The AMP-Activated Protein Kinase Regulates Endothelial Cell Angiotensin-Converting Enzyme Expression via p53 and the Post-Transcriptional Regulation of microRNA-143/145

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ABSTRACT

**Rationale:** High angiotensin-converting enzyme (ACE)-levels are associated with cardiovascular disease, but little is known about the regulation of its expression.

**Objective:** To assess the molecular mechanisms regulating endothelial ACE expression focusing on the role of the AMP-activated protein kinase (AMPK) and miR-143/145.

**Methods and Results:** Shear stress decreased ACE expression in cultured endothelial cells, an effect prevented by downregulating AMPKα2 but not AMPKα1. AMPKα2−/− mice expressed higher ACE levels than wild-type littermates resulting in impaired hindlimb-vasodilatation to the ACE substrate, bradykinin. The latter response was also evident in animals lacking the AMPKα2 subunit only in endothelial cells. In cultured endothelial cells miR-143/145 levels were increased by shear stress in an AMPKα2-dependent manner and miR-143/145 overexpression decreased ACE expression. The effect of shear stress was unrelated to an increase in miR-143/145 promoter activity and transcription but could be attributed to post-transcriptional regulation of precursor-miR-143/145 by AMPKα2. The AMPK substrate, p53, can enhance the post-transcriptional processing of several microRNAs, including miR-143/145. We found that shear stress elicited the AMPKα2-dependent phosphorylation of p53 (on Ser15) and that p53 downregulation prevented the shear stress-induced decrease in ACE expression. Streptozotocin-induced diabetes in mice was studied as a pathophysiological model of altered AMPK activity. Diabetes increased tissue phosphorylation of the AMPK substrates, p53 and acetyl-coenzyme A carboxylase, changes that correlated with increased miR-143/145 levels and decreased ACE-expression.

**Conclusions:** The AMPKα2 suppresses endothelial ACE expression via the phosphorylation of p53 and upregulation of miR-143/145. Post-transcriptional regulation of miR-143/145 may contribute to the vascular complications associated with diabetes.

**Keywords:** Diabetes, angiotensin-converting enzyme, microRNA

**Nonstandard Abbreviations:**
- AMPK: AMP-activated protein kinase
- ACE: angiotensin converting enzyme
- Ang II: Angiotensin II
- eNOS: endothelial nitric oxide synthase
- KLF2: Krüppel-like factor 2
- miRNA: microRNA
- NO: nitric oxide

INTRODUCTION

Angiotensin II (Ang II) has been implicated in the pathobiology of atherosclerosis and the arterial response to injury and restenosis via mechanisms that include vascular hypertrophy, extracellular matrix production, and cytokine induction. Although the angiotensin converting enzyme (ACE) is only one of several enzymes that can generate Ang II, the enzyme has been allocated a central role in cardiovascular disease development mainly on the basis of the positive effects observed in response to ACE inhibitor therapy and experimental work involving protein downregulation. Certainly, ACE levels seem to
Correlate with disease development and ACE protein has been detected in atherosclerotic human coronary 
and carotid lesions. 

Relatively little is known about the signals that regulate ACE expression in vascular cells but the 
exposure of vascular smooth muscle cells to fluid shear stress or pulsatile pressure is reported to increase 
expression of the enzyme. Although an identical phenomenon was initially reported in endothelial cells,
several other studies reported exactly the opposite i.e. that “arterial levels” of shear stress attenuate ACE 
activity and decrease the expression of ACE mRNA in endothelial cells. How can fluid shear 
stress regulate ACE expression? The first hypothesis was that the effects could be attributed to increased 
transcription as the genes for human, rat, and rabbit ACE all contain a number of shear stress-responsive 
elements in their promoter regions. These were however found to be inactive and responsiveness to flow 
was then attributed to a combination of Barbie and GAGA response elements. The transcription factor 
Krüppel-like factor 2 (KLF2) has also been linked to ACE expression as overexpression of the 
transcription factor decreases endothelial cell ACE expression. Given that KLF2 is activated by shear 
stress to regulate the expression of proteins such as the endothelial nitric oxide (NO) synthase (eNOS), it 
initially seemed that KLF2 activation could directly explain the shear stress-induced down regulation of 
ACE. This was a particularly attractive hypothesis since ACE has been reported to be a target of the miR-
143/145 gene cluster in vascular smooth muscle cells and fluid shear stress was recently reported to 
increase the expression of miR-143/145 by a KLF2-dependent mechanism. However, siRNA directed 
against KLF2 failed to prevent the shear-stress induced downregulation of ACE RNA levels, indicating 
that the mechanisms must be distinct. Recently, we reported that ACE levels in monocytes can be 
regulated by the AMP-activated protein kinase (AMPK). Since this kinase is rapidly phosphorylated and 
thereby activated in endothelial cells exposed to shear stress, we set out to determine whether or not the 
AMPK is involved in the shear stress-induced downregulation of ACE expression and whether a link 
exists to miR-143/145.

