S100A1 Deficiency Impairs Post-Ischemic Angiogenesis via Compromised Proangiogenic Endothelial Cell Function and Nitric Oxide Synthase Regulation

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ABSTRACT

Rationale: Mice lacking the EF-hand Ca\textsuperscript{2+} sensor S100A1 display endothelial dysfunction due to distorted Ca\textsuperscript{2+} activated NO generation.

Objective: To determine the pathophysiological role of S100A1 in endothelial cell (EC) function in experimental ischemic revascularization.

Methods and Results: Patients with chronic critical lower limb ischemia (CLI) showed almost complete loss of S100A1 expression in hypoxic tissue. Ensuing studies in S100A1 knockout (SKO) mice subjected to femoral artery resection (FAR) unveiled insufficient perfusion recovery and high rates of autoamputation. Defective in vivo angiogenesis prompted cellular studies in SKO ECs and human ECs with siRNA-mediated S100A1 knockdown demonstrating impaired in vitro and in vivo proangiogenic properties (proliferation, migration, tube formation), and attenuated vascular endothelial growth factor (VEGF)- and hypoxia-stimulated eNOS activity. Mechanistically, S100A1 deficiency compromised eNOS activity in ECs both by interrupted stimulatory S100A1/eNOS interaction and PKC hyperactivation that resulted in inhibitory eNOS phosphorylation and enhanced VEGF-receptor 2 (VEGFR2) degradation with attenuated VEGF signaling. Ischemic SKO tissue recapitulated the same molecular abnormalities with insufficient in vivo NO generation. Unresolved ischemia entailed excessive VEGF accumulation in SKO mice with aggravated VEGFR2 degradation and blunted in vivo signaling through the proangiogenic PI3K/Akt/eNOS cascade. NO supplementation strategies rescued defective angiogenesis and salvaged limbs in SKO mice post-FAR.

Conclusions: Our study shows for the first time downregulation of S100A1 expression in patients with CLI and identifies S100A1 as critical for EC function in postnatal ischemic angiogenesis. These findings link its pathological plasticity in CLI to impaired neovascularization prompting further studies to probe S100A1’s microvascular therapeutic potential.

Keywords: Angiogenesis, S100A1, endothelium, nitric oxide

Non-standard Abbreviations:

Akt    v-Akt murine thymoma viral oncogene homolog
CaM    calmodulin
CLI    critical limb ischemia
DETA/NO diethylenetriamine/NO
EC    endothelial cell
eNOS    endothelial nitric oxide synthase
FAR    femoral artery resection
GAPDH glyceraldehyde 3-phosphate dehydrogenase
GM    gastrocnemius muscle
HGF    hepatocyte growth factor
HIF1α hypoxia inducible factor 1 alpha
IGF    insulin-like growth factor
IP    immunoprecipitation
PAOD peripheral arterial occlusive disease
IP3(R) inositol-1,4,5 trisphosphate (receptor)
PDK phosphoinositide dependent protein kinase
PIP3 phosphatidylinositol 1,4,5 trisphosphate
PI3K phosphoinositide-3-kinase
PKCε protein kinase C epsilon
PIGF placental growth factor
INTRODUCTION

Chronic critical limb ischemia (CLI), the most advanced form of peripheral arterial occlusive disease (PAOD), is a common clinical problem with poor clinical prognosis. Without successful revascularization, up to 40% of patients will require major limb amputation within a year of diagnosis exceeding annual mortality rates of 20%. Though we have seen rapid evolution of endovascular revascularization and improvement in medical treatment over the past 15 years, therapeutic options to improve bridge-collateral (arteriogenesis) and/or capillary formation (angiogenesis) are limited. The prevailing lack of microvascular-targeted treatments fostered novel cell- and molecular-based strategies being tested in phase I and II clinical trials with promising preliminary results.

In this regard, the most recent discovery of the EF-hand calcium (Ca²⁺) binding protein S100A1 as virtually indispensable for postnatal endothelial cell (EC) function has sparked interest in its potential significance in vascular pathophysiology. Originally identified in the brain as a hydrophobic low molecular weight (22 kD) dimeric protein, S100A1 belongs to the largest subgroup of Ca²⁺ sensors within the EF-hand Ca²⁺ binding protein superfamily. The group, referred to as S100 proteins, comprises 21 isoforms each of which is characterized by a cell- and tissue-type specific expression pattern. S100A1, known for its high abundance in cardiomyocytes, controls a Ca²⁺-driven functional network orchestrating contractile performance, cell survival and metabolism.

In ECs, S100A1 likewise seems to be an important part of the cells versatile molecular toolkit to relay intracellular Ca²⁺ oscillations that regulate vascular tone. Lack of endothelial S100A1 attenuates in vitro and in vivo arterial vasorelaxation basally and in response to B₂-kininergic and M₂-muscarinic stimuli. The Ca²⁺ sensor interacts with the inositol-triphosphate receptor (IP3R) and enhances intracellular Ca²⁺ release from the endoplasmic reticulum (ER) that indirectly accounts for enhanced endothelial nitric oxide synthase (eNOS) activation. However, it is unclear whether the EF-hand Ca²⁺ sensor might also affect eNOS function directly. Hence, lack of S100A1 conveys endothelial dysfunction and suggests potential pathophysiological relevance in human vascular diseases.

In this study, we advance our understanding of S100A1’s role in vascular disease biology and identify the Ca²⁺ sensor as indispensable factor for postnatal ischemic neovascularization with potential clinical relevance. First time demonstration of a near complete loss of S100A1 expression in ischemic tissue from patients with chronic CLI spurred a comprehensive proof-of-concept study in S100A1 genetically-ablated (SKO) mice to determine its impact on postnatal ischemic neovascularization and underlying molecular mechanisms.
METHODS

A detailed description of the Materials and Methods used in this study is available in the expanded online Data Supplement. For the assessment of hindlimb ischemia and protein-protein interactions, we kindly refer to the online Data Supplement.

Patients.
Briefly, in patients with chronic CLI and indication for surgical revascularization or major amputation, and in control patients, a surgical biopsy of the gastrocnemius muscle was taken. Patients gave their written informed consent to participate in the study. The study protocol has been approved by the ethics committee of the Medical Faculty at the Technische Universität Dresden.

Mice.
SKO mice were derived on a C57Bl/6 background as described previously. All experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals. For femoral artery resection (FAR), mice were anesthetized and the right femoral artery was resected as described. In the course of the study, a modified minimal surgery was used. In some cases WT and SKO mice were treated with the NO donor diethylenetriamine/NO (DETA/NO, 2mg/kg) for three consecutive days immediately following FAR.

In vivo matrigel plug assay.
Mice were anesthetized, then two small subdermal pockets were created and each pocket received one matrigel plug. Plugs were harvested 16 days later. In some cases mice received injections of DETA/NO (2mg/kg) or vehicle (PBS). Recovered plugs were cryoemdedded, sliced, fixed, stained and subsequently imaged (Image J).

RNA isolation, reverse transcription and quantitative real-time PCR.
The procedures for RNA isolation, reverse transcription and quantitative real-time PCR were essentially carried out as described previously.

Endothelial cell preparation and siRNA-mediated endothelial S100A1 knockdown.
Primary ECs from WT or SKO mice were prepared as described, with modifications mentioned in the Data Supplement. Human cardiac microvascular endothelial cells (HMVECs, CC-7030) (LONZA) were cultured according to the manufacturer’s instruction in EGM-2 MV BulletKit media (CC-3162). S100A1 knockdown was achieved transfecting cells with siRNA against human S100A1 (Sigma 231-791-2) using the Lipofectamine RNAiMAX reagent (Invitrogen).

Skeletal muscle cell isolation.
Gastrocnemius muscle bundles of male WT mice (2-3 months old) were dissected. The isolated muscles bundles were treated with collagenase and muscle cells were then incubated under normoxic and hypoxic (2%O2) conditions.

[3H]Thymidine incorporation assay.
Agonist-induced [3H]-thymidine incorporation into endothelial cells was assessed as described.

Chemokinesis assay.
EC migration was assessed according to a modified protocol from Liang et al. ECs were seeded and allowed to attach overnight. Following 24h of serum starvation (0.2%FBS), the cell monolayer was denuded with a pipette tip and further incubated with 1%FBS, ECs were then photographed to record a starting point. After 48h of incubation, cells were photographed again and “wound closure” was quantified.
**Matrigel tube formation assay.**
ECs were seeded on matrigel-covered wells and stimulated with 30μg/ml ECGS in DMEM containing 0.2%FBS. After 24h of incubation, cells were photographed and tube formation was quantified by counting the branching points.

**Immunoblot analysis.**
Assessment of protein expression and phosphorylation from tissue and cellular extracts was carried out as described in details elsewhere, details can be found in the Data Supplement.

**Measurement of NO and nitrite in cell culture supernatants and tissue homogenates.**
NO in medium from EC cultures subjected to VEGF stimulation or hypoxia was measured using a NO/Nitrite/Nitrate assay (R&D systems) according to manufacturer’s instructions. The PKC inhibitors chelerythrin (Tocris, Cat.No 1330) and calphostin-C (Tocris, Cat.No. 1626) were used at 1μM and 100nM, respectively. Nitrate levels in skeletal muscle tissue homogenates were measured with the Nitrate/Nitrite Colorimetric Assay Kit (Caymen Chemicals 780001).

**Measurement of NOS/eNOS activity and PKC activity.**
Nitric oxide synthase activity from skeletal muscle tissue and ECs was measured with the Nitric Oxide Synthase Assay Kit (EMD) following manufacturer instructions. Protein Kinase C activity from WT and SKO skeletal muscle tissue was measured with the PepTag Protein Kinase Assay (Promega) following manufacturer’s instructions. PKC inhibitors were used as indicated.

**Statistical analysis.**
Student’s t-test and one-way ANOVA with Tukey’s post-test for multiple comparisons were used to analyze the appropriate data using GraphPad PRISM software. Fisher’s exact test was used to compare clinical score data by comparing mice with no evidence of hindlimb necrosis to mice with any evidence of necrosis. Data are presented ±SEM in the Figures. A p-value <0.05 was considered statistically significant.

