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ORIGINAL CONTRIBUTION

Endothelial α1AMPK modulates angiotensin II-mediated vascular inflammation and dysfunction

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Abstract

Mice with a global deletion of α1AMPK are characterized by endothelial dysfunction and NADPH oxidase subunit 2 (NOX-2)-mediated vascular oxidative stress. However, the underlying mechanisms are incompletely understood and may involve endothelial NOX-2 upregulation or facilitated vascular infiltration of phagocytic cells. Therefore, the current study was designed to investigate the vascular effects of chronic angiotensin II (AngII) infusion in mice with an endothelial-specific α1AMPK deletion. A mouse strain with endothelial-specific α1AMPK deletion was generated by breeding α1AMPK$^{flox/flox}$ mice with TekCre^{+} or Cadh5Cre^{+} mice. Chronic AngII infusion (0.5 mg/kg/day for 7day) caused mild endothelial dysfunction in wild-type mice that was significantly aggravated in endothelial α1AMPK knockout mice. Aortic NOX-2 and CD68 expression were increased, indicating that infiltrating leukocytes may significantly contribute to enhanced vascular oxidative stress. Flow cytometry revealed a higher abundance of aortic CD90.2^{+} T-cells, CD11b^{+}F4/80^{+} macrophages and Ly6G^{-}Ly6C^{+} monocytes. Vascular mRNA expression of monocyte chemoattractant protein 1, CCL5 and vascular cell adhesion molecule 1 was enhanced in AngII-infused mice lacking endothelial α1AMPK, facilitating the recruitment of inflammatory cells to the vessel wall. In addition, AngII-induced upregulation of cytoprotective heme oxygenase 1 (HO-1) was blunted in mice with endothelial α1AMPK deletion, compatible with an impaired antioxidant defense in these animals. In summary, endothelial expressed α1AMPK limits the recruitment of inflammatory cells to the vessel wall and maintains HO-1 mediated antioxidant defense. Both mechanisms reduce vascular oxidative damage and preserve endothelial function during chronic AngII treatment.

Keywords α1AMPK · Macrophages · Vascular inflammation · Reactive oxygen species · Endothelial dysfunction

Abbreviations

ACh	Acetylcholine
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
AngII	Angiotensin II
CCR2	C–C chemokine receptor type 2
CCL5	CC-chemokine ligand 5
DHE	Dihydroethidium
eNOS	Endothelial nitric oxide synthase
GTN	Glycerol trinitrate
HO-1	Heme oxygenase 1
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
MCP-1	Monocyte chemotactic protein 1

Swenja Kröller-Schön and Thomas Jansen contributed equally to this work.

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NO	Nitric oxide
ROS	Reactive oxygen species
PEG-SOD	Polyethylene-glycolated-superoxide dismutase
TBP	TATA box binding protein
VCAM-1	Vascular cell adhesion protein 1

Introduction

Oxidant injury of the endothelium is a key mechanism for the development of endothelial dysfunction and is associated with established cardiovascular risk factors such as arterial hypertension or diabetes [28]. Accordingly, patients with poor endothelial function are at risk of experiencing cardiovascular events [8]. On the other hand, drugs that limit the oxidative burden (e.g., ACE-inhibitors) improve endothelial function [36] and have beneficial effects on patient outcome [38]. Therefore, our understanding of the factors that create a pro-oxidant milieu in the vasculature will help to develop novel treatment strategies.

Important vascular sources of reactive oxygen species (ROS) include the mitochondrial respiratory chain [13], NADPH oxidases and uncoupled endothelial nitric oxide synthase (eNOS). Among these, the NADPH oxidase has gained particular interest, as it is expressed in various vascular cell types including the endothelium and vascular smooth muscle layer. In addition, inflammatory processes are another hallmark of vascular disease, and vascular recruitment and/or augmentation of immune cells bearing a phagocytic NADPH oxidase may significantly contribute to overall vascular ROS production [33]. To date, the relative importance of the vascular vs. phagocytic NADPH oxidase in vascular disease is still a matter of debate. Recent publications suggest a causal role of vascular infiltration with T cells [9], macrophages [5, 35] or monocytes [35] in the development of angiotensin II (AngII)-induced vascular oxidative stress, dysfunction and hypertension. Since these cells are fundamental parts of the innate and adaptive immune response, their ablation or suppression is not feasible as it might hamper the elimination of microbial pathogens. Therefore, strategies that target the interaction of inflammatory cells with the vascular endothelium are of greater interest, as they aim to prevent vascular inflammation more specifically. Following this idea, previous studies have identified the AMP-activated protein kinase (AMPK) as an important modulator of vascular inflammation. For example, pharmacological or genetic activation of AMPK inhibited vascular inflammation by suppression of STAT-1 [10], while AMPK inhibition was associated with endothelial barrier dysfunction [37] and a proinflammatory cytokine pattern [27]. However, the precise role of *endothelial* AMPK for the recruitment of inflammatory cells to the vasculature is still unclear at present. Therefore, the aim of the current study was to investigate the consequences of an endothelial-specific α1AMPK deletion on vascular function, oxidative stress and inflammation during a proinflammatory milieu caused by chronic AngII infusion.

Materials and methods

Experimental animal protocol

All animal treatment was in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the US National Institutes of Health and was granted by the Ethics Committee of the University Hospital Mainz and Landesuntersuchungsamt Koblenz (23 177-07/G-13-1-032). To examine the role of endothelial-expressed α1AMPK, mice with the α1AMPK allele flanked by *LoxP* sites (α1AMPK$^{flox/flox}$) [23] were bred with TekCre^{+} or Cadh5Cre^{+} mice on a C57/Bl6 background in order to obtain mice with an endothelial-specific α1AMPK deletion (α1AMPK EC KO mice). Corresponding wild-type littermates (TekCre^{+}/Cadh5Cre^{+}) served as controls. Age-matched male animals (6 weeks of age) were infused with 0.5 mg/kg/day AngII or NaCl (0.9% B. Braun Melsungen AG) by implanting osmotic minipumps (Alzet 1007d, DURECT Corporation, Cupertino).

Reagents

Antibodies against α1AMPK, p-AMPK (Thr172), ACC, NFκB, p-NFκB (Ser536), A20 and MCP-1 were purchased from Cell Signaling (Boston, MA, USA), eNOS and VCAM-1 from BD Biosciences (Heidelberg, Germany) and HO-1 from Enzo Life Sciences (Lörrach, Germany). Immunohistochemistry was performed with a NOX-2 specific antibody purchased from BD Biosciences (Heidelberg, Germany) as well as F4/80 from Thermo Fisher (Frankfurt, Germany). All other reagents and chemicals were of analytic grade and purchased from Sigma-Aldrich, Fluka or Merck (Germany).

