Chloride Intracellular Channel-4 Is a Determinant of Native Collateral Formation in Skeletal Muscle and Brain

Dan Chalothorn, Hua Zhang, Jennifer E. Smith, John C. Edwards, James E. Faber

Abstract—The capacity of the collateral circulation to lessen injury in occlusive vascular disease depends on the density and caliber of native (preexisting) collaterals, as well as their ability to outwardly remodel in ischemia. Native collateral conductance varies widely among healthy individuals, yet little is known about what specifies collateral formation. Chloride intracellular channel (CLIC)4 protein is required for endothelial cell hollowing, a process necessary for vessel formation during embryogenesis and ischemia. Whether CLIC4 has other physiological roles in vascular biology is uncertain. We studied collateral formation and remodeling in mice deficient in CLIC1 and CLIC4. Vascular responses to femoral artery ligation were similar in Clic1−/− and wild-type mice. In contrast, immediately after ligation perfusion dropped more in Clic4−/− than wild-type mice, suggesting fewer preexisting collaterals, a finding confirmed by angiography, greater ischemia, and worse recovery of perfusion; however, collateral remodeling was unaffected. Likewise, native cerebral collateral density in Clic4−/− (but not Clic1−/−) mice was reduced, resulting in severe infarctions. This was associated with impaired perinatal formation and stabilization of nascent collaterals. Clic4 hemizygous mice had intermediate deficits in the above parameters, suggesting a gene–dose effect. Ischemia augmented CLIC1 and CLIC4 expression similarly in wild-type mice. However, CLIC1 increased 3-fold more in Clic4−/− mice, suggesting compensation. Despite greater ischemia in Clic4−/− mice, hypoxia-inducible factor-1α, vascular endothelial growth factor (VEGF) and angiopoietin-2 increased less compared to wild-type, suggesting CLIC4 exerts influences upstream of hypoxia-inducible factor-1α–VEGF signaling. Hence, CLIC4 represents the second gene that, along with VEGF shown by us previously, specifies native collateral formation. (Circ Res. 2009;105:89-98.)

Key Words: arteriogenesis ■ intracellular chloride channels ■ cerebral circulation ■ angiogenesis ■ vascular development

Ischemic vascular disease of the heart, brain, and peripheral limbs are leading causes of morbidity and death. In dependent tissues, angiogenesis can only improve distribution of the remaining arterial inflow, whereas collateral remodeling (“arteriogenesis”) is capable of restoring the original resting flow.1,2 Thus, the density and diameter of native (preexisting) collaterals in healthy tissues and their capacity to remodel in ischemia are major determinants of the severity of tissue injury in obstructive vascular disease.3–5 Evidence suggests that native collateral conductance varies widely in healthy individuals.5,6 This presumably extends from the influence of as yet unknown genetic and environmental factors.

Identification of such factors requires an understanding of how these unique intertree artery-to-artery connections are formed. However, whereas the mechanisms that direct collateral remodeling are receiving considerable attention,3–5 no studies have examined when or how the native collateral circulation forms, although formation has been suggested to occur embryonically.7 We recently reported that collaterals are present at birth and mature/stabilize rapidly thereafter and that vascular endothelial growth factor (VEGF) is important in both processes.8 VEGF is critical in vascular development, wherein VEGF isoform gradients, in concert with hemodynamic and other factors, expand and remodel the primary embryonic plexus into the mature network present at birth,9,10 through new vessel sprouting, intussusception, branching morphogenesis, pruning, and remodeling of existing vessels and subsequent mural cell recruitment.11,12 Variation in these processes may underlie the involvement of VEGF in the wide variation in collateral conductance among healthy individuals.8

Undoubtedly, molecules in addition to VEGF are involved in collateral formation. Chloride intracellular channel (CLIC)4 belongs to a newly described family of proteins with actions distinct from traditional cell membrane chloride channels that include formation of anion channels in intracellular organelles and involvement in membrane trafficking,13 apoptosis,14 and cell differentiation.15 CLIC4 is highly expressed in endothelial cells of renal16 and retinal17 blood vessels, as well as in formation of new capillary sprouts in embryoid bodies and tumors.16 Interestingly, Bohman et al
have implicated CLIC4 in VEGF-induced tubulogenesis in mammalian endothelial cells. A similar process (epithelial cell hollowing and formation of the alimentary canal in Caenorhabditis elegans) is mediated by a CLIC homolog denoted exc-4. Moreover, Ulmasov et al recently demonstrated that Clic4 mice show defective angiogenesis both in a Matrigel plug assay and during oxygen-induced retinal neoangiogenesis, that endothelial cells from Clic4 mice show impaired tubulogenesis in culture, that intracellular endothelial vacuoles along the tubulogenic pathway acidify, and that vacuolar acidification is defective in Clic4 endothelial cells. These studies identify important functions of CLIC proteins in vessel formation, ie, endothelial cell hollowing and tubulogenesis.