**RESULTS**

**Loss of S100A1 expression in patients and mice with critical lower limb ischemia.**

Ischemic gastrocnemius muscle (GM) tissue biopsies from from five patients with advanced chronic CLI (Rutherford stages 4-6) exhibited decreased S100A1 mRNA amount to approximately 5% of S100A1 levels in control biopsies (Figure 1A). Control non-ischemic GM samples were derived from three patients with indication for elective knee arthroplasty and exclusion of peripheral arterial occlusive disease (PAOD; for detailed patient characteristics see Supplement methods Table I.). Surgically-induced hindlimb ischemia in C57BL/6 wild type (WT) mice due to right-sided femoral artery resection (FAR) mirrored the loss of S100A1 expression in ischemic GM tissue (Figure 1B). S100A1 expression levels in hypoxic ECs being relevant to ischemic neovascularization was determined next. Murine arterial ECs exposed to 2%O₂ over 48 hours displayed rapid S100A1 mRNA downregulation (Figure 1C) similar to human iliac artery ECs (HIAECs, data not shown). S100A1 levels in isolated adult murine skeletal muscle (SM) fibers (Supplement Figure I.) involved in contractility regulation, however, exhibited lesser attenuation when treated with the same protocol (Figure 1C). Indicative of a greater susceptibility of ECs to hypoxia-induced loss of S100A1, our clinical observation prompted further investigation of the pathophysiological role of S100A1 in ECs in regenerative ischemic neovascularization taking advantage of S100A1 gene-ablated mice (SKO).
High rate of autoamputation in SKO mice with experimental hindlimb ischemia.

To determine the in vivo significance of our findings, we chose the model of experimentally-induced CLI due to FAR in 8-12 week-old WT and SKO mice. Recovery from hindlimb ischemia was classified employing a clinical score as previously described (Supplement methods). In WT mice, active use of the right foot declined after surgery but improved by day seven, and activity of the preserved limb ultimately returned to near-normal levels with an average score below one by day 15 (Figure 2A). A similar abrupt but persistent reduction in hindlimb use occurred in SKO mice that developed into partial or total autoamputation of the affected limb (average score greater than three) in 80% of SKO mice between seven and 14 days post-FAR (Figure 2A). Suggestive of a lack of regenerative reperfusion and/or revascularization, we next measured arterial blood flow recovery in ischemic SKO hindlimbs post-FAR.

Impaired reperfusion and neovascularization in ischemic hindlimbs of SKO mice.

Arterial limb perfusion recovery was examined by pulsed-wave (PW) Doppler ultrasonography (Supplement Figure II.). SKO mice at risk for autoamputation never achieved more than 25-35% perfusion in the tibialis posterior artery of ischemic hindlimbs one and two weeks post-FAR (Figure 2B). Accordingly, direct measure of ischemic tissue perfusion using colored microspheres at day 15 post-FAR unveiled an 80% blood flow reduction in SKO ischemic limbs compared with controls (Figure 2C). Structural analysis of postischemic bridge-collateral formation by immunofluorescent smooth muscle actin (SMA) staining and infra-abdominal aortic corrosion cast, respectively, revealed no evidence for decreased arteriogenesis in SKO animals when compared with WT-FAR (Figure 3A, Supplement Figure III.). However, histological and immunofluorescent analyses of the EC marker von Willebrand factor (vWF) and CD31, respectively, yielded a significant reduction of the capillary/fiber ratio in ischemic SKO muscle tissue (Figure 3B). Indicative of a predominant defect in postischemic angiogenesis, we raised the question whether loss of S100A1 per se might compromise proangiogenic properties of ECs.

Blunted proangiogenic properties of SKO ECs and rescue of defective capillary formation by NO.

Isolated SKO ECs displayed significantly lower proliferation (Figure 4A) and migration rates (Figure 4B) than WT ECs under normoxic conditions. Likewise, formation of capillary-like tube networks on growth factor supplemented matrigel was compromised in SKO ECs (Figure 4C). We next determined whether these defects might translate into distorted angiogenesis in vivo. To this end, we employed subcutaneously implanted matrigel plugs in 12-week-old WT and SKO mice that were harvested 16 days later. Bases of matrigel plugs explanted from WT mice were densely populated by CD31-positive ECs and SMA-stabilized capillaries were traceable up to the plug’s center sections (Figure 4D). In contrast, matrigel plugs recovered from SKO mice unveiled a significantly lower number of organized capillaries at the bottom and fewer penetrations towards the center (Figure 4D). Importantly, intraperitoneal NO supplementation with diethylenetriamine-NO (DETA/NO) was sufficient to rescue this defect. A DETA/NO dosage was used that was previously described not to significantly change blood pressure or heart rate in mice (2mg/kg) but exert NO-dependent cardiac protection. Explanted matrigel plugs 16 days after DETA/NO treatment displayed marked improvement of capillary neoformation that was virtually indistinguishable from WT mice (Figure 4E). The NO-mediated rescue directed our attention to molecular abnormalities in S100A1-deficient ECs that contribute to distorted postischemic NO and capillary formation.

Abrogated NO generation in ischemic hindlimbs of SKOs and abnormal eNOS regulation in S100A1-depleted ECs.

SKO ischemic GM tissue samples showed blunted postischemic NO production compared to WT FAR mice (Figure 5A). Abnormalities in VEGF- and hypoxia-mediated eNOS function in SKO ECs are unknown though. Here, we demonstrate for the first time an attenuated NO generation in response to
VEGF and hypoxia in cultured SKO ECs when compared with WT ECs (Figure 5B and Supplement Figure IV-B, resp.). To elaborate on potential mechanisms, human microvascular ECs (HMVECs) were subjected to siRNA-mediated knockdown of S100A1 (HMVEC-SKD) that resulted in an approximately 80% reduction of the protein after 48 hours (Supplement Figure IV-A). Alike SKO-derived ECs, HMVEC-SKDs responded with abrogated NO generation to stimulation by VEGF or hypoxia (2%O2) (Figure 5C and Supplement Figure IV-C, resp.). Subsequent phosphorylation analysis of the inhibitory eNOS threonine 495 (p-T495) site revealed significantly augmented levels in HMVECs-SKDs, whereas control HMVECs demonstrated enhanced phosphorylation of the stimulatory serine 1177 (p-S1177) site (Figure 5D). The previously demonstrated ability of S100 proteins to attenuate PKC activity, which plays a critical role in p-T495 phosphorylation,15 prompted us to study PKC activity.16,17 In line with abnormal eNOS regulation, HMVEC-SKDs exhibited significantly greater PKC activity under VEGF stimulation than control cells (Figure 5E). Inhibition of PKC with the chemical compound calphostin-C prevented the increase of eNOS pT-495 (Supplement Figure V-A). Consequently, HMVEC-SKDs experienced a significantly greater relative increase in NO generation upon PKC inhibition than control cells (Supplement Figure V-B). The partial restoration of NO generation by PKC inhibition suggested an additional mechanism and spurred the question whether S100A1 might directly interact and modulate eNOS activity in ECs and ischemic tissue.

**S100A1 directly interacts with eNOS in ECs in a Ca2+-dependent manner.**

In support of this argument, S100A1 co-precipitated with eNOS in a Ca2+-dependent manner using murine EC lysates (Figure 6A). Abrogated eNOS/S100A1 interaction by EGTA corroborated Ca2+-dependency of their binding. Increasing cytosolic Ca2+ concentrations in these cells either using bradykinin or the Ca2+ ionophore A23187 prior to extract preparation and immunoprecipitation further enhanced S100A1-eNOS interaction (Figure 6A). Addition of increasing concentrations of recombinant human S100A1 protein (rhS100A1) to homogenates of human umbilical vein ECs (HUVEC) showed dose-dependency of the Ca2+-dependent binding between both human proteins, and similar results were obtained with adenovirally-overexpressed S100A1 in HUVECs (data not shown). Brief hypoxia (2%O2, for 2 hours), which does not appreciably result in loss of S100A1 protein, similarly augmented S100A1/eNOS binding in control HMVECs (Supplement Figure VI). When examined by confocal microscopy both in murine WT ECs as well as HIAECs, S100A1 displayed a granular cytosolic pattern and co-localized with eNOS throughout the cell (Figure 6B). Employing the in situ duolink assay, the distinct S100A1/eNOS interaction pattern could be confirmed in intact HUVECs that corroborated the immunoprecipitation studies (Figure 6B).

**S100A1 protein positively modulates activity of eNOS.**

To determine whether this interaction actually modulates function of the enzyme, we carried out reconstituted eNOS activity assays both using recombinant bovine eNOS and immunoprecipitated eNOS from EC lysates. As shown in Figure 6C, addition of increasing concentrations of rhS100A1 dose-dependently augmented the Ca2+/CaM-dependent arginine-citrulline conversion rate both of bovine recombinant and EC-derived eNOS. Interestingly, quantitative assessment of the CaM-eNOS interaction in S100A1-overexpressing and control HUVECs applying the duolink assay provided no evidence for enhanced CaM-eNOS binding through increased cellular S100A1 protein levels (Supplement Figure VII). Vice versa, increasing concentrations of recombinant CaM did not attenuate S100A1 co-precipitating with eNOS (data not shown). These results indicate an additive activation of eNOS through both Ca2+-sensors rather than mutually competitive or facilitated binding. Incubation of immunoprecipitated eNOS from both groups with recombinant CaM resulted in a significantly greater increase in eNOS activity from S100A1-competent WT tissue than SKO. Ensuing administration of rhS100A1 protein further increased eNOS activity from SKO, corroborating the additive effect of both Ca2+-sensors (Figure 6D). Indicating that S100A1 deficiency both directly and indirectly impairs eNOS function, we continued to investigate how
these molecular abnormalities might affect in vivo responsiveness and efficiency of the VEGF-PI3K-Akt-eNOS signaling axis in ischemic tissue.