**METHODS**

Detailed methods can be found in the Online Supplemental Materials.

**Cell culture.**

Human umbilical vein endothelial cells were isolated and cultured as described previously. Human 
coronary artery endothelial cells were obtained from CellSystems Biotechnologie Vertrieb GmbH 
(Troisdorf, Germany) and human aortic endothelial cells from Lonza (Basel, Switzerland). Confluent 
cultures of endothelial cells were washed twice in culture medium containing 2% fetal calf serum and 
were either maintained under static conditions or exposed to shear stress (12 dyn/cm²) in a temperature-
controlled cone-plate viscosimeter, as described.

**Animals.**

Genetically modified mice lacking either the AMPKα1 or the AMPKα2 subunits, their respective wild-
type littermates and floxed AMPKα2 mice were kindly provided by Benoit Viollet (INSERM, U1016, 
Paris, France) and bred at the Goethe University Hospital animal facility. Floxed AMPKα2 mice were 
crossed with animals expressing the Cre-deleter under the control of the VE-cadherin promoter to generate 
mice lacking the α2 subunit specifically in endothelial cells (AMPKα2−/−). Mice in which the miR-
143/145–coding genomic region was replaced with a lacZ reporter (miR-LacZ) were generated as 
described, and bred at the animal facility of the Max-Planck-Institut für Herz- und Lungenforschung 
(Bad Nauheim, Germany).

Experimental diabetes was induced in C57BL/6 mice (Charles River, Sulzfeld, Germany) by a single 
intraperitoneal injection of streptozotocin (150 mg kg⁻¹ body weight) 8-10 weeks prior to experiments.
Experiments conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23).

**Statistical analysis.**
Data are expressed as mean ± SEM. Statistical evaluation was performed on the absolute values or on log transformed data with Student’s t test for unpaired data, one-way ANOVA followed by a Bonferroni t test or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.

**RESULTS**

*Regulation of ACE expression by shear stress.*

The application of shear stress to cultured human umbilical vein endothelial cells time-dependently decreased ACE mRNA expression (Figure 1A). The effect was relatively modest after 24 hours (28±8%) but increased with prolonged stimulation (up to 72 hours). A similar time-dependent decrease in ACE protein expression was detected with significant changes being recorded after 48 hours (Figure 1B). As eNOS has previously been characterized as a shear stress-regulated gene, the effectiveness of cell stimulation was assessed by monitoring eNOS expression in the same cell lysates. NO has also previously been reported to negatively regulate ACE expression, but despite the upregulation of eNOS in the samples analyzed, we were unable to detect any consequence of NOS inhibition on the shear stress-induced downregulation of ACE (data not shown). Shear stress also decreased ACE expression in human coronary artery endothelial cells (Online Figure IA), indicating that the response is not restricted to cells of venous origin.

*Effect of AMPK activation/downregulation on ACE expression.*

The AMPK is reported to regulate ACE expression in monocytes and as the kinase is activated in endothelial cells exposed to shear stress, we analyzed ACE protein expression in human endothelial cells after pharmacological AMPK activation. The AMP analogue, AICAR (Figure 1C), and the AMPK activators metformin and thiopental all decreased ACE mRNA (Online Figure IB) and protein levels (Figure 1D); albeit with slightly different time courses.

Next we determined the consequences of AMPK downregulation on the shear stress-induced decrease of ACE expression in human endothelial cells. We found that downregulation of the AMPK 1 subunit slightly increased basal ACE expression but did not alter the sensitivity of the enzyme to shear stress (Figure 1E). Decreasing endothelial expression of the AMPKα2 subunit, on the other hand, significantly increased ACE expression under static conditions and abolished the response to shear stress.

To determine whether the negative regulatory effect of the AMPK observed in vitro is physiologically relevant, we assessed the expression of ACE in different arteries from AMPKα1+/+ or AMPKα2−/− mice and their wild-type littermates (i.e. AMPKα1+/− and AMPKα2+/−). While ACE expression was similar in AMPKα1+/− and AMPKα1−/− mice, the genetic deletion of AMPKα2 significantly increased ACE expression in the thoracic aorta (Figure 2A), kidney (Figure 2B), femoral artery (Figure 2C) and aortic arch (Online Figure IIA). Consistent with the findings obtained in human endothelial cells, the application of shear stress failed to affect ACE expression in cultured endothelial cells isolated from lungs of AMPKα2+/− mice, while ACE expression decreased in response to shear stress in cells isolated from AMPKα2−/−, AMPKα1−/− or AMPKα1+/− mice (Online Figure IIB).

*Consequences of AMPKα deletion on vasodilator responses to Bradykinin and ACE Inhibition.*

ACE not only generates Ang II but also degrades the vasodilator peptide bradykinin. Therefore, we assessed the consequences of AMPKα2 deletion on the bradykinin-induced vasodilatation of an entire...
vascular bed i.e. the murine hindlimb, which includes areas of high as well as low flow. Consistent with our observation that ACE expression was elevated in femoral arteries of AMPK\(\alpha_2\)-/- mice, the bradykinin-induced, endothelium-dependent vasodilatation of the hindlimb vasculature was significantly impaired (Figure 3A). The effects observed could be attributed to differences in ACE expression/activity rather than altered expression/sensitivity of the B\(_2\) kinin receptor as the vasodilatation in both strains was comparable in the presence of an ACE inhibitor (Figure 3B). We found no difference in the acetylcholine-induced vasodilatation in AMPK\(\alpha_2\) and AMPK\(\alpha_2^{-/-}\) animals (Online Figure IIIA) as well as no differences in the bradykinin- or acetylcholine-induced vasodilatation in AMPK\(\alpha_1^{-/-}\) and AMPK\(\alpha_1^{+/-}\) animals (Online Figure IIIB&C). Although bradykinin is an endothelium-dependent vasodilator, the AMPK may affect ACE expression in vascular smooth muscle cells and thus affect vascular responses to the kinin. Therefore, we re-assessed bradykinin-induced vasodilatation in AMPK\(\alpha_2\)\(_{-EC}\) mice and found that the response to bradykinin was also attenuated (Figure 3C). Again the differences in bradykinin-induced vasodilatation between AMPK\(\alpha_2^{+/-}\) and AMPK\(\alpha_2^{AEC}\) mice were abolished by ramiprilat (Figure 3D).

**Regulation of endothelial ACE expression by miR-143 and miR-145.**

ACE is a target of the miR-143/145 gene cluster and the protein is upregulated in vascular smooth muscle cells from miR-143/145\(^{-/-}\) mice.\(^1\)\(^1\) Moreover, fluid shear stress increases miR-143/145 levels in commercially available human endothelial cells.\(^1\)\(^2\) We found that freshly isolated and cultured (passage 1) human endothelial cells expressed low but detectable levels of miR-143 and miR-145 and that fluid shear stress increased the expression of both miRNAs over 72 hours (Figure 4A). In cells cultured under static conditions the overexpression of precursor (pre)-miR-143 or pre-miR-145 decreased endothelial ACE mRNA (Online Figure IVA) and protein levels (Figure 4B). Moreover, a mixture of specific antagonirs decreased miR-143/145 levels in human umbilical vein endothelial cells (Figure 4C), and prevented the shear stress-induced downregulation of ACE mRNA (Figure 4D) and protein (Figure 4E). AMPK\(_2\) levels were not affected by the pre-miRs (data not shown). In human aortic endothelial cells, siRNA directed against AMPK\(\alpha_2^{-}\) and antagonirs directed against miR-143/145 also increased basal ACE protein levels (Online Figure IVB) and prevented the decrease in ACE expression in response to shear stress (Online Figure IVC).

**Shear stress, AMPK\(\alpha_2\) and miR-143/145.**

To determine whether the shear stress-induced, AMPK-dependent regulation of ACE could involve miR-143/145, we assessed the consequences of AMPK\(\alpha_2\) downregulation on miRNA levels. Downregulation of the AMPK\(\alpha_2\) but not the AMPK\(\alpha_1\) subunit decreased the expression of miR-143 and miR-145 in cells maintained under static conditions (Online Figure IVD) and largely prevented the shear stress-induced increase in miR-143/145 levels (Figure 5A). Consistent with these in vitro findings, miR-143 and 145 levels were decreased in aortae from AMPK\(\alpha_2^{-/-}\) versus AMPK\(\alpha_2^{+/-}\) mice (Figure 5B).

Fluid shear stress was recently reported to increase the expression of miR-143/145 by a KLF2-dependent mechanism.\(^1\)\(^2\) Although siRNA-based experiments previously revealed no link between KLF2 and shear stress-induced changes in ACE expression\(^1\)\(^0\) we investigated a possible link between KLF2 and AMPK activation. While shear stress clearly increased KLF2 expression (Figure 6A), there was no effect of AMPK downregulation on the shear stress-induced increase in KLF2 mRNA (Figure 6B) or protein levels (Figure 6C). KLF2 was also not upstream of AMPK activation as the downregulation of KLF2 (Online Figure VA) was without effect on AMPK\(\alpha_2\) expression (Figure 6D). Moreover, in primary human umbilical vein endothelial cells KLF2 siRNA did not affect the shear stress-induced increase in either miR-143 or miR-145 (Figure 6E).

The involvement of an additional shear stress-sensitive transcription factor seemed unlikely as the primary (pri-) miR-143/145 transcripts were unaffected by shear stress over a 72 hour period (Figure 7A&B). The failure to detect pri-miR-143/145 was not a technical problem as primary transcripts increased in cells treated with transforming growth factor-\(\beta\) (TGF-\(\beta\); Online Figure VB&C), which was previously classified as a transcriptional regulator of miR-143/145.\(^2\)\(^2\) To address this issue more closely.

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we assessed the consequences of shear stress on the activity of the miR-143/145 promoter using endothelial cells isolated from miR-LacZ mice in which the miR-143/145–coding region was replaced with a LacZ reporter. Even though TGF-β increased galactosidase activity in these cells, there was no detectable effect of shear stress (Figure 7C). As a consequence of miR-143/145 deficiency, ACE was more highly expressed in endothelial cells from homozygous miR-LacZ mice than in cells from their heterozygous littermates (Online Figure VI).

How can shear stress affect miR-143/145 levels independently of KLF2 and promoter activation? After being transcribed, the primary miRNAs are processed into precursor miRNAs (pre-miRs), of approximately 70 nucleotides that are subsequently exported to the cytoplasm. Interestingly, despite the lack of effect on pri-miR levels and miR-143/145 promoter activity, fluid shear stress increased pre-miR- and mature miR-143/145 levels in endothelial cells (Figure 7A&B), indicating regulation by a post-translational mechanism. In agreement with these findings, AMPKα2 downregulation in cultured endothelial cells, had no effect on pri-miR levels (Figure 7D), but decreased pre-miR-143/145 under static conditions (Figure 7E). Similarly, pre-miR-143 and -145 levels were reduced in endothelial cells isolated from lungs of AMPKα2-/- mice (Figure 7F). AMPKα1 siRNA was without any effect on pri- or pre-miR levels. In heterozygous miR-LacZ mice, mature miR-143/145 levels were increased by shear stress (Figure 7G) and ACE expression was attenuated (Online Figure VI).

**AMPK, p53 and miR-143/145.**

Relatively little is known about the post-transcriptional regulation of miRNAs, but the tumor suppressor p53 was found to interact with the Drosha-p68 processing complex to enhance the post-transcriptional processing of several miRNAs, including miR-143/145. Given that p53 is a well characterized AMPK substrate, and was phosphorylated (on Ser15) by human recombinant AMPKα2 in vitro (Figure 8A), we determined whether or not the AMPK-dependent phosphorylation and activation of p53 were involved in the regulation of ACE expression by shear stress.

Shear stress induced the translocation of p53 to the detergent-insoluble fraction of endothelial cell lysates (Figure 8B), reflecting its activation. Moreover, the phosphorylation of p53 on Ser15 was increased (141±18% over basal levels) in response to shear stress, an effect that was prevented by the downregulation of the AMPKα2 but not the AMPKα1 subunit (Figure 8C). Similar effects were also observed in human aortic endothelial cells (Online Figure VIIA). Although p53 activation is associated with apoptotic processes, endothelial cells exposed to shear stress were protected against the induction of apoptosis, and we were unable to detect any increase in caspase 3 cleavage in endothelial cells exposed shear stress (Online Figure VIIB). Pre-treatment with siRNA against p53, which reduced p53 mRNA expression to 23±2% of its basal level (Figure 8D), decreased pre- and mature miR-143/145 levels, without affecting levels of the primary transcript (Online Figure VIIIC) and increased ACE protein expression (Figure 8E). Moreover, p53 siRNA prevented the shear stress-induced downregulation of ACE mRNA (Online Figure VIIID) and protein (Figure 8F).

To determine whether the AMPK-p53-miR-143/145-ACE pathway is of pathophysiological relevance we studied AMPK activation, miRNA levels and ACE expression in a mouse model of diabetes. Streptozotocin-induced diabetes in wild-type mice was associated with the increased phosphorylation of the AMPK substrates, p53 and acetyl-coenzyme A carboxylase (Figure 8G), and a significant reduction in ACE expression (Figure 8H). In the same animals levels of mature miR-143/145 (Figure 8I) and pre-miR-143/145 (Figure 8J) increased while pri-miR143/145 levels were unaffected (Figure 8J).

**DISCUSSION**

Increased ACE expression is generally associated with a higher risk of cardiovascular disease development and progression, since it contributes to the development of hypertension, myocardial infarction, stroke and atherosclerosis as well as to the renal pathophysiology associated with several diseases including diabetes. ACE inhibitors are widely used clinically and effectively lower blood
pressure and promote the reverse remodeling of the vasculature, but the molecular mechanisms that regulate ACE expression are unclear. The results of the present study indicate that endothelial ACE expression is downregulated in response to shear stress by a mechanism that involves activation of the AMPK, the subsequent phosphorylation and activation of p53 and the post-transcriptional upregulation of miR-143/145. Deletion experiments with siRNA revealed that the downregulation of ACE is dependent on the α2 catalytic subunit rather than AMPKα1. Indeed, AMPKα2-deficient mice demonstrated increased ACE expression in several vascular beds, coupled to an impaired vasodilator response to the ACE substrate, bradykinin.

The AMPK is a “metabolite-sensing kinase” that, when activated, initiates steps to conserve cellular energy. While there is a strong link between the activity of the AMPK and metabolic control in muscle cells, the activity of the kinase in endothelial cells can be regulated by stimuli that affect cellular ATP levels (e.g. hypoxia), as well as by stimuli that activate upstream kinases. The latter include fluid shear stress, Ca2+ -elevating agonists and hormones such as adiponectin (for review see ). At the outset of this study, circumstantial evidence suggested a link between fluid shear stress, the AMPK and ACE expression as 1) ACE levels are decreased by shear stress, 2) shear stress elicits activation of the AMPK, 3) LKB1 (an AMPK kinase) was linked to ACE expression in the mouse intestine, and 4) AMPK inhibition prevents the increase in ACE expression in macrophages induced by adipocyte-derived factors. However, direct molecular links between the different events were missing. There are two AMPK catalytic subunits, and although the AMPKα1 has been the major focus of studies in the vasculature, the α2 subunit is also expressed in endothelium and has been implicated in the regulation of endothelial cell signaling and function. The AMPKα2 subunit also seems to be the isoform most closely linked to cardiovascular disease development, especially when accompanied by metabolic disturbances as the AMPKα2 determines whole body insulin sensitivity. Our first step was to determine which of the α subunits contributed to the regulation of ACE expression and found that the downregulation of AMPKα2, but not AMPKα1, increased ACE expression in human endothelial cells and prevented its downregulation by shear stress in vitro. A similar upregulation of ACE was evident in a number of vessels in AMPKα2−/− mice, findings that went hand in hand with the markedly attenuated vasodilator response to bradykinin in the hindlimb vascular bed. Bradykinin is an ACE substrate and the fact that attenuated vasodilatation was observed in AMPKα2−/− as well as in animals lacking the AMPKα2 specifically in endothelial cells (AMPKα2 EC), and was abolished in animals receiving an ACE inhibitor, underlined the importance of altered endothelial AMPK activity on vascular responsiveness.

How could the AMPKα2 regulate the expression of ACE? The AMPKα2 is translocated to the nucleus after activation and is known to modulate the activity of several transcription factors and co-activators. One transcription factor of particular interest was KLF2 as it is induced by shear stress, and KLF2 overexpression was initially reported to decrease endothelial cell ACE expression. However, the latter study also reported that siRNA directed against KLF2 failed to prevent the shear-stress induced downregulation of ACE RNA levels, indicating that the mechanisms must be distinct. We also found that the downregulation of KLF2 had no effect on the shear stress-induced downregulation of ACE and were unable to link KLF2 induction with AMPKα2. Given that miR-143/145 was found to regulate ACE expression in vascular smooth muscle cells, we concentrated on establishing a link between AMPK-miR-143/145 and ACE in endothelial cells. We found that shear stress increased endothelial miR-143/145 levels and in turn strongly downregulated ACE expression, but only in cells expressing AMPKα2. The specificity of the response was demonstrated in experiments involving the overexpression of the appropriate pre-miR as well as by experiments using a mixture of antagomirs that abolished the shear stress-induced changes in ACE levels.

After transcription, primary miRNAs (pri-miRs) are processed into precursor miRNAs (pre-miRs) of approximately 70 nucleotides that are subsequently exported to the cytoplasm where they are cleaved by Dicer to generate the mature 22 nucleotide miRNA. Interestingly, we found that although shear stress increased the precursor and mature forms of miR-143/145 there was no detectable effect on primary miR-
143/145 levels in endothelial cells. We were able to rule out a shear stress-induced increase in miR-143/145 transcription, since shear stress failed to affect miR-143/145 promoter activity in endothelial cells isolated from homozygous or heterozygous miR-143/145-deficient (miR-LacZ) mice. Consistently, primary miR-143/145 levels did not increase in response to shear stress in cells from heterozygous miR-LacZ mice, whereas precursor and mature forms of miR-143/145 did. These observations indicated that the effects of shear stress on miR-143/145 expression are mediated by a post-translational mechanism.

To establish how AMPK could modulate post-transcriptional processing of miR-143/145 we focused on p53, as it is a well characterized AMPK substrate, activated by shear stress and involved in the regulation of miRNA transcription and/or processing. Indeed, p53 activation has been demonstrated to increase the levels of pre-miR-143/145 and mature miR-143/145, without affecting the primary miRNA transcripts. Such findings were highly reminiscent of our observations in endothelial cells exposed to shear stress and it was possible to demonstrate the involvement of p53 in the regulation of endothelial miR-143/145 levels by downregulating p53, which reduced precursor and mature miR-143/145 expression and prevented the shear stress-induced decrease in ACE expression. Downregulation of the AMPKα2 also decreased pre-miR but not pri-miR levels in cultured endothelial cells leading us to conclude that the effects observed are related to the AMPKα2-dependent activation of p53. Although p53 is frequently associated with DNA damage, the shear stress-induced and AMPKα2-dependent phosphorylation of p53 was on Ser15, a phosphorylation site previously linked to cell survival. Indeed, rather than promoting apoptosis low levels of p53 activity have been linked with the induction of anti-apoptotic and anti-oxidant genes as well as genes involved in DNA repair and the promotion of survival. It is not entirely clear how p53 affects post-transcriptional miR levels but it is likely to interfere with the Drosha processing step as co-immunoprecipitation demonstrated that p53 was associated with Drosha complex through its interaction with p68/p72.

The AMPKα2 is reported to protect against the development of atherosclerosis and alterations in AMPK activity have been linked with diabetes. In agreement with other reports, we found that AMPK activity was increased in mice (evidenced by an increase in the phosphorylation of p53 and the acetyl-coenzyme A carboxylase) 10 weeks after the induction of diabetes with streptozotocin. These changes were paralleled by an increase in precursor and mature miR-143/145 levels, with no apparent change in the primary transcripts, and by a decrease in ACE expression. While diabetes-associated metabolic stress activates the AMPK, the situation is likely to be different in the later stages or more severe forms of diabetes in which AMPK activity is thought to be impaired and associated with the accelerated development of cardiovascular disease. In such a state the activation of the AMPK may help to prevent the vascular complications associated with the metabolic syndrome, e.g. by modulation of the miRNA profile and the consequent reduction in ACE expression. So far, the spectrum of miRs that can be affected by changes in AMPKα2 activity is not known. However, the anti-diabetic and AMPK-activating drug, metformin, alters the tumor suppressor miRNA let-7a and the TGF-β-induced oncomiR miR-181a in breast cancer epithelial cells. Metformin also upregulates miR-26a, miR-192 and let-7c in human pancreatic cancer cells. Although the role of p53 activation was not been addressed in the latter studies, it is striking that miR let-7a, miR-26a and miR-192 are all regulated by p53 in different forms of cancer.

Taken together, the results of this study suggest that the downregulation of ACE in endothelial cells exposed to “arterial” levels of shear stress could be attributed to the activation of the AMPK, the phosphorylation and activation of p53 and an alteration in miR processing that leads to an increase in miR-143/145 levels. The latter miRs were recently characterized as “anti-atherosclerotic” since miR-145 levels are reduced in patients with coronary artery disease, and targeting miR-145 exerts a pronounced effect on neointima formation inasmuch as the introduction of miR-145 into balloon-injured arteries inhibits neointimal growth. It is tempting to speculate that this effect might also be related to altered ACE expression, since a small increase in ACE levels can markedly influence the magnitude of cuff-induced neointima thickening in mice. The post-translational regulation of endothelial cell miRNA expression by a metabolically regulated kinase provides a potential link between metabolic conditions...
such as diabetes, altered vascular homeostasis and gene expression, and may help to account for the ability of metformin therapy to decrease cardiovascular events.\textsuperscript{56,57}

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DISCLOSURES
None

REFERENCES


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FIGURE LEGENDS

Figure 1. Effect of fluid shear stress and AMPK activation on the regulation of endothelial ACE expression. Time-dependent effect of fluid shear stress on (A) ACE mRNA and (B) ACE protein expression in cultured human endothelial cells. eNOS expression was shown as a positive control for shear stress. (C&D) Effect of (C) AICAR (1 mmol/L, 6-12 hours), (D) metformin (10 mmol/L, 8-24 hours) and thiopental (Thio, 1 mmol/L, 12 hours) on ACE expression in endothelial cells. The bar graphs summarize data obtained in 15-20 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus solvent (Sol) or unrelated si-Scr treated cells under static conditions.

Figure 2. ACE expression in arteries and kidneys from wild-type, AMPKα1−/− and α2−/− mice. ACE expression levels were assessed by Western blotting of freshly isolated (A) aorta and (B) kidneys from wild-type (AMPKα1+/+, AMPKα2+/+), AMPKα1−/− or AMPKα2−/− mice. (C) ACE expression levels of the femoral artery isolated from wild-type (AMPKα1EC/+/) versus AMPKα2−/− mice. The bar graphs summarize ACE/β-actin expression levels (as percentage of expression levels in wild-type mice) obtained from 7-15 different animals; *P<0.05 versus the appropriate wild-type.

Figure 3. Bradykinin-induced hindlimb vasodilatation in AMPKα2−/− and AMPKα2EC mice. Bradykinin-induced vasodilatation (changes in perfusion pressure, ΔPP) of the murine hindlimb of (A&B) wild-type AMPKα2 (+/+) or knock-out mice (-/-) or (C&D) wild type (+/+) and AMPKα2EC (EC), each in the absence (A&C) or presence (B&D) of ramiprilat (30 µmol/L). Results were obtained from 4-10 different animals in each group; **P<0.01, ***P<0.001 versus +/+.

Figure 4. Regulation of ACE expression by miR-143 and miR-145. (A) Time-dependent effect of fluid shear stress on miR-143 and miR-145 levels. (B) Effect of Pre-miR-143 and Pre-miR-145 on ACE protein expression (72 hours) in cultured human endothelial cells. (C-E) Effect of antagomiR (AmiR)-143 and -145 on (C) miR-143 and miR145 expression levels, (D) ACE mRNA and (E) ACE protein expression levels under static conditions and in response to shear stress. The bar graphs summarize data obtained in 5-15 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus solvent (CTL) or cells under static conditions.

Figure 5. Regulation of miR-143 and miR-145 by AMPKα2. (A) Effect of a control siRNA (si-Scr) or siRNAs directed against AMPKα1 (si-α1) or AMPKα2 (si-α2) on miR-143 and miR145 expression in human endothelial cells after exposure to fluid shear stress. (B) miR-143/145 levels in aortae from AMPKα2−/− versus AMPKα2+ mice. The bar graphs summarize data obtained in 5-15 independent experiments; *P<0.05, ***P<0.001 versus si-Scr-treated cells under static conditions; #P<0.05 versus si-Scr treated cells exposed to shear stress.

Figure 6. Shear stress, KLF2 and AMPKα2. (A) Time dependent effect of shear stress on KLF2 mRNA expression. (B&C) Effect of shear stress on (B) KLF2 mRNA and (C) protein expression after incubation with either an control siRNA (si-Scr) or siRNA directed against AMPKα1 (si-α1) or AMPKα2 (si-α2). (D&E) Effect of shear stress on (D) AMPKα2 mRNA expression and (E) miR-143/145 levels in presence of control siRNA (si-Scr) or siRNA against KLF2 (si-KLF2). The graphs summarize data obtained in 6-10 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus static.

Figure 7. Effect of shear stress and AMPKα2 downregulation on primary, precursor and mature miR-143 and miR-145 levels. (A&B) Time dependent effect of shear stress on the expression of the primary (pri-), precursor (pre-) and mature forms of (A) miR-143 and (B) miR-145. (C) Effect of shear stress and TGF-β on the activity of the miR-143/145 promoter in lung endothelial cells isolated from heterozygous (+/-) or homozygous (-/-) miR-LacZ mice. (D&E) Effect of either a control siRNA (si-Scr), or siRNAs against AMPKα1 (si-α1) or AMPKα2 (si-α2) on (D) pri- and (E) pre-miR-143/145 levels. (F) Levels of pre-miR143/145 in endothelial cells isolated from lungs of AMPKα2−/− or AMPKα2−/− mice. (G)
Effect of shear stress and TGF-β on miR-143/145 levels in the endothelial cells from heterozygous miR-LacZ mice. Graphs summarize data obtained in 6-10 independent experiments; *$P$<0.05, **$P$<0.01 ***$P$<0.001 versus static or si-Scr.

**Figure 8. Role of p53 in the regulation of miR-143/145 and ACE in response to shear stress, AMPK activation and streptozotocin-induced diabetes.** (A) Phosphorylation of p53 on Ser15 by human recombinant AMPKα2. (B) Translocation of p53 from the detergent-soluble to the detergent-insoluble cell fraction in response to shear stress. (C) Consequences of AMPK downregulation on the phosphorylation of p53 on Ser15 in response to shear stress. (D&E) Effect of control siRNA or siRNA specific for p53 (si-p53) on p53 mRNA (D) and ACE protein levels (E). (F) Effect of shear stress on ACE protein expression after incubation with si-Scr or si-p53. (G-J) Consequences of streptozotocin (STZ)-induced diabetes on (G) phosphorylation of p53 and the acetyl-coenzyme A carboxylase (ACC), (H) ACE protein expression, (I) levels of mature miR-143/145 and (J) levels of Pre-and Pri-miR-143/145. The bar graphs summarize data obtained in 5-10 independent experiments; *$P$<0.05, **$P$<0.01 versus CTL/Static, ## $P$<0.01 versus shear, si-Scr.
Novelty and Significance

What Is Known?

- Increased expression of the angiotensin-converting enzyme (ACE) is associated with a higher risk of cardiovascular disease development and progression.
- Fluid shear stress/increased blood flow protects the vasculature and decreases endothelial cell ACE expression.
- In developing vascular smooth muscle cells, ACE is targeted by the mouse microRNA (miR)-143/145 cluster, which is required for acquisition of the contractile phenotype.

What New Information Does This Article Contribute?

- Activation of the $\alpha_2$-subunit of the AMP-activated kinase (AMPK) by hemodynamic (shear stress) or pharmacological stimulation (e.g. metformin) decreases endothelial ACE expression in vivo and in vitro.
- AMPK activation determines endothelial levels of miR-143/145 by a mechanism that involves p53 phosphorylation.
- AMPK$\alpha_2$ activation and p53 phosphorylation (on serine 15) do not increase miR-143/145 transcription or levels of the microRNA primary transcript, but increase the expression of mature microRNA by a post-transcriptional mechanism.

Metabolic diseases, like diabetes, are associated with dysregulation of the AMPK as well as the accelerated development of cardiovascular disease, which is also linked to increased ACE expression and activity. In endothelial cells, ACE expression can be attenuated by “athero-protective” levels of fluid shear stress, but the underlying molecular mechanisms involved remain unclear. Because the miR-143/145 cluster regulates ACE expression in developing vascular smooth muscle cells, we tested whether a link exists between fluid shear stress, AMPK activation, miR-143/145 levels and ACE in endothelial cells. We found that in response to fluid shear stress, the AMPK$\alpha_2$ subunit is activated and phosphorylates p53. The latter is then involved in increasing premature and mature miR-143/145 levels without influencing the activity of their promoter or levels of the primary transcripts. Given that the AMPK is also activated by an imbalance in the cellular AMP:ATP ratio, these results indicate a direct link between endothelial cell metabolism, post-transcriptional microRNA maturation and the expression of proteins implicated in cardiovascular disease development. Although the spectrum of microRNAs regulated by this mechanism are unknown, targeting AMPK$\alpha_2$, rather than a single specific miR, may be a novel approach for the treatment of cardiovascular disease.
Figure 2

A

\[ \text{ACE/\beta-actin} \]

\begin{align*}
\text{Aorta} & \\
+/+ & -/- & +/+ & -/- & \text{AMPK} \alpha 1 & \text{AMPK} \alpha 2
\end{align*}

B

\[ \text{ACE/\beta-actin} \]

\begin{align*}
\text{Kidney} & \\
+/+ & -/- & +/+ & -/- & \text{AMPK} \alpha 1 & \text{AMPK} \alpha 2
\end{align*}

C

\[ \text{ACE/\beta-actin} \]

\begin{align*}
\text{Femoral} & \\
+/+ & -/- & \alpha 2
\end{align*}
Figure 3

(A) Graph showing the effect of BK (in log mol/L) on ΔPP (mm Hg) for different genotypes (+/+ and -/-).

(B) Graph showing the effect of BK (in log mol/L) on ΔPP (mm Hg) for different genotypes (+/+ and -/-) with Ramiprilat.

(C) Graph showing the effect of BK (in log mol/L) on ΔPP (mm Hg) for different genotypes (+/+ and -/-) with ΔEC.

(D) Graph showing the effect of BK (in log mol/L) on ΔPP (mm Hg) for different genotypes (+/+ and -/-) with Ramiprilat and ΔEC.
The AMP-Activated Protein Kinase Regulates Endothelial Cell Angiotensin-Converting Enzyme Expression via p53 and the Post-Transcriptional Regulation of microRNA-143/145
Karin Kohlstedt, Caroline Trouvain, Thomas Boettger, Lei Shi, Beate Fisslthaler and Ingrid Fleming

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Supplemental Material

Expanded Methods Section

Materials

Cell culture media were purchased from Gibco (Invitrogen, Karlsruhe, Germany). The monoclonal antibody against human ACE was provided by Dr. