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Endothelial S100A1 Modulates Vascular Function via Nitric Oxide

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Abstract—S100A1, a Ca^{2+}-binding protein of the EF-hand type, is known to modulate sarcoplasmic reticulum Ca^{2+} handling in skeletal muscle and cardiomyocytes. Recently, S100A1 has been shown to be expressed in endothelial cells (ECs). Because intracellular Ca^{2+} ($[Ca^{2+}]_i$) transients can be involved in important EC functions and endothelial NO synthase activity, we sought to investigate the impact of endothelial S100A1 on the regulation of endothelial and vascular function. Thoracic aortas from S100A1 knockout mice (SKO) showed significantly reduced relaxation in response to acetylcholine compared with wild-type vessels, whereas direct vessel relaxation using sodium nitroprusside was unaltered. Endothelial dysfunction attributable to the lack of S100A1 expression could also be demonstrated in vivo and translated into hypertension of SKO. Mechanistically, both basal and acetylcholine-induced endothelial NO release of SKO aortas was significantly reduced compared with wild type. Impaired endothelial NO production in SKO could be attributed, at least in part, to diminished agonist-induced $[Ca^{2+}]_i$ transients in ECs. Consistently, silencing endothelial S100A1 expression in wild type also reduced $[Ca^{2+}]_i$ and NO generation. Moreover, S100A1 overexpression in ECs further increased NO generation that was blocked by the inositol-1,4,5-triphosphate receptor blocker 2-aminoethoxydiphenylborate. Finally, cardiac endothelial S100A1 expression was shown to be downregulated in heart failure in vivo. Collectively, endothelial S100A1 critically modulates vascular function because lack of S100A1 expression leads to decreased $[Ca^{2+}]_i$ and endothelial NO release, which contributes, at least partially, to impaired endothelium-dependent vascular relaxation and hypertension in SKO mice. Targeting endothelial S100A1 expression may, therefore, be a novel therapeutic means to improve endothelial function in vascular disease or heart failure. (*Circ Res*. 2008;102:786-794.)

Key Words: S100A1 ∎ vascular function ∎ NO ∎ hypertension ∎ endothelial function ∎ calcium

S100A1 is a low-molecular-weight ($\approx$10-kDa) Ca^{2+}-sensing protein of the EF-hand type known to modulate intracellular Ca^{2+} ($[Ca^{2+}]_i$) handling in skeletal muscle and cardiomyocytes.[1–3] S100A1 has been shown to affect cardiac contractile performance through enhanced sarcoplasmic reticulum Ca^{2+} cycling, modulation of myofilament function, and regulation of mitochondrial function.[4–10] S100A1 is especially interesting with respect to cardiovascular diseases because cardiac S100A1 expression levels are significantly downregulated in end-stage heart failure (HF) and the normalization of S100A1 protein expression has been shown to rescue the failing myocardium in vivo.[3,11–14]

Recently S100A1 expression has been described in endothelial cells (ECs).[15] A well-characterized and critical regulator of endothelial function is NO, which is generated by endothelial NO synthase (eNOS or NOS3).[16] Activation of eNOS is classically dependent on increased $[Ca^{2+}]_i$, which can be induced by agonists such as acetylcholine (ACh) or bradykinin (BK).[17,18] NO contributes to endothelium-dependent vascular relaxation and has additional functional roles, such as leukocyte adhesion and antiproliferative and antiapoptotic effects on the vascular wall.[17,19] Importantly, endothelial dysfunction occurs in a variety of cardiovascular diseases and was found to be associated with adverse effects such as inflammation, impaired vascular function, and long-term vascular remodeling.[20] Moreover, recent data provide evidence that endothelial dysfunction in HF is associated with an increased mortality risk in patients with both ischemic and nonischemic HF.[21]

The significant role of S100A1 in cardiac function, especially in disease, coupled with the importance of the endothelium for normal and compromised vascular function including HF, led us to hypothesize that S100A1 in ECs plays a key role in the regulation of vascular function. This hypothesis is supported by the fact that $[Ca^{2+}]_i$ plays a critical role in the regulation of eNOS activity and endothelial function. Because the physiological role of S100A1 in ECs has not been examined to date, in this study, we investigated the impact of endogenous S100A1 in ECs by taking advantage of S100A1 knockout mice (SKO) and found that endogenous S100A1 in ECs is critically involved in the endothelial-dependent regulation of vascular function.

Materials and Methods

Mice with a deletion of the S100A1 gene (SKO) were derived on a C57BL/6 background and characterized as described previously.[22] SKO and wild-type (WT) mice of either sex and 3 months of age were used for this study. All animal procedures and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Thomas Jefferson University or with the Norwegian Council of Animal Research.

Isolation of Mouse Aortic and Rat Cardiac Endothelial Cells

Mouse aortic endothelial cells (MAECs) from WT and SKO mice and rat cardiac endothelial cells (RCECs) were isolated and phenotyped as described previously.[23–25] MAECs were used at passage 1, whereas RCECs were used between passages 4 and 8. Detailed procedures for isolation and characterization of MAECs and RCECs are given in the online data supplement, available at http://circres.ahajournals.org.

Immunofluorescence and Immunohistochemistry

Immunofluorescence and immunohistochemistry procedures for human coronary artery endothelial cells (HCAECs) (Cambrex), MAECs, and RCECs were performed as described previously using appropriate antibodies.[12,26,27] Detailed procedures are described in the online data supplement.

In Vitro Physiology

The relaxation/contraction response of thoracic aortas of WT or SKO mice was examined as described before.[28] Detailed procedures are described in the online data supplement.

Telemetric Blood Pressure Measurement and Agonist-Induced Hypotensive Response In Vivo

Experimental design and surgical procedures are given in detail in the online data supplement.

NO Release From Mouse Aorta

NO release from the endothelial surface of aortas was measured in vitro using an Apollo 4000 Free Radical Analyzer (WPI, Sarasota, Fla) as described previously with some modifications.[25] Thoracic aortas of WT and SKO mice were carefully isolated and pinned down in KH solution after connective tissue was thoroughly removed. NO was measured under baseline conditions and after ACh stimulation (10^{-5} mol/L). Two measurements were recorded per animal and condition, and NO values were normalized to the weight of the vascular tissue.

Silencing or Upregulation of S100A1 Expression and NO Production in Endothelial Cells

S100A1 expression was silenced in RCECs by use of custom-made small interfering (si)RNA (Eurogentec) and Lipofectamine 2000 reagent (Invitrogen), as described previously.[4] S100A1 overexpression in HCAECs was achieved by electric transfection of plasmid DNA using Nucleofactor (AMAXA biosystems). NO in the supernatant was measured indirectly using the Nitrate/Nitrite fluorometric assay kit (Cayman Chemical) according to the recommendations of the supplier. Experimental design is described in detail in the online data supplement.

Adenoviral Vectors

Adenoviral vectors were obtained by the use of the pAdTrack-CMV/pAdEasy-1 system as described previously.[12,26] Detailed procedures are described in the online data supplement.

Ca^{2+} Transient Analyses of Isolated RCECs and MAECs

$[Ca^{2+}]_i$ transients of MAECs derived from SKO and WT mice at passage one were measured using the IonOptix MyoCam system (IonOptix Corp) as described recently.[13,26] RCECs (between passage 4 and 8) were used 96 hours after $siRNA_{S100A1}$ or $siRNA_{scramble}$ treatment for BK-induced (10^{-5} mol/L) $[Ca^{2+}]_i$ transient measurement using T.I.L.L. Vision software (version 4.01) as described previously.[12] Detailed experimental design and procedures are given in the online data supplement.