**Excessive postischemic VEGF levels and attenuated VEGFR2 signal relay in SKO ischemic hindlimbs.**

Analysis of the oxygen sensor hypoxia-inducible factor 1α (HIF-1α), which is known to drive vascular endothelial growth factor (VEGF) expression, revealed significantly greater protein levels in ischemic SKO than ischemic WT GM (Figure 7A). Concordantly, postischemic abundance of VEGF protein in SKO hindlimbs exceeded the VEGF rise in WT tissue by approximately 10-fold (Figure 7B). Similar abnormalities were found for other hypoxia-regulated growth factors including hepatocyte growth factor (HGF), placental growth factor (PIGF) and insulin-like growth factor (IGF) (Supplement Figure VIII) indicating unresolved ischemia in SKO hindlimbs. Previous studies that reported rapid degradation of VEGF receptor-2 (VEGFR2) in response to sustained VEGF stimulation via a PKC-dependent mechanism prompted investigation of abundance and phosphorylation status of key effectors along the VEGFR2 signaling axis. Both total and phosphorylated VEGFR2 protein levels were hardly detectable in ischemic SKO tissue extracts compared with only a slight decrease in ischemic WT (Figure 7C). When HMVEC-SKDs were exposed to VEGF, faster in vitro VEGF2 degradation was observed compared with controls and this effect was blocked by the PKC inhibitor calphostin-C (Supplement Figure IX.). Interestingly, ischemic SKO tissue unveiled significantly higher postischemic PKC activity (Supplement Figure X.), akin to enhanced PKC activity in hypoxic HMVEC-SKDs. Accordingly, relative active phosphorylation status of VEGFR2 downstream effectors such as PI3Kγ, PDK and Akt was significantly lower in ischemic SKO–derived GM compared with ischemic WT, whereas total levels were significantly higher (Figure 7D, Supplement Figure XI-A.). VEGFR1 in vivo levels were indeed unchanged in both groups (Figure 7C) while downstream activity of the PLCγ-ERK1/2 axis was significantly higher in ischemic SKO than WT tissue (Supplement Figure XI-B.). In line with published reports, we confirmed that VEGFR1 remains unaltered in ECs in response to prolonged VEGF stimulation (Supplement Figure XII.). Besides evidence for blunted postischemic VEGFR2 signaling in SKO, the postischemic VEGF overshoot might also entail dysbalanced VEGFR1 activity.

**Inhibitory eNOS phosphorylation pattern in SKO ischemic hindlimbs.**

Driven by abnormal in vitro eNOS regulation and attenuated in vivo VEGFR2-PI3K-Akt signaling, we next raised the question whether eNOS phosphorylation pattern and expression was similarly modified in ischemic tissue from SKO mice. Interestingly, ischemic SKO GM tissue unveiled an approximately two-fold increase in total eNOS protein expression over WT (Figure 8A). In line with our in vitro results, the enzyme exhibited a more than 7-fold increase in phosphorylation of the inhibitory phospho-threonine 495 (p-T495) over the stimulatory phospho-serine 1177 (p-S1177) site (Figure 8A) when compared with WT. Among PKC isoforms that are known to attenuate Ca²⁺/CaM-dependent eNOS activity through p-T495 phosphorylation, expression analysis showed a marked increase in PKCe protein and phosphorylation levels (Figure 8B) in accordance with elevated PKC activity in ischemic SKO tissue homogenates (Supplement Figure X.). Expression analysis of PP1, which dephosphorylates the p-T495 site, showed no difference between groups (data not shown).

**Direct NO substitution salvages ischemic limb loss in SKO mice.**

Given the fact that NO administration is sufficient to rescue defective in vivo angiogenesis in SKOs, we finally sought to answer the question whether the observed lack of NO bioavailability is actually causative for tissue necrosis in SKO FAR mice. To this end, WT and SKO animals were treated with DETA/NO for three consecutive days immediately post-FAR. Using the same dosage that rescued in vivo angiogenesis in SKO mice, NO treatment completely prevented autoamputation in all SKO mice (clinical score below one), while five out of six saline injected SKO mice still displayed evidence of limb necrosis 15 days post-FAR (Figure 8C). Our in vivo rescue corroborates defective NO bioavailability as
key for the postischemic SKO phenotype and NO supplementation as sufficient to bypass abnormal eNOS regulation and restore defective angiogenic activity.

DISCUSSION

This is the first report providing direct genetic evidence for a critical role of S100A1 in postnatal ischemic angiogenesis. Most salient findings of this study, that originates from the clinical finding of diminished S100A1 expression in patients with CLI, are the identification of endothelial S100A1 as i) an indispensable factor for mounting an adaptive angiogenic response to ischemia, and ii) a stimulatory eNOS-interacting protein critical for postischemic NO generation. To determine S100A1’s role in postischemic revascularization and decipher underlying cellular and molecular mechanisms, we took advantage of the hindlimb ischemia model in SKO mice. Among the relevant differentiated cell types expressing S100A1 in muscle tissue, ECs emerged as particular susceptibility to a hypoxia-induced loss of S100A1 expression. Our focus was thus drawn on S100A1’s impact on in vitro and in vivo endothelial proangiogenic properties being essential to reparative neovascularization.

After surgical blood flow interruption, SKO mice exhibited a high rate of tissue necrosis and subsequent auto-amputation of ischemic limbs due to insufficient NO generation. Similarly severe phenotypes have been observed in mice with homozygous ablation of Akt1 and eNOS, which groups S100A1 alongside genes that are crucial for postnatal neovascularization through the modulation of NO homeostasis. In support of this notion, assessment of changes in postischemic blood flow revealed failure of arterial perfusion recovery in SKO mice akin to Akt1- and eNOS-knockout mice undergoing FAR. However, ensuing investigation of bridge-collateral formation that fuels distal blood flow and occurs under normoxic conditions even showed a trend towards augmented collateral wall formation in SKO. This implicates at least normal smooth muscle cell (SMC) expansion compared with WT, and is consistent with reports on unchanged function of S100A1-deficient SMCs. It is thus conceivable that normal amounts of collateral arteries grew in SKOs post FAR but were unable to dilate adequately due to dysfunctional S100A1-deficient endothelium. The endothelial defect could explain in part diminished arterial blood flow recovery in SKO mice. Notwithstanding, evaluation of exercise- and shear-stress-based vasodilation of SKO arteries is needed to fully understand the role of S100A1 in EC-dependent arterial reserve mechanisms.

Turning our attention to angiogenesis then unveiled a striking difference in postischemic microvasculature formation that clearly distinguished post-FAR SKO from WT mice. Ischemic limbs of WT mice exhibited the expected hypoxia-driven enhancement of capillary formation. In contrast, SKO mice failed to mount an adaptive angiogenic response. Hence, SKO that suffered autoamputation may not have even reached the critical threshold of newly formed microvasculature necessary to preserve their limbs. In line with this argument, essential steps of the fine-tuned sequence of postischemic capillary formation including EC proliferation, migration and capillary-like tube network formation were all severely compromised in SKO ECs. Use of an integrative in vivo angiogenesis assay substantiated the postnatal inability of SKO mice to create three-dimensional capillary networks. Although these assays are not representative of the site and environment where postischemic angiogenesis occurs, they nevertheless unearth a basic defect in SKO ECs that might explain in part defective postischemic angiogenesis seen in vivo. These results further imply a pathophysiological role of the hypoxia-driven loss of endothelial S100A1 expression for impaired revascularization seen in patients with chronic CLI. Molecular mechanisms attenuating S100A1 expression in hypoxic ECs remain to be determined.

Despite the complexity of postischemic capillary neoformation, NO emerged as an ultimate signaling molecule governing this process. In line with this notion, we found impaired NO generation...
both in S100A1-deficient ischemic tissue, as well as in hypoxic and VEGF-stimulated S100A1-depleted ECs. Previous reports have already attributed abnormal Ca\textsuperscript{2+}-mobilization from intracellular stores in SKO ECs to diminished Ca\textsuperscript{2+}-dependent NO generation in response to B\textsubscript{2}-kininergic and M\textsubscript{2}-muscarinic stimuli.\textsuperscript{3,4} Although VEGF-mediated eNOS activation relies in part on Ca\textsuperscript{2+}-mobilization,\textsuperscript{25} the severity of the SKO phenotype suggested an even more substantial role of S100A1 in posts ischemic eNOS regulation. Ensuing molecular and imaging studies actually revealed first evidence for a direct Ca\textsuperscript{2+}-dependent stimulatory interaction of S100A1 with eNOS in ECs. S100A1 protein dose-dependently augmented eNOS activity and potentiated the effect of Ca\textsuperscript{2+}/CaM-dependent stimulation without evidence for facilitated CaM-eNOS interaction in ECs. These novel results support the notion that S100A1 might be a direct and positive modulator of eNOS function and advance our current understanding of S100A1's molecular actions in ECs. These data provide the rationale for continued studies determining the relevant binding domains between eNOS and S100A1 and its impact on other accessory eNOS modulators such as Hsp90 or caveolin-1 in greater detail.\textsuperscript{15}

In addition, our study unveiled another novel regulatory mechanism by which S100A1 can indirectly modulate eNOS function that involved PKC. S100 proteins have been reported to attenuate PKC activity by interfering directly with PKC, or indirectly with PKC substrates.\textsuperscript{16,17} The kinase has previously been described to negatively interfere with eNOS activity by p-T495 phosphorylation.\textsuperscript{15} Here, we firstly demonstrate that S100A1 is necessary to attenuate PKC-dependent inhibitory phosphorylation of eNOS in VEGF-stimulated ECs. Partial restoration of NO generation in S100A1-depleted ECs by chemical PKC inhibition corroborates the significance of this finding. Since it is still not entirely clear how S100 proteins can mitigate PKC activity, more work is required to uncover the mechanism that confers S100A1-mediated inhibition of PKC in ECs. Nonetheless, from the deepened mechanistic insight, we conclude that compromised posts ischemic eNOS function in SKO and S100A1-depleted ECs is most likely orchestrated both by interrupted stimulatory S100A1/eNOS interaction and augmented p-T495 eNOS phosphorylation due to PKC hyperactivation.