Isolation of mouse lung endothelial cells

Murine lungs were digested using Collagenase purchased from Worthington (LS 004216) as described previously to obtain mouse lung endothelial cells (MLECs) [18, 31]. Two separation steps were performed to isolate endothelial cells using CD31 MACS beads and ICAM Dynabeads according to the manufacturer's protocols. To further purify the mouse lung endothelial cells, FACS sorting experiments were performed using FACSAria (BD, Heidelberg, Germany). After the isolation steps, a single cell suspension was stained

for CD31 PE-CF594 (BD, Heidelberg, Germany), CD326 BV421, CD45 PE-Cy7 (Biolegend, San Diego, CA), and 7-AAD PerCP-Cy 5.5 (Thermo Fisher, Frankfurt, Germany) as described previously [32].

Isometric tension studies and aortic NO production

Isometric tension studies were performed in organ chamber experiments using intact mouse aortic rings (3 mm in length) and increasing doses of vasodilator acetylcholine (ACh, endothelial-dependent relaxation) and nitroglycerin (GTN, endothelial-independent relaxation) upon preconstriction with prostaglandin $F_{2\alpha}$ as described previously [24]. To determine the production of vascular NO, isolated mouse aortic rings (2 × 5 mm in length) were used in electron paramagnetic resonance (ESR) spectroscopy measurements using colloid Fe(II)-diethyldithiocarbamate [$Fe(DETC)_2$] as spin trap [14] with an X-band table-top spectrometer MS200 (Magnettech, Berlin, Germany). The instrument settings were as follows: 10 mW microwave power, 0.8 mT amplitude modulation, 100 kHz modulation frequency, 327 mT center field, 10 mT sweep width, 60 s sweep time and three scans. Total NO production was assessed by measurement of the amplitude of the characteristic triplet EPR signal and expressed in arbitrary units, AU/(mg dry weight × h) [30].

Vascular oxidative stress

Vascular hydrogen peroxide formation was measured by an HPLC-based Amplex Red assay as described earlier using isolated aortic rings (3 mm in length) [17]. To localize ROS production topographically and determine the ROS source in the vascular wall dihydroethidium (DHE) and MitoSOX staining of aortic cryosections was used in situ as described previously [4, 11].

Reverse transcription real-time PCR (qRT-PCR)

mRNA expression of isolated aortic tissue as well as isolated mouse lung endothelial cells were analyzed by quantitative real-time RT-PCR as previously described [25]. RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) was used according to the manufacturer's protocol to isolate total RNA. 50 ng was used for real-time RT-PCR analysis with the QuantiTect™ Probe RT-PCR kit (Qiagen). TaqMan® Gene Expression assays for *α1Ampk* (Mm01296695_m1), *Nos3* (Mm00435204_m1), *Nox2* (Mm00432775_m1), *Mcp1* (Mm00441243_g1), *Hmox1* (Mm00516004_m1) and *Ccl5* (Mm0132427_m1) were purchased as probe-and-primer sets (Applied Biosystems, Foster City, CA). The mRNA quantification was determined by the comparative $\Delta\Delta Ct$ method [19]. For normalization of the specific gene expression *TATA box binding protein* (*TBP*; MM00446973_m1) was used as an endogenous control and the amount of target gene mRNA expression was expressed as the percentage of $TekCre^+$.

Immunoblotting

Shock-frozen MLECs or aortic tissue was homogenized in cell lysis buffer [Tris–HCl 20 mM, saccharose 250 mM, EGTA 3 mM, EDTA 20 mM, PMSF 0.5 mM, Triton-X100 1%, Na-vanadate 0.5 mM, Na-fluoride 2.5 mM, protease inhibitor cocktail (P8340 Sigma-Aldrich) 1%, phosphatase inhibitor cocktail (P2850, Sigma-Aldrich) 1%]. Separation of proteins (30 µg per lane) was done by SDS-PAGE, blotted onto nitrocellulose or PVDF membranes and immunostaining was performed as described previously [30].

Immunohistochemistry

α1AMPK and NOX-2 staining of paraformaldehyde-fixed aortic sections (5 µm) was performed using the Vector M.O.M. immunodetection kit (Vector Laboratories, Burlingham, CA). Antibodies against NOX-2 ($gp91^{phox}$) and α1AMPK were purchased from Millipore and Biozol. A negative and positive control was included in every experiment to rule out unspecific antibody binding. The visualization of the vascular structure was performed using hematoxylin background staining.

Intravital microscopy

Animals were anesthetized by intraperitoneal injection of midazolam (5 mg/kg body weight), medetomidine (0.5 mg/kg body weight) and fentanyl (0.05 mg/kg body weight) and were fixed on a custom-built stage to maintain a physiological body temperature. The microscopic observation was performed on the right and left common carotid arteries of the mice. To stain circulating leukocytes in vivo, 100 ml of acridine orange (0.5 mg/ml; Sigma-Aldrich) was injected via a jugular vein catheter (inner diameter 0.28 mm; outer diameter 0.61 mm; Smiths Medical Deutschland GmbH, Grasbrunn, Germany). Assessment of leukocyte adhesion was performed by a high-speed wide-field Olympus BX51WI fluorescence microscope using a long-distance condenser and a 10× (numerical aperture, 0.3) water immersion objective with a monochromator (MT 20E; Olympus Deutschland GmbH, Germany) and a charge-coupled device camera (ORCA-R_2, Hamamatsu Photonics). We used a real-time imaging system (eXcellence RT software, Olympus Deutschland GmbH, Germany) for image acquisition and analysis. Cell recruitment was quantified in four fields of view (100 × 150 µm) per carotid artery. Cells that did not move or detach from the endothelial lining within an observation period of 10 s were defined as adherent cells and were counted per millimeter square. In part of the experiments, the

CCR2 chemokine receptor antagonist RS504393 (5 mg/kg; Tocris Bioscience) was injected i.p. 1 h prior to recordings.

Flow cytometry

The whole aorta was cleaned of the surrounding tissue and digested with collagenase I. The single cells were stained with CD11b-PECy7, F4/80-FITC, B220-APC-Cy7-Fluor, CD90.2 PERCP, Ly6C and Ly6G and Po-Pro-1-Pacific Blue monoclonal antibodies. Non-specific binding of Fc receptor was blocked by Fc block. Living cells were acquired and analyzed as reported previously [16].