Western Blot Analysis

The procedures are described in detail in the online data supplement.

Regulation of S100A1 Expression in Experimental HF and on G_q Protein–Coupled Receptor Stimulation

After growing HCAECs to 90% confluence, full medium was replaced by basal EBM-2 medium with 0.5% serum, and cells were stimulated with endothelin (ET)-1, angiotensin (Ang) II (both 10^{-7} mol/L), or PBS for 60 hours. Cells were harvested for mRNA isolation, and S100A1 expression was standardized against 18S rRNA.

Detailed experimental procedures for experimental post–myocardial infarction rat HF model and hemodynamic characterization are described in the online data supplement.

RNA Isolation and Real-Time RT-PCR

The procedures for RNA isolation and real-time PCR are described in detail in the online data supplement.

Statistical Analysis

Data are expressed as means±SEM. Unpaired Student's t test and 1-way repeated-measures ANOVA, including the Bonferroni test for all subgroups, were performed for statistical comparisons when appropriate. For dose–response curves ANOVA for repeated measures was used. For all tests, a value of $P<0.05$ was accepted as statistically significant.

Results

S100A1 Is Expressed in Endothelial Cells

Because S100A1 expression was recently described in rat cerebral endothelial cells,[15] we confirmed S100A1 expression in HCAECs, RCECs, and MAECs. Indirect immunofluorescence staining for S100A1 was consistently observed in the perinuclear region accentuated at 1 pole of the nucleus (Figure 1A, 1C, and 1E). Expression of endogenous S100A1 in ECs could also be detected by Western blotting (Figure 1G). Importantly, S100A1 was found in WT-derived MAECs in aortic vessel cryosections by immunohistochemistry, whereas S100A1 was not detectable in cryosections of SKO-derived MAECs (data not shown).
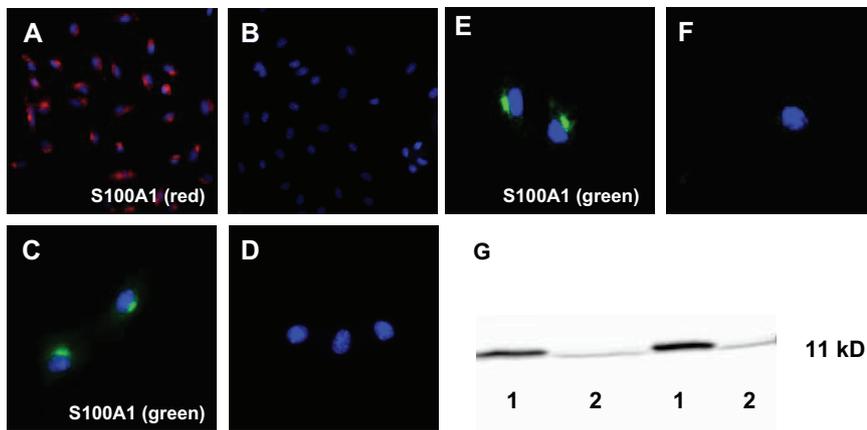
Figure 1. S100A1 is expressed in ECs. Representative indirect immunofluorescence for S100A1. A, Anti-S100A1 antibody treatment reveals endogenous S100A1 expression (red) in HCAECs, whereas IgG-negative control shows only 4′,6-diamidino-2-phenylindole (DAPI) staining (blue) (B) (both, ×20). S100A1 expression (green) was also detectable in anti-S100A1 antibody–incubated RCECs (C) and MAECs (E), whereas only DAPI staining (blue) was detectable in IgG-negative controls (RCECs [D] and MAECs [F]). Magnification, ×60 (C through F). G, Representative Western blot of RCECs reveals endogenous S100A1 expression (2) and positive control (1).

Expression of S100A1 in ECs Is Pivotal for Endothelium-Dependent Vessel Relaxation In Vitro

To investigate the role of endogenous S100A1 in ECs, we took advantage of the availability of SKO mice.[22] The effect of S100A1 expression independent of autonomic influences on the vasculature was examined in isolated thoracic aorta ring segments. Phenylephrine (PE)-mediated constriction of aorta rings from both the WT and SKO mice were similar. Pretension was established using 3×10^{-7} mol/L PE, which corresponded to 60% maximum PE stimulation in both SKO and WT mice (data not shown). Interestingly, endothelium-dependent relaxation in response to increasing dosages of ACh was significantly impaired in thoracic aortas from SKO compared with WT (Figure 2A). Moreover, the maximal response to ACh was significantly attenuated in SKO aortas compared with WT controls (Figure 2A). To verify endothelium dependence of the reduced relaxation in response to ACh and to rule out an inability of the smooth muscle cells (SMCs), which also express the S100A1 protein, to respond to NO, relaxation was induced by the direct NO donor sodium nitroprusside (SNP). No significant difference in direct SNP-induced vessel relaxation was observed between SKO and WT (Figure 2C). Moreover, β-adrenergic receptor (AR) agonist-induced, SMC-dependent relaxation of aorta rings using isoproterenol (Iso) was unaltered between WT and SKO (Figure 2E). Mechanical scraping of ECs resulted in similar vessel function in SKO or WT mice in response to ACh, SNP, Iso (Figure 2B, 2D, and 2F), or PE (data not shown).