Both molecular defects seem to have the potential to negatively amplify each other and might essentially contribute to blunted in vivo proangiogenic properties of S100A1-deficient ECs. We therefore focused next on in vivo consequences by examining the VEGF signaling-axis and eNOS post translational modifications. Reflecting unresolved tissue ischemia,\textsuperscript{18} protein levels of the main oxygen sensor HIF-1\textalpha were significantly higher in ischemic SKO tissue, and ensuing VEGF accumulation in operated SKO limbs exceeded the ischemic response in WT mice by more than 10-fold. But excessive VEGF production is not a genuine defect of SKO ECs since in vitro hypoxic VEGF production was indistinguishable from WT ECs (data not shown). Interestingly, eNOS mice subjected to FAR responded with a posts ischemic decrease in VEGF mRNA expression due to a proposed concurrent genetic defect,\textsuperscript{26} indicating that the SKO phenotype might differ substantially with respect to adaptive proangiogenic pathways. The concomitant increase of the angiogenically active cytokines HGF, IGF and PlGF in SKO post-FAR further substantiates the view of a rather futile secondary VEGF overshoot that might in turn disturb the finely regulated proangiogenic cross-talk between growth factors.\textsuperscript{19}

This is important to note since sustained VEGF stimulation can result in rapid downregulation and degradation of its receptor tyrosine kinase VEGFR2.\textsuperscript{19} In contrast to WT, assessment of VEGFR2 protein and phosphorylation uncovered virtually undetectable levels in ischemic SKO tissue. A potentially causal role for endothelial S100A1 herein emerged from our in vitro results showing accelerated VEGF2 degradation in VEGF-stimulated S100A1-depleted ECs. Attenuation of this defect by a chemical PKC inhibitor once more implies a role for PKC, which is involved in VEGF-mediated VEGFR2 downregulation and degradation.\textsuperscript{19,20} Enhanced PKC activity in S100A1-depleted ECs might therefore contribute to in vivo VEGFR2 degradation in SKO mice. In line with prevailing literature,\textsuperscript{19,27} it is tempting to speculate that loss of S100A1 feeds into a vicious cycle of reduced in vivo VEGF-responsiveness nurtured in part by endothelial VEGFR2 degradation, which again fuels a secondary VEGF overshoot and compromises residual angiogenic capacity in SKOs. Accordingly, the downstream

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PI3K-Akt-eNOS signaling cascade, which accounts for many of the VEGF actions, including EC proliferation, migration, tube formation, and promotion of the release of NO\(^{23}\) yielded marked abnormalities in ischemic SKO tissue. Relative active site phosphorylation levels for PDK and Akt were reduced supporting the notion of abrogated activation. In this regard, permanent upregulation of PI3K, PDK, Akt and eNOS protein expression might be viewed as compensatory attempts to uphold efficacy. Interestingly, eNOS\(^{-/-}\) mice subjected to FAR displayed similarly decreased postischemic Akt activation, although opposed to SKOs, total Akt levels were reported to be unchanged.\(^{28}\)

Most importantly, however, the balance between activating and inhibitory phosphorylation of eNOS in ischemic SKO samples was significantly shifted in favor of the p-T495 site. This observation is consistent with diminished in vivo ischemic NO bioavailability and mirrors our in vitro results of an abnormal eNOS phosphorylation pattern in S100A1-depleted ECs upon VEGF stimulation.\(^ {15,21,25}\) In view of our in vitro data, enhanced in vivo activity of PKC in ischemic SKO tissue might also be in part accountable for inhibitory eNOS phosphorylation. In support of this notion, indistinguishable expression and phosphorylation of PP1 between groups rather excludes a role for abnormal activity of the phosphatase assigned to the p-T495 site.\(^ {15}\) The role of other phosphatases potentially regulated by S100A1 such as PP5,\(^ {29}\) however, requires further evaluation. Increased PLC\(\gamma\) expression and ERK1/2 activity might finally result from a relative increase in VEGFR1 signaling\(^ {19}\) due to imbalances between VEGFR2 and VEGFR1 levels in ischemic SKO tissue. This might add to signaling abnormalities in ischemic SKO tissue.

Ultimate proof for the pathophysiological key role of compromised eNOS function in the SKO phenotype came from rescue experiments using pharmacological NO supplementation. NO was sufficient both to salvage ischemic SKO limbs and to restore in vivo microvasculature formation. Interestingly, a previous study showed that DETA/NO did not improve angiogenesis in ischemic hindlimbs of eNOS\(^{-/-}\) mice.\(^ {13}\) But this study used a 25-fold higher DETA-NO concentration, which suppressed in vivo angiogenesis in WT mice in our hands (data not shown). Hence, it seems reasonable to assume that endothelial S100A1-deficiency might blunt in vivo ischemic NO generation and angiogenesis both through interrupted S100A1-facilitated eNOS activation, unopposed PKC-mediated p-T495 phosphorylation and VEGFR2 degradation.

Despite the potential relevance of these novel mechanistic findings, important limitations of our study are noteworthy. First, FAR-induced hindlimb ischemia in otherwise healthy mice is not equivalent to CLI in humans which is frequently associated with comorbid illnesses\(^ {30}\) and thereby warrants evaluation of S100A1’s role in the context of hyperglycemia and hypercholesterolemia. Second, in light of previously reported functions of S100A1 in endothelial Ca\(^{2+}\) homeostasis,\(^ {3,4}\) we expect dysfunctional eNOS not to be the sole mediator of the postischemic SKO phenotype.\(^ {23}\) Besides NO, Akt suppresses various pro-apoptotic pathways and given attenuated Akt activity extended analysis of the potential susceptibility of SKO ECs to cell death is certainly needed. Third, oxidative stress resulting from inflammation and potential eNOS uncoupling may also play a role in defective angiogenesis in SKO mice.\(^ {25}\) Fourth, NO generation in cellular assays was measured only indirectly by assessing NO\(_2^-\)/NO\(_3^-\) levels. More sensitive assays are certainly needed in subsequent studies deciphering the impact of the S100A1/eNOS interaction on NO homeostasis. Finally, a contribution of altered S100A1 skeletal muscle expression to ischemic limb loss is possible. The observed marginal attenuation of S100A1 expression in differentiated hypoxic skeletal muscle fibers and barely detectable S100A1 expression in WT skeletal myoblasts (Supplement Figure XIII.) nevertheless weakens the notion of a relevant role of skeletal muscle S100A1, at least in angiogenesis.

Overall, our clinically driven experimental study identifies S100A1 as critical for postnatal ischemic angiogenesis and eNOS function. Our molecular results, summarized in Supplement Figure XIV., provide advanced mechanistic insight demonstrating that the lack of endothelial S100A1 might attenuate in vivo eNOS activity by at least three distinct but additive/synergistic pathomechanisms –
firstly, interrupted stimulatory S100A1/eNOS interaction; secondly, PKC hyperactivity that triggers inhibitory eNOS phosphorylation and abrogates VEGFR2 signal relay; and thirdly, as previously shown, insufficient IP3R-mediated Ca\(^{2+}\) release required for eNOS activation. These findings will prompt further studies to probe the microvascular therapeutic potential of restoring S100A1 levels in ischemic vascular disease.

**SOURCES OF FUNDING**  
This work was funded by NIH grants R01 HL 07842 (to KP), R01 HL 092130-01 and HL 092130-02S1 (to PM), and R01 HL56205 (to WJK), a postdoctoral fellowship from the American Heart Association (Great Rivers Affiliate) (to GR), the Rahel Goitein-Straus Scholarship from the Heidelberg University Medical Faculty (to CL), and grants from the Deutsche Forschungsgemeinschaft (#562 to PM and SP) and the German Cardiovascular Research Center (DZHK to PM and HAK).

**DISCLOSURES**  
None of the authors have any financial interests to disclose.

**REFERENCES**


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FIGURE LEGENDS

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Figure 2. S100A1 deficient mice undergo auto-amputation following femoral artery resection (FAR). (A) WT or SKO mice underwent FAR of the right hind limb (red circles). Representative examples of animals 15 days post-op are shown. Clinical score was assessed 15 days post-op (n=19 for each genotype, see Methods section for description of clinical scores). * p< 0.05, Fisher's exact test. (B) Hind limb perfusion was assessed by determining the velocity time integral (VTI) ratio of the blood flow in the tibial posterior artery of the ischemic and the contra-lateral non-ischemic limb using a pulsed wave doppler (n=10 for each genotype, p<0.05 WT vs. SKO). (C) Perfusion ratio of the ischemic to non-ischemic hind limb was assessed in mice 15 days post FAR using colored microspheres (n=5 for each genotype, * p<0.05 ischemic SKO vs. ischemic WT)

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Figure 4. Blunted proangiogenic properties of SKO ECs in vitro and defective capillary formation in vivo. Primary murine ECs were isolated from WT and SKO mice and were assayed for: (A) [3H] thymidine incorporation in response to different concentrations of FBS with or without endothelial cell growth supplement (ECGS). (B) The chemokinetic response to 1%FBS using an in vitro wound repair (Scratch-) assay (24h), (C) EC tube formation on Matrigel matrix. All experiments were repeated at least three times with different primary isolates and performed in duplicate. Representative images are shown in (B) and (C). The stippled area in (B) denotes the extent of the originally denuded area. *p<0.05 vs WT. (D) Matrigel plugs were implanted into WT and SKO mice (n=3) and harvested after 16 days. Recovered plugs (two for each animal) were cryoembedded and stained for the EC marker CD31 and smooth muscle actin (SMA). Nuclear DNA was visualized with Hoechst 33342 (10x magnification, scale bar=200µm, *p<0.05 WT vs SKO, n=6 plugs each genotype). Representative images (60x magnification, scale bar=20µm) are shown, CD31 (green), SMA (red), DNA (blue). (E) Matrigel plugs were implanted into WT or SKO mice. Mice received DETA/NO (2 mg/kg, i.p.) or saline three times weekly for two weeks. Plugs were harvested 16 days post implantation, cryoembedded and stained as in (D) at a depth of 1 mm. Scale bar=200µm. *p<0.05 vs SKO +DETA/NO.

