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:e28-e32.)

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

Vascular smooth muscle cell (VSMC) proliferation is a key event in the pathogenesis of atherosclerosis and many other vascular diseases.1–3 Endothelin-1 (ET-1), a potent vasoconstrictor and mitogen/proliferation factor for VSMCs, has been shown to induce VSMC proliferation in cultured rat and human aortic smooth muscle cells.4,5 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.6–9 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.10,11 Recent growing evidence indicates that Cl− channels may also play critical role in regulation of the cell cycle.12–17 In our previous studies,17 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′-disothiocyanostilbene-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 (ICl,Ca) and a volume-regulated Cl− channel (ICl,Vol), have been previously described in VSMCs.18–20 It is still not clear which type of Cl− channel is associated with VSMC proliferation, although recent studies have shown the involvement of ICl,Vol in cell proliferation in many other cell types, such as rat C6 glioma,15 liver,16 T lymphocytes,21 and endothelial cells.22 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.23,24 The ClC-3 current exhibits the properties including outward rectification, activation by extracellular hypotonicity, inhibition by DIDS, and tamoxifen, similar to that of ICl,Vol in cardiac myocytes and VSMCs.23,25 Inhibition of endogenous ClC-3 expression using antisense oligonucleotide against CIC-325 or anti-CIC-3 antibody26 causes a significant decrease in ICl,Vol in smooth muscle cells. We hypothesized, therefore, that Cl− channels encoded by CIC-3 might contribute to ET-1–induced VSMC proliferation. To test this hypothesis, we used multiple proliferation assays, immunoblot, and antisense strategy to examine (1) whether CIC-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 ClC-3 VSMC proliferation.

Materials and Methods

Cell Culture

Wistar rats were supplied by the Experimental Animal Center of Sun Yat-Sen University in Guangzhou, China. All procedures complied with the standards for the 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). VSMCs were isolated and cultured from rat thoracic aorta as previously described. Passages 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. The antisense sequence was 5'-TCCATTTGCTATTG-3'. The sense oligonucleotide had the sequence 5'-ACAATGACAAATGGA-3'. We designed missense oligonucleotide 5'-TCTATTCCTGTATTG-3'. As shown in Figure 1, ET-1 (10^{-8} mol/L) stimulated ClC-3 protein expression time-dependently and produced maximal effects at 48 hours.

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, NaNO_3 0.02%, Nonidet P-40 1%, SDS 0.1%, sodium deoxycholate 0.5%, 5 μg/mL leupeptin, and 1 μg/mL aprotinin. 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, Na_2 HPO_4 10, KH_2 PO_4 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 target bands were 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, 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 fluorescence. 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 CIC-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 CIC-3 antisense oligonucleotides, respectively. To exclude the nonspecificity of antisense oligonucleotide, the sense and missense oligonucleotides were synthesized and their effects on CIC-3 protein expression were examined. The sense or missense probe had no significant effects on ET-1–induced CIC-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 CIC-3 protein expression (P>0.05, n=4).

**Figure 3.** Effects of oligonucleotide transfection on CIC-3 protein expression induced by 10−8 mol/L ET-1. A, Densitometric analysis shows CIC-3 antisense oligonucleotide concentration-dependently inhibited CIC-3 protein expression induced by 10−8 mol/L ET-1 (n=6). B, Densitometric analysis shows 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 lipofectamine alone for 24 hours or with sense and missense oligonucleotide respectively for 48 hours. No significant inhibition of ET-1–induced [3H]-thymidine incorporation were observed (Figure 4; P>0.05, n=9).

**Discussion**

Our results provide compelling evidence that CIC-3 Cl− channels are critically linked to ET-1–induced VSMC proliferation. We first used Western blot and proliferation assay to demonstrate that CIC-3 is endogenously expressed in rat aortic VSMCs. Then, we found that ET-1–induced cell proliferation was accompanied by an increase in CIC-3 expression in the same concentration- and time-dependent pattern. Finally, antisense, but neither sense nor missense, oligonucleotides against CIC-3 not only inhibited the ET-1–induced expression of CIC-3 protein but also decreased [3H]-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 fact, recent studies have clearly shown that Cl\(^-\) channel blockers inhibit not only \(I_{\text{Cl,vol}}\) but also cell proliferation and \(^{[\text{H}]}\)-thymidine incorporation in many cell types, including glioma cells,\(^{15}\) hepatocytes,\(^{16}\) fibroblasts,\(^{18}\) T lymphocytes,\(^{21}\) endothelial cells,\(^{22}\) breast cancer,\(^{27}\) and microglial cells.\(^{28}\) Similarly, our previous study indicates that a widely used Cl\(^-\) channel blocker, DIDS, also inhibits cell proliferation and \(^{[\text{H}]}\)-thymidine incorporation in rat aortic VSMCs.\(^{17}\) Due to the lack of specificity of the Cl\(^-\) channel blockers used in these studies and the concomitant expression of several types of Cl\(^-\) channel in the same cell, however, it is almost impossible to draw a definitive conclusion that the effects of these channel blockers are due to the blockade of \(I_{\text{Cl,vol}}\) alone. For example, DIDS has been found to block not only \(I_{\text{Cl,vol}}\) but also \(I_{\text{Cl,Ca}}\) and several cation channels (such as L-type Ca\(^{2+}\) channels) as well.\(^{29,30}\)

In cardiac and vascular smooth myocytes and many other cell types, Cl\(^-\) 3 Cl\(^-\) channel has been confirmed to encode \(I_{\text{Cl,vol}}\);\(^{18,20,21,24,26,31,35}\) although conflicting results have been reported on the function and cellular localization of Cl\(^-\)3 channels.\(^{25}\) When expressed in mammalian cell lines, Cl\(^-\)3 mediates a DIDS-sensitive outwardly rectifying Cl\(^-\) current.\(^{18}\) In human blood vessels, Cl\(^-\)3 is the most abundantly expressed Cl\(^-\) channel that may underlie the native swelling-activated Cl\(^-\) current found in these tissues.\(^{23}\) Antisense oligonucleotide against Cl\(^-\)3 has been shown to significantly inhibit the swelling-activated Cl\(^-\) current and the functional regulatory volume decrease in HeLa cells,\(^{31}\) and nonpigmented ciliary epithelial cells.\(^{25}\) Using the previously reported Cl\(^-\)3 antisense oligonucleotide\(^{25}\) to specifically inhibit the functional expression of Cl\(^-\)3 in the cultured rat aortic VSMCs, we now find that deficiency in Cl\(^-\)3 expression prevents cell proliferation and \(^{[\text{H}]}\)-thymidine incorporation. Because it has been consistently reported from many independent investigators, as 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 Cl\(^-\)3 may be the gene responsible for \(I_{\text{Cl,vol}}\) in these cell types. Although it is still not clear how the Cl\(^-\)3 channels are involved in VSMC proliferation, our results strongly suggest that Cl\(^-\)3 channels, or \(I_{\text{Cl,vol}}\), are important regulators of the cell cycle and may play substantial crucial role in such pathogenic processes as hypertension and arteriosclerosis when VSMC proliferation is important in the development of vascular modeling.

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