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Induction of microRNA-138 by pro-inflammatory Cytokines causes Endothelial Cell Dysfunction:

Cytokines induce Endothelial Cell dysfunction via miR-138

Anagha Sen, PhD¹, Patrick Most, MD^{1,2,3}, and Karsten Peppel, PhD^{1,*}

¹ Center for Translational Medicine, Jefferson Medical College, Philadelphia PA, USA

² Laboratory for Molecular and Translational Cardiology, Department of Internal Medicine III, University of Heidelberg.

³ DZHK (German Center for Cardiovascular Research), Partner site Heidelberg/Mannheim, Heidelberg University Hospital, Heidelberg, Germany.

Abstract

Exposure to pro-inflammatory cytokines, such as angiotensin II, endothelin-1 or TNF leads to endothelial dysfunction, characterized by the reduced production of nitric oxide via endothelial nitric oxide synthase (eNOS). We recently identified the Ca²⁺ binding protein S100A1 as an essential factor required for eNOS activity. Here we report that pro-inflammatory cytokines down-regulate expression of S100A1 in primary human microvascular endothelial cells (HMVECs) via induction of microRNA-138 (miR-138), in a manner that depends on the stabilization of HIF1- α . We show that loss of S100A1 in ECs reduces stimulus-induced NO production, which can be prevented by inhibition of miR-138. Our study suggests that targeting miR-138 might be beneficial for the treatment of cardiovascular disease.

Keywords

Endothelial dysfunction; Endothelial nitric oxide synthase; Cytokines; Angiotensin II; Endothelin-1; TNF

1. Introduction

Dysfunction of the arterial vascular endothelium is associated with the most significant predisposing risk factors for cardiovascular disease (CVD) such as smoking, hypertension, diabetes, hyperglycemia, hypercholesterolemia and advanced age [1]. The endothelium serves a source of vasoactive factors that maintain – in its healthy state – the endothelium's anti-inflammatory, anti-thrombotic, anti-proliferative, and stimulus-dependent vasodilatory functions. Induction of chronic vascular inflammation, as characterized by the persistent elevation of circulating inflammatory cytokines, correlates with endothelial dysfunction

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* Corresponding Author: Karsten Peppel, PhD 1025 Walnut St., Suite 308, Center for Translational Medicine, Department of Medicine, Jefferson Medical College, Philadelphia, PA 19107 Tel.: ++1 (215) 503-9428 Fax.: ++1 (215) 503-5731 Karsten.Peppel@Jefferson.edu.

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(ED) and is recognized as a major contributor to the development of atherosclerosis and cardiovascular disease [2-4]. ED can be seen as a shift towards a chronic pro-coagulative and vaso-constrictive state that correlates with, but typically precedes, clinical manifestations of coronary, cerebrovascular and peripheral vascular diseases [1,5]. Central to the quiescent state of healthy endothelium is the stimulus-dependent generation of the vaso-relaxant factor nitric oxide (NO) by the endothelial isoform of the nitric oxide synthase enzymes (eNOS, NOS3). Chronic elevation of pro-inflammatory cytokines, most notably Angiotensin II leads to a reduction in stimulus-induced NO release from the endothelium with a consequent loss of vasoreactivity [6].

S100A1 is a small EF-hand Ca^{2+} binding protein that relays intracellular Ca^{2+} oscillations and regulates vascular tone [7-10]. Previously, we were able to show that S100A1 directly interacts with eNOS, prevents PKC-mediated phosphorylation of the eNOS inhibitory Thr-495 site and augments eNOS enzymatic activity in ECs [11]. S100A1 levels are rapidly lost in hypoxic ECs or in ischemic tissues and this correlates with a loss of stimulus-induced NO production [11]. We recently published that endothelial cell-type specific regulation of S100A1 in response to hypoxia is achieved via the HIF1- α dependent induction of miR-138, which specifically targets a conserved 22 nucleotide sequence in the 3'UTR of S100A1. Pro-inflammatory cytokines have been shown to activate HIF1- α in a variety of different cells types important to vascular biology [12-17]. Thus, here we sought to investigate if pro-inflammatory cytokines cause loss of eNOS activity and lead to ECs dysfunction via HIF1- α mediated induction of miR-138 and consequent loss of S100A1.

2. Materials and Methods

2.1 Cell Culture

EA.hy926 endothelial cells ATCC (CRL-2922) were cultured in DMEM (4.5g/L glucose) supplemented with 10% FBS. Human microvascular endothelial cells (HMVEC) were purchased from Lonza (CC-7030) at passage 4-5 and cultured in ATCC vascular cell basal medium (PCS-100-030) supplemented with the ATCC endothelial cell VEGF growth kit (PCS-100-041). For chemical hypoxia cells were treated for 24h with CoCl_2 (250 $\mu\text{mol/L}$). Viability of EA.hy926 ECs or primary human ECs was not compromised at CoCl_2 concentrations less than 1 mmol/L for 24h (not shown). Where appropriate cells were incubated with Ang II (10 nmol/L), Et-1 (10 nmol/L) or $\text{TNF}\alpha$ (50 ng/ml) for the times indicated.