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**Figure 6.** S100A1 interacts with eNOS and regulates its activity. (A) Lysates were prepared from ECs pretreated, or not, with A23187 or bradykinin. In a separate experiment immunoprecipitations were performed in the presence of Ca^{2+} or EGTA. Immunoblots were probed for the presence of eNOS or S100A1. Representative blots are shown, the experiment was repeated at least three times with similar results. (B) ECs were immunofluorescently labeled with an antibody to eNOS (red) or S100A1 (green) and assayed by confocal microscopy (40x magnification, scale bar=40um); enlarged merged images show partial co-localization (yellow). Close protein contact between S100A1 and eNOS was also shown using the duolink assay (60x magnification, scale bar=10um). (C) Recombinant bovine eNOS (upper panel) or eNOS immunoprecipitated from HUVEC cell extract (lower panel) was incubated with 1 mM Ca^{2+} in the presence or absence of CaM and increasing amounts of rhS100A1, as indicated. eNOS activity was determined by measuring the conversion of [3H] L-arginine to L-citrulline. # p<0.05 vs reaction in the absence of added S100A1. *p<0.05 vs reaction in the absence of added CaM and rhS100A1. The experiment was performed in duplicate and repeated 3 times. (D) WT and SKO mice were subjected to FAR and extracts from ischemic muscles were prepared 3 days later and used to immunoprecipitate eNOS. eNOS activity in the immunoprecipitates was assayed with or without added CaM and/or S100A1 and adjusted to eNOS protein content (n=6, * p<0.05 SKO vs WT).

**Figure 7.** Augmented activation of HIF-1α and VEGF in the ischemic hind limb of SKO mice disturbs VEGFR2 signaling, leading to eNOS inhibition. SKO and WT mice were subjected to FAR and gastrocnemius muscles of the ischemic and contra-lateral non-ischemic hind limb were procured and homogenized after three days (except for VEGF). Representative samples are shown for all immunoblots. Muscle extracts were immunoblotted for (A) HIF-1α, (B) VEGF (at times indicated), (C) total and p-Y1175 VEGFR2 and VEGFR1, (D) total and p-S473-Akt. All expression levels were normalized to GAPDH (n=4 for each genotype and condition, * p<0.05 vs ischemic WT).

**Figure 8.** NO donor administration prevents auto-amputation of the ischemic SKO hind limb and restores angiogenesis. (A) Muscle extracts from WT and SKO mice were prepared 3 days post FAR and immunoblotted for eNOS (total, p-T495 and p-S1177) and (B) total and p-PKC-ε. (C) SKO mice underwent FAR and received the NO donor DETA/NO (2 mg/kg, i.p.) or saline daily for the following three days. Clinical score was assessed at day 15 post-op (n=6, * p< 0.05 vs. non-ischemic saline). *p<0.05 vs SKO +DETA/NO.
Novelty and Significance

What Is Known?

- The EF-hand calcium (Ca2+) sensor protein S100A1, expressed in endothelial cells (ECs), facilitates endoplasmic reticulum (ER) mediated Ca2+ release which promotes Ca2+-dependent eNOS activity.

- Under normoxic conditions, the lack of endothelial S100A1 results in endothelial dysfunction by attenuating NO generation and agonist-induced vascular relaxation.

- In ischemic tissue, the endothelial NO synthase (eNOS) is generated as a component of the vascular endothelial growth factor (VEGF) signaling pathway that is involved in postischemic revascularization.

What New Information Does This Article Contribute?

- S100A1-deficient mice demonstrate postischemic hindlimb loss after arterial blood flow interruption due to defective postischemic angiogenesis. The latter related to defective VEGF-mediated eNOS activation and proangiogenic EC properties.

- S100A1 promotes postischemic NO generation in ECs by directly activating eNOS via a Ca2+-dependent mechanism as well as by preventing the protein kinase C (PKC) mediated phosphorylation of the inhibitory threonine-495 eNOS site and VEGF receptor 2 degradation.

- Molecular insights into defective vascular repair in S100A1-deficient mice links downregulated S100A1 expression in patients with advanced critical lower limb ischemia to impaired ischemic angiogenesis.

Here we describe a novel molecular pathomechanism with potential clinical significance for chronic critical limb ischemia (CLI), the most advanced form of peripheral arterial occlusive disease (PAOD). From our study, endothelial S100A1 emerged as an indispensable molecular factor for postischemic eNOS activation and angiogenesis, and which is therefore necessary for blood flow restoration and prevention of tissue necrosis, at least, in mice, and potentially in men. From a biological perspective, the central role of eNOS in angiogenic signaling in cardiovascular biology and disease highlights the importance of S100A1 as a direct regulator of eNOS activity. Its dual actions on Ca²⁺ and PKC activity, which can prevent the phosphorylation of eNOS on an inhibitors site and VEGF signaling, appear equally important. From a clinical standpoint, these observations link dysfunctional human ECs with hypoxia-induced loss of S100A1 and disturbed NO generation with insufficient reparative angiogenesis in patients with CLI and S100A1 expression loss in ischemic tissue. The advanced developmental stage of genetically-targeted therapies against heart failure that exploit S100A1’s pleiotropic actions in cardiomyocytes, will prompt translational research to probe the microvascular therapeutic potential of preserving or restoring endothelial S100A1 expression.
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**S100A1 is downregulated in critical limb ischemia.** (A) Digital subtraction angiography of a representative patient with peripheral arterial occlusive disease (PAOD) due to femoro-popliteal arterial obstruction (arrow points to site of proximal arterial obstruction). Gastrocnemius muscle biopsies (arrowhead) were analyzed for S100A1 mRNA expression using qRT-PCR (n=3 control, 5 CLI, *p<0.05) (Scale bar=5cm). (B) WT mice were subjected to FAR and S100A1 protein levels in GM muscle samples were assessed by immunoblot analysis before (NI) or 3 days post FAR (I) (n=3 each *p<0.05 vs NI). (C) Isolated primary murine WT EC or Skeletal muscle fibers were subjected to hypoxia for up to two days. mRNA was extracted and subjected to qRT-PCR for S100A1 (n=3, *p<0.05 vs EC).
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S100A1 Deficiency Impairs Post-Ischemic Angiogenesis via Compromised Proangiogenic Endothelial Cell Function and Nitric Oxide Synthase Regulation
Patrick Most, Carolin Lerchenmüller, Giuseppe Rengo, Adrian Mahlmann, Julia Ritterhoff, David Rohde, Chelain Goodman, Cornelius J. Busch, Felix Laube, Julian Heissenberg, Sven T. Pleger, Norbert Weiss, Hugo A. Katus, Walter J. Koch and Karsten Peppel

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Data Supplement (unedited) at:
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Circulation Research online-supplement: S100A1 deficiency impairs post-ischemic angiogenesis via compromised proangiogenic endothelial cell function and nitric oxide synthase regulation

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B) Supplemental Figures……………………………..…………………………………….page 9-22

A) Supplemental Methods

Patients

CLI was defined according to the criteria defined by the Inter-Society Consensus for the management of Peripheral Arterial Disease (TASC II). This includes duration of ischemic rest pain and ischemic skin lesions for more than 2 weeks induced by objectively confirmed PAOD. To prove PAOD ankle brachial index (ABI) or toe-brachial index (TBI) were measured and pulse volume recordings at the forefoot and toe were performed. Patients were included with CLI (Rutherford stage 4-6) due to femoro-popliteal (+/- crural, +/- iliac) vascular obstructions and an indication and technical possibility for surgical revascularization (femoral-distal bypass graft with distal anastomosis to crural arteries or the infrageniculate popliteal segment), or patients with indication for major amputation. Before surgical revascularization or major amputation diagnostic angiography was performed, which proved involvement of the femoro-popliteal artery. Exclusion criteria included isolated obstructions of crural arteries without upstream iliaco-femoral-popliteal obstruction, isolated foot or acral ischemia, super-infected lesions with systemic signs of infection, peripheral vascular disease of non-atherosclerotic causes (e.g. vasculitis, vascular injury, aneurysmal disease with peripheral embolization, cardioembolic occlusions). In the control group, ABI and, in addition, pulse-volume recordings were performed to exclude PAOD. Patients gave their written informed consent to participate in the study. The study protocol had been approved by the ethics committee of the Medical Faculty at the Technische Universität Dresden.

Table I. shows the characteristics of the patients whose samples were analyzed.

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<th>Control</th>
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<tr>
<td>age</td>
<td>58.0 ± 6.2</td>
<td>78.8 ± 7.3</td>
</tr>
<tr>
<td>gender: female / male</td>
<td>66.7 % / 33.3 %</td>
<td>60 % / 40 %</td>
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<td>33.3 %</td>
<td>0 %</td>
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<tr>
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<td>0 %</td>
<td>20 %</td>
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<tr>
<td>arterial hypertension</td>
<td>100 %</td>
<td>100 %</td>
</tr>
<tr>
<td>hyperlipoproteinemia</td>
<td>100 %</td>
<td>100 %</td>
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Muscle biopsies from the proximal gastrocnemius muscle (1cm³) were collected during surgery and immediately immersed in RNAlater solution according the manufacturer's instruction (Ambion) and stored at -80°C until further analysis. Total RNA was extracted using the RNeasy Plus Universal Kit (Qiagen).

Detailed Patient characteristics and co-morbidities:

Patients with chronic critical limb
G1  87, female, arterial hypertension, hyperlipoproteinemia
G7  79, female, arterial hypertension, hyperlipoproteinemia
G8  81, male, arterial hypertension, hyperlipoproteinemia, diabetes mellitus
G9  67, female, arterial hypertension, hyperlipoproteinemia
G14  80, male, arterial hypertension, hyperlipoproteinemia

Control group
O1  53, male, arterial hypertension, hyperlipoproteinemia, smoker
O3  56, female, arterial hypertension
O10  65, female, arterial hypertension

RNA Isolation, Reverse Transcription and Quantitative Real-Time PCR

Total RNA isolation from human and murine skeletal muscle tissue samples was performed using Trizol Reagent (Invitrogen) according to the manufacturer’s protocol. Quality of RNA was assessed by running an aliquot on a denaturating agarose (1%) gel. First strand cDNA synthesis from 1µg of total RNA was carried out by the use of the iScript cDNA Synthesis Kit (BioRad). For real-time PCR, 6.5 µl of diluted cDNA (1/100) was added to a 15µl mixture that contained a 1× concentration of iQ SYBR Green Supermix (BioRad) and 100nM of gene-specific oligonucleotides. Subsequently, real-time PCR was carried out on a MyiQ Single-Color Real-Time PCR detection system (BioRad) for murine and human 18s and S100A1 expression levels as indicated. Sequences of gene-specific oligonucleotide primers (Eurofins MWG) (Table 2) - based on cDNA sequences in the National Center for Biotechnology database - were generated by the use of PRIMER3 software. After each run, saturation of each amplification cycle was controlled by the use of MyiQ software (version 1.0) and, subsequently a melting curve acquired by heating the product to 95°C, cooling to and maintaining at 55°C for 20 seconds, then slowly (0.5°C/s) heating to 95°C was used to determine the specificity of the PCR products, which were then confirmed by gel electrophoresis.

