A Critical Role for Chloride Channel-3 (CIC-3) in Smooth Muscle Cell Activation and Neointima Formation

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Objective—We have shown that the chloride-proton antipporter chloride channel-3 (CIC-3) is required for endosome-dependent signaling by the Nox1 NADPH oxidase in SMCs. In this study, we tested the hypothesis that CIC-3 is necessary for proliferation of smooth muscle cells (SMCs) and contributes to neointimal hyperplasia following vascular injury.

Methods and Results—Studies were performed in SMCs isolated from the aorta of CIC-3-null and littermate control (wild-type [WT]) mice. Thrombin and tumor necrosis factor-α (TNF-α) each caused activation of both mitogen activated protein kinase extracellular signal–regulated kinases 1 and 2 and the matrix-degrading enzyme matrix metalloproteinase-9 and cell proliferation of WT SMCs. Whereas responses to thrombin were preserved in CIC-3-null SMCs, the responses to TNF-α were markedly impaired. These defects normalized following gene transfer of CIC-3. Carotid injury increased vascular CIC-3 expression, and compared with WT mice, CIC-3-null mice exhibited a reduction in neointimal area of the carotid artery 28 days after injury.

Conclusion—CIC-3 is necessary for the activation of SMCs by TNF-α but not thrombin. Deficiency of CIC-3 markedly reduces neointimal hyperplasia following vascular injury. In view of our previous findings, this observation is consistent with a role for CIC-3 in endosomal Nox1-dependent signaling. These findings identify CIC-3 as a novel target for the prevention of inflammatory and proliferative vascular diseases. (Arterioscler Thromb Vasc Biol. 2011;31:345-351.)

Key Words: cytokines ■ metalloproteinases ■ reactive oxygen species ■ restenosis ■ signal transduction

There is widespread appreciation that atherosclerosis is a chronic inflammatory disease.1 Proliferation of smooth muscle cells (SMCs) is fundamental to the development of vascular diseases, including arteriosclerosis, restenosis, and bypass graft failure.2,3 In addition to cell division, these processes require SMCs to secrete proteases to degrade extracellular matrix and facilitate the subsequent migration of cells from the media into the forming neointima. A variety of signaling molecules have been implicated in stimulating SMCs to undergo this process.

See accompanying article on page 240

Thrombin is a serine protease in the clotting cascade that converts soluble fibrinogen to insoluble fibrin. Thrombin also cleaves and activates the G-protein–coupled protease-activated receptor 1, which causes proliferation and increased motility via activation of the epidermal growth factor receptor (EGFR).4,5 Protease-activated receptor 1 activation contributes to the pathogenesis of atherosclerosis and restenosis via a variety of mechanisms,6 many of which are mediated via activation of the EGFR.8 Mice deficient in the protease-activated receptor 1 receptor display an altered response to vascular injury.7

The proinflammatory cytokine tumor necrosis factor-α (TNF-α) localizes to areas of arterial injury8,9 and contributes to development of neointimal hyperplasia.10,11 Although macrophages are a primary source of cytokines, vascular endothelium and SMCs also secrete TNF-α.12,13 On exposure to TNF-α, quiescent SMCs express matrix metalloproteinases (MMPs) and increase migration and proliferation by intracellular signaling pathways which involve activation of the transcription factor nuclear factor–κB (NF-κB).14,15 Animals lacking TNF-α display remarkably reduced activation of NF-κB and neointima formation after carotid injury.10

CIC-3 is a member of the CLC family of chloride-transporting proteins, which includes both anion channels (CIC-1 and CIC-2) and Cl−/H+ antiporters (CIC-3 through 7).16 CIC-3 is present in all cell types and localized in plasma membranes and in intracellular vesicles.17–19 In early endosomes of SMCs and in secretory vesicles of neutrophils, CIC-3 is necessary for activation of NADPH oxidase by cytokines.20–22