Endothelial Dysfunction in SKO Is Associated With Hypertension

Because the lack of S100A1 expression causes a deficit in endothelium-dependent vascular relaxation in vitro, we determined the impact of S100A1 expression on blood pressure (BP) in vivo. Interestingly, conscious SKO mice showed a significant 24.1% increase in systolic BP in vivo compared with conscious WT mice (Figure 3A and 3B). Moreover, diastolic BP and mean arterial BP were also significantly elevated in SKO compared with WT, whereas heart rate was unaltered (Figure 3C through 3E). Therefore, loss of S100A1 is associated with hypertension in vivo and S100A1 expression in ECs may at least contribute to regulate and maintain normal BP.
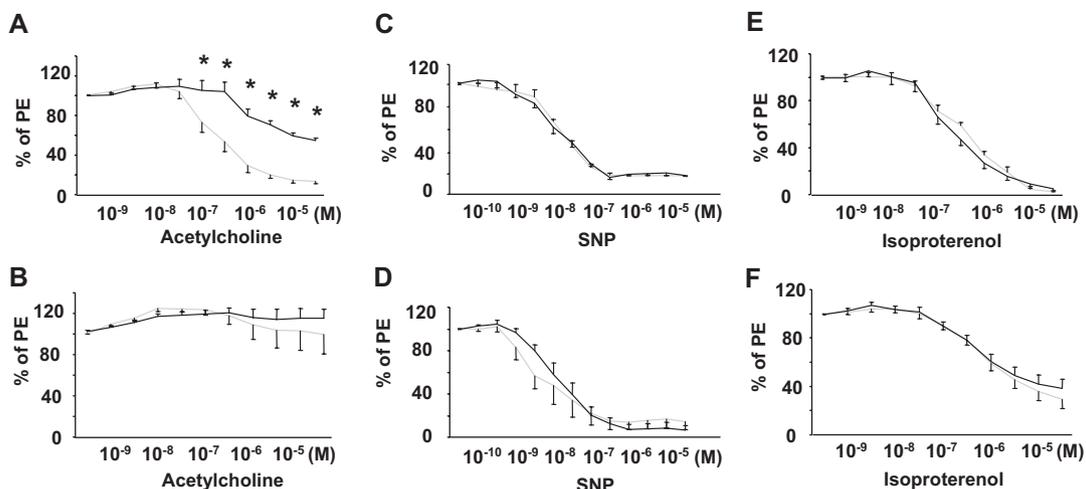
Figure 2. Impaired endothelium-dependent vessel relaxation in aortas from SKO mice. A, ACh-induced relaxation of PE-preconstricted (EC₆₀) thoracic aortas was significantly reduced in SKO-derived aortas (black; n=6) compared with WT control aortas (gray; n=6). Direct SMC-mediated vessel relaxation of preconstricted aortas using either SNP (C) or Iso (E) was not different between SKO (black; n=6) and WT (gray; n=6) groups. Removal of ECs resulted in similar responses of SKO- and WT-derived aortas to ACh (B), SNP (D), or Iso (F). *$P<0.05$.
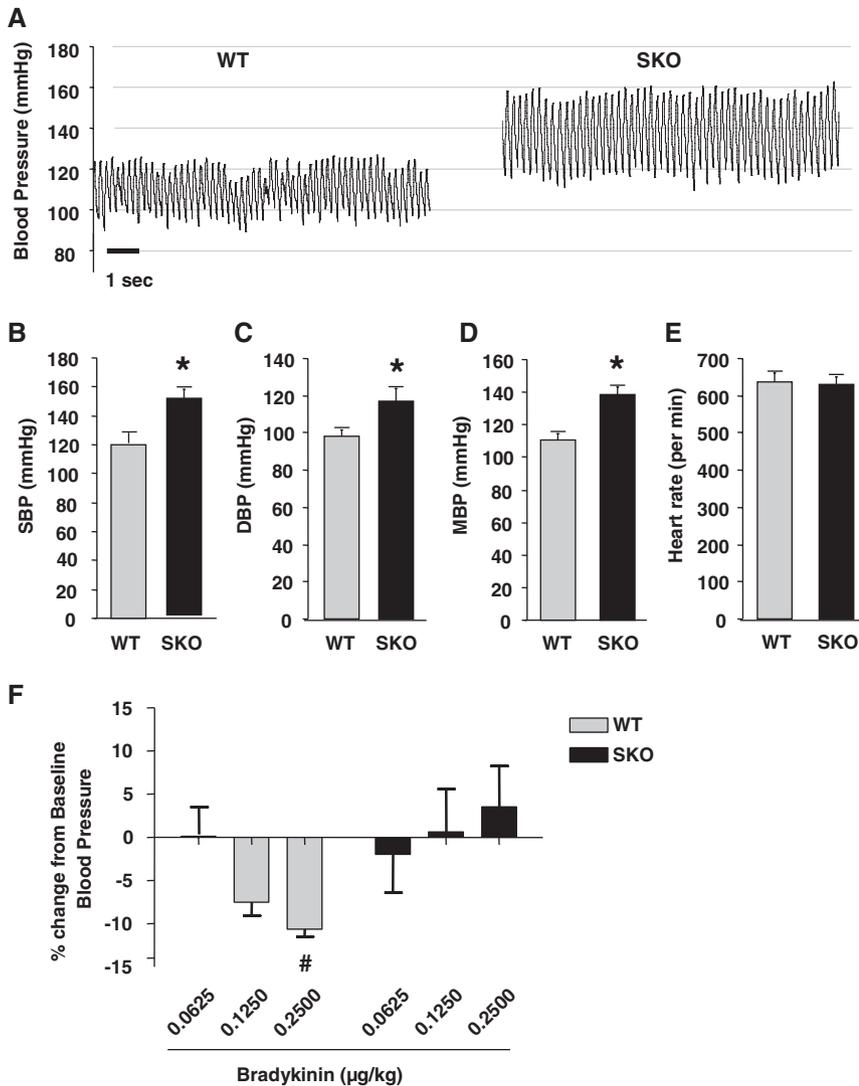
Figure 3. Loss of S100A1 expression causes endothelial dysfunction and hypertension in vivo. A, Representative raw traces of BP recordings of WT and SKO mice. B through E, Systolic BP (SBP) (B), diastolic BP (DBP) (C), and mean arterial BP (MBP) (D) were significantly increased in SKO mice (n=7) in vivo compared with WT controls (n=6), whereas heart rate was similar in both groups (E). F, Endothelium-dependent hypotensive response to increasing amounts of BK is attenuated in SKO (n=4) compared with WT (n=4) mice in vivo. *$P<0.05$ vs WT, #$P<0.05$ vs SKO. Data are presented as means±SEM.

Loss of Endothelium-Dependent Hypotensive Response in SKO In Vivo

To investigate the consequences of the lack of S100A1 expression on endothelial function in vivo BK (0.0625 to 0.25 μg/kg body weight) was injected systemically in sedated WT and SKO mice. WT mice showed a dose-dependent decrease of in vivo BP, whereas agonist-induced attenuation of BP was completely abolished in SKO mice (Figure 3F).

eNOS Expression Is Unaltered in SKO Mice

Lack of S100A1 expression was confirmed in SKO mice by Western blot for S100A1 in cardiac tissue (Figure 4A). Equal expression of eNOS and its critical regulator heat shock protein (Hsp)90 in WT and SKO mice was confirmed by Western blotting in lung tissue (Figure 4A).

## Endothelial S100A1 Expression Affects Basal and Agonist-Induced NO Release

Endothelial NO generation affects vascular tone and contributes to BP regulation and vascular function.[19] Accordingly, we measured levels of aortic endothelial NO release in SKO and WT mice to gain mechanistic insight in endothelial

dysfunction and impaired vascular relaxation observed in SKO. Interestingly, basal aortic NO levels were significantly reduced by ≈50% in SKO compared with WT mice (Figure 4B). Additionally, the ACh-induced (10^{-5} mol/L) increase in aortic endothelial NO release was significantly ablated in SKO mice (Figure 4C). Thus, loss of endogenous S100A1 expression in endothelial cells leads to a significant reduction of both basal and ACh-induced NO levels.

To further corroborate the specificity of our findings, S100A1 protein expression was downregulated in normal RCECs using small interfering (si)RNA, and effects on basal and agonist-induced NO generation were examined. Use of siRNA$_{S100A1}$ resulted in significant downregulation of S100A1 mRNA and protein compared with siRNA$_{Scramble}$ in RCECs 96 hours after transfection (Figure 4D through 4F). Decreased endothelial S100A1 expression caused both significantly diminished basal and agonist-induced NO generation using BK or thrombin (Figure 4G).