Table II. shows sequences of gene-specific oligonucleotide primers.

<table>
<thead>
<tr>
<th></th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
<tbody>
<tr>
<td>18s mouse/human</td>
<td>5′-TCAAGAACGAAA GTCGGAGG -3′</td>
<td>5′-GGACATCTAAGG GCATCAC-3′</td>
</tr>
<tr>
<td>S100A1 mouse</td>
<td>5′-CCATGGAGACCC TCATCAAT -3′</td>
<td>5′-TTGAAGTCCACT TCCCCATC -3′</td>
</tr>
<tr>
<td>S100A1 human</td>
<td>5′-CGATGGAGACCC TCATCAAC-3′</td>
<td>5′-TGGAAGTCCACC TCCCCGTC -3′</td>
</tr>
</tbody>
</table>

Mice

S100A1 deficient (SKO) mice, backcrossed at least 10 generations onto C57Bl/6, have been described, and were kept as inbred colonies. C57Bl/6 (WT) mice were purchased from Jackson Labs (Bar Harbor, ME stock # 664). All experiments were performed according to protocols approved by the Institutional Animal Care And Use Committee and complied with the Guide for the Care and Use of Laboratory Animals. SKO mice appear to have no defect in vascular development.

Femoral artery resection (FAR)

Mice were anesthetized with isofluorane, and the right common femoral artery was resected as described. In the course of our study, a modified minimal surgery by removal of only about 2 mm of the upper femoral artery that excluded the saphenous arterial branch was
used for all experiments except those depicted in Figures 2A and 8A, C to minimize necrosis and allow for adequate recovery of ischemic muscle tissue from SKO mice. Using this minimal surgery SKO mice did not experience necrosis beyond their digits. Clinical score severity was determined visually 15 days post operation and ascribed as follows: 0= normal; 1= pale foot or gait abnormality; 2= toe necrosis or auto-amputation; 3= half foot necrosis or auto-amputation; 4= entire foot necrosis or auto-amputation; 5= lower limb necrosis or auto-amputation. In some cases WT and SKO mice were treated with the NO donor diethylenetriamine/NO (DETA/NO (Sigma), 2 mg / kg in 200 µl PBS, i.p.) for 3 consecutive days immediately following FAR.

Assessment of hindlimb ischemia

**Blood flow measurement.** Blood Flow (BF) was evaluated by pulsed-wave Doppler (PW-doppler)\(^6\) using a VisualSONICS VeVo 770 imaging system with a 10Mhz scanhead in isofluorane-anesthetized mice (2% v/v). BF was measured before FAR, immediately after FAR (2-3 hours), seven days, and 15 days post-FAR. BF was evaluated on the tibial posterior artery of the ischemic and non-ischemic hindlimb by measuring maximal velocity (Vmax). Data are expressed as ischemic to non-ischemic hindlimb BF ratio for each animal at each time point. Mice undergoing ischemic hindlimb auto-amputation/necrosis were not included for Echo Doppler measurements.

**Microsphere uptake.** Fifteen days post-surgery, mice were anesthetized with isofluorane, and a PE-10 catheter was advanced into the abdominal aorta through the left common carotid, as previously described.\(^7\) Maximal vasodilatation was obtained by nitroglycerin (2 µg i.a.) followed by injection of 3x10\(^6\) orange dyed beads (15µm diameter, Triton Technologies). Animals were then sacrificed; gastrocnemius samples of the ischemic and non-ischemic hindlimb were collected, frozen in liquid nitrogen and stored at -80°C. Next, samples were homogenized and digested according to manufacturer protocol, the beads were collected and suspended in N,N-dimethylthioformamide (DMTF). Dye release was assessed by light absorption at 450 nm. Data are expressed as ischemic to non-ischemic hindlimb ratio. Mice undergoing ischemic hindlimb auto-amputation/necrosis were not included in the analysis.

**Immunohistochemistry.** Mouse gastrocnemius muscles were harvested 15 days post-FAR and paraffin embedded using standard histology techniques. 5µm sections were rehydrated and stained for endothelial cells using an anti vWF antibody (DAKO #A0082), followed by counterstaining with hematoxylin and eosin to visualize muscle fibers.

**Immunofluorescent analysis of collateral formation.** Mouse gastrocnemius muscles were harvested 40 days post FAR, placed in tight fitting plastic tubes, flash frozen in liquid N\(_2\) and sectioned (10µm) on a cryostat (Leica). Sections were fixed in ice-cold MeOH:Acetone (1:1), blocked in Rockland NIR-buffer and incubated with a Cy3 conjugated anti SM-actin antibody (clone 1A4, Sigma, 1:400 dilution). All specimens were imaged at 1.6x using a Sensicam high-resolution camera and Streampix image software (Norpix) using same illumination and acquisition conditions. Conversion to binary images was done using Image J and collaterals of different sizes were counted using Image J. The diameter of a collapsed collateral was estimated by measuring the total area of a sm-actin positive collateral and assuming a spherical representation in vivo.

**Corrosion casting of hindlimb circulation.** Corrosion casting was done as described before.\(^8\) Briefly, mice were anesthetized and anticoagulated by i.p injection of heparin (10 Units/g body weight) prior to experimentation. Subsequently, the thoracic aorta was excised and cannulated using a 21-gauge needle, and the tissue was flushed with buffered saline at a monitored pressure of 80–100mmHg. Following the removal of blood, a prefixation of the vascular system was done using 2% glutaraldehyde and tissue was then infused with approx 600µl of Mercox resin (Ladd Research Industries, Burlington, VT) until the onset of polymerization. Tissue was removed by maceration in 3M KOH in 50% EtOH alternated with water rinses, and the resulting corrosion casts were cleaned in distilled water, and air dried.
In vivo Matrigel Plug assay

Three WT and three SKO mice (age matched) received each two matrigel plugs as follows: The rim of a 0.5 ml snap cap tube was removed with a razor blade and used as a vessel of 60μl of matrigel (BD biosciences) and incubated for 30min to allow matrigel to solidify. Mice were anesthetized with averin (0.5ml i.p.). Two small incisions were placed on either side of the abdominal midwall and a small subdermal pocket was created by blunt dissection. Each pocket received one matrigel plug inside the plastic rim so that the matrigel was in contact with the abdominal skin and muscle layer. Each incision was then closed and mice were allowed to recover. Matrigel plugs were harvested 16 days later. In some cases mice received injections of DETA/NO (2mg/kg in 200μl PBS i.p.) or vehicle (200μl PBS, i.p.) on day 2, 6, 9 and 13 after plug implantation. Mice were then sacrificed by CO2 and the matrigel plugs were recovered from their respective subdural pouches by fine dissection. Each plug was freed from its plastic rim and covered with OCT compound and sliced at approximately 0.1mm, 0.75mm and 1.5mm from the surface of the matrigel plug. Following this, each slide was fixed in 1:1 Acetone: MeOH at -20°C for 10min. Following washes with H2O, the specimens were blocked with Rockland Blocking buffer for 30min at RT. Antibodies were prepared as follows: 1600μl of Rockland Blocking Buffer + 8μl anti CD31-FITC (BD biosciences) + 4μl 1A4-Cy3 (anti-sm actin, Sigma). Each specimen received 40μl of antibody solution and was incubate for 45min in a light tight container in a humidified atmosphere. Slides were then washed in TBS and covered with mounting medium containing Hoechst 33342. Specimens were imaged using an Olympus I90 fluorescent microscope. All images were analyzed and quantitated using Image J (NIH) with identical parameters for all images.

Endothelial cell preparation

Primary ECs from WT or SKO mice were prepared as described, with modifications. Briefly, mice were sacrificed by CO2 asphyxiation, the left ventricle was perfused with PBS and the thoracic aorta excised. The lumen of the aorta was perfused with collagenase D (10 mg/ml, Worthington) dissolved in Dispase II (2.4 U/ml, Roche). Both ends of the aorta were tied and the aorta was incubated for 20 min at 37°C in DMEM (1g/l glucose). Subsequently the ties were removed and the aorta was flushed forcefully with 5ml of EC culture medium (DMEM containing glucose (1g/l), heat inactivated FBS (20%), endothelial growth supplement (ECGS, 30µg/ml, Sigma), Penicillin (100U/ml), Streptomycin (100µg/ml) supplemented with ciprofloxacin (25µg/ml), and amphotericin B (0.6µg/ml). The ECs contained in the flow through were cultured on 60 mm Primaria™ (Falcon) dishes. After one week of culture, the medium was changed to EC medium without ciprofloxacin and amphotericin B. Cultures typically obtained confluency after approximately 2 weeks and were used between passages 3 and 4. Purity of EC preparation was routinely assessed by immunostaining for CD31 and smooth muscle actin, respectively. All cultures used were at least 90% pure.

Skeletal muscle cell isolation

Gastrocnemius muscle bundles of male WT mice (2 - 3 months old) were dissected using a dissection microscope (Olympus SZ60). The isolated muscles bundles were treated with collagenase (Type IA, Sigma; 1·5 mg/ml) for 30 min at 30 °C in an incubator with gentle stirring. Collagenase was washed out with normal saline. The muscles were then incubated in DMEM under normoxic and hypoxic (2% O2) conditions as indicated. For RNA isolation and RT-PCR, muscle cells were collected, briefly washed twice in ice-cold PBS and analyzed with the RNeasy Plus Universal Kit from Qiagen according to the manufacturer’s protocol.