2.2 Immunoblot assay

Cell samples were lysed in Laemmli buffer and 30 $\mu\text{g/Lane}$ whole cell extract was loaded on 4-20% Tris-Glycine gel, (Life Technologies, CA) and transferred to nitrocellulose membrane. Membranes were probed for S100A1 (Acris, San Diego, CA, cat # SP5355P, β -actin (SantaCruz cat # sc-8432), HIF1- α (SantaCruz cat# sc-10790) total eNOS (BD Biosciences cat# 610297) or phospho Thr-495 eNOS (BD Biosciences cat# 612707). Protein expression was quantitatively assessed using an Odyssey scanner (Li-Cor Biosciences, Lincoln NE). All immunoblot assays were done in duplicate and repeated a minimum of three times.

2.3 Plasmids

All constructs were as described [18]. The S100A1-3'UTR reporter construct comprised the 3'UTR of the human S100A1 gene cloned downstream of a constitutive ribosomal protein L10 (RPL10) promoter and renilla luciferase gene. A 3'UTR control plasmid with SV40 T antigen 3'UTR and β -actin control plasmid with β -actin promoter were used as controls for transfection efficiency. A gene block (IDT) comprising the entire S100A1-

3'UTR, but lacking specifically the 22 nucleotide putative miR-138 target site, was subcloned NheI to XhoI into the luciferase reporter vector to generate the Δ miR-138 construct as described [18]. All constructs were verified by sequencing using Jefferson's genomics facility.

2.4 Transfection

EA.hy926 ECs were cultured in 24 well plates in DMEM with 10% FBS. Cells were transfected with 1 μ g of luciferase reporter constructs mixed with Lipofectamine 2000 in serum-free DMEM, according to the manufacturer's instruction (Life Technologies). Transfection media was changed after 2-3 hours and replaced with DMEM, 10% FBS. Cells were harvested 40h after transfection. HIF1- α siRNA was purchased from SantaCruz (Dallas, TX, cat # SC-35561). To inhibit activity of microRNA-138, we used a cholesterol-conjugated antagomir-138 (10 μ g/ml, Fidelity Systems, Gaithersburg, MD) [19] to inhibit miR-138. To test activation of MAPKs via miR-138, primary HMVECs were transfected in DMEM with 20 nmoles (per well of a 6-well dish) microRNA mimic (Dharmacon ThermoFisher, cat # C- 300605-05-0005) or Control mimic (cat # CP-004500-01-05) using Lipofectamine 2000 and media was changed after 1–2 hours. Cell extracts were prepared 24h later.

2.5 Real time PCR for miR-138 expression

RNA was isolated from cell samples using RNazol RT according to manufacturer's instruction (MRC, Inc cat# RN190). 1 μ g of input RNA was used to reverse transcribe cDNA using Origene (Rockville, MD) kit (cat # HP100042). Human miR-138 real time PCR primer pair (cat # HP300151) was from Origene which consisted of a universal primer-5'-CTCTATGCGTCTGTACAAG-3' and microRNA-138 specific primer 5'-AGCUGGUGUUGUGAAUCAGGCCG-3'. Quantitative real time PCR was performed using BioRad (Hercules,CA) SYBR Green master mix and a Stratagene (La Jolla, CA) real time PCR cycler. Expression of U6 RNA, which did not change by cytokine treatment, was used to normalize for input RNA. In order to allow for better comparison between different batches of primary HMVECs the maximum expression of miR-138 induced by 24h treatment with Ang II (10nmol/L) in HMVECs was arbitrarily set as 1.

2.6 Luciferase assays

Light Switch luciferase assay reagents (cat # LS100) were from SwitchGear Genomics. ECs were cultured and transfected in 24 well plates. To measure luciferase expression, 100 μ l of assay reagents were added to each well and enzyme activity was measured, according to the manufacturer's instruction (SwitchGear Genomics) using a Wallac Victor³ Multilabel counter (Perkin Elmer, MA). Luciferase expression is given as relative light units (RLU). All luciferase experiments were done in triplicate and repeated a minimum of 3 times.

2.7 Matrigel tube formation assay

Growth-factor reduced Matrigel Matrix (BD Biosciences, cat# 356231) was diluted 1:1 with ATCC vascular cell basal medium (no additives). HMVEC (approx 200,000 cells) were treated with Angiotensin II (10 nmol/L) and either incubated with antagomir-138 or control or infected with recombinant adenovirus expressing S100A1 and GFP from a bicistronic insert [10] or control (ctr) as described [18] at a multiplicity of infection (MOI) of 17. After 24h cells were detached from the plastic support with trypsin and seeded onto the Matrigel matrix in ATCC medium supplemented with ATCC endothelial cell VEGF growth kit (PCS-100-041) and Ang II (10 nmol/L). Images were taken 24h later and digitized using NIH-ImageJ software. Original images of the matrigel tube formation assays are included as online supplement Figure S11.

