Hypoxia inducible factor stabilization improves defective ischemia-induced angiogenesis in a rodent model of chronic kidney disease

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Chronic kidney disease (CKD) is associated with increased risk and worse prognosis of cardiovascular disease, including peripheral artery disease. An impaired angiogenic response to ischemia may contribute to poor outcomes of peripheral artery disease in patients with CKD. Hypoxia inducible factors (HIF) are master regulators of angiogenesis and therefore represent a promising target for therapeutic intervention. To test this we induced hind-limb ischemia in rats with CKD caused by 5/6 nephrectomy and administered two different treatments known to stabilize HIF protein *in vivo*: carbon monoxide and a pharmacological inhibitor of prolyl hydroxylation 2-(1-chloro-4- hydroxyisoquinoline-3-carboxamido) acetate (ICA). Expression levels of pro-angiogenic HIF target genes (*Vegf, Vegf-r1, Vegf-r2, Ho-1*) were measured by qRT-PCR. Capillary density was measured by CD31 immunofluorescence staining and HIF expression was evaluated by immunohistochemistry. Capillary density in ischemic skeletal muscle was significantly lower in CKD animals compared to sham controls. Rats with CKD showed significantly lower expression of HIF and all measured pro-angiogenic HIF target genes, including VEGF. Both HIF stabilizing treatments rescued HIF target gene expression in animals with CKD and led to significantly higher ischemia-induced capillary sprouting compared to untreated controls. ICA was effective regardless of whether it was administered before or after induction of ischemia and led to a HIF expression in skeletal muscle. Thus, impaired ischemia-induced angiogenesis in rats with CKD can be improved by HIF stabilization, even if started after onset of ischemia.

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Chronic kidney disease (CKD) is a growing health burden worldwide.[1–3] Premature cardiovascular disease accompanies even early stages of CKD and leads to increased mortality among CKD-patients.[4] Among these cardiovascular comorbidities in CKD, peripheral arterial disease (PAD) plays an important role.[5] Sprouting of new capillaries (angiogenesis) is an important compensatory mechanism when stenosis or occlusion of large arteries restricts blood supply (ischemia) and oxygen supply (hypoxia).[6] This mechanism appears to be impaired in CKD.[7,8]

Stimulating hypoxia inducible factors (HIFs) represents a promising concept to improve angiogenesis because HIFs are master transcriptional regulators of a protective response to hypoxia.[9] HIF target genes include VEGF and its receptors, as well as hemoxygenase-1 (HO-1)[10,11] which are all crucial for vessel formation.[12] There is some evidence that CKD may cause HIF downregulation and disturbed HIF dependent signaling.[13–15] An *in vivo* model revealed that ischemia does not prompt upregulation of pro-angiogenetic genes in CKD animals.[16] Furthermore, CKD patients exhibit elevated plasma levels of anti-angiogenetic factors, such as endostatin.[17]

Animal models confirmed that HIF upregulation via viral vectors or transgenic overexpression can promote angiogenesis without systemic VEGF-induced side effects.[18–20] Prolyl hydroxylase inhibitors provide a pharmacological approach to stabilize HIF. Such compounds are in clinical trials for the treatment of renal anemia.[21–24] In animal models, deficiency or pharmacological inhibition of PHD improved angiogenesis, especially in the setting of acute ischemia.[25–28] However, the effects of PHD inhibitors on angiogenesis in the presence of CKD remain unknown.

We therefore tested the hypothesis that HIF stabilization in CKD animals before or after the onset of hind-limb ischemia would improve ischemia-induced angiogenesis. Two different methods of HIF stabilization were used: carbon monoxide (CO) administration, which induces the hypoxia-response by blocking oxygen transport capacity, and a recently described pharmacological inhibitor of HIF degradation, 2-(1-chloro-4-hydroxyisoquinoline-3-carboxamido) acetate (ICA).[29] We hypothesized that a short, transient HIF stabilization at the time of ischemia induction could exert a lasting beneficial effect on capillary supply because the most important angiogenesis-related genes peak very early after hind-limb ischemia.[30,31]

RESULTS

Compensatory capillary sprouting is compromised in ischemic limbs of rats with renal impairment

Male rats were used to explore angiogenesis (capillary sprouting) in a model of CKD. CKD was induced by removing 5/6 of total kidney mass (5/6 nephrectomy model / ablation model). Eight weeks later, unilateral hind-limb ischemia was induced by femoral artery ligation and the effects on angiogenesis were studied. In the nonischemic limbs we could not find differences of capillary density as determined by CD31 staining (Figure 1a and b) between sham-operated rats with normal kidney function (SHAM) and subtotally nephrectomized rats with impaired kidney function (SNX), suggesting that CKD in this setting had no effect on vascularization at baseline.

