Deficiency in ClC-3 Chloride Channels Prevents Rat Aortic Smooth Muscle Cell Proliferation

Guan-Lei Wang, Xue-Rong Wang, Mo-Jun Lin, Hua-He, Xiu-Jian Lan, Yong-Yuan Guan

Abstract—Recent growing evidence suggests that chloride (Cl⁻) channels are critical to the cell cycle. In cultured rat aortic vascular smooth muscle cells (VSMCs), we have previously found that Cl⁻ channel blockers inhibit endothelin-1 (ET-1)–induced cell proliferation. The present study was designed to further identify the specific Cl⁻ channels responsible for VSMC proliferation. Due to the lack of a specific blocker or opener of any known Cl⁻ channels, we used the antisense strategy to investigate the potential role of ClC-3, a member of the voltage-gated Cl⁻ channel gene family, in cell proliferation of cultured rat aortic VSMCs. With [³H]-thymidine incorporation and immunobLOTS, we found that ET-1–induced cell proliferation was parallel to a significant increase in the endogenous expression of ClC-3 protein. Transient transfection of rat aortic VSMCs with antisense oligonucleotide specific to ClC-3 caused an inhibition in ET-1–induced expression of ClC-3 protein and cell proliferation of VSMCs in the same concentration- and time-dependent pattern, whereas sense and missense oligonucleotides resulted in no effects on ClC-3 protein expression and cell proliferation. These results strongly suggest that ClC-3 may be the Cl⁻ channel involved in VSMC proliferation and thus provide compelling molecular evidence linking a specific Cl⁻ channel to cell proliferation. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2002;91:1111–1116.)

Key Words: vascular smooth muscle ■ chloride channel ■ proliferation ■ gene expression

Vascular smooth muscle cell (VSMC) proliferation is a key event in the pathogenesis of arteriosclerosis and many other vascular diseases. Endothelin-1 (ET-1), a natural potent vasoconstrictor and comitogen/proliferation factor for VSMCs, has been shown to induce VSMC proliferation in cultured rat and human aortic smooth muscle cells. The mitogenic effect of ET-1 has also been related with the vascular remodeling involved in the pathogenesis of vascular diseases such as hypertension and atherosclerosis. Many ion channels may be involved in ET-1–induced cell proliferation. For example, electrophysiological studies in systemic vascular smooth muscle cells demonstrate that ET-1 can activate L-type Ca²⁺ channels and receptor-operated Ca²⁺ channels and promote cell proliferation. Recent growing evidence indicates that Cl⁻ channels may also play critical role in regulation of the cell cycle. In our previous studies, we have examined the effects of different kinds of Cl⁻ channel blockers on the proliferation of VSMCs induced by ET-1. We found only 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) significantly inhibited ET-1–induced proliferation of VSMCs in a concentration-dependent manner (0.1 μmol/L to 1 mmol/L), whereas other Cl⁻ channel blockers such as IAA-94, NPPB, DPC, and furosemide had no significant inhibitory effect on VSMC proliferation under the same concentrations. These data indicate that DIDS-sensitive Cl⁻ channels may be involved in ET-1–induced VSMC proliferation.

At least two types of DIDS-sensitive Cl⁻ channels, including a calcium-activated Cl⁻ channel (IC ca) and a volume-regulated Cl⁻ channel (IC Vol), have been previously described in VSMCs. It is still not clear which type of Cl⁻ channel is associated with VSMC proliferation, although recent studies have shown the involvement of IC Vol in cell proliferation in many other cell types, such as rat C6 glioma, liver, T lymphocytes, and endothelial cells.

At a molecular level, among the identified Cl⁻ channels in VSMCs, ClC-3, a member of the ClC superfamily of chloride channels, has been found to be the dominant Cl⁻ channel in human vascular smooth muscle. The CIC-3 current exhibits the properties including outward rectification, activation by extracellular hypotonicity, inhibition by DIDS, and tamoxifin, similar to that of IC Vol in cardiac myocytes and VSMCs. Inhibition of endogenous CIC-3 expression using antisense oligonucleotide against CIC-3 or anti-CIC-3 antibody causes a significant decrease in IC Vol in smooth muscle cells. We hypothesized, therefore, that Cl⁻ channels encoded by ClC-3 might contribute to ET-1–induced VSMC proliferation. To test this hypothesis, we used multiple proliferation assays, immunoblot, and antisense strategy to examine whether ClC-3 protein is endogenously ex-
pressed in cultured rat aortic VSMCs; (2) whether the endogenous expression of ClC-3 is enhanced by ET-1; and (3) what the relationship between the expression of ClC-3 and VSMC proliferation induced by ET-1 would be. Our results demonstrate that ET-1 stimulates VSMC proliferation and ClC-3 protein expression, and deficiency in endogenous ClC-3 caused by antisense oligonucleotide prevented the ET-1–induced CIC-3 VSMC proliferation.