2.8 Nitric oxide assay

HMVEC (approx 100,000 cells) were incubated with antagomir-138 or control and either Ang II (10 nmol/L) or TNF α (50 ng/ml) for 24h. Following this, cells were starved for 4h in ATCC vascular cell basal medium without supplements. Cells were then incubated with ATCC vascular cell basal medium supplemented with all components of the ATCC endothelial cell growth kit (PCS-100-041) except VEGF. NO production was then stimulated with the addition of 50 ng/ml VEGF (R&D systems) and Ang II or TNF α . Medium was collected 24h later and NO levels were measured using a fluorescent NO/Nitrite/Nitrate assay (Cayman Chemical, cat# 780051) according to manufacturer's instructions. In other experiments HMVECs were infected with recombinant Adenovirus expressing S100A1 and GFP from a bicistronic insert, or control, at a MOI of 17. The infection medium was removed 8h later and incubation was continued overnight. Next day cells were treated with Ang II (10nmol/L) or TNF α (50 ng/ml). After 24h cells were starved for 4h in ATCC medium without supplements. Cells were then incubated with ATCC vascular cell basal medium supplemented with all components of the ATCC endothelial cell growth kit (PCS-100-041) except VEGF. NO production was then stimulated with the addition of 50 ng/ml VEGF (R&D systems) and Ang II or TNF α . Medium was collected 24h later and NO levels were measured using a fluorescent NO/Nitrite/Nitrate assay (Cayman Chemical, cat# 780051) according to manufacturer's instructions.

2.9 Statistical Analysis

One-way ANOVA with Tukey's post-test for multiple comparisons was used to analyze the appropriate data using GraphPad PRISM software. Data are shown as \pm SEM in the figures. A *P* value of < 0.05 was considered statistically significant. Experiments were done independently a minimum of three times. Each set-up was done at least in duplicate for each repetition.

3. Results

3.1 Pro-inflammatory cytokines reduce S100A1 expression and cause dysfunction in ECs

Endothelial cells isolated from patients with Heart Failure (HF) display reduced levels of S100A1 [10], while increased expression of vasoactive pro-inflammatory cytokines is known to correlate with HF [20,21]. In order to investigate if pro-inflammatory cytokines would change expression of S100A1 we treated primary human microvascular endothelial cells (HMVECs) with angiotensin II (Ang II), endothelin-1 (Et-1) or tumor necrosis factor (TNF α) for 24h. All of these cytokines reduced S100A1 protein levels to less than 25% of untreated controls, while simultaneously increasing expression of the intercellular adhesion molecule-1 (ICAM-1), a molecule known to participate in vascular pathologies by promoting the attachment of leucocytes to injured endothelium [22,23] (Figure 1A, see Figure S1 for quantification of Immunoblot). The magnitude of the effect was comparable to that induced by hypoxia (induced by treatment with CoCl $_2$) in ECs, a treatment we have recently shown to cause EC dysfunction via the reduced expression of S100A1 [18].

Reduced bioavailability of nitric oxide (NO) is a hallmark of cytokine-induced EC dysfunction [24]. We have previously shown that S100A1 is an essential regulator of eNOS (NOS3) activity in ECs [11] and that lack of S100A1 impairs endothelial NO production and endothelial-dependent vasodilation [10]. Treatment of HMVECs with Ang II, Et-1 or TNF α all strongly impaired both basal as well as stimulus-induced NO production (Figure 1B). Re-expression of S100A1 via infection with recombinant adenovirus restored both basal as well as VEGF-induced NO production in cytokine-treated ECs, demonstrating the essential role of S100A1 to eNOS activation (Figure 1C).

3.2 Pro-inflammatory cytokines decrease S100A1 via increased HIF1- α and miR-138 levels in HMVECs

We recently reported that hypoxia-induced HIF1- α stabilization in ECs led to the induction of miR-138 with consequent loss of S100A1 [18]. Ang II as well as Et-1 and TNF have been shown to increase the stability of HIF1- α in different cell types, [25-27] albeit not primary human ECs. All three different cytokines tested increased HIF1- α levels (Figure 2A, see also Figure S2 for quantification) in HMVECs, comparably to those induced by chemical hypoxia via CoCl₂, and greatly induced miR-138 (Figure 2B), (see Figure S3 for a timecourse of miR-138 induction in ECs). The importance of the HIF1- α - miR-138 axis to the expression of S100A1 in ECs was demonstrated by measuring expression of a luciferase reporter construct that contained the human S100A1-3' untranslated region (3'UTR) [18] in cytokine treated EA.hy926 cells, which are easily transfectable, immortalized human endothelial cells. Expression of this reporter construct was greatly diminished when cells were exposed to Ang II, Et-1 or TNF (Figure 2C) whereas expression of a control construct having the 3'UTR of the SV40-T-ag instead of S100A1 was not affected by cytokine treatment (Figure S4). The 3'UTR of the S100A1 mRNA has a strong miR-138 binding site [18]. Deletion of the 22 nucleotide miR-138 target site within the S100A1-3'UTR also completely prevented any downregulation of reporter gene expression by cytokine treatment (Figure 2C). Finally, siRNA mediated knockdown of HIF1- α reduced miR-138 expression in Ang II treated ECs (Figure S5) As expected, this inhibition of HIF1- α by siRNA knockdown restored expression of the S100A1-3'UTR reporter construct in cytokine or CoCl₂ treated cells (Figure 2D), while expression of a control 3'UTR luciferase reporter was not changed by HIF1- α inhibition (Figure S6).

3.3 Inhibition of miR-138 restores normal function to cytokine-treated ECs

The importance of miR-138 to cytokine-induced EC dysfunction was also demonstrated by direct inhibition of miR-138. When miR-138 function was silenced by incubation of cytokine-treated ECs with a cell-permeable, cholesterol-conjugated antimir (antagomir-138) [18] expression of a luciferase S100A1-3'UTR reporter gene was restored to normal (Figure 3A) in a dose dependent fashion (Figure S7), while expression of a control-3'UTR was unaffected (Figure S8). Moreover, the antagomir-138 restored normal S100A1 protein expression levels to cytokine-treated HMVECs (Figure 3B). We had previously shown that loss of S100A1 reduced NO production by ECs via the increased phosphorylation of eNOS on Thr495 [11], a known inhibitory site [28]. Incubation of Ang II treated HMVECs with the antagomir-138 reduced the increased phosphorylation of eNOS pT495 within 5h (Figure 3B, see Figure S9 for quantification of immunoblots), and was able to restore both basal as well as VEGF-stimulated NO production in cytokine-treated HMVECs (Figure 3C) and reduced expression levels of ICAM back to normal (Figure S10). In addition, using the EC tube formation assay on matrigel matrix, we were able to show that both inhibition of miR-138 with the antagomir-138, as well as restoration of S100A1 levels by recombinant adenovirus, was sufficient to restore tube formation capability to Ang II treated HMVECs. (Figure 3D, original, non-digitized images are provided as Figure S11).

3.4 Inhibition of miR-138 prevents chronic activation of cytokine-induced MAPK phosphorylation

We next investigated the effects of miR-138 inhibition on cytokine signal transduction. As might be expected from the timecourse of miR-138 induction, activation of the immediate, receptor proximal, signal transduction cascades are unimpeded by the inhibition of miR-138. Thus the MAPKs p38 and ERK1/2 are both rapidly and strongly activated by treatment of HMVECs with Ang II (Figure 4A, see Figure S12 for quantification). However, continued activation of MAPKs by Ang II at later timepoints (i.e. post 5h) was strongly dependent on miR-138, as treatment with the antagomir-138 completely prevented phosphorylation of p38

and ERK1/2 at 5h and 24h. Moreover, incubation of HMVECs with a miR-138 mimic for 24h also led to MAPK activation, even in the absence of cytokine treatment (Figure S13). The reduction of this late MAPK activation by miR-138 inhibition was due to the restoration of eNOS activity, since incubation with the NOS-inhibitor L-NAME increased the phosphorylation of both p-p38 and p-ERK, even in the presence of the miR-138 inhibitor (Figure 4B, see Figure S14 for quantification).

4. Discussion

Chronic vascular inflammation is recognized as significantly predisposing to the development of cardiovascular disease. This condition correlates with reduced bioavailability of cardioprotective NO and consequent diminished endothelium-dependent dilation leading to a chronic pro-coagulative and vaso-constrictive state. The most salient finding of our work here is the identification of a signaling cascade (Figure 5) whereby vasoactive or pro-inflammatory cytokine signaling increases stability of HIF1- α , induces miR-138 expression that leads to loss of S100A1 and consequent inactivation of eNOS. This signaling axis induces and maintains the reduced bioavailability of NO, characteristic of the dysfunction observed in ECs chronically exposed to vasoactive cytokines, such as Ang II, Et-1 or TNF [29-35].

A large number of regulatory factors have been identified that control the activity of eNOS (see ref [24] for a recent review). Aside from the required cofactors Heme, NADPH, FAD, FMN, and BH4 [36-40], Ca²⁺/calmodulin (CaM) serves to enhance the catalytic activity of eNOS in response to Ca²⁺ oscillations [41]. S100A1, belonging to the same EF-hand Ca²⁺ binding protein family as CaM, also directly interacts with eNOS and serves as a required cofactor, as we have recently shown [11]. Not surprisingly, S100A1 deficient mice display reduced stimulus-induced endothelium-dependent vasorelaxation and are hypertensive [10,42], similar to eNOS deficient mice [43,44].

Activity of eNOS is enhanced by increased Ser-1177 phosphorylation by Akt [45], and possibly other kinases [24] and decreased by phosphorylation of Thr-495 [28]. Pointing towards a mechanism revealing how S100A1 may control eNOS activity, we have shown that siRNA knockdown-induced loss of S100A1 in ECs leads to increased PKC activity and phosphorylation of eNOS on the inhibitory Thr-495 site, with resultant loss of stimulus-induced NO generation [11].