Cell culture experiments

Immortalized hybridoma EA.hy926 cells that share key features with human endothelial cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) with 10% fetal calf serum, 2 mM L-glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin and 1 mM sodium pyruvate in 10% CO_2. For experiments, the cells were seeded in six-well plates at a density of 1.5×10^5 cells/well and grown to 60–80% confluence.

siRNA transfection

siRNA experiments were performed with silencer select siRNA for *α1Ampk* using Lipofectamine according to the manufacturer's protocol (Thermo Fisher, Frankfurt, Germany). Lipofectamine RNAiMAX Reagent (9 µl) was diluted with 150 µl Opti-MEM Medium and 10 µM *α1Ampk* siRNA. Scrambled siRNA served as negative control. The diluted siRNA and the diluted Lipofectamine were mixed in a 1:1 ratio and incubated for 5 min at room temperature. The siRNA–lipid complex was added to the cells and incubated for 24 or 48 h. After incubation, cells were washed and total RNA or proteins were isolated for further analysis.

Statistical analysis

Results are expressed as mean ± S.E.M. For analysis of numerical data, one-way ANOVA was used. Newman–Keuls or Bonferoni correction was used for comparison among the groups. p values of < 0.05 were considered significant.

Results

Characterization of endothelial-specific α1AMPK knockout mice

Effective α1AMPK gene silencing in endothelial cells was confirmed by isolation of mouse lung endothelial cells (MLEC). We observed more than 80% reduction of α1AMPK protein expression (Fig. 1a,b) in FACS-sorted MLEC of α1AMPK EC KO mice (TekCre-specific deletion). Immunohistochemistry for α1AMPK showed no signal in the endothelial layer of α1AMPK EC KO mice, thus confirming effective gene deletion (Fig. 1c). There were no other phenotypical changes in α1AMPK EC KO mice with respect to blood glucose levels, heart weight or body weight (data not shown).

Endothelial α1AMPK deletion results in impaired endothelial function and NO production during chronic angiotensin II treatment

AngII infusion (0.5 mg/kg/day for 7day) resulted in mild endothelial dysfunction in wild-type animals, whereas α1AMPK EC KO animals (TekCre-specific deletion) displayed a significant further impairment (Fig. 1d). Endothelial-independent relaxation in response to nitroglycerin (GTN) showed a similar impairment in AngII-treated α1AMPK EC KO mice (Suppl. Figure 1a). Since Tek recombinase activity may also be present in hematopoietic cells, we used the CadhCre model as an alternative approach to achieve endothelial-specific α1AMPK gene silencing and observed a similar effect on vascular function (Suppl. Figure 1b,c).

Aortic NO levels assessed by EPR spectroscopy were decreased in AngII-treated wild-type mice, but significantly further diminished in α1AMPK EC KO mice treated accordingly (Fig. 1e). Subpressor doses of AngII did not alter blood pressure among both groups (Fig. 1f). These data suggest that loss of endothelial α1AMPK leads to compromised endothelial function through decreased NO bioavailability.

Endothelial-specific α1AMPK deletion increases angiotensin II-induced vascular oxidative stress

Since endothelial dysfunction may also be a consequence of ROS-triggered NO consumption, vascular ROS formation was analyzed by using a HPLC-based Amplex Red assay with intact aortic rings and dihydroethidium staining of aortic cryosections. While chronic AngII infusion caused a mild increase of ROS production in wild-type animals, it led to a further elevation in α1AMPK EC KO mice (TekCre-specific deletion) (Fig. 2a,b).

To assess the functional relevance of the observed increase in vascular ROS production, aortic rings were incubated with PEG-SOD (polyethylene glycolated superoxide dismutase) in vitro before the assessment of endothelial function. While PEG-SOD pretreatment had only minor impact on endothelial dysfunction in aortas from

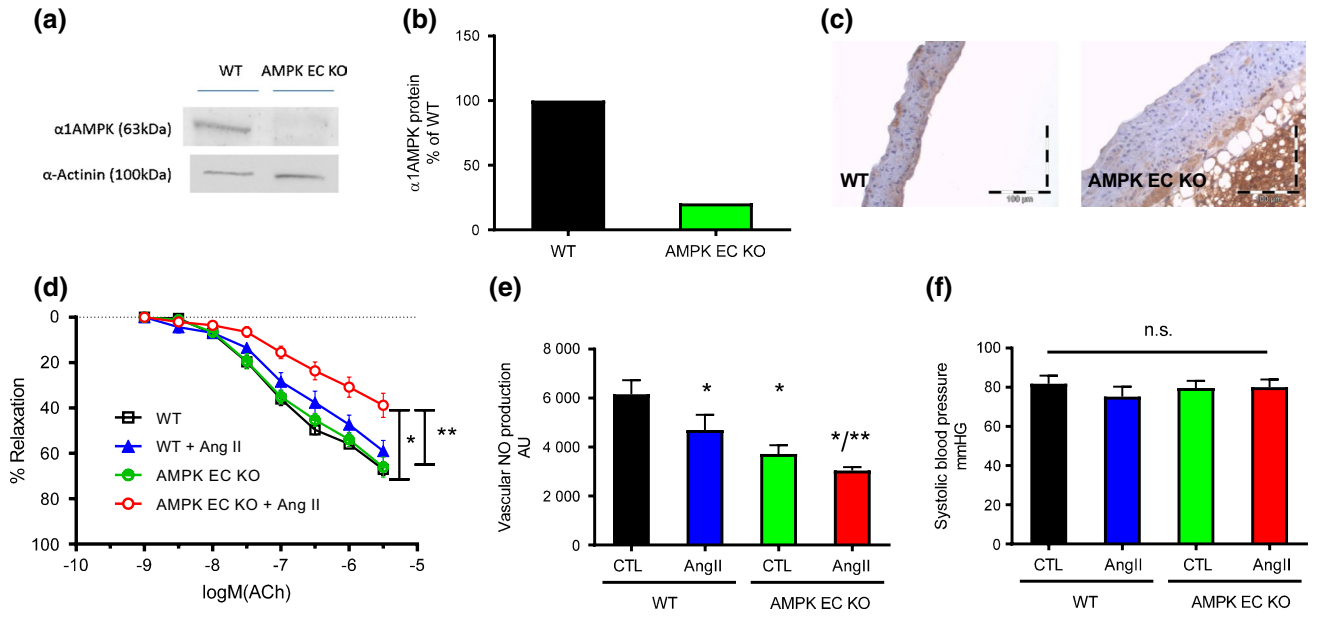

Fig. 1 Endothelial-specific α1AMPK deletion enhances AngII-induced vascular dysfunction and impairs NO signaling. Representative Western blot for α1AMPK in FACSAria-sorted MLECs ($n = 10$–12/group) (**a**) and densitometric analysis ($n = 6$/group) (**b**). Immunohistochemistry of aortic sections (**c**) confirming specific deletion of α1AMPK in the endothelium (TekCre model). Endothelial-dependent relaxation in response to acetylcholine (ACh, **d**) was analyzed by isometric tension studies (TekCre model) ($n = 10$–12/group). Tissue NO levels in aortic sections from α1AMPK EC KO and corresponding wild-type mice treated with AngII (0.5 mg/kg for 7day; $n = 8$–11/group) (**e**). Systolic blood pressure measurement after AngII treatment (0.5 mg/kg for 7day; $n = 7$–11/group) was similar among all groups (**f**). $^{*}p < 0.05$ vs. WT, $^{**}p < 0.05$ vs. WT + AngII