Materials and Methods

Cell Culture

VSMCs were isolated and cultured from rat thoracic aorta as previously described.3,17 Passage 8 to 14 VSMCs at 70% to 90% confluence in 35-mm dishes or 96-well plates were growth-arrested by incubation in 0.2% calf serum/DMEM for 72 hours. Cells were identified as smooth muscle cells by morphology and immunostaining with monoclonal antibody specific for smooth muscle α-actin.

Transfection of the VSMCs With Antisense, Sense, or Missense Oligonucleotides

The antisense and sense oligonucleotides corresponding specifically to the initiation codon region of the human ClC-3 mRNA were synthesized (Sangon, Shanghai, China) as reported previously.23 The antisense sequence was 5′-TCTATTCCTGTATTG-3′. The sense oligonucleotide had the sequence 5′-ACAATGACAAATGGA-3′. The antisense sequence was 5′-TCCATTTGTCATTGT-3′. The sense oligonucleotide was phosphorothioated. To examine the uptake of oligonucleotides by VSMCs, the oligonucleotides were labeled with fluorescent. For transient transfection, the cells in the quiescent state were transfected with oligonucleotides by incubation for 24 hours with lipofectamine 2000 (5 μg/mL, Life Technologies, Inc.). Cells were switched to the 0.2% FCS-containing medium with or without various concentration of ET-1.

[3H]-Thymidine Incorporation

To examine the DNA synthesis, rat aortic VSMCs, either rendered quiescent or transfected with antisense or sense oligonucleotides, were incubated with ET-1 in DMEM/0.2% FBS medium for 18 hours, then 1 μCi/mL [3H]-thymidine was added to the medium for a further 6-hour incubation. TCA-insoluble radioactivity was counted with Beckman liquid scintillation counter. To exclude possible toxicity, lipofectamine 2000, antisense, sense, and missense oligonucleotides were incubated with VSMCs, respectively, and radioactivity was counted. No toxicity was detected at the concentrations used in present study.

Western Blot Analysis

To examine ClC-3 protein expression, rat aortic VSMCs were washed with PBS and lysis buffer: Tris-Cl 50 mmol/L, NaCl 150 mmol/L, NaHCO3 0.02%, Nonidet P-40 1%, SDS 0.1%, sodium deoxycholate 0.5%, 5 μg/mL leupeptin, and 1 μg/mL aprotilin. The protein content of cell lysates was quantified with Coomassie Brilliant Blue, and separated by SDS-PAGE and transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked at room temperature (24°C to 26°C) for 1 hour in PBST (in mmol/L: NaCl 130, KCl 2.5, Na2HPO4 10, KH2PO4 1.5, 0.1% Tween 20, and 5% BSA, pH 7.4), incubated initially with primary antibodies, (anti-ClC-3, Alomone Labs, 1 hour at room temperature or overnight at 4°C), and then with the appropriate secondary peroxidase-conjugated antibodies (HRP-linked anti-rabbit secondary antibody and HRP-linked anti-biotin antibody, 1 hour at room temperature). Final detection was carried out with Lumiglo chemiluminescent reagent (New England Biolabs) as described by the manufacturer. The density of band was accurately determined by the computer-aided 1-D gel analysis system.

Statistical Analysis

All data are expressed as mean±SEM. Statistical analyses were performed using Student’s t test or ANOVA. Values of P<0.05 were considered significant.

Results

ET-1 Induced Endogenous ClC-3 Expression

Expression of ClC-3 protein in VSMCs was detected by immunoblotting with the use of a polyclonal antibody directed against ClC-3. The anti–ClC-3 antibody recognized a major band at 80 to 90 kDa (Figure 1). Then, we examined whether ET-1 could functionally enhance endogenous ClC-3 expression. As shown in Figure 1, ET-1 (10−8 mol/L) stimulated the expression of ClC-3 protein in a time-dependent manner. Compared with relative controls, at 24, 48, and 72 hours, the ClC-3 protein expressions were elevated 1.05±0.11-fold (P>0.05, n=4), 2.89±0.43-fold (P<0.01, n=4), and 2.72±0.50-fold (P<0.01, n=4), respectively. The ClC-3 protein expression reached the maximal expression level at 48 hours. These results suggest that ET-1–induced VSMC proliferation is closely associated with a corresponding increase in endogenous ClC-3 protein expression. To be consistent with the studies in proliferation assay,17 we chose ET-1 stimulation for 48 hours in subsequent experiments.