Overall expression levels of S100A1 are down-regulated in several pathologies linked to tissue malperfusion. Patients in heart failure [9], as well as those with critical limb ischemia [11] show reduced myocardial and gastrocnemius muscle S100A1 levels, respectively. The ECs of these tissues appear especially susceptible to hypoxia-induced loss of S100A1 [11]. We recently identified miR-138 as the hypoxia-induced negative regulator of S100A1 in microvascular ECs [18]. In this cell type, the rapid induction of miR-138 by hypoxia critically depends on the stabilization of HIF1- α [18]. Although most prominently regulated by the action of oxygen-sensing prolylhydroxylases (PHDs), stability of the limiting α -subunit of HIF1 can also occur under normoxic conditions (see [46] for review), and has been demonstrated in response to pro-inflammatory cytokines, including angiotensin II and TNF in different cell types [14,26,27] and by endothelin-1 in lymphatic ECs [25]. Here we find that at least 3 different vasoactive pro-inflammatory cytokines (Ang II, Et-1 and TNF) are able to increase stability of HIF1- α in primary human microvascular ECs with consequent induction of miR-138, followed by down-regulation of S100A1 and resultant loss of eNOS activity.

We found that even basal NO generation in HMVECs is strongly reduced upon cytokine treatment, with complete loss of stimulus-induced NO generation (Fig. 1B), while inhibition of miR-138 restores normal S100A1 expression to HMVECs (Fig. 3B) and, in turn, is capable of restoring both basal and VEGF-induced levels of NO generation to HMVECs (Fig. 3C). This occurs even in the continued presence of cytokines, and coincides with reduced phosphorylation of the inhibitory Thr-495 site of eNOS (Fig. 3B), an event we have shown to correlate with restored S100A1 function in ECs [18].

Finally our study demonstrates the importance of miR-138 induced loss of S100A1, and subsequent eNOS inhibition, to the maintenance of cytokine-induced pro-inflammatory signal transduction in ECs. While, as expected, the immediate angiotensin II-induced activation of the MAPKs p38 and ERK1/2 is not impacted by inhibition of either miR-138 (via antagomir-138) or eNOS (via L-NAME), the long term (post 5h) activation of these signaling intermediates is dependent upon the inactivation of eNOS brought on by the miR-138 induced loss of S100A1, as phosphorylation of ERK1/2 or p38 is abrogated at these later timepoints upon miR-138 inhibition but restored when eNOS is inhibited (Fig. 4B). These studies may provide an explanation for the deleterious effects of chronic, rather than acute, cytokine exposure in ECs.

Taken together our study provides a compelling rationale to further investigate the therapeutic potential of selective miR-138 inhibition for the treatment of cardiovascular pathologies linked to endothelial dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Ang II	Angiotensin II
Akt	v-Akt murine thymoma viral oncogene homolog
BH4	(6R-)5,6,7,8-tetrahydro-L-biopterin
CaM	calmodulin
CLI	critical limb ischemia
EC	endothelial cell
Et-1	Endothelin-1
eNOS	endothelial nitric oxide synthase
FAD	flavin adenine dinucleotide
FMN	flavin mononucleotide
GM	gastrocnemius muscle
HIF1-α	hypoxia inducible factor 1 alpha

HMVEC	human microvascular endothelial cell
NADPH	nicotinamide-adenine-dinucleotide phosphate
PKC	protein kinase C
SKO	S100A1 knockout
TNF	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
WT	wild-type

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Highlights

- Pro-inflammatory cytokines activate HIF1- α in endothelial cells.
- Activation of HIF1- α induces microRNA-138 and consequent loss of S100A1.
- Loss of S100A1 inactivates eNOS to cause EC dysfunction.
- Inhibition of miR-138 restores S100A1 expression and reverses EC dysfunction.

