Endophilin A2 Influences Volume-Regulated Chloride Current by Mediating CIC-3 Trafficking in Vascular Smooth Muscle Cells

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Background: Previous research has demonstrated that CIC-3 is responsible for volume-regulated Cl⁻ current (I_{Cl.vol}) in vascular smooth muscle cells (VSMCs). However, it is still not clear whether and how CIC-3 is transported to cell membranes, resulting in alteration of I_{Cl.vol}.

Methods and Results: Volume-regulated chloride current (I_{Cl.vol}) was recorded by whole-cell patch clamp recording, and Western blotting and co-immunoprecipitation were performed to examine protein expression and protein-protein interaction. Live cell imaging was used to observe CIC-3 transporting. The results showed that an overexpression of endophilin A2 could increase I_{Cl.vol}, while endophilin A2 knockdown decreased I_{Cl.vol}. In addition, the SH3 domain of endophilin A2 mediated its interaction with CIC-3 and promotes CIC-3 transportation from the cytoplasm to cell membranes. The regulation of CIC-3 channel activity was also verified in basilar arterial smooth muscle cells (BASMCs) isolated from endophilin A2 transgenic mice. Moreover, endophilin A2 increase VSMCs proliferation induced by endothelin-1 or hypo-osmolality.

Conclusions: The present study identified endophilin A2 as a CIC-3 channel partner, which serves as a new CIC-3 trafficking insight in regulating I_{Cl.vol} in VSMCs. This study provides a new mechanism by which endophilin A2 regulates CIC-3 channel activity, and sheds light on how CIC-3 is transported to cell membranes to play its critical role as a chloride channel in VSMCs function, which may be involved in cardiovascular diseases. (Circ J 2016; 80: 2397–2406)

Key Words: CIC-3; Endophilin A2; Vascular smooth muscle cells; Volume-regulated Cl⁻ current; Volume-regulated chloride channel
in the pathology of hypertensive cerebrovascular remodeling,10–12 arterogenesis,13 and vascular inflammatory responses.14,15 Our previous studies revealed that a hypotonic solution evoked a native volume-regulated outwardly rectifying Cl⁻ current (I_{Cl,vol}), with a decrease in cytoplasmic Cl⁻ concentration ([Cl⁻]) in vascular smooth muscle cells (VSMCs). The response of [Cl⁻] and I_{Cl,vol} to hypotonic challenge can be enhanced by CIC-3 overexpression, and inhibited by CIC-3 antisense. The currents in VSMC (I_{Cl,vol}) and in CIC-3-VSMC (I_{CIC-3}) are prohibited by intracellular dialysis of the anti-CIC-3 antibody. In addition, current evidence indicates that I_{Cl,vol} and I_{Cl,CIC-3} share the same pharmacological properties and regulatory mechanisms, such as PKC and PTK regulation of the Cl⁻ currents.16

We demonstrated that CIC-3 protein is distributed broadly in the cytoplasm and cell membrane of A10 VSMCs and primary culture of BASMCs. Even though CIC-3 is primarily localized in the cytoplasm of some cell types, CIC-3 protein could be transiently trafficked to the cell membrane during clathrin-mediated endocytosis,17 suggesting the existence of a dynamic mechanism regulating subcellular distribution of CIC-3 protein. The trafficking of ion channel protein members from the intracellular compartment to the cell membrane via a shuttle protein is emerging as a key factor involved in the regulation of the ion channel.18,19

Endophilins are a family of evolutionarily conserved proteins, which are well documented in the pathway of endocytosis, where endophilins can bind to the cell membrane, induce membrane bending to form a molecular scaffold, and then promote efficient endocytic protein trafficking.20 Among the most extensively studied endophilin A family members, endophilin A2, is ubiquitously expressed in eukaryotic cells. All forms of endophilins contain 2 major functional domains: an N-terminal Bin-amphiphysin-Rvs (BAR) domain and C-terminal Src homology 3 (SH3) domain. During the membrane remodeling process, the N-BAR domains are sensors of membrane curvature and provide a molecular scaffold that combine bending activities with specific protein-protein interacting processes; and the SH3 domain is the linker region by which endophilins can recognize proline-rich domains (PRDs) and recruit the essential membrane-trafficking proteins. Accumulating evidence also support an emerging role for endophilins in the functions of cell membrane receptors via protein trafficking mechanisms.21–22 For example, a direct interaction between endophilin A2 and a voltage-dependent Ca²⁺ channel are required for synaptic vesicle endocytosis, and the PRDs within endophilin A2 serve to sense the intracellular Ca²⁺ concentration and regulate the formation of the endophilin-Ca²⁺ channel complex.23 To date, whether and how endophilin A2 is involved in regulating I_{Cl,vol}, subcellular distribution of CIC-3 proteins, and VSMC proliferation remains unclear.