AngII-treated wild-type animals, it completely restored endothelial function in AngII-treated α1AMPK EC KO mice (Fig. 2c). These results suggest that the exacerbation of oxidative stress by endothelial α1AMPK deletion is responsible for the observed impairment of endothelial function.

Lack of endothelial α1AMPK facilitates recruitment of inflammatory cells to the vascular wall by an upregulation of endothelial adhesion molecules

Angiotensin II is a proinflammatory stimulus that attracts immune cells to the vascular wall by enhancing the expression of cytokines and adhesion molecules [33]. Interestingly, we found increased levels of phagocyte-type NADPH oxidase, NOX-2 mRNA and protein in AngII-treated mice lacking endothelial α1AMPK (TekCre-specific deletion), pointing to a possible involvement of myeloid cells regarding the observed increase of oxidative stress (Fig. 2d,e). In accordance with this notion, further experiments revealed increased expression of the macrophage marker CD68 in aortas of these animals (Fig. 2f).

Recruitment of inflammatory cells is preceded by increased expression of chemokines which initiate adhesion and internalization of cells to the vascular wall. Accordingly, we observed a significant increase in MCP-1 expression after AngII administration, while this response was significantly stronger in aortas and MLECs from endothelial-specific α1AMPK knockout animals (Fig. 3a, b). To assess whether MCP-1/CCR2 interaction is required for α1AMPK-dependent inflammatory cell recruitment, we performed intravital microscopy experiments. During chronic AngII treatment, endothelial rolling of leukocytes was significantly increased in AngII-treated α1AMPK EC KO mice (Fig. 3c,d). This effect was prevented by pretreatment with anti-CCR2, indicating a causal link between endothelial α1AMPK and MCP-1-mediated leukocyte recruitment.

In a next step, we aimed to investigate the abundance of specific inflammatory cell subsets in our model. By using flow cytometry of aortic tissue, we identified an increased number of $CD45^{+}$ leukocytes, CD90.2 + T-cells, $CD11b^{+}F4/80^{+}$ macrophages as well as both $Ly6C^{hi}$ and $Ly6C^{lo}$ monocytes that infiltrated the vascular wall (Fig. 4a–g, Suppl. Figure 2). Since Tek expression is also observed in myeloid cells and may therefore affect the inflammatory response, we used another genetic model of endothelial-specific α1AMPK deletion ($Cadh5Cre^{+}$ vs. $\alpha1AMPK^{fl/fl}$ x $Cadh5Cre^{+}$). Likewise, the results on increased leukocyte, T-cell and myeloid cell infiltration were confirmed by flow cytometry in aortic tissue of AngII-treated Cadh5Cre-specific α1AMPK EC KO mice (Suppl. Figure 3a–k).