Antisense Oligonucleotide Inhibits ET-1–Induced ClC-3 Protein Expression

Because there exists no specific ClC-3 chloride channel blockers, we used antisense oligonucleotide specifically...
against ClC-3 to inhibit endogenous ClC-3 protein expression. To examine the uptake of oligonucleotide by VSMCs, the oligonucleotides were labeled with fluorescein. As shown in Figure 2, under resting conditions the fluorescence in the cells was negligible, but the fluorescence in cells treated with antisense, sense, or missense were greatly increased, which confirmed the uptake of oligonucleotides by these cells. Figure 3 illustrates ET-1–induced ClC-3 protein expression was decreased by 36±8%, 59±7%, and 68±8% (n=7) in 5 μL/mL lipofectamine plus 25, 50, and 100 μg/mL ClC-3 antisense oligonucleotides, respectively. To exclude the nonspecificity of antisense oligonucleotide, the sense and missense oligonucleotide were synthesized and their effects on ClC-3 protein expression were examined. The sense or missense probe had no significant effects on ET-1–induced ClC-3 protein expression (Figure 4; P>0.05, n=5). Figure 3 also illustrates that there was no significant effect of transfection agent lipofectamine (5 μL/mL) alone on ET-1–induced ClC-3 protein expression (P>0.05, n=4).

**Inhibition of ET-1–Induced Cell Proliferation by Antisense Oligonucleotides Against ClC-3**

The misogynic effect of ET-1 on VSMCs was examined by cell count and [3H]-thymidine incorporation into DNA synthesis as described in our previous studies. ET-1 stimulated the proliferation of VSMCs in a concentration-dependent manner (10⁻¹⁰ mol/L to 10⁻⁶ mol/L). As shown in Figure 4, 10⁻⁸ mol/L ET-1 increased [3H]-thymidine incorporation to 3.6±0.4-fold higher than that in ET-1–free medium. To test the efficacy of antisense oligonucleotide as an inhibitor of proliferation, we transfected the VSMCs with the sense, antisense, or missense oligonucleotides, respectively. The incorporation of [3H]-thymidine induced by ET-1 was significantly inhibited by ClC-3 antisense oligonucleotide, also concentration-dependently (Figure 4), 200 μg/mL antisense oligonucleotide produced maximal effects, with the maximal inhibitory rate of 70±7% (n=9). To exclude the nonspecificity of the oligonucleotides and/or transfection reagent had antiproliferative effects on VSMCs, cells were incubated with

![Figure 2](https://example.com/image2.png)

*Figure 2. Representative images of fluorescence in VSMCs. All oligonucleotides were labeled with fluorescein as described in Materials and Methods. There was no fluorescence detectable in VSMCs before transfection. After 6-hour incubation with oligonucleotides labeled with fluorescein, the fluorescence can be detected in these VSMCs, indicating oligonucleotides have been taken by VSMCs. A, Untransfected VSMCs; B, VSMCs transfected with 200 μg/mL sense oligonucleotide; C, VSMCs transfected with 200 μg/mL missense oligonucleotide; and D, VSMCs transfected with 200 μg/mL antisense oligonucleotide.*

![Figure 3](https://example.com/image3.png)

*Figure 3. Effects of oligonucleotide transfection on ClC-3 protein expression induced by 10⁻⁸ mol/L ET-1. A, Densitometric analysis shows ClC-3 antisense oligonucleotide concentration-dependently inhibited ClC-3 protein expression induced by 10⁻⁸ mol/L ET-1 (n=6). B, Densitometric analysis shows 200 μg/mL missense or sense oligonucleotide plus transfecting agent (5 μL/mL lipofectamine 2000) had no significant effects on 10⁻⁸ mol/L ET-1–induced ClC-3 protein expression (n=5); 5 μL/mL lipofectamine 2000 alone had no significant effects on ET-1–stimulated ClC-3 protein expression (n=4). Results are the mean±SEM in bar graphs. C and D, Representative Western blots are shown. §P>0.05 vs ET-1; #P<0.01 vs control; **P<0.01 vs ET-1.*
lipofectamine alone for 24 hours or with sense and missense oligonucleotide respectively for 48 hours. No significant inhibition of ET-1–induced [3 H] thymidine incorporation were observed (Figure 4; \( P < 0.05, n = 9 \)).