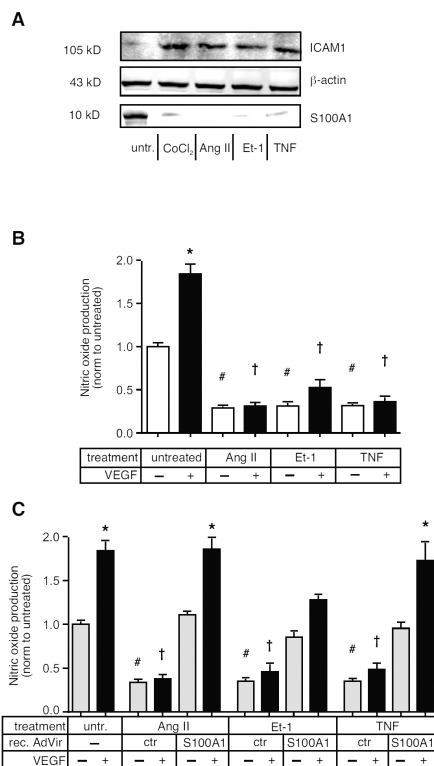


Figure 1. Pro-inflammatory cytokines reduce S100A1 expression and cause dysfunction in ECs
A) Primary HMVECs were incubated with Ang II (10 nmol/L), Et-1 (10 nmol/L), TNF α (50 ng/ml) or CoCl₂ (250 μ mol/L) for 24h before extract preparation and SDS-PAGE analysis for ICAM and S100A1. Representative image is shown. Experiment was repeated twice more, for quantification see Figure S1. **B)** HMVECs were incubated with cytokines for 24h, then treated \pm VEGF (50 ng/ml) as indicated, for a further 24h. eNOS produced nitric oxide levels in the supernatant were measured using a nitrate/nitrite assay. Experiment was done 3 times, each at least in duplicate. **C)** HMVECs were infected with recombinant adenovirus expression S100A1 (or ctr. Adenovirus) and treated for 24h with Ang II, Et-1 or TNF as indicated. Cells were then stimulated, or not, with VEGF (50 ng/ml) for 24h before assaying supernatant nitrate/nitrite levels. *, P<0.05 vs untreated, no VEGF. # P<0.05 vs untreated, no VEGF; †, P<0.02 vs untreated + VEGF. Experiment was performed 3 times, each at least in duplicate.

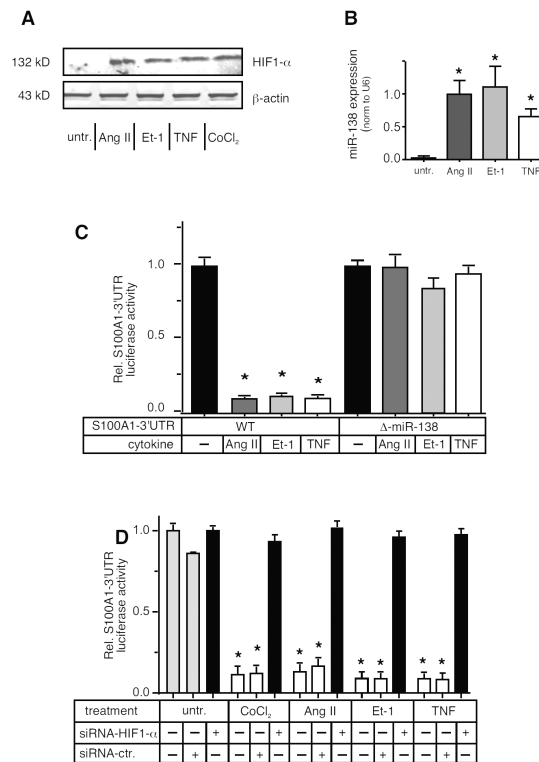


Figure 2. Pro-inflammatory cytokines decrease S100A1 via increased HIF1- α and miR-138 levels in ECs

A) Primary HMVECs were incubated with Ang II, Et-1, TNF α or CoCl₂ (250 μ mol/L) for 24h before extract preparation and SDS-PAGE analysis for HIF1- α . Representative immunoblot is shown. Experiment was repeated twice more, for quantification see Figure S2. **B)** Expression levels of miR-138 were assessed by qPCR in EC extracts subjected to 24h treatment with Angiotensin II (Ang II, 10 nmol/L), Endothelin-1 (Et-1, 10 nmol/L) or human TNF α (TNF, 10ng/ml). Expression levels of the small nuclear RNA U6 were assessed in parallel. U6 levels were not changed by cytokine treatment. *, P<0.05 vs untreated. The experiment was performed 4 times, each at least in duplicate. **C)** EA.hy926 ECs were transfected with the WT S100A1 luciferase reporter construct or the miR-138 binding site deleted reporter (Δ -miR-138) and treated for 24h with Ang II, Et-1 or TNF α , prior to luciferase assay. *, P<0.05 vs untreated. Experiment was done 3 times, each in triplicate. **D)** EA.hy926 ECs were transfected with the S100A1-3'UTR reporter gene construct and co-transfected with either siRNA against HIF1- α (Dharmacon) or scramble control and subsequently treated with cytokines as indicated. Treatment with CoCl₂, a chemical inducer of the hypoxia response was included as a control. *, P<0.02 vs untreated. Experiment was done 3 times, each at least in duplicate.