[3H]Thymidine incorporation assay

Agonist-induced [3H] thymidine incorporation into endothelial cells was assessed as described.
Chemokinesis assay

EC migration was assessed according to a modified protocol from Liang et al. ECs (80,000/well) were seeded in a 24-well plate and were allowed to attach overnight. Following 24h of serum starvation (0.2% FBS), the cell monolayer was denuded with a P1000 pipette-tip. Cells were washed and further incubated with DMEM containing 1% FBS, a concentration that allows for migration, but not proliferation. At that point the ECs were photographed (Leica SensiCam, at 40x magnification) to record a starting point. After 48h of incubation, cells were stained with Crystal Violet, photographed and “wound closure” was quantified using ImageJ software.

Matrigel tube formation assay

ECs (50,000/well) were seeded on Matrigel-covered wells of a 24-well plate and stimulated with 30μg/ml ECGS in DMEM containing 0.2% FBS. After 24h of incubation, cells were photographed (Olympus, MVX10) and tube formation was quantified by counting the branching points.

Immunoblot analysis

Assessment of protein expression and phosphorylation from tissue and cellular extracts was carried out as described in details elsewhere. Briefly, non-ischemic and ischemic gastrocnemius skeletal muscle from WT and SKO mice were harvested and homogenized in 5ml ice-cold extraction buffer (1% Nonidet-P-40, 10% glycerol, 135mM NaCl, 5mM KCl, 1mM CaCl₂, 20mM TRIS HCl pH7.4) per gram tissue containing a mixture of 2% (v/v) phosphatase inhibitors (Sigma; phosphatase inhibitor mixture I/II) and protease inhibitor (1 tablet/5ml) (Roche Applied Science; Mini Complete EDTA free protease inhibitor). Samples were kept on ice for 30 min, transferred to microcentrifuge tubes and centrifuged in a microcentrifuge at 4°C at 13,000 rpm for 20 min. Supernatants were transferred to fresh microcentrifuge tubes and protein content was measured. 50μg protein per lane was subjected to electrophoresis, transferred to a PVDF membrane, and probed with appropriate sets of primary (1:1000) and secondary antibodies to assess protein levels of S100A1 protein (Acris #SP5355P), anti eNOS, anti phospho-T495 eNOS, anti phospho-S1177 (Becton Dickenson), anti VEGF, anti PKCε, anti phospho-PKCε (Santa Cruz), anti PI3Kγ, anti PDK1, anti phospho-ser 241 PDK1, anti Akt, anti phospho-S473 Akt, anti VEGFR2, anti phospho-T1175 VEGFR2, anti p44/42, anti phospho-44/42 (Cell Signaling), anti VEGFR1 (abcam), anti GAPDH (Millipore). Proteins were visualized with a LI-COR infrared imager (Odyssey), quantitative densitometric analysis was performed by applying Odyssey version 1.2 infrared imaging software and signals were normalized to GAPDH.

Assessment of protein-protein interactions

Immunoprecipitation. Co-Immunoprecipitation for eNOS was carried out to investigate S100A1 protein interaction with eNOS. All steps were carried out on ice. WT EC cultures were rinsed and scraped off the dish in PBS. After brief centrifugation (13,000 rpm, 4°C for 5 min), pellets were resuspended in non-denaturing lysis buffer (1% Nonidet-P-40, 10% glycerol, 135mM NaCl, 1mM CaCl₂, 20mM TRIS HCl pH7.4), incubated for 30min at 4°C and centrifuged again (13,000 rpm, 4°C for 15min). Supernatants were diluted to 1μg/1μl protein with lysis buffer and rotated with bovine serum albumin-treated A/G-PLUS-Agarose (20µl/500µl; Santa Cruz Biotechnology) for 4 hours and centrifuged at 13,000 rpm for 15min at 4°C to remove protein non-specifically bound to A/G-Sepharose. The supernatants were then mixed with a precipitating antibody for eNOS (BD-Biosciences) and incubated for 8 hours at 4°C. Again, A/G-PLUS-Agarose was added and samples were rotated for additional 4 hours and centrifuged (13,000 rpm, room temperature for 15min). Pellets were washed three times with lysis buffer supplemented with mercaptoethanol (2% v/v) was added. Samples were heated at 95°C for 1min and centrifuged (800 rpm, room temperature for 15min) and 45µl of the
supernatant were transferred in a new microcentrifuge tube without aspirating the pelleted beads. 45μl of loading buffer were added and the sample divided in two parts that were resolved by SDS-PAGE on a 4-20% Tris-Glycin gel, transferred to a PVDF, and stained for co-precipitating S100A1 (Acris).

**Immunoﬂuorescence.** Cells were seeded overnight on gelatine-coated glass coverslips, fixed with 4% Paraformaldehyde and permeabilized using Acetone. Human iliac artery endothelial cells (HIAEC; purchased from Promocell) and WT ECs were labeled with a rabbit anti-S100A1 antibody (Acris, 1:200) followed by a 488 donkey anti-rabbit (Alexa Fluor, 1:300). Co-immunoﬂuorescence for S100A1 and eNOS was performed using S100A1 staining as described above and, consecutively, a mouse anti eNOS (BD, 1:200) followed by probing with a 568 donkey anti-mouse and (Alexa Fluor, 1:300). Coverslips were mounted using Vectashield medium with DAPI (Vector Laboratories). Images were obtained with an Olympus IX71 microscope. Background correction was performed using the appropriate negative controls.

**Duolink assay.** Human umbilical vein endothelial cells (HUVEC; purchased from Promocell) were seeded overnight on gelatine-coated glass coverslips and fixed and permeabilized as described above. The Duolink assay was purchased from OLink Bioscience and protein-protein interactions were detected by performing the protocol according to the manufacturer’s instructions. Briefly, after fixation and permeabilization the cells were incubated with the two antibodies of interest (1:200 in blocking buffer; eNOS (BD Biosciences) and either CaM (Santa Cruz), or S100A1 (Acris)) for a minimum of four hours. After first antibody incubation, cells were washed with PBS three times and then incubated for one hour with the corresponding PLA probes (plus and minus probes) diluted in blocking buffer 1:5. For detection, cells were again washed three times with PBS and a Ligation-Ligase mixture was added and incubated on each sample for 30 minutes. After removal of the Ligation-Ligase mixture and a washing step, an Amplification-Polymerase solution was added following an incubation period of 100 minutes. After a final washing step, the samples were mounted (Vectashield with DAPI, Vector Laboratories) over night and subsequently imaged (Olympus IX71). Quantification was done using ImageJ Software by measuring duolink pixels per cell (normalized to cell size). Negative controls were obtained by using PLA probes only on samples.

**S100A1 overexpression in HUVECs**

5,000 human umbilical vein endothelial cells (HUVECs) were seeded on a glass cover slip precoated with gelatine and incubated at 37°C, 95%O2/5%CO2 over night. Adenoviral transfection (MOI 10) of either GFP as a control or S100A1-GFP was carried out the next day in complete growth medium 2 (Promocell), and cells were maintained for another 24hours. Subsequently the duolink assay was performed (see above).

**S100A1 knockdown in human HMVECs**

Human cardiac microvascular endothelial cells (HMVECs, CC-7030) (LONZA) at passage 4-5 were cultured to confluence according to the manufacturer’s instruction in EGM-2 MV BulletKit media (CC-3162) in 6-well plates. S100A1 knockdown was achieved transfecting cells with siRNA against human S100A1 (Sigma 231-791-2, 50 nM) using the Lipofectamine RNAiMAX reagent (Invitrogen) according to manufacturer instructions while scramble siRNA (50 nM) served as control. Efficiency of S100A1 protein knockdown was assessed by Western blotting 48 hours after siRNA transfection. Stimulation with human recombinant VEGF (50 ng/ml; R&D Systems 293-VE) was carried out both under normoxic (21%O2) and hypoxic (2%O2) conditions. Medium was changed to 21%O2 and 2%O2 equilibrated Endothelial Basal Medium 2 (EBM-2) without supplements 6 hours prior to VEGF stimulation. For eNOS phosphorylation and PKC activity assay, VEGFR2 degradation and NO supernatant analysis, cells were stimulated with VEGF for 15 min, 2 hours and 16 hours, respectively. Cells were then lysed in buffer containing PBS (pH 7.4), 2% SDS, 2 mM EGTA/EDTA and 2% (v/v) phosphatase inhibitors (Sigma; phosphatase inhibitor mixture I/II) and protease inhibitor (1 tablet/5ml) (Roche Applied
Science; Mini Complete EDTA free protease inhibitor). Immunoblotting analysis and NO measurements from the 24 h supernatant were carried out as described in the corresponding methods sections. Co-immunoprecipitation experiments between S100A1 and eNOS in HMVECs were performed as described in the appropriate methods section. 250 μg lysate each from control and S100A1-depleted HMVECs were used both cultured under normoxic (21%O₂) and hypoxic (2%O₂) conditions for 2 hours. Antibodies were affinity-purified anti-human eNOS (BD 610297) and the corresponding affinity-purified IgG1 (BD 647451).

**Measurement of NO and Nitrite in cell culture supernatants and tissue homogenates**

NO in medium (DMEM 1g/l glucose, FBS 0.2%, penicillin 100U/ml, streptomycin 100μg/ml) from confluent WT and SKO EC cultures subjected to VEGF stimulation (50 ng/ml, 24hours, 21%O₂/ 5%CO₂/ 74%N₂, 100% humidity, 37°C) or hypoxia (6hours, 2%O₂/ 5%CO₂/ 93%N₂, 100% humidity, 37°C) was measured using Parameter™ NO/Nitrite/Nitrate assay (R&D systems, KGE001) according to manufacturer’s instructions. Nitrate levels in skeletal muscle tissue homogenates were measured with the Nitrate/Nitrite Colorimetric Assay Kit (Caymen Chemicals 780001) following the manufacture’s instructions for tissue homogenates. Briefly, skeletal muscle tissue samples were homogenized in PBS (pH 7.4) and 2 ml supernatant obtained after 10,000 × g centrifugation (20 min) was then subjected to 7500 × g centrifugation for 30 min using a 4 ml Amicon Ultra 4 filtration device (UFC8010) with 30,000 molecular weight cut off. 40 μl of the filtrate was assayed in duplicates for each sample after conversion of nitrate to nitrite following the instruction of the manufacturer.