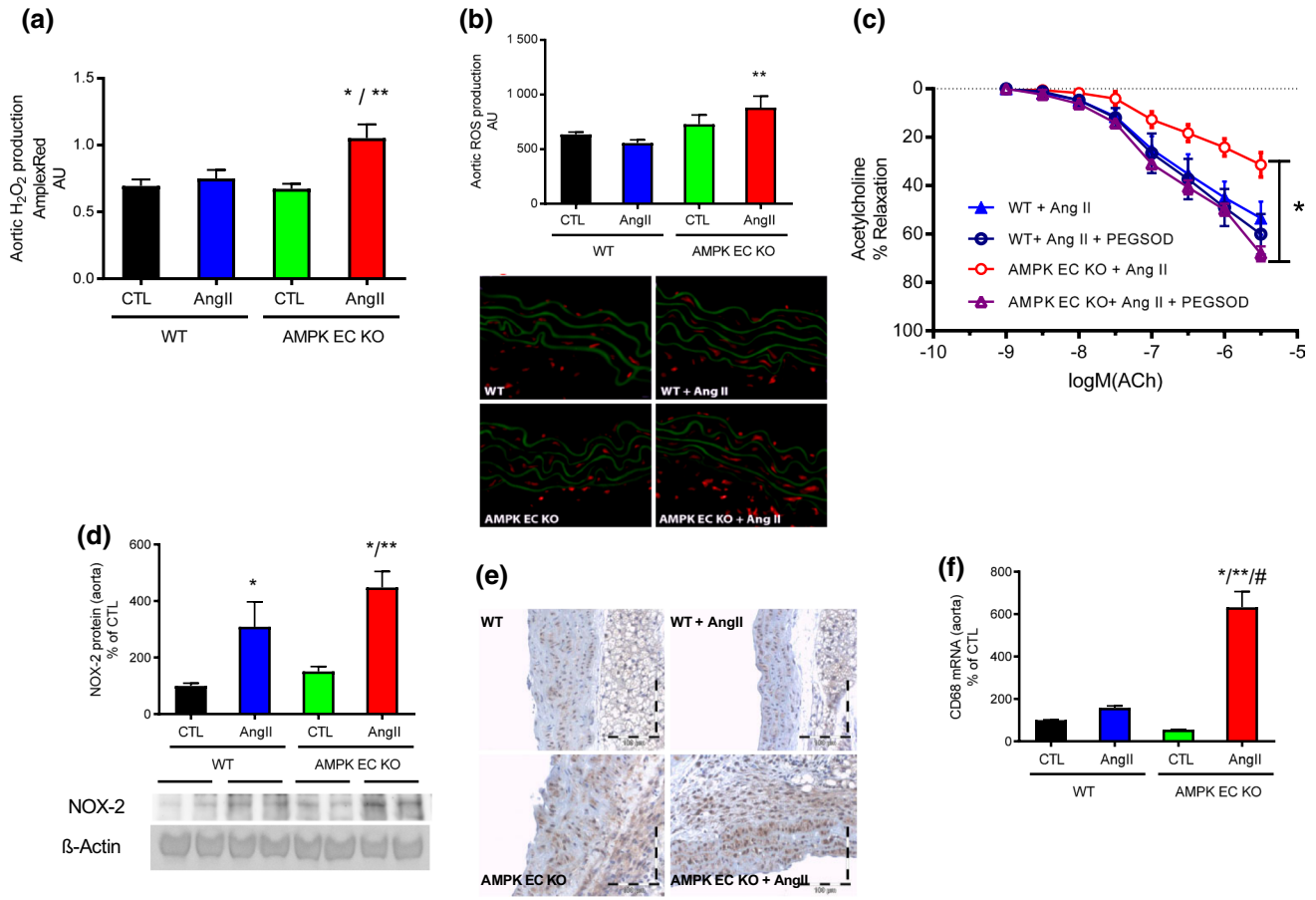

Fig. 2 Vascular oxidative stress is increased in mice lacking endothelial cell α1AMPK and mediates AngII-mediated endothelial dysfunction. Aortic H_2O_2 production was assessed by Amplex Red assay in α1AMPK EC KO (TekCre model) and WT littermates treated with AngII (0.5 mg/kg for 7day; $n=4$/group) (**a**). Vascular superoxide anion production was analyzed by dihydroethidium staining of aortic cryosections and densitometric analysis (**b**). Intact aortic rings from α1AMPK EC KO and WT littermates treated with AngII (0.5 mg/kg for 7day) were incubated with PEG-SOD ex vivo; endothelial-dependent relaxation in response to acetylcholine (ACh) was analyzed by isometric tension studies (**c**). $n=6$, $^{*}p<0.05$ vs. WT, $^{**}p<0.05$ vs. WT + AngII. NOX-2 protein expression in α1AMPK EC KO (TekCre model) and WT littermates treated with AngII (0.5 mg/kg for 7day; $n=4$/group) (**d**) and immunohistochemistry (**e**). CD68 mRNA expression in aortic tissue (**f**). $^{*}p<0.05$ vs. WT, $^{**}p<0.05$ vs. WT + AngII, $^{\#}p<0.05$ vs. α1AMPK EC KO

To assess whether macrophages are the predominant source of vascular NOX-2 and oxidative stress in our model, we performed immunohistochemical double staining for NOX-2 and F4/80 in aortic sections. In AngII-treated α1AMPK EC KO mice (TekCre-specific deletion), we found a significant co-localization of NOX-2 (green fluorescence) and F4/80 (red fluorescence), especially in the adventitia (Fig. 4h). Taken together, these results suggest that vascular infiltration of inflammatory cells significantly contributes to increased NOX-2 levels and oxidative stress observed in the setting of endothelial α1AMPK deficiency.

Deletion of endothelial α1AMPK disturbs the antioxidant defense governed by HO-1 induction

Whenever the organism cannot prevent ROS generation itself, detrimental biological effects of ROS can be attenuated by several detoxifying enzyme systems. Among these, induction of heme oxygenase-1 (HO-1) is an important stress response that confers cellular protection against oxidative damage. In endothelial cells isolated from wild-type mice, we observed a strong HO-1 induction after in vivo AngII treatment that was completely blunted in endothelial cells (Fig. 5a,b) as well as in aortic tissue (Fig. 5c,d) from α1AMPK EC KO mice. These data indicate that endothelial α1AMPK supports the antioxidant defense by induction of HO-1 in response to AngII, besides its fundamental role for the endothelial barrier function.

To obtain further mechanistic insights whether HO-1 may also be responsible for the proinflammatory response observed in the setting of endothelial α1AMPK deficiency, we performed additional experiments in EAhy926 cells. siRNA-mediated gene silencing of either α1AMPK or