Endothelial S100A1 Regulates Agonist-Induced Ca²⁺ Transients

Classically, eNOS activation is dependent on [Ca^{2+}]$_i$ and the binding of Ca^{2+}/calmodulin to the enzyme, whereas chelation
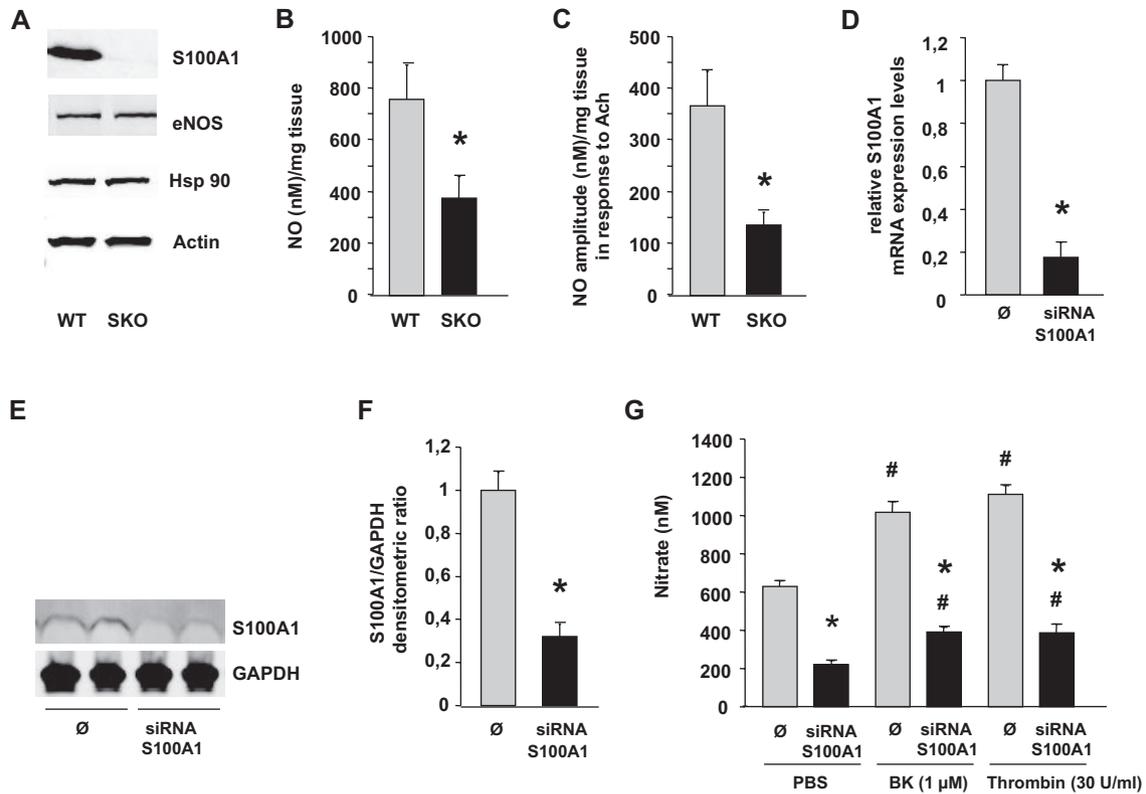
Figure 4. Endothelial S100A1 modulates basal and agonist-induced NO generation. A, Representative Western blots for S100A1, eNOS, Hsp90, and actin in SKO and WT mice. NO production was measured at the endothelial surface of thoracic aortas and found to be significantly reduced in SKO mice (n=4) compared with WT mice (n=4) under both basal conditions (B) and ACh stimulation (C). siRNA$_{S100A1}$ treatment resulted in significant downregulation of S100A1 mRNA (D) and protein (E and F) after 96 hours in RCECs compared with siRNA$_{scramble}$ controls (Ø). G, Silenced S100A1 expression in isolated RCECs caused significantly reduced basal and agonist-induced NO generation. *$P<0.05$ vs WT or siRNA$_{scramble}$ (Ø), #$P<0.05$ vs PBS. Experiments were run in triplicates; 3 individual experiments. Data are presented as means±SEM.

of extracellular calcium abolishes agonist-induced NO generation and vascular relaxation.[18] Because, in cardiomyocytes, S100A1 is known to mechanistically act via a significant gain in $[Ca^{2+}]_i$,[1–3] we investigated the impact of S100A1 on agonist-induced $[Ca^{2+}]_i$ in ECs. siRNA$_{S100A1}$-mediated S100A1 downregulation in RCECs resulted in significantly decreased BK-induced $[Ca^{2+}]_i$ transients compared with siRNA$_{scramble}$-treated RCECs (Figure 5A and 5B). Consistently, SKO-derived MAECs showed a significantly reduced response to ACh compared with WT (Figure 5C). To ensure that the observed effects were attributable to the lack of S100A1 expression in SKO, we expressed S100A1 in SKO using an adenoviral construct (AdS100A1). AdGFP was used as a control and infection of MAECs with both vectors were confirmed by green fluorescent protein (GFP) coexpression. Interestingly, S100A1 expression in SKO-ECs significantly increased ACh-induced $[Ca^{2+}]_i$ compared with SKO ECs treated with the control virus (Figure 5C). Endothelial S100A1 may therefore regulate agonist-induced $[Ca^{2+}]_i$ transients.

S100A1 Colocalizes With Both the Inositol-1,4,5-Triphosphate Receptor and SERCA2

Because lack of S100A1 expression reduces agonist-induced endothelial $[Ca^{2+}]_i$ transients, and to identify potential target proteins for S100A1 in ECs, we studied the intracellular localization of S100A1, the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), and the inositol-1,4,5-triphosphate receptor (IP$_3$R). Figure 6 shows representative images of endogenous expression of SERCA2 (red; Figure 6A), IP$_3$R (red; Figure 6D), and S100A1 (green, Figure 6C and 6F) in RCECs. Merging the corresponding pictures revealed that endogenous S100A1 partly colocalizes both with SERCA2 and IP-$_3$R (Figure 6B and 6E).

S100A1 Overexpression Increases Agonist-Induced NO Generation in HCAECs

We demonstrated that endogenous S100A1 expression is essential for endothelial NO generation. To further investigate the functional role of S100A1 in ECs, we tested whether an increased S100A1 expression in ECs would stimulate NO generation. Additionally, because decreased endothelial NO generation in SKO was associated with reduced $[Ca^{2+}]_i$ transients, we studied the impact of the blockage of the IP$_3$R on the S100A1-mediated increase in NO generation. Transfection of the plasmid pAdTrack-S100A1 yielded in ≈50% GFP-positive HCAECs and a 1.6-fold overexpression of S100A1 compared with GFP and PBS control groups (Figure 7A through 7E). Under all conditions tested, there was no significant difference in NO production between the PBS and the GFP groups. Importantly, ACh-induced NO generation was significantly increased in S100A1-overexpressing
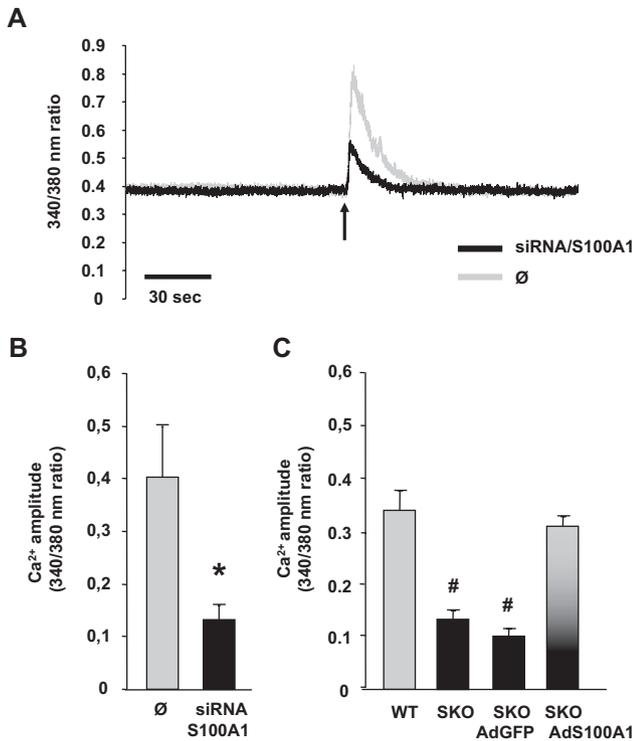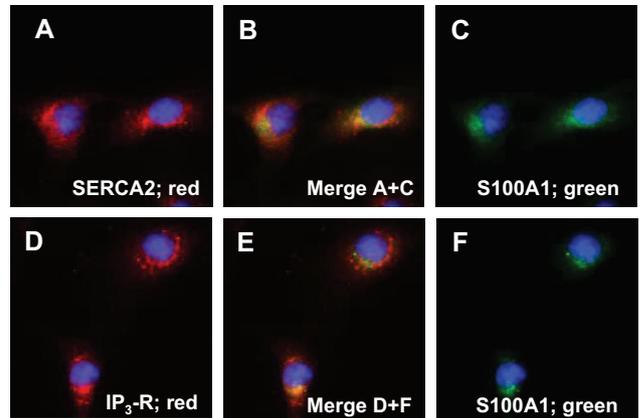
Figure 5. S100A1 expression modulates agonist-induced $[Ca^{2+}]_i$ transients in ECs. A, Representative raw traces of BK-induced $[Ca^{2+}]_i$ transients from siRNA$_{scramble}$-treated (Ø; gray) and siRNA$_{S100A1}$-treated (black) RCECs. B, Silencing of S100A1 expression (n=48) significantly reduced BK-induced $[Ca^{2+}]_i$ transients in RCECs compared with siRNA$_{scramble}$ controls (Ø; n=49). C, $[Ca^{2+}]_i$ transients in response to ACh stimulation were significantly decreased in MAECs from SKO mice (n=15) compared with WT MAECs (n=15). Adenoviral expression of S100A1 (AdS100A1) significantly increased ACh-induced $[Ca^{2+}]_i$ transients in SKO-derived MAECs (n=15), whereas an AdGFP control virus did not affect $[Ca^{2+}]_i$ transients (n=13). *$P<0.05$ vs siRNA$_{scramble}$ (Ø), #$P<0.05$ vs WT or SKO/AdS100A1. Data are presented as means±SEM. Three individual experiments each using different animals.