Presently, we examined involvement of CLIC4, as well as CLIC1, which is expressed widely, in determining collateral density and diameter in healthy tissue and in collateral remodeling in hindlimb ischemia. Given the above evidence for the role of CLIC4 in VEGF-mediated vessel formation, together with findings that VEGF contributes to collateral formation in the embryo, maturation in the neonate, and remodeling in ischemia, we hypothesized that CLIC proteins contribute to all 3 processes.

Materials and Methods

Procedures were conducted blindly. Clic4−/− were on CD-1 background; Clic1−/− mice were constructed similarly (manuscript in preparation). Both lack an apparent phenotype (see Results). Wild-type littermates served as controls. The right femoral artery was ligated distal to the lateral caudal femoral and superficial epigastric arteries (latter also ligated) and proximal to the genu, followed by transection. Histology, angiography, and arteriography were performed after pressure-perfused maximal dilation and fixation. Postmortem arterial microangiography was done with lead-based latex with a viscosity sufficient to minimize capillary transit on separate groups after right femoral ligation 7 days earlier and left femoral ligation on day-7. Data (±SEM) were tested by ANOVA, Dunn–Bonferroni, Student’s t tests, or Mann–Whitney U tests.

An expanded Materials and Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

Results

Clic4−/− Mice Have Reduced Hindlimb Perfusion Immediately After Femoral Artery Ligation and Reduced Recovery of Perfusion

Mice with CLIC1 and CLIC4 disruption (Clic1−/−, Clic4−/−) have minimal apparent phenotype in unstressed laboratory conditions, except that Clic4−/− have less dense retinal vasculature and lower body weight (herein, Clic4−/−, 29±2 g; CD-1 wild-type, 32±2 g; Clic1−/−, 30±2 g). Immediately after ligation, perfusion in the plantar foot (Figure 1a and 1c), which correlates with overall hindlimb blood flow, dropped to similar values in Clic1−/− and wild-type mice. However, perfusion decreased more in Clic4−/−. Lower conductance of the native collateral circulation results in lower plantar perfusion immediately after ligation. Thus, these data suggest that Clic4−/− mice (but not Clic1−/−) have lower perfusion immediately after femoral artery ligation and reduced recovery of perfusion. Doppler images (a and b) showing regions of interest (outlined area) for quantification (c and d) of perfusion of the plantar foot (index of hindlimb perfusion) and adductor thigh (index of combined collateral and adductor perfusion). Plantar and adductor perfusion decreased more in Clic4−/− immediately after ligation, suggesting lower collateral conductance, but recovered at a similar rate relative to wild-type, suggesting similar collateral remodeling. *P<0.05, **P<0.01 vs wild type. In this and subsequent figures, values are means±SEM for n number of mice per group, time point, or bar.

Figure 1.

Wild-type littermates served as controls. The right femoral artery was ligated distal to the lateral caudal femoral and superficial epigastric arteries (latter also ligated) and proximal to the genu, followed by transection. Histology, angiography, and arteriography were performed after pressure-perfused maximal dilation and fixation. Postmortem arterial microangiography was done with lead-based latex with a viscosity sufficient to minimize capillary transit on separate groups after right femoral ligation 7 days earlier and left femoral ligation on day-7. Data (±SEM) were tested by ANOVA, Dunn–Bonferroni, Student’s t tests, or Mann–Whitney U tests.

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fewer and/or narrower native hindlimb collaterals than wild-type. Over 10-day recovery following femoral ligation, plantar perfusion in Clic4<sup>-/-</sup> mice increased in parallel with wild-type, suggesting similar collateral remodeling in both strains.

We also measured perfusion in the adductor region (ie, the “collateral zone”) that contains collaterals supplying the lower leg (Figure 1b and 1d).<sup>8,21,22</sup> Collaterals are presumed to have little or no net flow in the absence of obstruction. Thus, flow in small arterioles and capillaries supplied by the distal branches of the saphenous and lateral caudal femoral artery trees, which are also present in this region, determine the perfusion signal before femoral artery ligation. Immediately after ligation, measured perfusion reflects the combined effects of a drop in flow in arterioles and capillaries of the saphenous artery tree, together with an increase in flow in collaterals interconnecting it with the lateral caudal femoral artery tree. The increase in perfusion over subsequent days reflects primarily collateral enlargement (remodeling), because no angiogenesis occurs in this region in the ligation model used here.<sup>2,8,22</sup> Adductor perfusion showed a pattern similar to plantar: Baseline perfusion before ligation was comparable among the 3 groups. Perfusion after ligation followed comparable patterns in Clic1<sup>-/-</sup> and wild-type, but decreased more in Clic4<sup>-/-</sup> mice immediately after ligation and thereafter followed a parallel recovery. These plantar and adductor patterns suggest that germline deletion of CLIC4 (but not CLIC1) results in fewer and/or narrower native collaterals, which, however, undergo comparable remodeling (confirmed below).