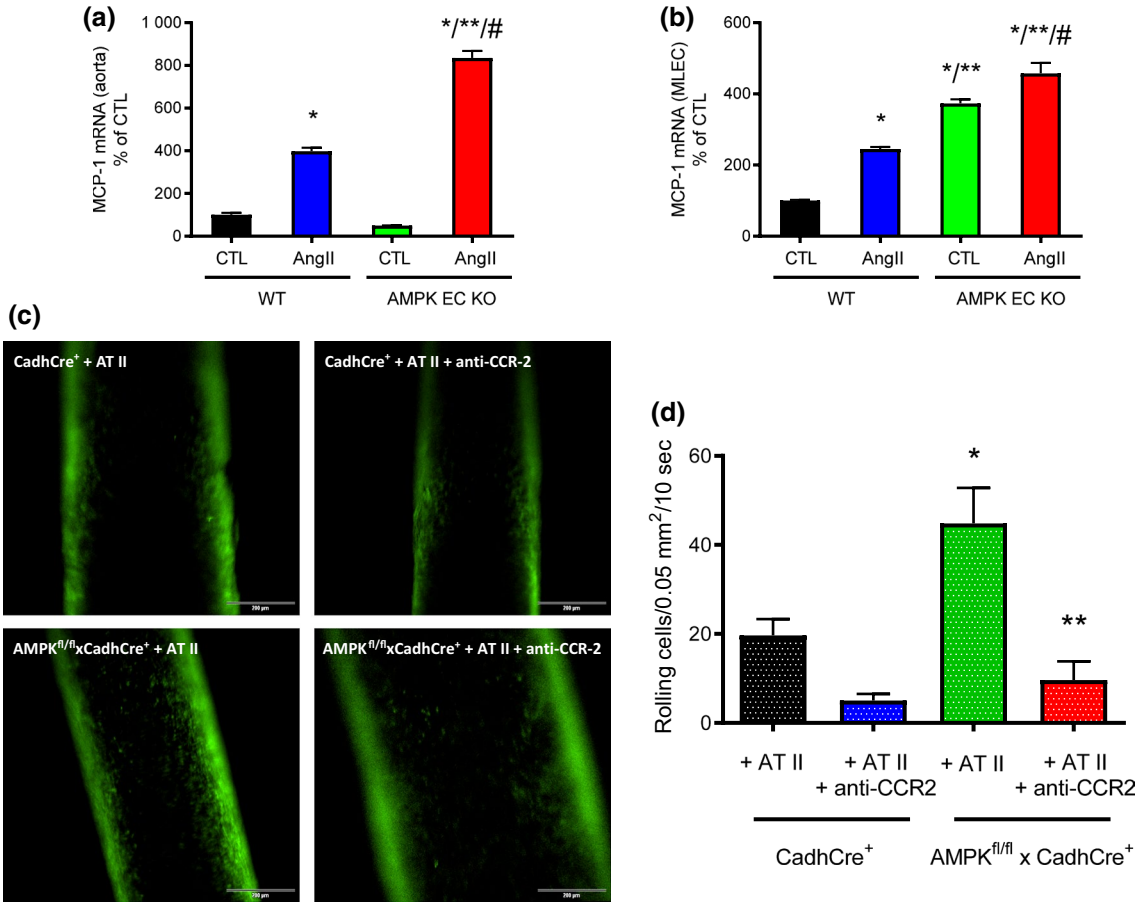

Fig. 3 Endothelial α1AMPK deficiency enhances MCP-1-dependent leukocyte rolling during chronic AngII infusion. MCP-1 mRNA expression in aortic tissue (**a**) and MACS-sorted MLECs (**b**) were significantly increased in AngII-treated AMPK EC KO mice. Intravital microscopy was used to investigate the consequences of increased MCP-1 signaling. Blocking of MCP-1/CCR-2 interaction by intraperitoneal injection of a CCR2 chemokine receptor antagonist (RS504393) completely prevented the increased leukocyte rolling observed in AMPK EC KO mice (CadhCre model) (**c**, **d**). $^{*}p < 0.05$ vs. WT, $^{**}p < 0.05$ vs. WT + AngII, $^{\#}p < 0.05$ vs. AMPK EC KO

HO-1 both led to an induction of VCAM-1 and MCP-1 (Suppl. Figure 4). In the setting of α1AMPK knockdown by siRNA, upregulation of HO-1 by hemin as well as preincubation with PEG-SOD were able to prevent the induction of VCAM-1 and MCP-1 (Fig. 6a–e).

These data suggest that indeed the suppression of HO-1 and increased oxidative stress are crucial steps how endothelial α1AMPK deficiency leads to a proinflammatory vascular phenotype.

Discussion

The data presented here identify novel mechanisms how endothelial α1AMPK protects the vasculature against oxidative damage in vivo. These involve specifically the endothelial cell barrier function as well as the ROS-detoxifying machinery. We demonstrate that the loss of endothelial α1AMPK enhances recruitment of inflammatory cells to the vascular wall by an upregulation of cytokines and vascular adhesion molecules including VCAM-1, MCP-1 and CCL5. This was accompanied by an increased vascular abundance of the phagocytic NADPH oxidase isoform NOX-2 in aortic tissue, oxidative stress and endothelial dysfunction. In vitro scavenging of excess superoxide levels in α1AMPK EC KO mice by PEG-SOD normalized endothelial function, pointing to a causal role of oxidative stress in this setting. In addition, AngII-mediated induction of cytoprotective HO-1 was blunted, indicating an increased susceptibility towards oxidative damage in mice lacking endothelial α1AMPK. In EAhy926 cells, induction of HO-1 or ROS scavenging by PEG-SOD were able to prevent the proinflammatory phenotype caused by α1AMPK deficiency.

Endothelial dysfunction is often caused by oxidative stress rather than diminished NO production and has

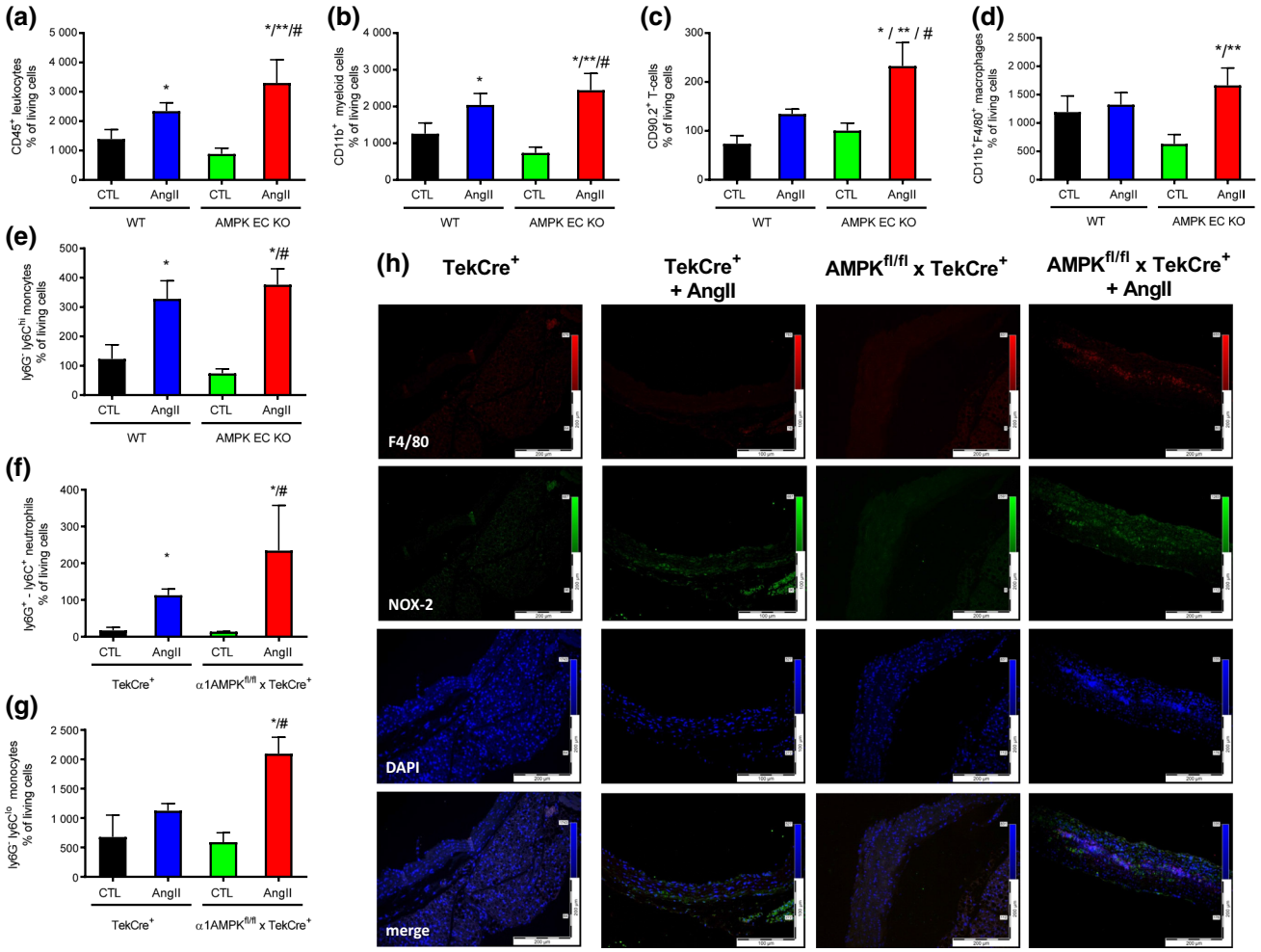

Fig. 4 Endothelial α1AMPK deletion increases vascular abundance of inflammatory cells during chronic AngII infusion. Flow cytometry of single cell suspension from aortic tissue homogenates was used to identify $CD45^+$ leukocytes (**a**), $CD11b^+$ myeloid cells (**b**), $CD90.2^+$ T-cells (**c**) $CD11b^+F4/80^+$ macrophages (**d**), $Ly6G^-Ly6C^{hi}$ monocytes (**e**), $Ly6G^+Ly6C^+$ neutrophils (**f**) and $Ly6G^-Ly6C^{lo}$ monocytes (**g**) ($n = 6$/group). **h** Double staining of F4/80 and NOX-2 in aortic sections (representative images of $n = 3$–6 mice). $^*p < 0.05$ vs. WT, $^{**}p < 0.05$ vs. WT + AngII, $^{\#}p < 0.05$ vs. AMPK EC KO