Figure 6. S100A1 colocalizes with both SERCA2 and the IP$_3$R. A, C, D, and F, Representative immunolabeling of SERCA2 (red) (A), IP$_3$R (red) (D), and S100A1 (green) (C and F). Overlays of A and C (B) and D and F (E) reveal similar intracellular localization of both SERCA2 and IP$_3$R with S100A1 protein. Original magnification, ×60.

HCAECs compared with control groups (Figure 7F). Consistently, blockage of the agonist-induced $[Ca^{2+}]_i$ transients by use of 2-amino-4-phosphonobutyrate (2-APB) abolished NO generation in all groups and masked the difference between S100A1 and control groups (Figure 7F). Thus, S100A1 overexpression enhances endothelial NO generation, and this effect is, at least in part, $[Ca^{2+}]_i$-dependent.

Endothelial S100A1 Is Downregulated in Experimental HF and on G$_q$ Protein–Coupled Receptor Stimulation

To investigate whether reduced S100A1 expression in ECs could potentially be involved in endothelial dysfunction in cardiovascular diseases, we stimulated HCAECs and RCECs with Ang II or ET-1, both known to be increased in a variety of cardiovascular diseases and involved in cardiac and vascular remodeling.[29,30] Ang II and ET-1 stimulation (both 10^{-7} mol/L) significantly reduced S100A1 mRNA levels after 60 hours in vitro (Figure 8A). Importantly, ECs isolated from failing rat hearts 56 days after myocardial infarction showed a significant 45±8% decrease of S100A1 expression compared with sham controls in vivo (Figure 8B).

Discussion

The EF-hand-type Ca^{2+}-sensing protein S100A1 plays a significant role in cardiovascular function because it has been characterized as a novel positive inotropic factor and regulator of myocardial contractility in vitro and in vivo.[1–3] Moreover, S100A1 gene therapy and preservation of cardiac S100A1 expression have been shown to rescue and prevent HF in animal models in vivo, mainly by maintaining $[Ca^{2+}]_i$ cycling in cardiomyocytes.[12,13,26] Importantly, vascular function and one of its major regulators, eNOS, are also critically regulated by $[Ca^{2+}]_i$.[16–18]

Endothelial dysfunction, defined as impaired endothelium-dependent vascular relaxation, is linked to a large number of cardiovascular diseases and has been shown to be associated with an increased mortality risk in patients with both ischemic and nonischemic HF.[20,21] Because S100A1 has been reported recently to be expressed in ECs,[15] and given the relevance of ECs in cardiovascular diseases, we investigated the impact of S100A1 on vascular function and, importantly, found a critical role for this Ca^{2+}-sensing protein because loss of S100A1 in ECs results in vascular dysfunction that actually includes hypertension.

Specifically, in the present study, we demonstrate a critical role for endothelial S100A1 on vascular function because endothelium-dependent relaxation of SKO thoracic aortas was significantly reduced compared with WT. Direct vessel relaxation using SNP was not different between both groups, revealing that SMCs also lacking S100A1 expression in SKO mice relax normally in response to NO. Of note, our data suggest no major contribution of S100A1 on vascular SMC contraction coupling because both protein kinase G–dependent and β-AR agonist–induced relaxation of SMC and PE-induced absolute contraction of thoracic aortas were similar between SKO and WT mice. This is somewhat surprising because S100A1 has been shown to bind caldesmon, which is an important regulator of SMC performance.[31] Lack of S100A1 expression also caused endothelial dysfunction under in vivo conditions because systemic injection of BK resulted in loss of hypotensive response in SKO com-
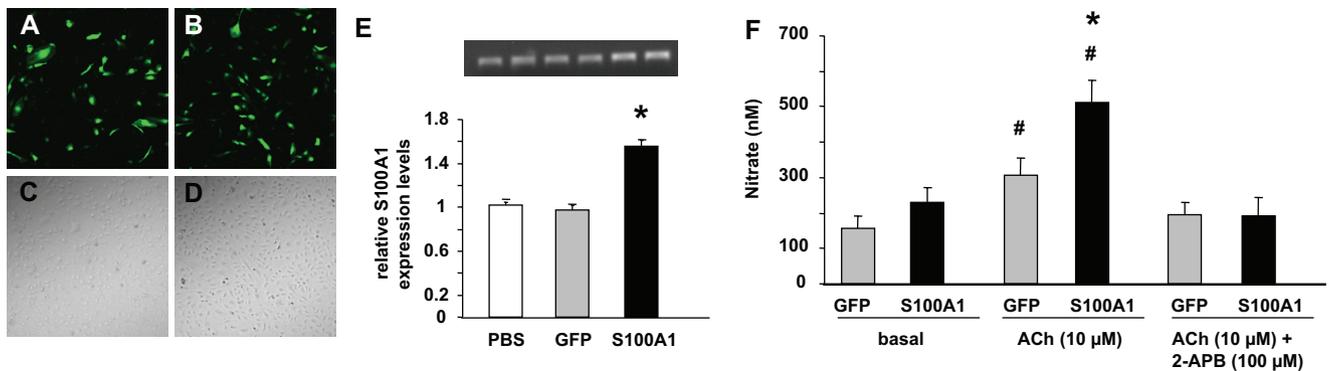
Figure 7. S100A1 overexpression increases agonist-induced NO generation in HCAECs. A, Transfection of the plasmid pAdTrack-S100A1 in HCAECs was controlled by GFP coexpression. B, Transfection of the plasmid pAdTrack-GFP resulted in similar GFP expression. C and D, Light microscopy of A and B. E, RT-PCR reveals a 1.6-fold increase in S100A1 mRNA expression in HCAECs attributable to pAdTrack-S100A1 transfection compared with both PBS and GFP control groups (interrupted PCR reaction). F, Basal NO generation, as measured by the stable end product nitrate, was unaltered in all groups after 1 hour in cultured HCAECs. GFP and S100A1 groups showed a significant agonist-induced increase in NO generation. S100A1 overexpression significantly increased ACh-induced NO generation compared with GFP controls groups. Blockage of IP$_3$R-mediated Ca^{2+} release (by use of 2-APB) significantly reduced ACh-stimulated NO production and masked S100A1-mediated effects. *$P<0.05$ vs GFP/ACh, #$P<0.05$ vs basal. Data are presented as means±SEM of 3 individual experiments.