In the present study, we found endophilin A2 is expressed abundantly in BASMCs, we also demonstrated that endophilin A2 participates in the regulation of hypotonic-activated I_{Cl,vol} in BASMCs. BASMCs isolated from endophilin A2 transgenic mice exhibited a high degree of CIC-3 protein accumulation on the cell surface of BASMCs, a marked enhancement of hypotonic-induced I_{Cl,vol}, and elevated endothelin-1 or hypotonic-induced BASMC proliferation. By constructing three deletion mutants that failed to induce the function of the SH3, PRD or BAR domain in endophilin A2, the CIC-3 binding site in endophilin A2 was ascribed to the C-terminal SH3 domain. The SH3 deletion mutant of endophilin A2 completely abolished its association with CIC-3 and resulted in a significant decrease in cell membrane localization of CIC-3. Our findings suggest an endophilin A2-mediated protein trafficking mechanism, coupling intracellular CIC-3 to cell membrane I_{Cl,vol} for VSMC functions.

**Methods**

**Plasmid Construction**

The full-length endophilin A2 sequence was amplified from cultured rat BASMCs using TRizol reagent (Invitrogen). Then the coding sequence of endophilin A2 was amplified using full-length cDNA as a template, and the primers were ATGTCCGTTGGCggggtgtaagag (forward) and TCACGTGAGCAGGGCACCCAG (reverse). The construction of the recombinant plasmid pCMV-endophilin A2-flag was performed by using an overlap extension poly-merase-chain-reaction (PCR) cloning method.

**Endophilin A2 Transgenic Mice**

The full-length murine endophilin A2 cDNA coding sequence was infused into an ExE3d-CMV-IRES vector. After linearization and subsequent gel-purification, this vector was microinjected into the pronuclei of fertilized C57BL/6j mouse eggs. PCR was used for genotyping. The primers used for genotyping were: 5′-CCTAGGTGTAACACTCCC-3′ (forward) and 5′-GGCCTTACGGGTGATATCC (reverse).

**siRNA Transfection**

The siRNA (small interfering RNA) duplexes against the rat endophilin A2 gene (Gene ID: 81922) was designed and constructed by QIAGEN and transfected with Hyperfect Transfection Reagent, according to the manufacturer’s instructions (QIAGEN). The working concentration of endophilin A2 siRNA was 40nmol/L. A negative siRNA (QIAGEN) was used as a negative control. The cells were then used for western blot and patch-clamp studies after 48h.

**BASMC Isolation and Culture**

Basilar arterial smooth muscle cells were isolated and cultured, as previously described.31 Freshly isolated BASMCs were stored at 4°C and used for patch-clamp experiments within 10h. The BASMC cells in passage 8 through 12 were used for cell proliferation assays.

**Cell Culture and Plasmid Transfection**

The A10 vascular smooth muscle cells (American Type Culture Collection) were cultured at 37°C under 5% CO₂ in DMEM/F12 supplemented with 10% fetal bovine serum (Sigma-Aldrich) and 1% penicillin-streptomycin, as previously described.18 Plasmids were transfected into the cells at 80% confluence with Lipofectamine 2000, according to the manufacturer’s instruction (Invitrogen).