We have recently demonstrated that both thrombin and TNF-α signaling are dependent on production of reactive oxygen species (ROS) via activation of the Nox1 NADPH oxidase.23 Nox1 plays an essential role in SMC proliferation,
motility, and the inflammatory cascade associated with NF-κB activation. It is therefore not surprising that Nox1 signaling contributes to neointima development after acute vascular injury. However, we have demonstrated that the mechanism and subcellular location of Nox1 activation by thrombin and TNF-α is very different. Whereas TNF-α induces a Nox1-dependent, CIC-3-dependent, endosomal generation of intracellular ROS, thrombin activates Nox1 such that ROS does not occur within endosomes and does not require CIC-3. We have proposed that CIC-3 provides charge neutralization for the NADPH oxidase electron current that is required for generation of ROS in signaling endosomes following cytokine activation. On the basis of these findings, we hypothesized that CIC-3 is critical to the activation of SMCs by TNF-α but not to thrombin. We therefore determined whether CIC-3 was required for activation of extracellular signal-regulated kinases 1 and 2 (ERK1/2) and MMP-9 or for cell proliferation in response to thrombin or TNF-α. In addition, because the contribution of CIC-3 to the development of vascular disease has not been previously tested, we assessed the requirement of this protein for neointimal hyperplasia following carotid injury.

Materials and Methods

A full description of all methods can be found in the Supplemental Materials, available online at http://atvb.ahajournals.org. SMCs were isolated from aorta of mice lacking the Clcn3 gene encoding CIC-3 chloride channels (CIC-3-null) and from wild-type (WT) littermate controls as previously described. For experiments, SMCs were serum deprived (0.5% serum) for 24 hours before stimulation with TNF-α (10 ng/mL) or thrombin (2 U/mL). The long N-terminal isoform of human CIC-3 (P51790) was polymerase chain reaction amplified and cloned into the adenovirus shuttle plasmid pacAd5 cytomegalovirus behind the cytomegalovirus promoter and used for reconstitution of CIC-3 expression in the null cells. Cell proliferation was determined by measuring [³H]thymidine incorporation. MMP activity within the media was measured by gelatin zymography. The blood flow cessation model was used to induce carotid neointimal hyperplasia as previously described in CIC-3-null and littermate controls.
control mice and carotid arteries harvested 28 days later. Results are expressed as mean ± standard error of the mean. Statistical comparisons were performed by the Student 2-tailed t test or analysis of variance with Tukey multiple comparison post test as appropriate.

Results

Role of CIC-3 in Proliferation of SMCs

In response to either thrombin or TNF-α, WT SMCs increased [3H]thymidine incorporation more than 2-fold. In contrast, SMCs deficient in CIC-3 responded similarly to thrombin but failed to respond to TNF-α (Figure 1A and 1B). However, adenovirus-mediated gene transfer of CIC-3 to CIC-3-null SMCs normalized [3H]thymidine incorporation in response to TNF-α (Figure 1C), suggesting a requirement for CIC-3 in TNF-α-mediated proliferation. The overexpression of CIC-3 in WT SMCs increased [3H]thymidine uptake under basal conditions without affecting the maximal response to TNF-α (Figure 1C). Further supporting a role for CIC-3 in SMCs growth, the chloride channel inhibitor niflumic acid (NFA, 0.3 mmol/L) inhibited TNF-α-dependent [3H]thymidine incorporation in WT SMCs (2.1 ± 0.3 versus 0.2 ± 0.1 for dimethyl sulfoxide + TNF-α versus NFA + TNF-α, n=4, P < 0.05).

Role of CIC-3 in Secretion of MMP-9

As detected by gelatin zymography, MMP-9 activity in the culture media increased when SMCs were treated for 24 hours with thrombin (Figure 2A) or TNF-α, respectively (Figure 2B). SMCs deficient in CIC-3 responded normally to thrombin but failed to produce the normal increase in MMP-9 activity in response to TNF-α. This response was corrected following gene transfer of CIC-3 (Figure 2B). Consistent with these observations, pretreatment of WT SMCs with NFA markedly inhibited TNF-α-induced MMP-9 activation (4.2 ± 0.3 versus 0.7 ± 0.1 for dimethyl sulfoxide + TNF-α versus NFA + TNF-α, n=4, P < 0.05).

We next confirmed that these observations reflected changes in MMP-9 activation and not in expression by CIC-3-null SMCs. WT and CIC-3-null mice were serum deprived for 24 hours before stimulation with TNF-α or thrombin for 24 hours, and cells were collected for real-time polymerase chain reaction and Western blotting. There were no differences in the mRNA levels of MMP-9 between the genotypes and treatments (n=4, data not shown). Expression of MMP-9 protein was also similar in untreated WT and CIC-3-null cells (Figure 2C). Following TNF-α and thrombin treatment, protein levels of MMP-9 were reduced in WT cells, consistent with its secretion into the media (as shown in Figure 2A and 2B). In contrast, expression of MMP-9 by CIC-3-deficient cells was not significantly reduced by TNF-α.

Role of CIC-3 in TNF-α-Induced Activation of ERK1/2

Thrombin and TNF-α have been previously shown to activate multiple signaling pathways in SMCs, including ERK1/2.29,30 We tested the hypothesis that CIC-3 is necessary for activation of ERK1/2 by these agonists. Five minutes after treatment of SMCs with thrombin (Figure 3A), phosphorylation of ERK1/2 was increased approximately 2-fold. This effect was unaltered in cells lacking CIC-3. In response to TNF-α (Figure 3B), phosphorylation of ERK1/2 was increased in WT SMCs, but CIC-3-null SMCs did not respond. To exclude a delay in signaling, we also found that TNF-α failed to activate ERK1/2 in CIC-3-null SMCs at 10 and 15 minutes after stimulation (data not shown). However, adenovirus-
mediated gene transfer of ClC-3 rescued activation of ERK1/2 in ClC-3-deficient SMCs (Figure 3B). Furthermore, NFA inhibited TNF-α-induced ERK1/2 activation in WT SMCs (1.7 ± 0.1 versus 0.6 ± 0.1 for dimethyl sulfoxide + TNF-α versus NFA + TNF-α, n = 4, P < 0.05). We next examined the role of ERK1/2 in cell proliferation and activation of MMP-9 in SMCs following stimulation by TNF-α. The mitogen activated protein kinase (MEK) 1/2 inhibitor U012631 reduced baseline [3H]thymidine incorporation and prevented the proliferative response to TNF-α in WT SMCs (Figure 3C). Overexpression of ClC-3 failed to restore proliferation in the presence of MEK1/2 inhibition (data not shown). U0126 had a similar effect on MMP-9 activation (Figure 3D), which is consistent with previous reports.32 Taken together, these observations suggest that in response to TNF-α, ClC-3 is proximal to activation of ERK1/2, which is necessary for the signaling of proliferation and MMP-9 activation.

Role of ClC-3 in Neointimal Hyperplasia

Expression of Nox1 is increased early after balloon injury of rat arteries and remains elevated in association with the development of neointima.33 We recently demonstrated that activation of NF-κB by Nox1 requires ClC-3.20 Therefore, we next examined expression of ClC-3 in vascular tissues following cytokine activation or vascular injury. SMC expression of ClC-3 increased more than 3-fold following exposure to TNF-α (Figure 4A). Extending these observations in vivo, the left carotid artery was ligated in mice as a model of neointimal hyperplasia.28 Ten days following surgery, which is before the development of neointima,34 ClC-3 expression was increased approximately 2.5-fold in the injured carotid artery, as compared with the contralateral noninjured artery (Figure 4B).

The observation that SMCs modulate expression of ClC-3 in response to cytokines and vascular injury, together with our findings that SMCs are dependent on ClC-3 for cellular signaling, matrix degradation, and proliferation, suggests a role for ClC-3 in the vascular response to injury. To test this hypothesis, we performed carotid ligation in WT and ClC-3-null mice. Twenty-eight days following ligation, WT mice developed a robust neointima that was significantly attenuated in the ClC-3-null mice (Figure 5A). Deficiency of ClC-3 resulted in significant reduction in intimal area (Figure 5B) and the intima:media ratio (Figure 5C) as compared with WT mice. Flow-induced outward remodeling is characteristic of the right carotid artery after ligation of the left carotid.35 The external elastic lamina perimeter was greater in ClC-3-null mice as compared with WT mice (1.08 ± 0.01 versus 1.01 ± 0.02, n = 6, P < 0.01).

Discussion

Inflammation, matrix remodeling, and SMC proliferation are key features in the development of atherosclerosis, restenosis after angioplasty, vein-graft stenosis, and transplant arteriosclerosis. In this study, we show that exposure of cultured SMCs to thrombin or TNF-α enhances secretion of the matrix degrading enzyme MMP-9, activates the mitogen-activated protein kinase signaling cascade ERK1/2, and increases cell

Figure 4. ClC-3 expression increases in response to TNF-α and carotid injury. ClC-3 mRNA levels were measured by real-time polymerase chain reaction in WT SMCs treated with TNF-α for 6, 24, or 48 hours (*P < 0.05 versus control, n = 3) (A) and carotid arteries collected 10 days following ligation (*P < 0.05 versus noninjured, n = 3) (B).

Figure 5. Deficiency of ClC-3 protects from neointimal formation. Twenty-eight days following common carotid ligation, right (noninjured) and left (injured) carotid arteries were collected, sectioned 0.5 mm proximal to the ligation, and stained. A, Representative photomicrographs. Original magnification, ×10; scale bar represents 50 μm. B, Intimal area of injured carotid artery. Each symbol represents the mean intimal area of 3 sections of 0.5 mm proximal to the ligation from 1 animal. Mean ± SEM is shown by bar to right of group data. *P < 0.05 versus WT. C, Summary data of the ratio of intimal area to medial area of injured carotid in WT and ClC-3-null mice. *P < 0.05 versus WT, n = 6.
proliferation. The primary novel finding of this study is the specific and distinct requirement for the CIC-3 Cl⁻/H⁺ antiporter in mediating these effects. Whereas responses to thrombin are maintained, those to TNF-α are markedly attenuated in SMCs deficient in CIC-3. Overexpression of CIC-3 augments the growth of WT SMCs, carotid injury induces vascular expression of CIC-3, and neointima development is impaired after carotid injury in CIC-3-null mice. These observations demonstrate a role for CIC-3 in agonist-specific activation of SMCs and provide the first evidence that CIC-3 contributes to neointimal hyperplasia in response to vascular injury. Our results identify CIC-3 as a potential therapeutic target in inflammatory and proliferative vascular disease.

The majority of CIC-3 protein is localized intracellularly but cycles through the plasma membrane, where it has a half-life of less than 10 minutes in resting cells. In various cell types, CIC-3 has been localized to intracellular vesicles and proposed to act as an anion channel and regulate vesicular acidification by providing charge neutralization for the V-ATPase proton pump. However, we have recently provided direct evidence that CIC-3 is an antiporter and have proposed that CIC-3 is required for charge neutralization of electron flow through the NADPH oxidase when generation of ROS is within vesicles.

NADPH oxidases are a primary source of signaling ROS in vascular cells that activate both normal physiological and pathological processes. We have identified spatially distinct pathways of Nox1 activation and ROS production by TNF-α and thrombin. Whereas TNF-α results in dynamin-dependent Nox1 generation of ROS within early endosomes, Nox1 activation by thrombin involves extracellular shedding of epidermal growth factor–like ligands and activation of the receptor tyrosine kinase EGFR independent of endocytosis. It is expected that these distinct differences in activation of Nox1 provide specificity in cell signaling, possibly distinguishing between inflammatory and noninflammatory stimuli. Activation of G-protein–coupled receptors causes shedding of ligands and subsequent transactivation of EGFR. This process may represent a feed-forward mechanism necessary for amplification of intracellular ROS production and cell signaling.