To test functional significance, scores were obtained for hindlimb use (index of muscle function) and appearance (index of ischemia).<sup>8,21,22</sup> In agreement with the perfusion data, use and appearance scores were similar in wild-type and Clic1<sup>-/-</sup> mice but significantly greater/worse in Clic4<sup>-/-</sup> (Figure 2a and 2b).

**Collateral Remodeling Is Unaffected in Clic4<sup>-/-</sup> Mice**

Lower conductance of the native collateral circulation in Clic4<sup>-/-</sup> mice could reflect reduced collateral number and/or lumen diameter. We thus measured diameter of collaterals in the anterior and posterior gracilis muscles by histomorphometry after maximal dilation and fixation. After ligation, flow in these superficial collaterals is a primary contributor to Doppler perfusion measured in the adductor collateral zone (Figure 1b and 1d). Wall thickness (intima-to-outer limit of media) was also determined to confirm lumen measurements,<sup>21</sup> because it varies in proportion to diameter according to Laplace’s equation. Baseline diameter and wall thickness did not differ in the nonligated contralateral limbs of wild-type and Clic4<sup>-/-</sup> mice (Figure 2c and 2d). This suggests that the lower perfusion in Clic4<sup>-/-</sup> mice immediately after ligation (Figure 1c and 1d) is not attributable to narrower native collaterals but, instead, reflects fewer collaterals (see below). After ligation, gracilis collaterals in Clic4<sup>-/-</sup> and wild-type mice underwent similar lumen enlargement and medial thickening (Figure 2c and 2d). This indicates that arteriogenesis is not impaired in Clic4<sup>-/-</sup>, which is consistent with the parallel increases in plantar and adductor perfusion in wild-type and Clic4<sup>-/-</sup> mice (Figure 1c and 1d).

**Clic4<sup>-/-</sup> Mice Have Reduced Density of Native Collaterals in the Hindlimb**

Microangiography was obtained in separate groups to determine whether Clic4<sup>-/-</sup> mice have fewer preexisting collaterals. Microfil, with a viscosity adjusted to impede capillary
transit (8:1 latex-to-diluent), was infused (aorta) after acute ligation of the left femoral artery in mice that received right femoral ligation 7 days earlier. We then counted the number of arterial vessels crossing a line drawn through the center of the thigh collateral zone in x-ray angiograms (Figure 3a).22 Detection of both native and remodeled vessels (H11350/11350 25/H9262 m diameter resolution8,21,22) were reduced in Clic4 mice (Figure 3b). This agrees with the lower perfusion in the plantar and adductor regions immediately after femoral ligation in Clic4 mice (Figure 1c and 1d). Arteries in the calf of the immediately ligated limb had reduced filling, compared to the 7-day ligated limb, because upstream collaterals had not undergone remodeling (Figure 3a).22 These data suggest that absence of CLIC4 results in reduced density of native collaterals in skeletal muscle.

Ischemia-Induced Angiogenesis in Clic4 Mice

Although baseline collateral conductance and their subsequent enlargement primarily determine limb perfusion after ligation, reduced baseline capillary density and ischemic angiogenesis after ligation could also contribute, respectively, to the lower plantar and adductor perfusion immediately after ligation and subsequent recovery in Clic4 mice. The prior observation that CLIC4 deficiency results in reduced angiogenesis in vitro16,18 and in Matrigel plug and retinal neangiogenesis models in vivo17 would support this hypothesis. To address this possibility, we measured capillarity/muscle fiber ratio and fiber area/size in the gastrocnemius muscle of the left (nonligated) and right leg ligated 10 days earlier (Figure 3c through 3e). The gastrocnemius experiences ischemic angiogenesis and atrophy after femoral ligation.8,21,22 Muscle fiber size and the ratio of capillary-to-muscle fiber number before ligation (nonligated limb; “baseline”) were similar in wild-type and Clic4 mice (Figure 3d and 3e). This strengthens the suggestion from the preceding analyses that lower preexisting collateral number in Clic4 mice, rather than baseline capillary-to-fiber ratio (capillary density), accounts for the larger drop in perfusion immediately after ligation. Clic4 mice had greater atrophy and a smaller increase in capillary-to-fiber ratio in the ligated limb (Figure 3d and 3e), although the latter was not significant. Greater atrophy and less angiogenesis is consistent with the greater ischemia and use–impairment scores in Clic4 mice (Figure 2a and 2b) and may contribute, along with reduced collateral density, to the lower recovery of plantar perfusion in Clic4 mice (Figure 1c). The absence of a significant increase in capillary-to-fiber ratio in these CD-1 wild-type mice may reflect their smaller increase in VEGF induced by ischemia, compared to other strains such as C57BL/6,8 or the need to make measurements later than 10 days (eg, at 21 days).