pared with WT control mice. To address the pathophysiological consequences of impaired vascular function, we measured BP in conscious SKO and WT mice in vivo. Both systolic and diastolic BP were significantly higher in SKO mice. Therefore, endothelial dysfunction attributable to the lack of S100A1 expression in ECs may at least contribute to vascular dysfunction in vivo, and endothelial S100A1 may be critical for the maintenance of normal BP. Mechanistically, both basal and agonist-induced NO release analyzed directly at the endothelial surface of aortas were significantly decreased in SKO thoracic aortas compared with WT controls. Beyond this, our data suggest that reduced/lack of endothelial S100A1 expression causes diminished [Ca^{2+}]$_i$ transients, which contribute to reduced eNOS activation and, thus, NO generation. Of note, this could be demonstrated by use of SKO-derived MAECs and WT RCECs using siRNA$_{S100A1}$. Adenoviral-mediated expression of S100A1 in MAECs from SKO mice resulted in restoration of [Ca^{2+}]$_i$ transients, demonstrating that the observed effects were specifically mediated by the S100A1 protein.

S100A1 enhances [Ca^{2+}]$_i$ cycling in cardiomyocytes by an increase in SERCA2a activity and a biphasic modulation of the open probability of the ryanodine receptor.[2,5,10,12,32] Co-immunofluorescence revealed partial colocalization for S100A1 with both SERCA and IP$_3$R in the perinuclear region also in ECs. Therefore, SERCA and the IP$_3$R may be potential target proteins for S100A1 in ECs and modulation of SERCA or IP$_3$R activity may further be involved in altered endothelial [Ca^{2+}]$_i$ transients. Notably, the SERCA3 knockout mouse shows a phenotype of decreased ACh-induced [Ca^{2+}]$_i$ transients and impaired endothelium-dependent vessel relaxation similar to our results but lacks the development of high BP.[33] Moreover, the agonist-induced increased NO production in S100A1-overexpressing HCAECs was blocked by the use of the IP$_3$R blocker 2-APB, demonstrating that the S100A1-mediated effect is, at least in part, [Ca^{2+}]$_i$-dependent.

Noteworthy, various transduction pathways mediated by extracellular signals can modulate eNOS activity, including Hsps.[16–19] Importantly, eNOS protein expression and expression of Hsp90, known to interact with S100A1 and regulate eNOS activity,[34,35] were not altered in ECs from SKO mice compared with WT.

Additional mediators of endothelium-dependent relaxation, such as endothelium-derived hyperpolarization factor or prostacyclin, exist,[36] and the lack of S100A1 expression may additionally affect these pathways and contribute to vascular dysfunction in SKO. Future studies will be needed to determine the interplay between changes in S100A1 in ECs and these important endothelial factors.

Cardiac S100A1 protein expression is known to be down-regulated in HF in vivo.[11–13] Because endothelial dysfunction is a characteristic of a variety of cardiovascular diseases and risk factors such as hypertension, HF, chronic smoking, and hypercholesterolemia,[20,21,29] we investigated endothelial S100A1 expression in response to neurohumoral stimulation involved in vascular and cardiac remodeling. Decreased S100A1 expression following ET-1 and Ang II stimulation in vitro and reduced endothelial S100A1 expression in HF in vivo indicate that reduced endothelial S100A1 levels could potentially be implicated in the development of endothelial dysfunction in cardiovascular diseases. Because S100A1 overexpression in HCAECs caused a significant increase in agonist-induced NO generation in vitro, increasing/normalizing endothelial S100A1 could potentially add to existing therapeutic strategies to treat cardiovascular diseases.

To summarize, our study confirms S100A1 expression in ECs and reports on a novel critical role for S100A1 in vascular function. Importantly, the critical nature of S100A1 in EC function was found both in vitro and in vivo, and all data corroborate that the loss of S100A1 causes dysfunction. We found that endothelial S100A1 expression is essential for agonist-induced [Ca^{2+}]$_i$ transients, and this finding may at least contribute to reduced eNOS activity and decreased NO generation in ECs lacking S100A1 expression. Endothelial dysfunction in SKO mice translates into impaired endothelium-dependent vascular relaxation and increased systolic and
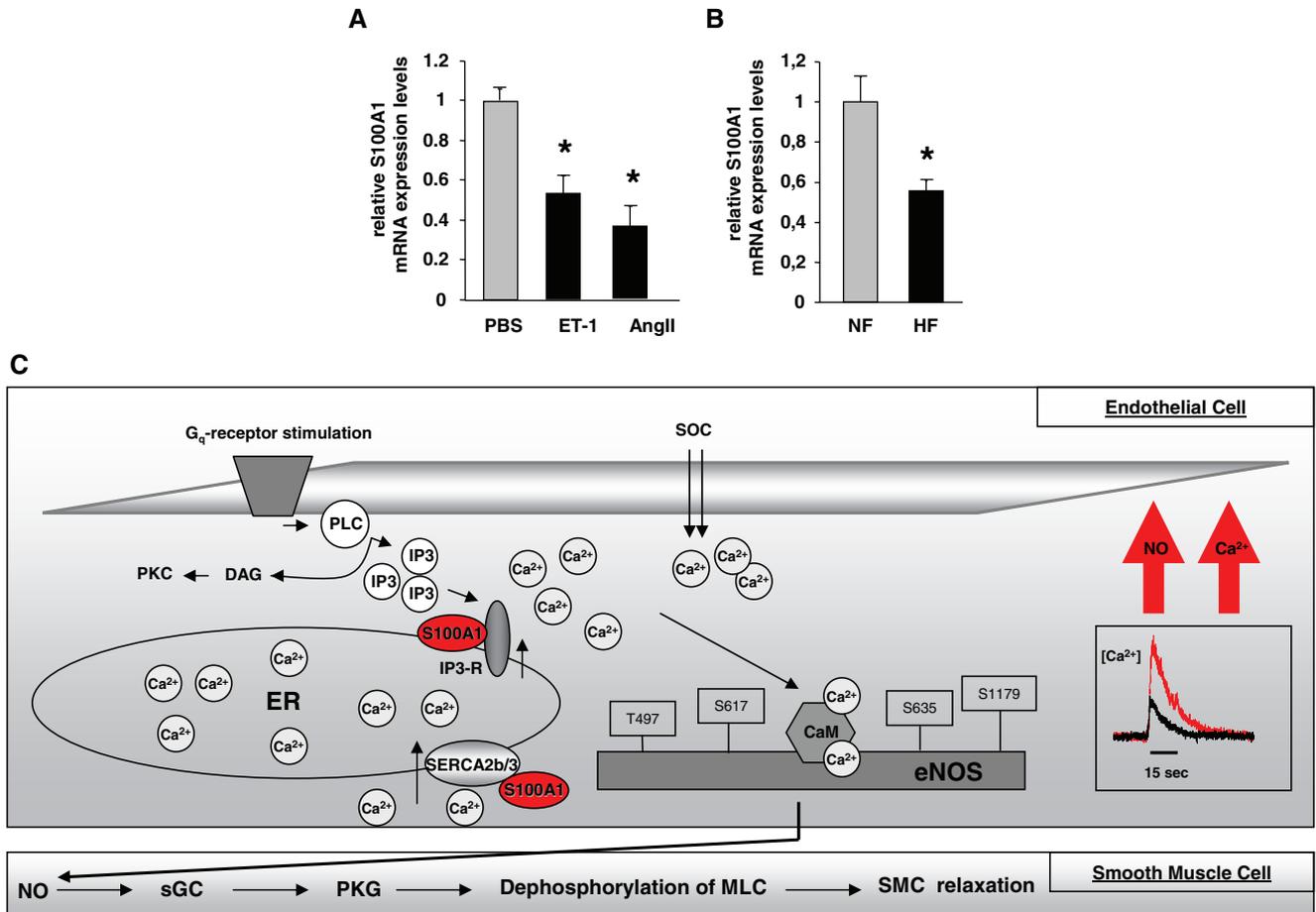
Figure 8. A, ET-1 or Ang II (both 10^{-7} mol/L) significantly reduce S100A1 mRNA expression in isolated RCECs after 60 hours. Experiments were done in triplicates; 3 individual experiments. B, Endothelial S100A1 expression is significantly downregulated in an experimental rat HF model (n=7) 56 days after myocardial infarction compared with endothelial cells isolated from nonfailing (NF) control hearts (n=7). *$P<0.05$ vs PBS or nonfailing. Data are presented as means±SEM. C, Proposed role of endothelial S100A1 on agonist-induced $[Ca^{2+}]_i$ transients. Activation of the G_q protein–coupled receptor stimulates phospholipase C (PLC) to split phosphatidylinositol into IP_3 and 1,2-diacylglycerol (DAG). Whereas DAG activates protein kinase C (PKC), IP_3 promotes the release of calcium (Ca^{2+}) from the endoplasmic reticulum (ER). Store-operated calcium (SOC) may contribute to agonist-induced $[Ca^{2+}]_i$ transients in ECs. Increased cytoplasmic Ca^{2+} levels activate calmodulin (CaM), which binds eNOS, leading to efficient NO generation. Ca^{2+} is pumped back into the endoplasmic reticulum by SERCA isoforms 2b and 3. Of note, endothelial S100A1 protein was shown to partly colocalize to both SERCA2b and IP_3R and to increase agonist-induced $[Ca^{2+}]_i$ transients in ECs, causing enhanced endothelial NO generation. eNOS activity is also regulated by several phosphorylation sites stimulating or inhibiting NO generation and by protein–protein interactions such as Hsp90. NO activates soluble guanylyl cyclase (sGC) and protein kinase G (PKG) in SMCs causing dephosphorylation of myosin light chain (MLC) and relaxation of SMCs.