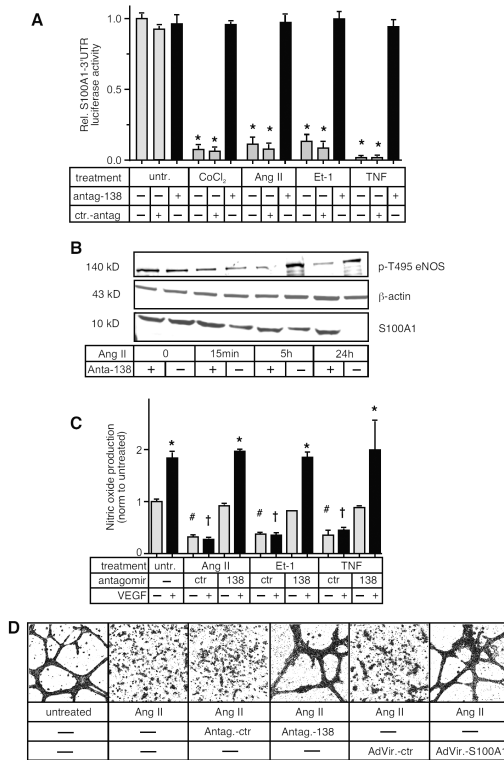


Figure 3. Inhibition of miR1-38 restores normal function to cytokine-treated ECs

A) EA.hy926 ECs were transfected with the S100A1–3’UTR luciferase reporter construct and incubated with either antagomir-138 or control and stimulated for 24h with cytokines or CoCl₂ as indicted prior to luciferase assay. *, P<0.02 vs untreated. **B)** Primary HMVECs were treated with Ang II ± antagomir-138 for times indicated and S100A1 and phospho-T495 eNOS levels were assessed by Immunoblot analysis. β-actin was assessed as loading control. Representative images are shown. Experiment was repeated twice more, for quantification see Figure S9. **C)** HMVECs were incubated with cytokines for 24h and co-incubated with either antagomir-138 or control, then treated ± VEGF (50 ng/ml) as indicated for a further 24h. eNOS produced nitric oxide levels in the supernatant were measured using a nitrate/nitrite assay. *, P<0.05 vs untreated. # P<0.05 vs untreated, no VEGF; ✕, P<0.05 vs untreated + VEGF. Experiment was performed 3 times, each at least in duplicate. **D)** Primary HMVEC were incubated with antagomir-138 or control and treated with Ang II for 24h. Next day later cells were infected (MOI=17) with either control Adenovirus or Adenovirus expressing S100A1. 24h later cells were seeded onto Matrigel matrix. Images of EC tube formation were taken 24h later and digitized using Image J (Original pictures of EC tube formation are included as supplemental Figure S11. Representative images are shown. The experiment was done 3 times, each in duplicate.

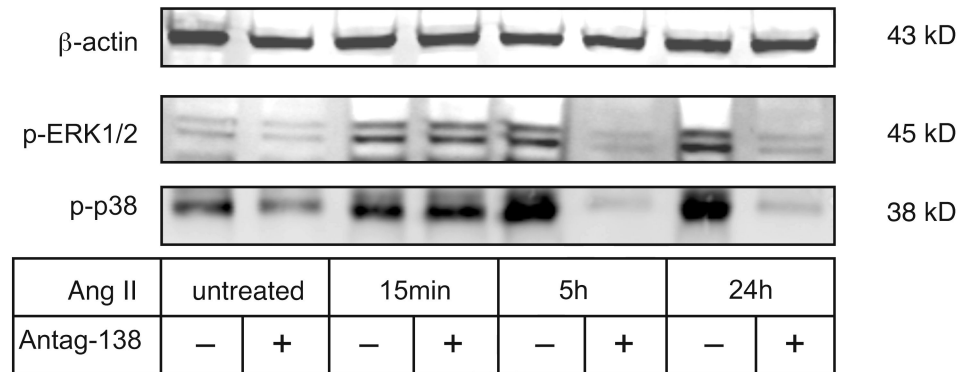
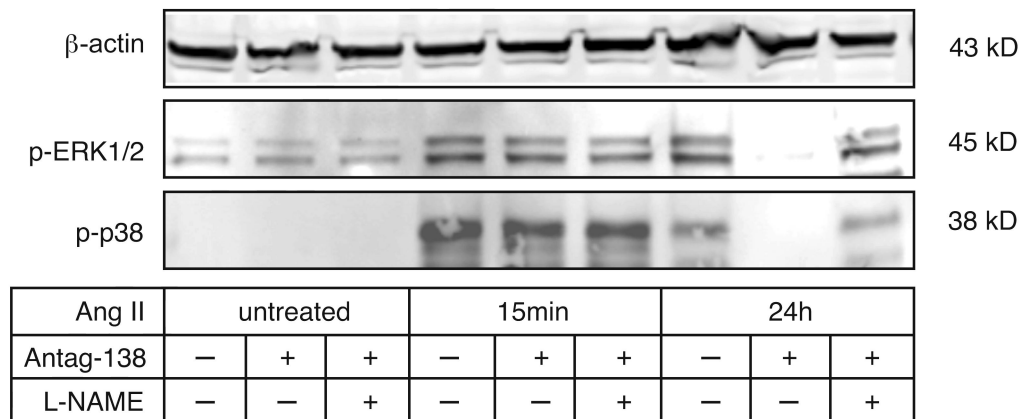
A**B**

Figure 4. Inhibition of miR-138 prevents chronic activation of cytokine-induced MAPK phosphorylation

A) Primary HMVECs were treated with Ang II ± antagomir-138 for times indicated and the phosphorylation state of the MAPK p38 and ERK1/2 were assessed by Immunoblot analysis. Representative images are shown. Experiment was repeated twice more, for quantification see Figure S12. **B)** Primary HMVECs were treated with Ang II ± antagomir-138 ± L-NAME (NOS inhibitor) for times indicated and the phosphorylation state of the MAPK p38 and ERK1/2 were assessed by Immunoblot analysis. Representative image is shown. Experiment was repeated twice more, for quantification see Figure S14.