important prognostic implications [8]. Previous data from cultured endothelial cells identified AMPK as a stimulator of eNOS by site-directed phosphorylation [3] or by promoting its association with heat shock protein 90 [29]. In addition, murine in vivo studies showed that pharmacologically activated AMPK (e.g., by AICAR, metformin) was able to prevent endothelial dysfunction and oxidative stress [30]. However, the functional relevance of AMPK-mediated eNOS activation in vivo remains unclear. Our data indicate that endothelial lack of the α1AMPK isoform is not critical to maintain endothelial function under basal conditions. However, during oxidative challenge by AngII infusion, endothelial α1AMPK deletion led to enhanced vascular dysfunction paralleled by a diminished NO bioavailability. Even though this finding may be explained by a loss of AMPK-mediated eNOS activation, it seems of less functional importance since scavenging of superoxide by PEG-SOD was able to correct endothelial dysfunction completely. Therefore, our data would support the concept that other upstream kinases or α2AMPK may compensate the loss of endothelial α1AMPK with respect to eNOS activation, but would strengthen the role of α1AMPK for the prevention of vascular oxidative damage. This is achieved by different mechanisms including the inhibition of pro-apoptotic signaling pathways like JNK [30], less recruitment of inflammatory cells to the vasculature and an induction of HO-1 or forkhead transcription factors [34] to improve the antioxidant defense.

Availability of endothelial targeted knockout models is limited by the presence and specificity of endothelial promoters. While the receptor tyrosine kinase promoter/enhancer (Tek/Tie2) is known to provide uniform expression

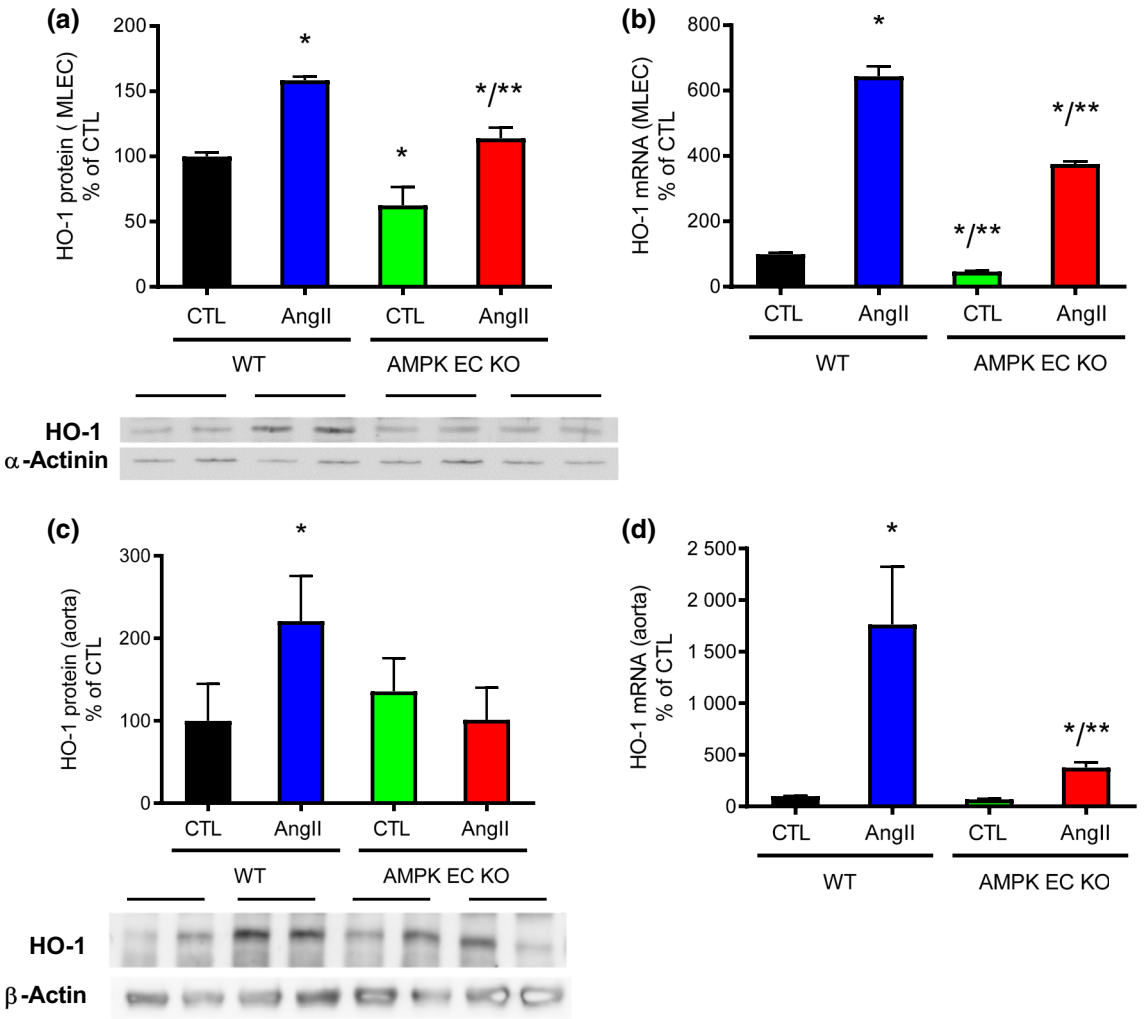

Fig. 5 Blunted HO-1 induction in α1AMPK EC KO mice indicates an impaired antioxidant defense in response to AngII. HO-1 protein and mRNA expression was assessed by Western blot and real-time qRT-PCR in mouse lung endothelial cells (**a**, **b**) and aortic tissue (**c**, **d**) from α1AMPK EC KO (TekCre model) and WT littermates treated with AngII. *$p < 0.05$ vs. WT, **$p < 0.05$ vs. WT + AngII)

in endothelial cells, it was also shown that its recombinase activity results in the deletion of target genes in hematopoietic cells [15]. Therefore, the TekCre model used here may have altered the phenotype of inflammatory cells with a possible impact on vascular inflammation observed in our study. To overcome this issue, we used the VE-cadherin-Cre promoter as another model of endothelial-specific gene silencing [2, 22] and found similar results for the most crucial experiments. Together, both approaches clearly support a fundamental role of endothelial α1AMPK in the regulation of vascular inflammation.