CLIC1 Expression Is Upregulated During Ischemia in Clic4 Mice

Clic1 and Clic4 mice have no apparent phenotype. In addition, CLIC4 is expressed in ECs,17,18 whereas CLIC1 is...
expressed ubiquitously. Thus, these proteins may provide functional redundancy or compensation. To examine this possibility, we compared expression of CLIC1 and CLIC4 in the calf muscle. CLIC1 and CLIC4 mRNA in the nonligated limb (baseline) was comparable among wild-type, Clic4/H11002/H11002, and Clic1/H11002/H11002 mice (data not shown). CLIC1 and CLIC4 increased similarly (6- to 7-fold) 1 day after ligation in all 3 strains (Figure 4a). Five days after ligation, expression of CLIC1 increased ~15-fold in Clic4/H11002/H11002 mice. These data suggest that CLIC1 may provide partial compensation for deficient CLIC4 expression and lessen the deficits we observed in Clic4−/− mice. However, CLIC1 appears to have little contribution when CLIC4 is intact, because CLIC4 expression in Clic1−/− mice was comparable to wild-type, in agreement with our failure to find deficiencies in Clic1−/− mice (Figures 1, 2a, and 2b).

Hypoxia-Inducible Factor-1α, VEGF-A, and Angiopoietin-2 Expression During Ischemia Are Reduced in Clic4−/− Mice

Besides its role in angiogenesis, VEGF is an important determinant of collateral formation in the embryo and neonate and collateral remodeling in adult occlusive disease. To determine whether VEGF-A is affected by CLIC4 deletion, we examined levels of mRNA encoding VEGF-120, -164, and -188 in the calf. Basal expression in the nonligated limb was similar in Clic4−/− and wild-type mice (data not shown). One day after ligation, VEGF-120 increased similarly in the ligated limb of wild-type and Clic4−/− mice and returned to control by 5 days, whereas the other 2 isoforms were reduced at both times in Clic4−/− (Figure 4b and 4c). These observations are consistent with the known importance of soluble VEGF-120 in ischemia. More importantly, they suggest that CLIC4 may positively regulate high-molecular-weight VEGF isoforms when their levels are augmented, eg, in ischemia and during embryonic growth. During the latter period, impaired CLIC4 expression could thus disturb VEGF gradients, resulting in the impaired collateral formation we observed.

Among several possible mechanisms for lower induction of VEGF in Clic4−/− mice, hypoxia-inducible factor (Hif)-1α expression could be reduced. Indeed, increased Hif-1α in the ischemic calf of wild-type mice was reduced in Clic4−/− mice (Figure 4d). Moreover, Hif-1α and VEGF were lower in Clic4−/− mice despite their greater ischemia (Figures 1, 2a, and 2b). Similarly, upregulation of angiopoietin-2 (like VEGF-A, regulated by HIF-1α) was reduced in Clic4−/−.
mice (Figure 4e). Thus, CLIC4 impacts upstream regulation of Hif-1α and its downstream targets, VEGF and angiopoietin-2.

Collateral Density and Diameter in the Cerebral Circulation Are Reduced in Clic4−/− Mice

The greater decrease in perfusion immediately after ligation (Figure 1c and 1d) plus fewer arteries in the collateral zone detected by angiography in the acute and chronically ligated leg (Figure 3b) suggest that Clic4−/− mice have reduced numbers of preexisting collaterals. To test this with higher-resolution methods and determine whether it extends to other tissues, we examined the collateral circulation that interconnects the anterior cerebral artery (ACA) and middle cerebral artery (MCA) trees (Figure 5a). Cerebral cortical collaterals are confined to the pial surface and can thus be imaged in approximately 2D with high fidelity. Native collateral density and diameter were unaffected in adult Clic1−/− mice, in agreement with hindlimb data (Figures 1, 2a, and 2b); thus, Clic1−/− were not examined at postnatal time points. In contrast, Clic4−/− were born with a lower collateral density that further declined by P21 to that present in adults wherein diameter was also smaller. These data indicate that CLIC4 positively regulates pial collateral formation in the embryo and is important for stabilization of nascent collateral number and size after birth. Similar results were obtained for VEGF-hypomorphic mice.8

Clic4−/− Mice Undergo Larger Infarctions After MCA Occlusion

To determine whether the lower density and diameter of pial collaterals in adult Clic4−/− mice is functionally significant, infarct volume was assessed 3 days after MCA occlusion. We used our modified laser Doppler imager21 to assess the success of the occlusion surgery by noninvasively monitoring transcranial perfusion (Figure 6a and 6b). The signal derives from perfusion of the skin, bone, and upper cortex. Although the former 2 are not supplied by the cerebral arteries and thus could lessen detection of differences after occlusion, a similar ∼40% decrease in perfusion after occlusion in mice was
obtained with a Doppler probe positioned over a sealed cranial window made in a similar location as our region of interest.24 Infarct volume was increased 4-fold in Clic4+/− mice (Figure 6c and 6d). Similar findings were obtained in VEGF hypomorphic mice.8 Larger infarct volume in Clic4+/− is not attributable to an increase cortical territory supplied by the MCA. Area of the MCA tree was comparable in Clic4+/− and wild-type mice, whereas ACA area was increased and posterior cerebral artery area was decreased (Figure 5d). Cerebral artery territories were also different in Clic1+/− mice (see Discussion).