**Discussion**

Our results provide compelling evidence that ClC-3 Cl\(^{-}\) channels are critically linked to ET-1–induced VSMC proliferation. We first used Western blot and proliferation assay to demonstrate that ClC-3 is endogenously expressed in rat aortic VSMCs. Then, we found that ET-1–induced cell proliferation was accompanied by an increase in ClC-3 expression in the same concentration- and time-dependent pattern. Finally, antisense, but neither sense nor missense, oligonucleotides against ClC-3 not only inhibited the ET-1–induced expression of ClC-3 protein but also decreased [3 H]-thymidine incorporation and prevented VSMC proliferation.

It is well known that most cells swell during the early phase of cell proliferation probably caused by water influx that accompanies changes in cell metabolism (such as obligatory uptake of amino acids) in the cell cycle. An increase in cell volume usually initiates the so-called regulatory volume decrease (RVD) process through activation of ion (K\(^{+}\) and Cl\(^{-}\)) channels and transporters, which moves K\(^{+}\) and Cl\(^{-}\) out of the cell to balance the water influx and returns the cell volume to its normal size. Theoretically, therefore, activation of \( I_{\text{Cl,vol}} \) may play an essential role in cell proliferation.

In heart and vascular smooth myocytes and many other cell types, ClC-3 Cl\(^{-}\) channel has been confirmed to encode \( I_{\text{Cl,vol}} \), although conflicting results have been reported on the function and cellular localization of ClC-3 channels. When expressed in mammalian cell lines, CIC-3 mediates a DIDS-sensitive outwardly rectifying Cl\(^{-}\) current. In human blood vessels, ClC-3 is the most abundantly expressed Cl\(^{-}\) channel that may underlie the native swelling-activated Cl\(^{-}\) current found in these tissues. Antisense oligonucleotide against CIC-3 has been shown to significantly inhibit the swelling-activated Cl\(^{-}\) current and the functional regulatory volume decrease in HeLa cells, *Xenopus laevis* oocytes, and nonpigmented ciliary epithelial cells. Using the previously reported ClC-3 antisense oligonucleotide to specifically inhibit the functional expression of ClC-3 in the cultured rat aortic VSMCs, we now find that deficiency in ClC-3 expression prevents cell proliferation and [3 H]-thymidine incorporation. Because it has been consistently reported from many independent investigators, as

![Figure 4. Effects of oligonucleotide transfection on VSMC proliferation induced by 10⁻⁸ mol/L ET-1. After different treatments (5 \( \mu \)L/ml lipofectamine alone, 200 \( \mu \)g/mL ClC-3 missense or sense oligonucleotides, and ClC-3 antisense oligonucleotide at different concentrations), the cells were incubated with 10⁻⁸ mol/L ET-1 for 18 hours. [3 H]Thymidine was added. Incorporation of [3 H]-Thymidine was then determined in 6 hours. Left bar graphs show that lipofectamine alone and missense had no effects on 10⁻⁸ mol/L ET-1–induced VSMC proliferation (\( P > 0.05, n = 9 \)). Right curves show that 12.5, 25, 50, 100, and 200 \( \mu \)g/mL ClC-3 antisense concentration-dependently inhibited the ET-1–induced VSMC proliferation, whereas the similar concentration of sense had no significant effects on VSMC proliferation (\( n = 9 \)). **\( P < 0.01 \) vs ET-1.**

![Graph showing effects of oligonucleotide transfection on VSMC proliferation induced by 10⁻⁸ mol/L ET-1.](http://circres.ahajournals.org/ Downloaded from)

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mentioned earlier, that $I_{\text{Cl,vol}}$ is closely associated with cell proliferation in many cell types, our present work provides further evidence to support the notion that CIC-3 may be the gene responsible for $I_{\text{Cl,vol}}$ in these cell types. Although it is still not clear how the CIC-3 channels are involved in VSMC proliferation, our results strongly suggest that CIC-3 channels, or $I_{\text{Cl,vol}}$, are important regulators of the cell cycle and may play substantially crucial role in such pathogenic process as hypertension and arteriosclerosis when VSMC proliferation is important in the development of vascular modeling.

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