In the affected ischemic limbs, capillary density significantly increased (by ~53%) in SHAM animals but not in SNX animals. In fact, in SNX there was no difference between the ischemic limb and the contralateral site, indicating that the angiogenic response was completely blunted (Figure 1b).

Ischemic HIF upregulation is defective in skeletal muscle of rats with renal impairment

We next hypothesized that insufficient capillary sprouting after ischemia in rats with renal impairment might be related to impaired HIF-dependent signaling. Thus, we performed immunohistochemical stainings for HIF1a in skeletal muscle of SHAM and SNX rats. HIF1a expression was significantly upregulated in ischemic versus nonischemic skeletal muscles in SHAM operated rats. In contrast, HIF expression was not different between ischemic and nonischemic limbs in SNX animals (Figure 2).

HIF stabilizing treatment increases HIF-1α protein abundance *in vitro*

To test whether impaired HIF expression in ischemic limbs of SNX rats might be susceptible to HIF inducing treatment, we

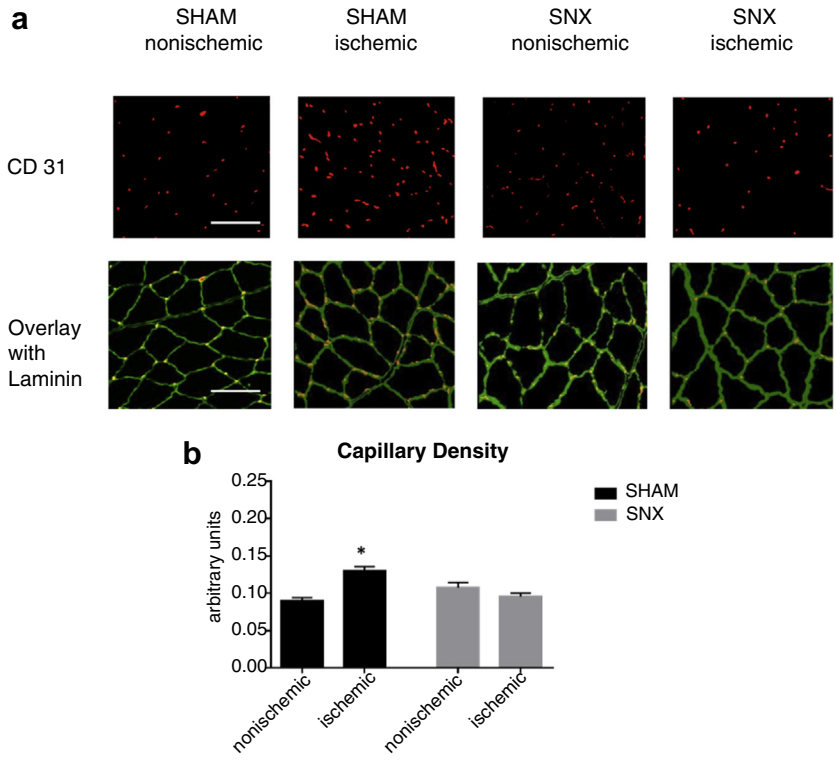

Figure 1 | Immunohistochemical analysis of skeletal muscle capillary density in sham-operated (SHAM) controls and in subtotally nephrectomized (SNX) rats following limb ischemia. (**a**) Frozen sections were obtained from gastrocnemius muscles and stained for Laminin (green) and CD31 (red). (**b**) Capillary density in nonischemic and ischemic limbs of sham-operated (SHAM) and subtotally nephrectomized (SNX) rats. Capillary density was measured as CD31-positive percentage/Laminin-positive percentage 14 days after induction of ischemia. Values are means ± SEM. N = 18 for SHAM and n = 17 for SNX. One-way Anova with Bonferroni correction *$P < 0.05$ versus all other groups. Bar = 100 μm.