Because MCA tree size trended smaller in Clic4+/− and Clic4+/+ (Figure 5d), this could be accompanied by fewer distal branches and a corresponding fewer number of collaterals. However, Clic4−/− have ≈21% fewer distal-most branches abutting the collateral zone, yet ≈75% fewer collaterals (this results in a commensurate increase in “terminal arterioles,” ie, distal-most branches that do not give rise to a collateral) (Figure 5e and 5f). Hence, the role of CLIC4 in “collaterogenesis” is not simply secondary to its influence on tree structure. This is consistent with our other studies showing no relationship between MCA tree size and collateral abundance.25

Clic4+/− Mice Display Collateral Deficits Intermediate to Clic4−/− Mice
To investigate whether a gene dosage effect could be detected, we compared Clic4 heterozygotes to homozygous wild-type and Clic4−/−. Immediately after femoral artery ligation, perfusion in Clic4+/− mice declined to values in the plantar as low as (and in the adductor intermediate to) Clic4−/− mice (Online Figure I, a and b). In the pial circulation, the number of nascent collaterals present at birth in Clic4+/− were comparable to wild-type mice, although diameter was smaller (Figure 5b and 5c). By 3 weeks postnatal (when the adult collateral density is established), loss of pial collaterals was intermediate compared to Clic4−/− mice (Figure 5b). These and the hindlimb adductor data provide evidenced for a gene dose–effect of CLIC4 expression on postnatal maturation and thus collateral anatomy in the adult.

Discussion
Recently, we reported that collaterals form in the murine pial circulation between embryonic days 13.5 and 18.5 and undergo maturation and stabilization over the first several weeks after birth; moreover, these processes are strongly impaired in the BALB/c strain, compared to C57BL/6.26 Thus, collaterogenesis in the cerebral circulation occurs during the embryonic and early postnatal period and is susceptible to genetic background. In the present study, CLIC4 disruption resulted in greater decrease in hindlimb perfusion immediately after femoral artery ligation. Clic4−/− mice also showed less recovery of perfusion, in association with worse ischemia, use impairment, and muscle atrophy, yet normal collateral remodeling. These data suggest Clic4−/− mice have fewer native collaterals. This was confirmed by arteriography, where we detected 40% fewer vessels crossing the collateral zone of the nonligated leg in Clic4−/− and a more dramatic 70% fewer native collaterals in the pial circulation that was associated with 4-fold larger infarctions after MCA occlusion. These findings demonstrate that CLIC4 is a major determinant of native collateral density in healthy skeletal muscle and brain. Because of technical
limitations, we do not know if our findings in newborn brain predict the situation in newborn hindlimb.

We did not measure arterial pressure, because like other groups, perfusion ratio between the 2 hindlimbs was measured to minimize the effect of any potential difference. Moreover, CLIC4 is expressed predominantly in endothelial cells, with little or no signal in vascular smooth muscle cells or cardiomyocytes (J Edwards, personal communication, 2008; also see Ulmasov et al17). In addition, collateral wall thickness did not differ between wild-type and Clic4−/− (Figure 2d), arguing against the existence of a difference in pressure.

We also found that CLIC4 expression increased in ischemic muscle, plus evidence suggesting CLIC4 promotes HIF-1α: VEGF signaling, based on reduced Hif-1α and VEGF expression in Clic4−/− mice despite their greater ischemia. Given the role of VEGF as a positive regulator of density and diameter of native collaterals,8 these findings provide a potential hypothesis for why CLIC4 deficiency leads to impaired collaterogenesis: reduced or absent CLIC4 results in lower VEGF expression during conditions when VEGF expression is induced rather than constitutive (eg, during perinatal development and ischemia). Interestingly, although VEGF regulates both collaterogenesis and collateral remodeling,8 no deficiency in remodeling was observed in Clic4−/− mice after femoral ligation. One possibility, in addition to potential compensation by other factors, is that the increase in VEGF around enlarging collaterals8 is not diminished enough in Clic4−/− mice to impair remodeling.

Significant variation in collateral conductance in healthy tissues and collateral remodeling in ischemic disease exists among humans and species.2,5,27–30 We previously reported that BALB/c mice have markedly fewer native collaterals in the hindlimb (as did Helisch et al), brain, and intestine; less remodeling in ischemia; and less induction of VEGF in the hindlimb (as did Helisch et al).21 In addition, CLIC1 has been reported to undergo nuclear translocation and bind transcription factors20 and, when induced by TGF-β1, to cause conversion of fibroblasts to myofibroblasts.32 In addition, 6 CGTG sequences that comprise the core sequence of the Hif-1α–binding site are present in 2 Kb flanking G-C repeats (Transcriptional Regulatory Element Database, http://rulai.cshl.edu/TRED).

Unlike CLIC4, we found no evidence that CLIC1 is required for collaterogenesis. However, Clic1−/− mice had a larger MCA tree and smaller ACA tree compared to wild-type (Figure 5d). This suggests that CLIC1 has a role in pre- or postnatal growth of these trees. The MCA tree was smaller in Clic4−/− and trended smaller yet in Clic4−/− mice, whereas the ACA trees were proportionately larger and posterior cerebral artery trees smaller. Although the smaller MCA trees in Clic4−/− and Clic4−/− mice mirror the successively smaller collateral number interconnecting them to the ACA trees, a comparison of 19 mouse strains found no correlation between collateral number and size of any of the cerebral artery trees.25 This indicates that the number of collaterals that form is not simply a result of tree size (which is specified in the embryo30). Native collateral number in skeletal muscle was also reduced in Clic4−/− mice (Figures 1 and 3B), but unlike in the pia, diameter was unaffected, at least in the gracilis muscles (Figure 2c). Our previous studies found that differences in collateral density in pial, hindlimb, and intestinal circulations, arising from strain differences or targeted mutation of VEGF, agree qualitatively; however, differences in the pial circulation are much larger than hindlimb and intestine beds.22 Likewise, in the present study,
deficits in native collaterals in Clic4−/− mice (Figure 5) appeared greater in the pia than in the hindlimb (Online Figure I). Thus, we did not examine Clic4 hemizygotes for differences in recovery of perfusion, etc (Figures 1 through 3) after femoral ligation.

Native pial collateral density and diameter and response to hindlimb ligation were unaltered in Clic1−/− mice, suggesting that Clic1 is not required for collateralgenesis or collateral remodeling. However, both Clic4 and Clic1 increased 7-fold in ischemic calf of wild-type mice, and Clic1 increased double this in Clic4−/− mice. This suggests that Clic1 could compensate for deficient Clic4 during collateral formation, stabilization, and remodeling. Thus, greater deficits in the former processes (and a frank deficit in collateral remodeling) might be observed when expression of both genes is reduced. The Clic family of proteins consists of multiple highly homologous members,20 Clic1 and Clic4 are expressed in virtually all tissues,20 whereas Clic2 is found in fetal liver and skeletal muscle,20 and Clic3 is expressed in placental and fetal membranes.33 Thus, it is possible that both Clic1 and Clic4 are involved in collateral remodeling and are able to compensate for the loss of the other, or that both do not participate in this process. Additional approaches, eg, double knockout (if viable) or cellspecific or conditional knockout mice, will be required to address the possibility of compensation.

In conclusion, our findings suggest that Clic4 contributes to formation and stabilization of collaterals during vascular development and thus native collateral density in adults. These effects may be due in part to its influence on HIF-1α-VEGF signaling, which is critical in formation of the general (ie, arterio-venous)11,12 and collateral18 circulations. Identification of how these and other factors interact to regulate collateralgenesis is needed to begin to understand the basis for the wide variance in collateral capacity in healthy humans and determine whether therapeutic strategies can be devised to induce formation of new collaterals in individuals that have, or are prone to, occlusive vascular disease.

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Disclosures
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References
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Supplement Material

Materials and Methods

Animals. As described previously\textsuperscript{1}, mice with targeted deletion of chloride intracellular channel-1 or -4 (\textit{Clic1}\textsuperscript{-/-}, \textit{Clic4}\textsuperscript{-/-}) were generated on the CD-1 strain. The wild-type littermates served as controls. Mice were studied at postnatal day 1 and 21 (P1, P21) and at 12-16 weeks of age. Animals were randomized and procedures and analyses were conducted blindly. Procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee.

\textit{Hindlimb ischemia model.} Femoral artery ligation was performed as described\textsuperscript{2,3}. Briefly, mice were anesthetized with 1.25\% isoflurane/O\textsubscript{2} and the hindlimbs depilated. Temperature was maintained at 37.0±0.5\degree C. The right femoral artery was exposed through a 2mm incision and ligated with two 7-0 ligatures placed distal to the origin of the lateral caudal femoral and superficial epigastric arteries (the latter was also ligated) and proximal to the genu artery. The artery was transected between the sutures and separated by 1-2 mm. The wound was irrigated with sterile saline and closed, and cefazolin (50mg/kg, im), furazolidone (topical) and pentazocine (10mg/kg, im) were administered.