**Current Recording**

The whole-cell Cl⁻ currents were recorded with an Axopatch 200B Amplifier (Axon Instruments, Foster City, CA, USA), as previously described.16 Briefly, the currents were elicited with voltage steps from -100mV to +120mV in +20mV increment for 400ms, and with an interval of 5s from a holding potential of -40mV. Currents were sampled at 5kHz using pCLAMP8.0 software (Axon Instruments) and filtered at 2kHz. The hypotonic bath solution contained (mmol/L): N-methyl-D-glucamine chloride (NMDG-Cl) 107, MgCl₂ 1.5, MnCl₂ 0.5, CdCl₂ 0.5, GdCl₃ 0.05, glucose 10, and Hepes 10; pH 7.4 was adjusted with NMDG. The osmolality of this solution, measured by a freezing-point depression osmometer (OsmOMAT030; Germany), was 230mOsm/L. The isosmotic bath solution was...
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a 2-way ANOVA. For western blot and cell proliferation experiments, “n” represents the number of independent experiments. For patch-clamp experiments, “n” represents the number of cells from at least 6 different batches of cells or 6 different rats. In all tests, P<0.05 was taken to be as statistically significant.

Results

Endophilin A2 Is Expressed Abundantly in BASMCs
To determine the expression profiles of endophilin A in BASMCs, western blotting analysis was performed using cultured rat BASMCs. Endophilin A2 protein was found to be abundantly expressed in BASMCs, while antibodies against endophilin A3 detected very faint bands, and endophilin A1 was not found (Figure S1), indicating that endophilin A2 is the predominant isoform expressed in BASMCs, and may play a functional role in the vasculature.

Endophilin A2 Regulates ICl.vol and the ClC-3 Chloride Channel
Consistent with our previous findings, whole-cell patch-clamp recording results showed a very small outward Cl− current in BASMCs in the resting state, and perfusion with a hypotonic solution (230 mosmol/kg H2O) caused cell swelling and activated ICl.vol (Figure 1). The amplitude and current density of ICl.vol was further increased by the overexpression of endophilin A2 (Figure 1A). Conversely, the hypotonic-induced ICl.vol was markedly decreased by endophilin A2 siRNA (20nmol/L) (Figure 1B), which could knockdown the endogenous endophilin A2 expression (Figure S2). Neither endogenous endophilin A2 protein expression nor ICl.vol was affected by the transfection of negative endophilin A2 siRNA. Moreover, intracellular dialysis of an anti-endophilin A2 antibody significantly decreased the activation of ICl.vol under isotonic and hypotonic conditions.

Figure 1. Endophilin A2 regulated ICl.vol in basilar arterial smooth muscle cells (BASMCs). (A) Representative traces of ICl.vol (a), I-V curves (b) and mean current densities measured at ±100mV (c) in the control (con), GFP vector (GFP) and GFP-endophilin A2 adenovirus transfecting (GFP-endo2) groups (n=10, **P<0.05 vs. control (iso) or GFP (iso) group, *P<0.05 vs. control (hypo) or GFP (hypo) group). (B) Representative traces of ICl.vol (a), I-V curves (b) and mean current densities measured at ±100mV (c) in the control (con), negative and endophilin A2-siRNA (endo2-siRNA) groups (n=10, **P<0.05 vs. control (iso) or neg (iso) group; *P<0.05 vs. control (hypo) or neg (hypo) group).
hypo-osmolarity. These results suggest that endogenous endophilin A2 may participate in the regulation of $I_{Cl.vol}$ in response to hypo-osmolarity. We have documented that $ClC-3$ is critical for regulation of $I_{Cl.vol}$ in VSMCs. BASMCs express $ClC-3$ and endophilin A2 proteins endogenously, and both are involved in hypotonic-induced $I_{Cl.vol}$. We then examined whether endophilin A2 is associated with $ClC-3$ in regulating $I_{Cl.vol}$.

Patch-clamp experiments were performed on A10 cells co-transfected with $ClC-3$ siRNA or GFP-tagged endophilin A2 plasmid. Overexpression of endophilin A2 resulted in a substantial increase in $I_{Cl.vol}$ under the hypotonic condition (Figure S4). At 100 mV, the current densities of $I_{Cl.vol}$ were increased from 42.6±8.7 pA/pF (control) to 87.6±10.1 pA/pF (GFP-endophilin A2 overexpression). Moreover, the increased hypotonic-induced $I_{Cl.vol}$ by endophilin A2 overexpression in VSMCs was inhibited by co-transfection with GFP-endophilin A2 and $ClC-3$ siRNA or GFP-tagged endophilin A2 plasmid. ClC-3 siRNA also inhibited hypotonic-induced $I_{Cl.vol}$, supporting our previous findings that endogenous ClC-3 is important for the activation of $I_{Cl.vol}$ in VSMCs. Together, these data suggest that ClC-3 may be associated with endophilin A2 in regulating $I_{Cl.vol}$.