Recent studies suggest that cells of the innate and adaptive immune system play a crucial role in vascular biology as their inhibition or ablation reverses AngII-induced vascular dysfunction and hypertension [9, 35]. In this respect, AMPK is known to exert anti-inflammatory effects mainly through a modulation of the inflammatory cell phenotype. For example, loss of α1AMPK in myeloid cells prevented the transition of macrophages from a proinflammatory M1 to an anti-inflammatory M2 phenotype [21]. With respect to the vasculature, it was recently shown that α2AMPK in myeloid cells plays a crucial role in vascular repair via hypoxia-inducible factor-1α [1]. Data from our own laboratory demonstrate that α1AMPK in myelomonocytic cells modulates vascular inflammation with implications for endothelial function [12]. Mechanistically, AMPK may modulate the inflammatory response by secretion of cytokines/chemokines [20] or the induction of several inflammatory pathways (JNK, JAK/Stat) [6, 7, 26]. However, strategies that target inflammatory cells to prevent or treat vascular disease may have undesired side effects attributable to a compromised host defense against harmful microbial pathogens. Therefore, a concept that involves the interaction between the endothelium and inflammatory cells to modulate the

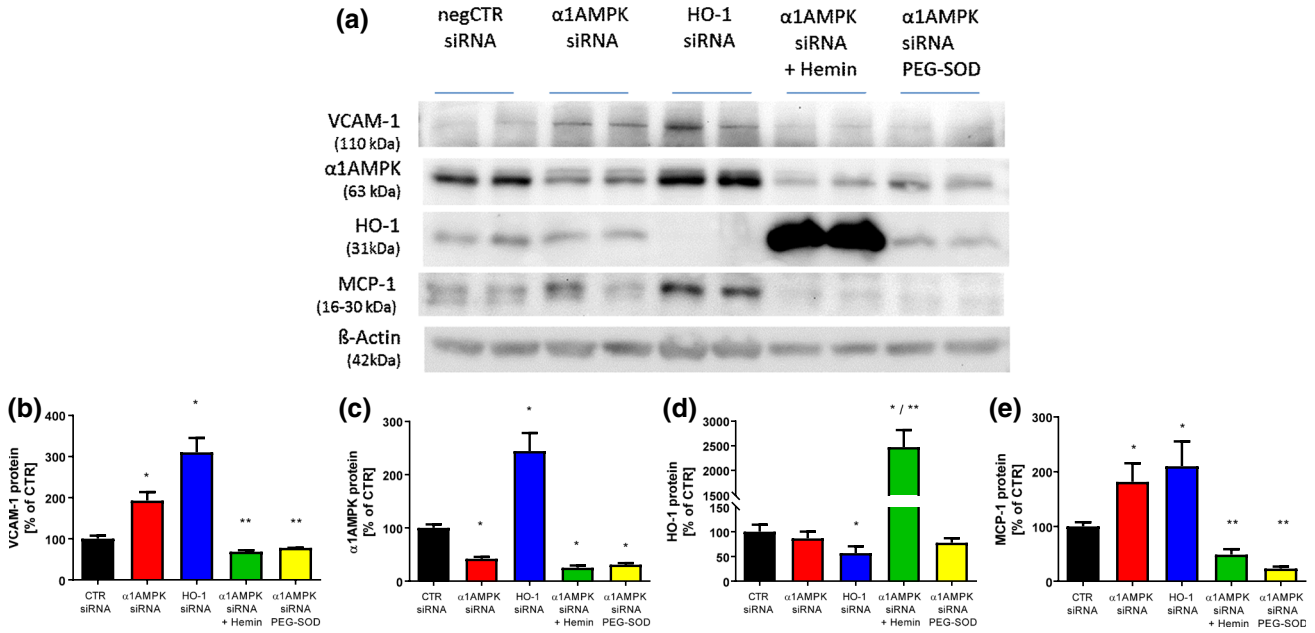

Fig. 6 HO-1 signaling and ROS are required to initiate a proinflammatory endothelial phenotype caused by α1AMPK deficiency in EA.hy926 cells. Gene silencing of α1AMPK or HO-1 in EA.hy926 was achieved by Lipofectamine-mediated siRNA transfection, while simultaneous HO-1 induction by hemin or PEG-SOD pretreatment was used to rescue the effects of α1AMPK deficiency. Representative Western blot (**a**) as well as densitometric analysis ($n = 3$–6) for VCAM-1 (**b**), α1AMPK (**c**), HO-1 (**d**) and MCP-1 (**e**) protein expression in EAhy926 cells. *$p < 0.05$ vs. neg.CTR siRNA; **$p < 0.05$ vs. α1AMPK siRNA

inflammatory reaction might be a more suitable approach to prevent vascular disease with less undesired side effects.

A unique and complex signaling cascade is required until the initiation of inflammation by cytokines and adhesion molecules eventually results in oxidative damage of the vasculature. In this respect, loss of α1AMPK may be an early signaling event to start such processes. Our study supports this concept, since α1AMPK deficiency led to upregulation of MCP-1, while the inhibition of MCP-1/CCR2 interaction prevented rolling of leukocytes in vivo, an early step in the vascular recruitment of inflammatory cells. In cell culture experiments, α1AMPK gene silencing led to enhanced VCAM-1 and MCP-1 expression, while this upregulation was prevented by HO-1 induction or ROS scavenging. These data suggest that α1AMPK may prevent inflammation at an early stage by a suppression of cytokine signaling and by mechanisms that involve ROS.

In summary, our study characterises endothelial α1AMPK as a crucial element for the preservation of vascular function in a pro-oxidative milieu. Mechanistically, we identified recruitment of inflammatory cells and associated oxidative stress as well as suppression of the HO-1 pathway as crucial steps how deficiency of endothelial α1AMPK leads to vascular dysfunction, while increased ROS and suppression of HO-1 seem to precede vascular inflammation. Our study may stimulate further research aiming at the endothelium to modulate inflammatory responses and identifies endothelial α1AMPK as a putative target for this purpose.

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Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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