\textit{Laser Doppler perfusion imaging.} As detailed previously\textsuperscript{2,3}, under 1.125\% isoflurane/O\textsubscript{2} anesthesia and 37±0.5\degree C, non-invasive perfusion imaging of the adductor thigh region and plantar foot of both limbs was performed before,
immediately after, and at 1, 3, 7, and 10 days after femoral ligation. Regions of interest were drawn to anatomical landmarks.

Muscle function and ischemia. At 1, 3, 7, and 10 days after femoral ligation, animals were evaluated for right hindlimb use with a "clinical use score: 0=normal use, 1=no toe flexion, 2=no plantar flexion, and 3=dragging foot. Mice were also scored for appearance (index of ischemia): 0=normal, 1-5=cyanosis or loss of nail(s), where the score is dependent on the number of nails affected, 6-10=partial or complete loss of digit(s), where the score reflects number of digits affected, 11=dry necrosis of forefoot; and 12=dry necrosis of foot up to ankle.

Histology and Morphometry. Ten days after femoral ligation, animals were cannulated via the descending aorta, heparinized, perfusion-cleared at 100 mmHg with phosphate-buffered saline (PBS, pH 7.4) containing adenosine (10 mg/ml) and papaverine (4 mg/ml), followed by fixation with 4% paraformaldehyde (PFA) as detailed previously\textsuperscript{2}. Hindquarters were post-fixed and tissue sections of the medial adductor and calf from the ligated and sham-ligated limbs were blocked and embedded in paraffin for sectioning (5 microns, modified cyanomassons elastin stain). Diameters of collaterals at their ~midpoints in the anterior and posterior gracilis muscles were determined at baseline and 10 days after femoral artery ligation\textsuperscript{2}. Capillaries in calf sections were labeled with biotinylated \textit{Griffonia simplicifolia} isolectin-1-B\textsubscript{4} (GSL-1- B\textsubscript{4}, 1:100; Vector Laboratories, Burlingame, CA) plus streptavidin horseradish peroxidase
(Vectastain ABC, Vector Laboratories) and diaminobenzidine (DAB React, Sigma, St. Louis, MO). Sections were lightly counterstained with methyl-green, and capillaries in the lateral and medial heads of the gracilis were counted as previously detailed\(^2\). Muscle fiber number and fiber size (index of atrophy) were determined for the fascicles in which capillary number was determined.

**Postmortem arterial micro-angiography.** Separate mice that received right femoral artery ligation 7 days earlier were heparinized, perfusion-cleared, vasodilated and fixed (as above), followed by acute ligation of the left femoral artery. X-ray opaque latex (MV-122, Flow Tech Inc, Carver, MA) with a viscosity sufficient to minimize capillary transit (8:1 latex-to-diluent) was injected into the cannulated abdominal aorta and allowed to cure. After post-fixation overnight, the skin was removed and arteriograms were obtained (MX-20, 22kV, 6s; Faxitron X-ray Corporation, Wheeling, IL). Films were digitized and a Rentrop-like line analysis was performed by counting vessels crossing the middle of the posterior thigh as described previously\(^3\).

**CLIC1, CLIC4, VEGF-A isoforms, HIF-1α and angiopoietin-2 expression.** Calf muscle of the ligated and sham-ligated limbs was harvested following perfusion of the hindquarters with RNAlater (Sigma). Samples were frozen in liquid nitrogen and stored at -80°C. Tissue was powdered with a tissue pulverizer cooled in liquid nitrogen and homogenized in a buffer containing guanidine thiocyanate (RLT buffer, RNeasy Fibrous Tissue Mini Kit, Qiagen, Valencia, CA).
Total RNA was extracted according to the manufacturer’s protocol. Genomic DNA was removed using RNase-Free DNase Set (Qiagen). cDNA was prepared from 1 μg of total RNA using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen, Carlsbad, CA). An equal amount of total RNA was included as a no-RT control for each tissue sample.

Samples for real-time PCR included cDNAs (1:50) in triplicate and the respective no-RT control, along with a no-template control for each target analyzed. Real-time PCR (RT-PCR) for mRNA transcripts was performed (Rotor-Gene 3000, Corbett Life Science, Sydney, Australia) using SYBR Green chemistry (SYBR Green JumpStart Taq ReadyMix, Sigma-Aldrich).