diastolic BP in vivo. Finally, endothelial S100A1 is downregulated after the neurohumoral stimulation involved in vascular and cardiac remodeling in vitro and in HF in vivo. Therefore, S100A1 in ECs plays a critical role for vascular function, and targeting endothelial S100A1 expression may be a novel therapeutic means to improve endothelial function in vascular disease or HF.

Disclosures
None.

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EXPANDED MATERIALS AND METHODS

Isolation of Mouse Aortic Endothelial Cells

Mouse aortic endothelial cells (MAEC) from wild-type (WT) and S100A1 knock-out mice (SKO) were obtained by outgrow from aortic patches on a collagen matrix (Sigma) for three days in basal EBM-2 media (Cambrex, Walkersville, MD) supplemented with vascular endothelial growth factor (VEGF) and 5% fetal calf serum (FCS) as described previously.[1] MAEC were identified morphologically and phenotyped by functional uptake of acetylated LDL and immunohistochemistry against vWF. Outgrow resulted in a mixture of cell types and proportion of EC as measured by vWF staining was ~50%. Cells were used at passage one.

Isolation of Rat Cardiac Endothelial Cells

Rat cardiac endothelial cells (RCEC) were isolated using magnetic microbeads (Miltenyi Biotech, Auburn, CA) ligated with an anti-rat platelet endothelial cell adhesion molecule-1 (PECAM-1) antibody.[2] RCEC were phenotyped by functional uptake of acetylated LDL and PECAM-1 staining as described previously.[3] RCEC were used between passage 4 and 8.

Immunofluorescence

Cells were seeded overnight on collagen-coated glass coverslips, fixed and permeabilized as described previously.[4] Human coronary endothelial cells (HCAEC; purchased from Cambrex), RCEC and MAEC were labelled with a rabbit anti-S100A1 antibody (AB) (Acris, SP5355P; 1:200) followed by a 488 donkey anti-rabbit AB (alternatively, 568 goat ant-rabbit AB, both Molecular Probes; 1:100). Co-immunofluorescence for S100A1 and sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2)

as well as for S100A1 and the inositol 1,4,5- triphosphate receptor (IP$_3$R) was performed in

RCEC using the S100A1 AB as described above and, consecutively, a goat-anti SERCA2 (sc-

8095, 1:100) or a goat-anti IP$_3$R AB (sc-7278, Santa Cruz, CA) followed by consecutive

probing with a 488 donkey anti-rabbit and a 555 donkey anti-goat AB's (Molecular Probes;

1:100).   Negative controls were done by using corresponding amounts of corresponding

IgG's.   Slides were mounted using Vectashield medium with dapi (Vector Laboratories,

Burlingame, CA).   Images were obtained by using an Olympus IX 71 microscope, a mercury

arc light and suitable filters and background correction was performed using the appropriate

negative controls.

Immunohistochemistry

Immunohistochemistry was performed as described previously.[5]   Briefly, MAEC were

seeded on collagen-coated glass coverslips and allowed to grow overnight in fully

supplemented EBM-2 medium.   Cells were permeabilized and incubated with rabbit anti-

vWF-AB (Dako; A-0082; 1:100).   A peroxidase-conjugated secondary antibody and Vector

VIP peroxidase substrat kit (Vector Laboratories, Burlingame, CA, USA) was used to reveal

the antigen.   Rabbit IgG (Santa Cruz Technologies, Santa Cruz, CA, USA) was used as a

negative control.   Additionally, aortas from WT or SKO were cryosectioned (7 µm), mounted

and immunostained for either vWF or S100A1 (Acris, SP5355P; 1:100) as described above.

In Vitro Physiology

The relaxation/contraction response of thoracic aortas of WT or SKO mice was

examined using a commercially available mounting apparatus attached to a force transducer.

Responses of aortas to phenylephrine (PE; 10^{-9} - 3×10^{-5} M), acetylcholine (ACh; 10^{-9} - 3×10^{-5}

M), sodium nitroprusside (SNP; 10^{-10} - 3×10^{-5} M) and isoproterenol (Iso; 10^{-9} - 3×10^{-5} M)

were tested in the presence and absence (mechanically scraped using a thin wire) of

2

endothelial cells at 37°C in bubbled (95% O_2/5% CO_2) Krebs-Henseleit (KH) buffer as described before.[6] For ACh, SNP and Iso responses, pretension was established at 60% of the maximum PE response (3×10^{-7} M). Data were normalized to the PE-induced contraction.

Telemetric Blood Pressure Measurement

Mice were anesthetized using ketamine (100 mg/kg BW) and xylazine (5 mg/kg BW) and a pressure transducer was placed in the aortic arch while miniaturized telemetry devices (DSI, St. Paul, MN, USA) were implanted on the nape of the neck of the animal. After implantation of the transmitter, mice were returned to their cages. Ibuprofen (10 mg/50ml) was given in the water of all animals post surgery for 48 hours and no local analgesic was provided. Systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial blood pressure (MAP) and heart rate (HR) were recorded from the aortic arch in conscious, unrestrained animals at the same time for four days.

Agonist-induced Hypotensive Response *in vivo*

Mice were anesthetized with ketamine (100 mg/kg body weight (BW)) and xylazine (5 mg/kg BW). After implantation of radiotelemetric catheters as described above acute blood pressure responses were analyzed. A second catheter was placed in the jugular vein to infuse BK. After an equilibration period, mice received bolus injections at 2- to 5-min intervals.

Silencing of endothelial S100A1 expression by small interfering RNA and NO production

S100A1 expression was silenced in RCEC by use of custom-made small interfering RNA (siRNA) (Eurogentec) and "Lipofectamine 2000 reagent" (Invitrogen) according to the manufacturer`s recommendations. Briefly, 700000 RCEC were treated with 100 nM $siRNA_{S100A1}$ for 6 h using 60 mm plates. Custom-made $siRNA_{scramble}$ (Eurogentec) was used

as control. 96 h after transfection of siRNA`s RCEC were treated with bradykinin (BK; 1 µM), thrombin (30 U/ml) or PBS for 72 h. NO in the supernatant was measured indirectly using the Nitrate/Nitrite fluorometric assay kit (Cayman, Chemical) according to the companies recommendations. Experiments were run in triplicates and 3 individual experiments were performed.

S100A1 Overexpression and NO Production in HCAEC

S100A1 overexpression in HCAEC was achieved by electrical transfection of the plasmid pAdTrack-S100A1 (10 µg), which encodes for green fluorescent protein (GFP) and S100A1 both driven by individual cytomegalovirus (CMV) promoters. PBS and the plasmid pAdTrack (10 µg) were used as controls. 500000 HCAEC were used for each transfection using Nucleofactor$^{®}$ (AMAXA biosystems) according to the manufacturer`s instructions and plated on 6 well dishes. Eighteen hours after transfection cells were stimulated using 10^{-5} M ACh or PBS for 1 hour. A third group of transfected HCAEC were pre-incubated with the IP$_3$R blocker 2-aminoethoxydiphenylborate (2-APB) (Cayman Chemical, Ann Arbor, MI) (10^{-4} M) 20 minutes prior to ACh stimulation. NO in the supernatant was measured indirectly using the Nitrate/Nitrite fluorometric assay kit (Cayman, Chemical) according to the companies recommendations. Transfection was controlled by GFP expression and cells were harvested for mRNA isolation. Experiments were run in triplicates and 3 individual experiments were performed.

Adenoviral Vectors

First-generation S100A1 adenovirus (AdS100A1) was obtained by the use of the pAdTrack-CMV/pAdEasy-1 system as previously described.[4,5,7] Expression of human S100A1 cDNA and green fluorescent protein (GFP) reporter gene were each driven by a CMV promoter. The same adenovirus devoid of S100A1 cDNA served as control (AdGFP).

The titre of stocks determined by plaque assays were 5×10^{11} pfu/ml for both AdGFP and AdS100A1 respectively.

Ca^{2+}-transient Analyses of Isolated RCEC and MAEC

EC used for Ca^{2+}-transient measurements were plated with a density of 20.000 cells/cm^2 on collagen-coated glass dishes (BT-CS Cult/stim chamber; Cellmc, Norfolk, VA) and loaded with Fura 2-AM (0.5 µmol/L for 20 minutes at 37°C). RCEC (between passage 4 and 8) were used 96 h after siRNA$_{S100A1}$ or siRNA$_{scramble}$ treatment and stimulated with BK (10^{-5} M) at room temperature. Ca^{2+}-transients of RCEC were measured using T.I.L.L. Vision software (version 4.01) as described previously.[4]

Intracellular Ca^{2+}-transients of MAEC at passage one were measured using the IonOptix MyoCam system (IonOptix Corporation) as recently described.[5,8] After stable recording MAEC were stimulated using ACh (10^{-5} M) at room temperature. Measurements were carried out for 300 seconds using an inverse Olympus microscope (IX 71) with a dual-excitation single-emission fluorescence photomultiplier system and custom-made settings for endothelial cells (IonOptix Corporation). AdS100A1 (10 pfu/cell) was used to express the S100A1 protein in SKO-derived MAEC and AdGFP (10 pfu/cell) was used as a control. GFP fluorescence was used in order to confirm AdS100A1 or AdGFP infection. MAEC were isolated from 3 mice for each group.

Western Blot Analysis

S100A1 protein, heat shock protein 90 (Hsp90) and eNOS expression was assessed in cardiac tissue, blood-free lung samples from WT or SKO mice or cultured RCEC and HCAEC cells as described previously using rabbit anti-S100A1-AB (Acris, SP5355P; 1:3000), mouse anti-Hsp90 (SC-13119; 1:1000) or mouse anti-eNOS (BD Transduction,

610297; 1:1000).[3,4] In addition to Bradford analysis probing against rabbit anti-actin-AB (Sigma, A-2066; 1:1000) was used in all western blots to control equal loading .

Rat Model of Experimental Heart Failure

Male Wistar rats (~300 g) were subjected to left coronary artery ligation or sham operation during isoflurane anesthesia (1% isoflurane in a mixture of one-third O_2 and two-thirds N_2O) as previously described.[9] Mortality of the rats with myocardial infarction (MI) was ~50% during the first 48 hours after surgery. Surviving rats were euthanized 56 days after the surgical procedure. Before the rats were euthanized, LV end-diastolic pressure (LVEDP) was measured under isoflurane anesthesia using a 2F micromanometer-tipped catheter (model SPR-407, Millar Instruments, TX) inserted through the right carotid artery. In rats that underwent ligation of the left coronary artery, only those with LVEDP >15 mmHg with transmural infarction of the left ventricular (LV) free wall comprising >40% of LV circumference were considered to have heart failure (HF) and included in the study (sham, n=7; HF, n=7). RCEC were isolated as described above.

RNA Isolation and Real-time RT-PCR

Total RNA was isolated from either HCAEC or RCEC using Trizol® (Life Technologies) according to the company's recommendations.

cDNA was synthesized by reverse transcription of the RNA with Superscript II® (Life Technologies) as recommended. Real-time PCR was performed in duplicates with a 1:100 dilution of the cDNA on a MyIQ real time PCR detection system (BioRad) with the SYBR® Green PCR master mix (Applied Biosystems). The oligonucleotide primers to examine expression of genes were as follows: S100A1 (forward primer 5`-CGATGGAGACCCTCATCAAC-3`, reverse primer 5`-TGGAAGTCCACCTCCCCGTC-3`).   For   normalization,   18S   rRNA   was   used   (forward   primer   5`-

TCAAGAACGAAAGTCGGAGG-3`, reverse primer 5`-GGACATCTAAGGGCATCAC-3`).

PCR conditions were 95ºC, 3 min, and 40 cycles of 95ºC, 10 sec; 63.5ºC, 45 sec. Specificity

of PCR products were confirmed by gel electrophoresis.

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