Endophilin A2 Regulates ClC-3 Protein Not Through Tyrosine Phosphorylation

We then examined whether endophilin A2 affected $ClC-3$ protein expression. As shown in Figures 2A and B, under isotonic conditions, neither over-expression nor knockdown of endophilin A2 could change $ClC-3$ protein expression in BASMCs. We have recently reported that hypo-osmolarity activates $I_{Cl.vol}$ through phosphorylation of tyrosine residues in the $ClC-3$ protein rather than through increasing $ClC-3$ protein expression. However, neither over-expression nor knockdown of endophilin A2 affected the hypotonic-induced enhancement in $ClC-3$ tyrosine phosphorylation (Figures 2C, D), suggesting that there might be another mechanism underlying the association of endophilin A2 and $ClC-3$ in regulating $I_{Cl.vol}$. 

Figure 2. Endophilin A2 did not influence CIC-3 expression and CIC-3 tyrosine phosphorylation induced by hypo-osmolarity. (A, B) Representative western blot illustrating that neither endophilin A2 siRNA nor adenovirus-mediated endophilin A2 overexpression (Ad-endo2) affected total CIC-3 protein levels in A10 cells (n=6). (C, D) Hypotonic-induced tyrosine phosphorylation of CIC-3 protein in A10 cells were not affected by endophilin A2 siRNA or adenovirus endophilin A2 expression. p-Y, phospho-tyrosine antibody (n=6).

Figure 3. Endophilin A2 facilitated CIC-3 trafficking from the cytoplasm to the plasma membrane in basilar arterial smooth muscle cells (BASMCs). (A) Co-immunoprecipitation analysis of endophilin A2 and CIC-3 protein interaction in cultured BASMCs. (B, C) Western blot analysis of the CIC-3 protein in the membrane and cytoplasm from the control BASMCs (con), BASMCs transfecting vector and endophilin A2 adenovirus (Ad-endo2), or BASMCs transfecting negative and endophilin A2 siRNA (endo2siRNA) (n=6, *P<0.05 vs. corresponding controls).
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The co-immunoprecipitation assay in BASMCs demonstrated that there is an interaction between CIC-3 and endophilin A2 proteins (Figure 3A). Endophilins are best known for their functions in membrane curvature induction and/or stabilization during endocytosis. In the endocytic pathway, endophilin A2 can bind to signaling proteins and assemble and traffic these proteins between the cytoplasm and cell membrane. The CIC-3 protein is ubiquitously expressed in mammalian cells, but was reported to be located primarily in the cytoplasm of several cell types. We therefore examine whether the interaction between CIC-3 and endophilin A2 may have a functional effect on the localization of CIC-3 protein in BASMCs. The Ad-mediated endophilin A2 overexpression caused CIC-3 protein expression levels to increase significantly in the cell membrane and decrease in the cytoplasm. Conversely, the knockdown of endophilin A2 caused an increase in CIC-3 protein accumulation in the cytoplasm (Figures 3B,C).

To study if endophilin A2 regulates CIC-3 trafficking and hypotonic-activated $I_{\text{Cl,vol}}$ ex vivo, we generated transgenic mice that overexpress endophilin A2 (Figures S5A,B). The results from endophilin A2 transgenic mice (endo$^{27g}$) support the idea that endophilin A2 facilitates CIC-3 trafficking from the cytoplasm to the plasma membrane in BASMCs. Primary cultures of BASMCs from WT endo$^{27g}$ groups treated with 10nmol/L ET-1 for 24 h (a) or by hypo-osmolarity for 24 h (b). (n=5, *P<0.05 vs. WT group, #P<0.05 vs. the ET-1-treated or hypotreated WT).