Murine CLIC1 and CLIC4 were amplified using forward and reverse primers. The following oligonucleotides were used (Bioneer, Alameda, CA): CLIC1 forward primer (5’-GCTCAAGAACAACCTCAGGTC-3’) and reverse primer (5’-TCTGAGAGATGCCCTCATCTTC-3’); CLIC4 forward primer (5’CTGAAGGAGGAGGACAAAGAG-3’) and reverse primer (5’GCGCTTCATTAGCCTCTGGT-3’). Murine VEGF-A was amplified using a forward primer common to all VEGF-A isoforms (exon3F) and reverse primers specific to each isoform. Primers were designed using VectorNTI (Invitrogen). The following oligonucleotides were used (Invitrogen): VEGF-A-exon3F (5’-ATCTTCAAGCCGTCCTGTGC-3’), VEGF-A isoform specific reverse primers: VEGF-A-120R (5’-TTGGCTTGTACATTTTTCTGG-3’), VEGF-A-164R (5’-CAAGGCTCACGATTCTTCTGG-3’), VEGF-A-188R (5’-ATCTTCAAGCCGTCTGTGC-3’). The forward primer for HIF-1α was 5’-
CAAGATCAGCCAGCAAGTCC-3'' and the reverse was 5'-GGGACTGTTAGGCTGGGAAA-3'. The forward primer for angiopoietin-2 was 5'-AGCAGATTTTGATCAGACCAG-3' and the reverse was 5'-GCTCCTTCATGGACTGTAGCTG-3'. The forward primer sequence for 18s (housekeeping gene) was 5'-TTGACGGAAGGGCACCACCAG-3' and the reverse was 5'-GCACCACCACCCACGGAATCG-3'.

PCR cycling parameters were: 95°C for 2m, 45 cycles of 95°C for 10s, 60°C for 20s, and 72°C for 25s. Fluorescence acquisition was performed at the end of each cycle. Specificity of the PCR was verified with melt-curve analysis. Fluorescence threshold was set and the cycle at which each sample crossed the threshold (Ct) was recorded in triplicate and averaged. mRNA was quantified using the $2^{-ΔΔCt}$ mathematical model, where $ΔΔCt = ΔCt_{Ligated} - ΔCt_{Non-ligated}$ ($ΔCt = Ct_{gene~of~interest} - Ct_{housekeeping~gene}$).

Cerebral collateral circulation. Separate groups of animals were cannulated via the descending abdominal aorta, heparinized, perfusion-cleared and maximally dilated (as above). The dorsal calvarium and adherent dura mater were removed to expose the pial circulation. A second catheter was placed retrogradely into the thoracic aorta, and a polyurethane solution with a viscosity sufficient to minimize capillary transit (1:1 resin-to-methylethyl ketone, PU4ii, Vasqtec, Zurich, Switzerland) was infused with the aid of a stereomicroscope. 4% PFA was applied topically, and the latex was allowed to cure. After post-fixation with 4% PFA, the pial circulation was imaged (Leica MZ16FA, Leica Microsystems,
Bannockburn, IL). Collaterals connecting the middle and anterior cerebral artery trees of both hemispheres were counted, and lumen diameters were determined at their midpoints (ImageJ, NIH).

**Middle cerebral artery occlusion (MCAO) and infarction volume assessment.** A 4-5 mm incision was made between the right eye and ear and the temporal muscle was separated at its midpoint and retracted to expose a small portion of the zygomatic and temporal bones. A ~2mm burr-hole was made over the trunk of the MCA with a handheld drill. The MCA was irreversibly cauterized, and the incision was closed with Vetbond (3M). Trans-cranial Doppler perfusion scanning was then done to verify occlusion of the MCA and to monitor recovery of perfusion on successive days. Three days after MCAO, the mouse received an overdose of ketamine (100 mg/kg ip) and xylazine (15 mg/kg ip). The brain was removed, sliced into 1 mm coronal sections, and incubated in a PBS solution containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C for 20 min. Sections were washed in PBS, fixed in 10% formalin and imaged (MZ16FA, Leica). Infarct volume was calculated as the sum of the cortical volume devoid of TTC in each section, and expressed as a percent of total left cortex volume.

**Statistics.** All data are reported as means ± SEM. Statistical significance (P < 0.05) was determined by two-way ANOVA followed by Dunn-Bonferroni Corrected t-tests, paired t-test for within-animal comparisons, and unpaired t-tests for group comparisons where appropriate. Parametric analysis was
conducted on all data except capillary density and capillary number-to-muscle fiber ratio, which were subjected to non-parametric analysis. For qRT-PCR, Student’s $t$-test was used to determine if the fold change was significantly different from 1.
Online Figure Legend

**Online Figure I.** Perfusion of the *Clic4<sup>+/−</sup>* hindlimb declined to levels comparable to the *Clic4<sup>+/−</sup>* plantar but intermediate to the *Clic4<sup>−/−</sup>* adductor immediately after ligation. Assessed Doppler perfusion in (a) the plantar region and (b) adductor region. The hindlimb adductor perfusion provides evidence for a gene dose-effect of CLIC4 expression on postnatal maturation and thus collateral anatomy in the adult. *p*<0.05 vs. WT.
References