Activation of mitogen-activated protein kinases is central to initiating cellular responses in vascular injury. ERK is rapidly phosphorylated in balloon-injured arteries and has been shown to be important in the activation of MMP-9 and in cell proliferation. This was confirmed by our observation that inhibition of ERK phosphorylation with U0126, a selective pharmacological inhibitor of MEK, prevented the TNF-α-dependent increase in MMP-9 and [³H]thymidine incorporation by SMCs. Further highlighting the importance of ERK in vascular injury, gene transfer of WT ERK increased, whereas gene transfer of a dominant-negative mutant ERK decreased neointimal hyperplasia in carotid arteries following balloon injury.

On the basis of pharmacological effects of anion channel blockers, anion currents have long been linked to proliferation of multiple cell types. Reduction of CIC-3 protein levels using antisense oligonucleotides decreased proliferation of SMCs in response to ET-1, and CIC-3 mRNA expression was proportional to [³H]thymidine incorporation. The current work supports these observations, because CIC-3-null cells clearly display impaired TNF-α-induced proliferation. We extend these observations in vivo and show reduced neointima formation in CIC-3-null mice following vascular injury.

In addition, we found that CIC-3 expression increased in cultured SMCs and in carotid arteries following TNF-α and carotid ligation, respectively. This is consistent with the previous report that CIC-3 expression is increased in SMCs of pulmonary arteries from rats with experimental pulmonary hypertension and following incubation with endothelin-1, platelet-derived growth factor, and interleukin-1β. These observations suggest that changes in CIC-3 expression or activity may be central in the regulation of cell growth.

Proliferation of SMCs in response to cytokines, including TNF-α, plays an important role in the formation of atherosclerotic and restenotic lesions. TNF-α activates the transcription factor NF-κB, which is widely recognized as a key regulatory step in vascular inflammation. In vivo transfection of NF-κB decoy oligodeoxynucleotides into rat carotid artery inhibits neointimal formation after injury. We previously showed that NF-κB activation was markedly inhibited in CIC-3-null cells, and that the defect was a function of impaired ROS production following cytokine stimulation. The current data showing defects in ERK1/2 and MMP-9 activation as well as proliferation are consistent with prior results. ERK1/2 activation is linked to ROS production and as we have demonstrated here, ERK1/2 is upstream of MMP-9 activation and proliferation.

The responses to vascular injury are complex and dependent on the model studied. The most commonly used murine..........................
models to induce neointimal hyperplasia are perivascular cuff, wire injury, and carotid ligation. A potential limitation of our in vivo findings is that ligation results in cessation of blood flow and thrombus formation, which may activate cellular processes distinct from models of mechanical injury. Nevertheless, vascular lesions in humans often develop at sites of low shear stress, and the carotid ligation model has advantages of reproducibility and simplicity.

In summary, this study presents in vitro data supporting a role for the chloride antiporter ClC-3 in MMP-9 activation and SMC proliferation via impaired TNF-α-mediated activation of ERK1/2 (Figure 6). In addition, in vivo data demonstrate that deficiency of ClC-3 inhibits neointimal hyperplasia in a model of carotid injury. Based on these observations, we propose that ClC-3 is a new therapeutic target for the prevention of vascular disease.

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Disclosures

None.