Figure 4. CIC-3 is localized at the cytomembrane. $I_{\text{Cl,vol}}$ and cellular proliferation is increased in basilar arterial smooth muscle cells (BASMCs) from endophilin A2 transgenic mice. (A) Immunofluorescence of CIC-3 in rat BASMCs from endophilin A2 transgenic mice (endo$^{27g}$) and their wild-type (WT) littermates. (B) Representative traces of $I_{\text{Cl,vol}}$ (a), I-V curves (b) and mean current densities measured at ±100mV (c) in primary cultured BASMCs from wide-type (WT) and endophilin A2 transgenic mice (endo$^{27g}$). (n=10, *P<0.05 vs. isotonic WT, #P<0.05 vs. hypotonic WT). (C) CCK-8 cell viability analysis in BASMCs from WT endo$^{27g}$ groups treated with 10nmol/L ET-1 for 24 h (a) or by hypo-osmolarity for 24 h (b). (n=5, *P<0.05 vs. WT group, #P<0.05 vs. the ET-1-treated or hypotreated WT).
domains in endophilin A2 may bind to ClC-3 and mediate its trafficking from the cytoplasm to cell membrane. To test this hypothesis, we constructed 3 endophilin A2 deletion mutants, which failed to induce the function of the: (1) SH3 domain (Endophilin A2-∆SH3); (2) BAR domain (Endophilin A2-∆BAR); and (3) PRD domain (Endophilin A2-∆PRD). As shown in Figure 5, co-immunoprecipitation analysis showed that endophilin A2-∆BAR and endophilin A2-∆PRD still had an interaction with ClC-3; however, endophilin A2-∆SH3 exhibited no association with ClC-3. These results indicate that endophilin A2 interacts with the ClC-3 protein through the C-terminal SH3 domain.

To verify whether the SH3 domain of endophilin A2 affects the subcellular distribution of ClC-3 protein, we first isolated the membrane and cytoplasmic protein and found that the membrane ClC-3 expression in endophilin A2 full-length and other deletion mutants groups (Figures 6A, B). We then performed live long-term fluorescence imaging to observe ClC-3 distribution dynamically. When full-length ClC-3 and endophilin A2 cDNAs were co-transfected into A10 cells, ClC-3 protein was found to be gradually recruited into the cell membrane. The transfection of full-length ClC-3 cDNA alone caused the ClC-3 fluorescence to reside mainly in the cytoplasm within the same observation period. Among the endophilin A2 mutants, the ∆PRD and ∆BAR mutants exhibited a similar capability to recruit ClC-3 protein to the plasma membrane; whereas the ∆SH3 mutant retained ClC-3 fluorescence in the cytoplasm (Figure S6). We confirmed our findings by detecting the ability of ClC-3 fluorescence recovery after photobleaching (FRAP). A zone near the cell membrane

BASMCs also exhibit a larger I_{Cl,vol} than those of age-matched WT mice. The mean current density of I_{Cl,vol} was significantly higher in endo2^Tg^ BASMCs than in the WT group, as shown in Figure 4B (~6.1±1.2 pA/pF in endo2^Tg^ vs. ~2.7±0.3 pA/pF in WT, at −100 mV and 19.7±2.7 pA/pF in endo2^Tg^ vs. 8.4±1.9 pA/pF in WT at +100 mV, n=6). There was no significant difference between basal currents in BASMCs from endo2^Tg^ and WT groups under the isotonic condition (P>0.05). Because I_{Cl,vol} and CIC-3 Cl− currents are critical for VSMC proliferation and cerebrovascular remodeling in hypertension, we then tested whether endophilin A2 affects VSMC proliferation. Figure 4C shows that cultured BASMCs from endo2^Tg^ had significantly higher proliferation rates compared with those from WT mice in the presence of ET-1 or hypo-osmolarity. However, we did not find a significant difference in the bodyweight and systolic blood pressure between endo2^Tg^ and WT during the 20 weeks of observation (Figures S5C,D).

Taken together, it is clear that endophilin A2 may function to enhance CIC-3 protein trafficking from the cytoplasm to the cell membrane, which could be intimately tied to the enhancement of I_{Cl,vol} and VSMC proliferation induced by ET-1 and hypo-osmolarity.

