIC-3 Cl⁻ channel prevents ET-1-induced cell proliferation through down-regulation of cyclins and up-regulation of CDKIs, and then results in restriction of G₁/S transition.

It has been shown that Akt mediates cell cycle G₁/S transition through increases in cyclin E and cyclin D1 expression (Suzuki *et al.* 1999; Haq *et al.* 2000; Hixon *et al.* 2000; Box & Demetrick 2004). Mutation of Akt at Thr308 and Ser473 abrogates vascular smooth muscle cell proliferation both *in vitro* and *in vivo* by delaying G₁/S exit (Stabile *et al.* 2003). GSK-3 β is a downstream target of Akt, and phosphorylation of GSK-3 β at Ser9 saves cell cycle regulators, including cyclin D1, cyclin E, β -catenin and c-Myc, from their ubiquitin-dependent destruction in many cancer cells (Haq *et al.* 2000; Park *et al.* 2003; Box & Demetrick 2004). Activation of Akt or overexpression of its constitutively active forms decreases levels of p27 (Medema *et al.* 2000; Sedding *et al.* 2003; Kotake *et al.* 2005; Barchiesi *et al.* 2006) and phosphorylation of GSK-3 β triggers p21 degradation (Rossig *et al.* 2002; Mitchell *et al.* 2004), thereby promoting cell proliferation. Our results in this study have shown that ET-1 failed to induce phosphorylation of Akt (Thr308, Ser473) and GSK-3 β (Ser9) when CIC-3 was depleted by siRNA, suggesting that the CIC-3 channel is one of the important regulators in this signal cascade. The data provide evidence to explain, at least partly, why CIC-3 knockdown affects cyclins, such as cyclin D1 and cyclin E, and CDKIs, such as p27^{KIP}, p21^{CIP} and subsequent cell cycle G₁/S progression. However, it is still not clear how CIC-3 knockdown affects the Akt/GSK-3 β signal cascade.

In people, development of hypertension and atherosclerosis are associated closely with vascular remodelling, which involves phenotypic changes to arterial smooth muscle cells from a contractile to a proliferative phenotype and enhancement of VSMC proliferation (Intengan & Schiffrin 2001; Mandegar *et al.* 2004). Recently, we have found that volume-regulated Cl⁻ movement is augmented in rat BASMCs in proportion to the severity of hypertension and cerebral vascular remodelling (Shi *et al.* 2007). Our present results provide further evidence to support the suggestion that CIC-3 Cl⁻ channel is a potential target modulating BASMC proliferation in the process of cerebrovascular remodelling.

In summary, depletion of CIC-3 Cl⁻ channel protein effectively suppressed phosphorylation of Akt/GSK-3 β signal pathway, resulting in down-regulation of cyclin D1 and cyclin E. In

contrast, depletion of CIC-3 Cl⁻ channel protein produced up-regulation of p27^{KIP} and p21^{CIP}. Integrated effects on these key cell cycle regulated proteins result in G₁/S cell cycle arrest in BASMCs.

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