References


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Supplemental Material

Chemicals. Fetal Bovine Serum (FBS), Dulbecco’s Modified Eagle’s Medium (DMEM), penicillin-streptomycin mixtures, glutamine, MEM amino acids solution, Trypsin/EDTA and HEPES buffer solution were purchased from Gibco BRL (Grand Island, NY). [Methyl-\(^{3}\)H] thymidine (1.0mCi/ml) was from PerkinElmer Life and Analytical Sciences (Boston, MA). Antibodies to phospho-ERK1/2 and to ERK2 were from Santa Cruz Biotechnology (Santa Cruz, CA), U0126 was from Calbiochem Chemicals Inc (San Diego, CA), and MMP-9 from ABCAM (Cambridge, MA). Horseradish peroxidase-conjugated goat anti-mouse, and rabbit anti-goat antibodies were from Bio-Rad Laboratories (Hercules, CA). ECL Western blotting detection reagents were from Amersham Bioscience (Piscataway, NJ). TRIzol and SuperScript II Reverse Transcriptase were obtained from Invitrogen (Carlsbad, CA). cDNA synthesis kit was purchased from Bioline USA inc.(Randolph, MA). All other chemicals were obtained from Sigma Chemical (St. Louis, MO).

Cell Culture. SMCs were isolated from aorta of mice lacking the Clcn3 gene encoding ClC-3 chloride channels \(^1\) (ClC-3 null) and from wild-type (WT) littermate controls as previously described \(^2\) and grown in 10% fetal calf serum in DMEM containing 1% BME, 1% glutamine, 1% penicillin/streptomycin, 1% MEM 100x non-essential amino acids and 2% HEPES. Studies were performed on three independent isolations of ClC-3 null SMCs at passages 5 to 10 and 70-90% confluence. For experiments, SMCs were serum-deprived (0.5% serum) for 24 hours prior to stimulation with TNF-\(\alpha\) (10ng/ml) or thrombin (2 U/ml).
Gene Transfer of ClC-3. The long N-terminal isoform of human ClC-3 (P51790) was PCR amplified and cloned into the adenovirus shuttle plasmid pacAd5 CMV. Bicistronic adenoviruses co-expressing ClC-3 (AdClC-3) behind the CMV promoter and enhanced green fluorescent protein (eGFP) behind the RSV promoter were prepared and titrated by the University of Iowa Gene Transfer Vector Core. Adenovirus encoding green fluorescent protein (AdGFP) or AdClC-3 was mixed with the cationic polymer poly-L-lysine (250 molecules/virus particle) and added to SMCs (70% confluence, 50 MOI) in serum-free DMEM. After 4 hours, the media was replaced with DMEM containing 10% serum and experiments were performed 48 hours later.

Cell proliferation. Cell proliferation was determined by measuring [\(^{3}\)H]-thymidine incorporation into SMCs. Following AdClC-3 or AdGFP, cells were serum-deprived for 24 hours then treated with thrombin or TNF-\(\alpha\) for 24 hours, incubated in 1 \(\mu\)Ci/ml of [\(^{3}\)H]-thymidine per well for the final 4 hours. Cells were washed with phosphate buffered saline and lysed in cold 10% (w/v) trichloroacetic acid. After 20 minutes at 4\(^\circ\) C, 1N sodium hydroxide was added at room temperature. After 20 minutes, 1N HCL was added and radioactivity measured in a liquid scintillation counter (Beckman LS6000 SC, Fullerton, CA). In some experiments, the chloride channel inhibitor niflumic acid (NFA) was added 2 hours prior to TNF-\(\alpha\) and equal concentration of DMSO used for vehicle control.
MMP-9 Gelatin Zymography. SMCs were serum-deprived for 24 hours then treated with thrombin or TNF-α for 24 hours. The culture medium was collected and centrifuged at 12 g for 5 minutes at 4°C. Following electrophoresis (10% Zymogram gels, Bio-Rad Laboratories, CA), gels were soaked in NOVEX™ zymogram renaturing buffer (Invitrogen, CA) for 1 hour at room temperature and incubated in NOVEX™ zymogram developing buffer (Invitrogen, CA) at 37°C for 48 hours, to allow proteinase digestion of its substrate. Gels were then stained in solution of 40% methanol, 10% acetic acid, and 0.25% brilliant blue R-250 for 1 hour and destained with solution containing 10% acetic acid glacial, 40% ethyl alcohol. Gelatinolytic activity appeared as clear bands of digested gelatin against a dark blue background of stained gelatin.

Detection of ERK1/2 by Immunoblotting. SMCs were grown to 80% confluence, then incubated with DMEM containing 0.5% serum for 24 hours. Cells were treated with thrombin or TNF-α for 5 minutes then lysed in radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM ethylenediamine tetra-acetic acid, 1 mM sodium orthovanadate, 1 mM sodium fluoride) and complete protease inhibitors cocktail (Roche Diagnostic, USA). The lysates were collected and centrifuged at 12,000 g for 5 min, the supernatants mixed with reducing sample buffer and boiled for 5 minutes. Proteins (15 μg) were separated by using 12% SDS-polyacrylamide gel electrophoresis and transferred to poly vinylidene difluoride (PVDF) membranes (Millipore Corporation, MA, USA). The membranes were blocked with 10% non-fat milk in TBS-T (20 mM Tris, pH 7.5, 0.9% NaCl and 0.1% Tween-20) for 1 hour at room temperature, and then
incubated overnight at 4°C with a primary antibody against ERK1/2 (1:5000) or phospho-ERK1/2 (1:5000). The membranes were washed with 10% non-fat milk or 1%BSA and then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated anti-goat or anti-mouse IgG (1:5000). After washing the membranes in TBS-T buffer, immunoreactive signals were detected by an ECL advance chemiluminescence reagent. Data are reported as ratios of p-ERK:ERK densitometry.

Real-time PCR. Total mRNA was isolated from cultured SMCs or from intact carotid arteries obtained ten days following ligation surgery (see below) using Tri-Reagent (Molecular Research Center). Relative copy number of ClC-3 was determined by real-time PCR using QuantiTect SYBR Green PCR kit (Qiagen Inc, Valencia, CA) and ABI Prism 7000 (Applied Biosystems, Foster City, CA) detection system, normalized to 18s ribosomal RNA (Applied Biosystems, Foster City, CA). For ClC-3, the forward and reverse primers were CGTGAGAACCGCGTTACTTTC and TGCGCTCTCCAAGGACTACA. For MMP-9, the forward primer and reverse primers were CAAGTGGGACCATCATAACATCA and CTCGCGGCAAGTCTTCAGA.

Model of carotid injury. The blood flow cessation model was used to induce carotid neointimal hyperplasia as previously described 4. At 20 to 24 weeks of age, male and female ClC-3 null and littermate control mice on a normal chow diet were anesthetized using intraperitoneal ketamine-HCl (80 mg/kg) and xylazine-HCl (10mg/kg) and placed on a warming pad. Following a midline neck incision, the left common carotid artery was isolated by blunt dissection using a dissecting microscope. A 7-0 silk suture was passed
under the common carotid artery just proximal to the bifurcation of the external and internal carotid branches, and tied to totally ligate the artery. The skin was closed with 5-0 Monocryl interrupted suture. At 28 days following surgery, the mice were euthanatized with pentobarbital sodium (50 mg/kg IP). Animals received intracardiac heparinized saline followed by 5% formalin at a constant perfusion pressure of 100 mm Hg. All procedures were approved by the Institutional Animal Care and Use Committee at University of Iowa and complied with the standards stated in the Guide for the Care and Use of Laboratory Animals.

*Morphometric analysis.* The left and right carotid arteries were removed, incubated overnight in 5% formalin, and paraffin-embedded. Sections were obtained at 0.5 and 1.0 mm proximal to the ligation and stained with Verhoeff-van Gieson. Images digitized and the luminal area, the area inside the internal elastic lamina (IEL), and the area encircled by the external elastic lamina (EEL) were measured in each section. The medial area was calculated as the area between the IEL and EEL, the intimal area was calculated by subtracting the luminal area from the area inside the IEL. Data from three adjacent sections were average for each specimen.

*Statistical Analysis.* Results are expressed as mean ± standard error of the mean (SEM). Statistical comparisons were performed by Student’s two-tailed t-tests or analysis of variance (ANOVA) with Tukey multiple comparison post-test as appropriate. A value of p<0.05 was considered statistically significant.
References