SH3 Domain Contributes to Endophilin A2-Mediated CIC-3 Trafficking

All endophilin proteins contain 2 well-documented functional domains: a BAR domain that mediates membrane remodeling, and a C-terminal SH3 domain that serves as protein-recognition modules. Because of the interaction between endophilin A2 and CIC-3 proteins, we hypothesized that some functional domains in endophilin A2 may bind to CIC-3 and mediate its trafficking from the cytoplasm to cell membrane. To test this hypothesis, we constructed 3 endophilin A2 deletion mutants, which failed to induce the function of the: (1) SH3 domain (Endophilin A2-∆SH3); (2) BAR domain (Endophilin A2-∆BAR); and (3) PRD domain (Endophilin A2-∆PRD). As shown in Figure 5, co-immunoprecipitation analysis showed that endophilin A2-∆BAR and endophilin A2-∆PRD still had an interaction with CIC-3; however, endophilin A2-∆SH3 exhibited no association with CIC-3. These results indicate that endophilin A2 interacts with the CIC-3 protein through the C-terminal SH3 domain.

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Discussion

In the present study, we found that ClC-3 protein expression is abundant in the cytosol and the cell membrane of BASMCs, in comparison with previous reports showing that ClC-3 was primarily localized to the cytoplasm in some cell types. We further demonstrated that endophilin A2, an adapter protein, interacted with ClC-3 through the SH3 domain, enhanced ClC-3 trafficking from the cytoplasm to the surface membrane.

Figure 6. The SH3 domain participated in ClC-3 trafficking from the cytoplasm to the cell surface in A10 cells. Western blot analysis of the ClC3 protein in the membrane (A) and cytoplasm (B) from the control basilar arterial smooth muscle cells (BASMCs) (con), BASMCs transfected with a vector, endophilin A2 full length or three deletion mutants (n=6, *P<0.05). (C) Monitoring the fluorescence recovery after photobleaching in the vector and endophilin A2 mutant transfecting groups (n=8, *P<0.01 vs. vector or ∆SH3).

was randomly selected and bleached by a strong excitation beam, where we monitored the capability of FRAP. As we had predicted, endophilin A2 enhanced the fluorescence recovery capability whereas the ∆SH3 mutant abolished this effect (Figure 6C). These results indicate that the SH3 domain is the interacting site between endophilin and ClC-3, which is also essential for trafficking ClC-3 from the cytoplasm to the cell membrane.
VSMCs when exposed to cell shape or volume challenges. It is therefore not surprising here to observe the association between endophilin A2 and ClC-3 during the hypotonic-induced cell volume challenge.

In contrast, a large number of previous studies have documented that ClC-3 is critical for adaptive remodeling in many cell types in response to physiological and pathophysiological stresses associated with osmotic stress or cell volume perturbations, such as vascular smooth muscle reactive oxygen species (ROS) generation and inflammatory responses, insulin secretion, and neutrophil infiltration. In VSMCs, ClC-3 protein is abundantly expressed and has functionally been associated with \( I_{\text{Cl,vol}} \) during the development of vascular remodeling. However, before the present study, the trafficking of intracellular ClC-3 to the surface membrane upon hypotonic-induced cell swelling or endophilin-A2 overexpression had never been described. An acute cell volume challenge activates \( I_{\text{Cl,vol}} \) and results in the efflux of Cl–, which is essential for the development of hypertensive cerebrovascular remodeling and other pathophysiological processes. These morphological alterations of VSMC under diseased conditions definitely include membrane bending or curvature. By using multiple approaches, we demonstrated here that endophilin A2 interacts with CIC-3 protein to enhance the surface membrane ClC-3 accumulation and upregulate \( I_{\text{Cl,vol}} \). It is important to note that BASMCs overexpressing endophilin A2 or from endophilin A2 transgenic mice exhibited no change in outward Cl– current and cell viability under the resting state or under isotonic conditions, but have significantly enhanced the

We presented several lines of evidence to support the idea that endophilin A2 functions as a new trafficking mechanism regulating CIC-3 and \( I_{\text{Cl,vol}} \) based on the following findings: (1) in BASMCs overexpressing endophilin A2 or from endophilin A2 transgenic mice, we observed a significant increase in both the magnitude of \( I_{\text{Cl,vol}} \) and CIC-3 distribution to the cytoplasmic membrane, which were accompanied by an enhanced ET-1 or hypotonic-induced cell proliferation in BASMCs from transgenic mice, while the \( I_{\text{Cl,vol}} \) activation by hypoviscosity and membrane CIC-3 trafficking was inhibited by endophilin A2 siRNA; (2) the intracellular dialysis of the anti-endophilin A2 antibody specifically inhibited hypotonic-induced \( I_{\text{Cl,vol}} \), and co-transfection with CIC-3 siRNA markedly inhibited the large \( I_{\text{Cl,vol}} \) induced by endophilin A2 expression; and (3) there is a molecular interaction between endophilin A2 and CIC-3 proteins. By employing 3 endophilin A2 deletion mutants, we found that the SH3 domain is necessary for the interaction between endophilin A2 and CIC-3, as well as for the CIC trafficking between the cytoplasm and cell membrane (Figure 7).

Endophilins have shown their prominent roles in the processes that require remodeling of the membrane structure. Endophilins can sense membrane curvature, form and expose a scaffold and large membrane surface for recruiting specific downstream interacting proteins to the membrane, which are responsible for coupling distinct plasma membrane remodeling to specific biological processes. The endophilin A2-mediated protein trafficking is evoked during the process of cell membrane bending or remodeling. Membrane bending or curvature is commonly induced in VSMCs when exposed to cell shape or volume challenges. It is therefore not surprising here to observe the association between endophilin A2 and CIC-3 during the hypotonic-induced cell volume challenge.

Figure 7. Schematic of endophilin A2 regulation of CIC-3-mediated \( I_{\text{Cl,vol}} \) in vascular smooth muscle cells (VSMCs). CIC-3 is evenly distributed in the cytoplasm and plasma membrane in VSMCs, and intracellular CIC-3 is primarily localized in the endosome and vesicle. Endophilin A2 could bind to CIC-3 by its SH3 domain and modulate CIC-3 trafficking from the cytoplasm to the plasma membrane and stabilize CIC-3 expression on the cell surface, which eventually leads to activation of the CIC-3-mediated swelling-regulated Cl– channel under hypotonic stress.
increased the proliferation rate in response to ET-1 or hypo-osmolarity. These results are in agreement with the phenotype of endophilin A2 transgenic mice where there was no abnormality in body weight and systolic blood pressure. That is, endophilin A2 may participate in the activation of IC\textsubscript{vol} during the development of cardiovascular diseases. Our results from a 2-kidney, 2-clip (2k2c) renopertensive rat model agree with this inference and demonstrated that endophilin A2 protein expression and the surface membrane CIC-3 accumulation in BASMCs from 2k2c hypertensive rats were increased within 4 weeks of operation (unpublished data), which was accompanied by the upregulation of the CIC-3 channel and IC\textsubscript{vol} in vascular SMC proliferation and hypertensive cerebrovascular remodeling. Together, our data indicate that an endophilin A2-associated trafficking abnormality may occur during hypertension, which may contribute to the alteration of CIC-3 function and expressions during the VSMC proliferation and hypertensive vascular remodeling.

N-terminal BAR domain and SH3 domain of endophilins are 2 key players in the regulation of membrane remodeling and reorganization of the protein-protein interaction partners.\textsuperscript{37,38} The SH3 domains of endophilin recognize and bind to the proline-rich domain (PRD), which is important for endophilin-dynamin interaction\textsuperscript{20} and endophilin-ion channel interaction. Our results from mutant analysis demonstrated that it was the SH3 domain of endophilin A2 that interact with CIC-3 and influence subcellular CIC-3 localization to the cell membrane. Whereas the N-BAR domain and PRD were not necessary. There is no PRD in CIC-3, and the ∆PRD mutant showed no effects on the interaction between endophilin A2 and CIC-3 or on the CIC-3 membrane trafficking. It therefore suggests an unknown intermolecular mechanism underlying the SH3-dependent protein-protein interaction in CIC-3 trafficking that is not reliant on PRD-recognition.

In addition to the SH3 domain, several membrane-binding mechanisms have been ascribed to CIC-3 channels, such as Src, phospholipase C-Ins(3,4,5,6) and P4.\textsuperscript{40,41} Several putative trafficking motifs within CIC-3 were also suggested for CIC-3 trafficking during diverse biological processes. For example, CIC-3 could interact with clathrin and regulate the endocytosis and surface expression of CIC-3.\textsuperscript{17} Our recent work has demonstrated that tyrosine 284 phosphorylation targeted by Src is associated with CIC-3 channel activation upon hypotonic stress in VSMCs.\textsuperscript{25,40} However, the tyrosine phosphorylation signaling appears not to be involved in the endophilin A2-mediated CIC-3 trafficking mechanism, which may be due to their different influences on the subcellular localization of CIC-3. In the basal state, exogenous endophilin A2 transfection could regulate CIC-3 subcellular localization, but it is not the case for tyrosine 284 phosphorylation.

One novel finding here is that we had recorded a larger hypotonic-activated outward Cl\textsuperscript{−} current in BASMCs overexpressing endophilin A2 or from endophilin A2 transgenic mice. The properties of the overexpressed or transgenic endophilin A2 Cl\textsuperscript{−} current were generally similar to those of VRACs. Given that we have a well-established association of CIC-3 with IC\textsubscript{vol} in VSMCs, particularly during the development of hypertension, endophilin A2 may be reasonably interpreted as a new CIC-3 trafficking mechanism for membrane IC\textsubscript{vol} regulation. The ability of CIC-3 siRNA to significantly reduce the endophilin A2-dependent, hypotonic-activated outward Cl\textsuperscript{−} current strengthened this notion in VSMCs. Currently, accumulating evidence has demonstrated that CIC-3 serves as one molecular component involved in the activation or regulation of IC\textsubscript{vol} in VSMCs and some other cell types.\textsuperscript{16}

As an essential component for outward Cl\textsuperscript{−} current or [Cl\textsuperscript{−}]\textsubscript{i} in VSMCs, CIC-3 has been well established to play a critical role in vascular inflammatory and proliferative diseases. Our present findings in VSMCs thus provide a potential mechanism for CIC-3 function as a cell membrane Cl\textsuperscript{−} channel. Another important factor is that different cells exhibit different subcellular distributions of CIC-3, even in resting states, which may be one reasonable explanation for the existence of cell type-specific interdependence of CIC-3 and IC\textsubscript{vol}. In addition, we previously demonstrated that inhibition of CIC-3 expression and IC\textsubscript{vol} contributes to the beneficial effects of simvastatin, which is clinically used for the prevention of a secondary stroke, on BASMC proliferation and vascular remodeling during 2k2c hypertension. Based on the observation that endophilin A2 expression and the surface membrane CIC-3 accumulation in BASMCs were gradually upregulated during the development of hypertension, the present findings suggest that endophilin A2-mediated CIC-3 trafficking may represent an attractive target for pharmacological or therapeutic interventions in vascular remodeling induced by hypertension.

In conclusion, the present study identified the SH3 domain of endophilin A2 as a CIC-3 channel partner, which serves as a new CIC-3 trafficking mechanism regulating IC\textsubscript{vol} in VSMCs. Endophilin A2 may be therefore involved in the development of cardiovascular diseases related to altered CIC-3 signaling (ie, hypertensive cerebrovascular remodeling). In addition, our data provides insight for new investigations of endophilin A2 in physiological and pathological functions of the vascular system.
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Supplementary Files

Supplementary File 1

Methods (Expanded)

Figure S1. Expression profile of the endophilin A subfamily in rat basilar arterial smooth muscle cells (BASMCs) and brain tissue.

Figure S2. Efficiency of endophilin A2 siRNA knockdown and 

Figure S3. Inhibition of a native hypotonic-activated Cl– current in basilar arterial smooth muscle cells (BASMCs) by anti-endophilin A2 antibody intracellular dialysis.

Figure S4. Large hypotonic-activated Cl– current induced by adeno-

Figure S5. Endophilin A2 in transgenic mice had no effect on body weight and systolic blood pressure compared to its littermate control.

Figure S6. The SH3 domain participated in CIC-3 trafficking from the cytoplasm to the cell surface in A10 cells. Please find supplementary file(s);

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