**Measurement of NOS/eNOS activity**

Nitric oxide activity from skeletal muscle tissue and ECs was measured with Nitric Oxide Synthase Assay Kit (EMD, Cat. # 482700) following manufacturer instructions. Briefly, non-ischemic and ischemic gastrocnemius skeletal muscle from WT and SKO mice were harvested and homogenized in 20ml ice-cold homogenization buffer (25mmol/L Tris-HCl, 1mmol/L CaCl₂, pH7.4) per gram tissue. 1ml aliquots were centrifuged in a microcentrifuge at 13,000 rpm at 4°C for 5min and supernatants were transferred to a fresh microcentrifuge tube and kept on ice until further assessment of NOS activity. Cultured WT and SKO ECs (100mm dishes) were washed once with phosphate-buffered saline (PBS), harvested in PBS containing 1mmol/L EDTA, and transferred to microcentrifuge tubes. Cells were pelleted by spinning in a microcentrifuge at full speed for 2min at 4°C. Supernatants were removed, pellets were resuspended in 100μl of 1x homogenization buffer and disrupted by trituration. Homogenates were centrifuged in a microcentrifuge at 13,000rpm for 5min and supernatants were kept for further use. Protein concentrations in skeletal muscle and EC supernatants were adjusted to 10μg/μl and 10μl of tissue and cell supernatants were added to 40μl of the reaction mixture (30mmol/L Tris-HCl, 3.75μmol/L Tetrahydrobiopterin, 1.25μmol/L FAD, 1.25μmol/L FMN, pH7.4, 1.25μmol/L NADPH, 0.025v/v% [³H]-arginine (1μCi/μl), 1mmol/L CaCl₂ where indicated). When immunoprecipitated murine and recombinant bovine eNOS activity were tested, immunoprecipitated eNOS from 300μg EC lysate and 4 units recombinant bovine eNOS (EMD, Cat. # 482732) were added together with calmodulin to a final concentration of 100nmol/L. rhS100A1 protein was added at indicated concentrations. Reactions were incubated at 22-37°C for 10 to 60min followed by addition of 400μl of stop buffer (50mmol/L HEPES, 5mmol/L EDTA, pH5.5) and 100μl of equilibrated cation exchange resin. Then, samples were centrifuged at 13,000rpm for 30s using provided spin cups and holders. The eluate was transferred to a scintillation vial, scintillation fluid was added and radioactivity quantified in a liquid scintillation counter. Counts were divided by the fluorescent intensity of immunoprecipitated eNOS protein.
**Measurement of PKC activity**

Protein Kinase C activity from WT and SKO skeletal muscle tissue and HMVEC extracts was measured with the PepTag® Non-Radioactive Protein Kinase Assay (Promega, Cat. #V5330) following manufacturer’s instructions.

**References**

Supplement Figure I
Representative picture of an isolated murine skeletal muscle fiber basally and under hypoxic conditions after 48 h (40x objective)
Supplement Figure II
Representative images of arterial blood flow analysis by pulsed wave (PW) doppler velocity-time integral (VTI) in the tibialis posterior artery of an ischemic and non-ischemic WT mouse hindlimb 15 days post FAR.
Supplement Figure III

Lack of S100A1 does not decrease ischemia-induced collateral formation.

Representative images of collateral formation 1 month post FAR in the ischemic and non-ischemic muscles of WT and SKO mice. Collaterals were visualized by staining cryoembedded muscle cross sections for smooth muscle actin (upper panel). Numbers of collaterals were counted using Image J. Diameters of collapsed collaterals were estimated assuming circularity in vivo (lower panel). There is a trend for increased collateral formation in the ischemic SKO limb (n=5 each genotype).
Supplement Figure IV

**Reduced hypoxia-stimulated NO production in S100A1 deficient ECs.** (A) HMVECs were subjected to S100A1 knockdown or treated with scrambled (scr) siRNA. siRNA against S100A1 reduced endogenous S100A1 levels by over 80%. WT or SKO ECs (B) or HMVECs subjected to S100A1 knockdown (C) were subjected to hypoxia (2%O₂). eNOS-derived nitrite was determined in the cell culture supernatant as described in the Methods section. (experiments done in quadruplicate, repeated 2 times).
Supplement Figure V
Increased PKC activity in ECs subjected to S100A1 knockdown inhibits eNOS activity via enhanced p-T495 phosphorylation. (A) HMVECs were subjected to S100A1 knockdown, treated or not with the PKC inhibitor Calphostin-C and treated with VEGF (50ng/ml) for 15 min before extract preparation and immunoblot analysis. (B) HMVECs were subjected to siRNA mediated knockdown of S100A1 or control (scr), treated, or not with Calphostin-C and stimulated with 50ng/ml VEGF or subjected to hypoxia. The ratio of Calphostin-C over vehicle treated nitrite levels in the supernatant is plotted. Overall nitrite levels in HMVECs not treated with Calphostin-C were as described in Figure 5 C and Supplement Figure IV C, resp. eNOS-derived nitrite was determined in the cell culture supernatant as described in the Methods section. (experiments done in quadruplicate, repeated 2 times).
Supplement Figure VI

Brief hypoxia enhances interaction of S100A1 and eNOS. HMVECs were subjected to brief (2 hour) hypoxia. Extracts were immunoprecipitated with an anti eNOS monoclonal antibody. Immunoblots were probed for eNOS and S100A1. Experiment was repeated twice.
Supplement Fig. VII, Most et al.

**Supplement Figure VII**

S100A1 overexpression does not change interaction of calmodulin with eNOS. Duolink analysis of interactions between calmodulin (CaM) and eNOS was performed in HUVEC infected with an adenovirus expressing S100A1 and GFP from a bicistronic promoter (Ad-S100A1-GFP) or GFP only (Ad-GFP). (n=3 for each interaction)
Supplement Figure VIII
Increased growth factor expression in ischemic muscle of SKO mice post FAR. SKO or WT mice were subjected to FAR and gastrocnemius muscles of the ischemic and contra-lateral non-ischemic hindlimb were procured after three days. mRNA prepared from muscle extracts was analyzed by qRT-PCR for expression levels of hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and placental growth factor (PIGF). Expression was normalized to 18S mRNA. (n=3 for each genotype and condition, *p< 0.05 vs ischemic WT).
Supplement Figure IX

Increased PKC activity in S100A1 knockdown HMVECs causes enhanced degradation of the VEGFR2. HMVECs were subjected to S100A1 knockdown, stimulated with VEGF (50ng/ml) and subjected to hypoxia for the times indicated, before extract preparation and immunoblot analysis for the VEGFR2. Pretreatment with the PKC inhibitor calphostin-C prevented S100A1-kd enhanced VEGF stimulated degradation of the VEGFR2.
Supplement Figure X

**Increased PKC activity in ischemic muscle of SKO mice.** SKO or WT mice were subjected to FAR and gastrocnemius muscles of the ischemic and contra-lateral non-ischemic hind limb were procured and homogenized after three days. PKC activity in skeletal muscle homogenates was determined by measuring the phosphorylation of the PKC substrate peptide. Specificity of the reaction was determined by the inclusion of the PKC inhibitor chelerythrine (1µM) in the reaction mix.
Supplement Figure XI
Disturbed Signal transduction in ischemic hindlimbs of SKO mice. SKO and WT mice were subjected to FAR and gastrocnemius muscles of the ischemic and contra-lateral non-ischemic hind limb were procured and homogenized after three days. Muscle extracts were immunoblotted for (A) PI3K-γ, total and phospho-Ser241 PDK1 and (B) total and phospho-ERK1/2. Expression levels were normalized to GAPDH, (n=4 for each genotype and condition, *p< 0.05 vs ischemic WT). Representative samples are shown for all immunoblots.
Supplement Figure XII

Differential downregulation of VEGFR2 vs VEGFR1. HUVEC were treated with 50 ng/ml VEGF for the times indicated. Representative receptor protein levels are shown for all immunoblots, expression levels were normalized to GAPDH (n=3, *p< 0.05 vs control).
Supplement Figure XIII
Low levels of S100A1 expression in skeletal muscle myoblasts. (A) Levels of S100A1 in skeletal muscle and circulating RBCs of WT mice were examined. (B) L6 rat skeletal myoblasts and C2C12 mouse skeletal myoblasts were immunoblotted for S100A1. Equal amounts of protein (20 µg) were loaded for each lane except the for WT heart sample in the left-most lane in (A).
Supplement Figure XIV:
Simplified scheme illustrating molecular abnormalities in S100A1-deficient ECs in response to ischemia. (A) Ischemic damage leads to (I) increased VEGF expression subsequently stimulating the (II) VEGFR2/PI3K/PDK/Akt and VEGFR1/PLC/ERK1-2 (not shown) axis. As a result, eNOS activation due to enhanced Akt-mediated ser-1177 phosphorylation (III) increases NO formation facilitating postischemic EC activation (migration, proliferation and sprouting). Former studies by our group showed that S100A1 is present at the ER (1) modulating IP3R-dependent ER calcium (Ca\(^{2+}\)) release required for Ca\(^{2+}\)/CaM-dependent eNOS activation (Supplement Ref. 2). This study provides advanced mechanistic insight into a (2) direct Ca\(^{2+}\)-dependent stimulatory interaction between S100A1 and eNOS, amplifying the activity of the enzyme, and postischemnic NO generation. In addition, (3) S100A1 constrains PKC activation by an unresolved mechanism in response to VEGF stimulation thereby preventing inhibitory eNOS thr-495 phosphorylation. (B) In hypoxic S100A1-deficient ECs, as our study indicates, VEGF-activated eNOS generation is impaired by (4) interrupted stimulatory S100A1/eNOS interaction, (5) unopposed PKC activity that results in inhibitory eNOS thr-495 hyperphosphorylation and VEGFR2 degradation. (6) Impaired ER-mediated Ca\(^{2+}\) release in the absence of S100A1 (Supplement Ref. 2) potentially contributes to insufficient eNOS activity due to impaired interaction between CaM and eNOS. Due to defective angiogenesis, ischemic S100A1-deficient tissue apparently both upregulates VEGF expression and signaling modules involved in eNOS activation including the PI3K/PDK/Akt and PLC/ERK1-2 (data not shown) axis as well as eNOS. VEGF overshoot might further accelerate VEGFR2 degradation entering a feed forward vicious cycle of VEGF unresponsiveness.