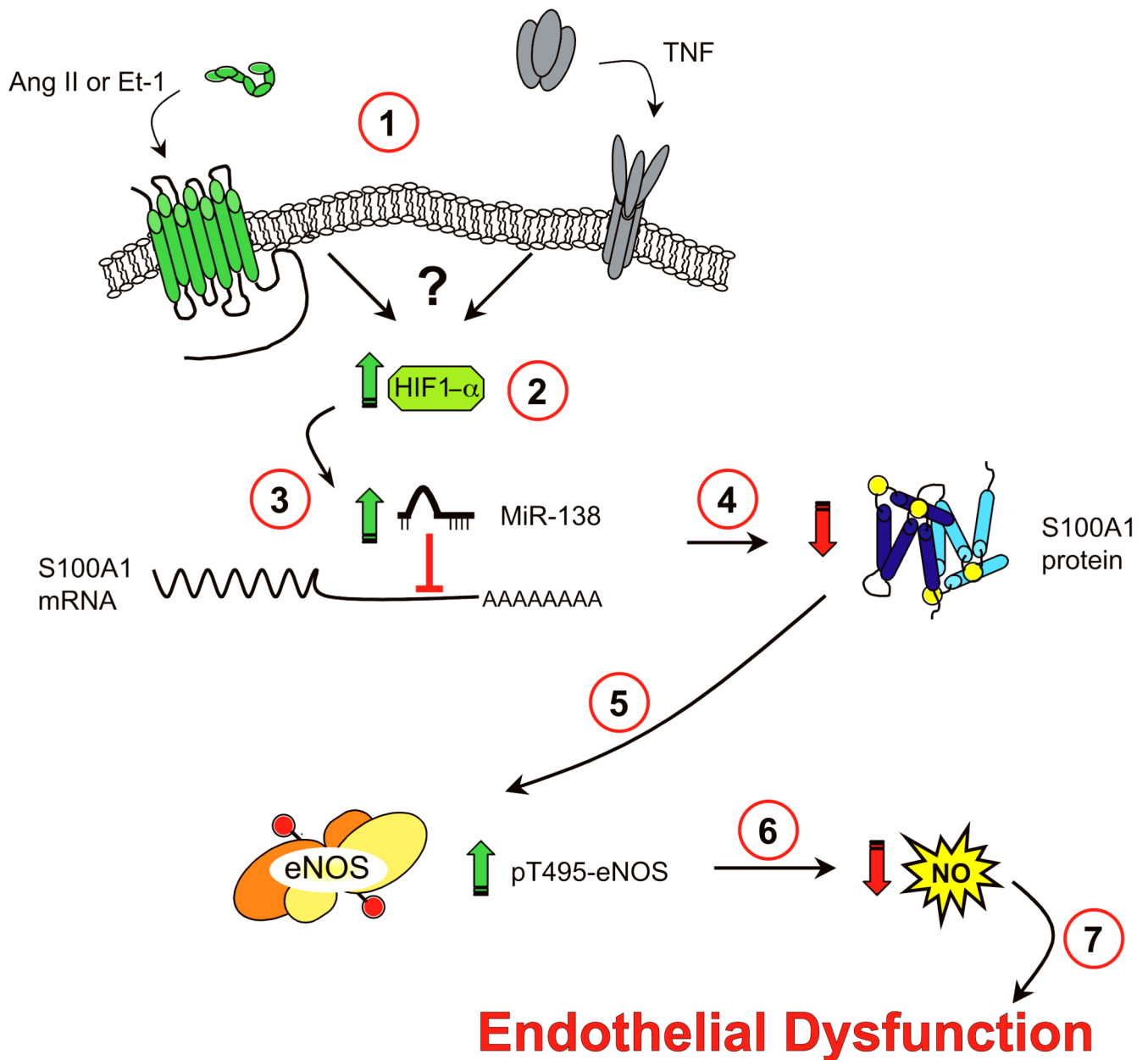


Figure 5. Proposed Model of cytokine induced, miR-138-mediated, EC dysfunction

(1) Engagement of vasocative or pro-inflammatory cytokine receptors by their respective ligands leads to stabilization of HIF1- α in ECs through as yet incompletely defined mechanisms (2). Stabilization of HIF1- α increases miR-138 levels (3), which destabilize S100A1 mRNA or suppress its translation, leading to rapid loss of S100A1 protein in ECs (4). This leads to increased phospho-Thr495 eNOS levels (5) with consequent reduction of NO generation (6). This loss of endothelial NO generation engenders endothelial dysfunction (7).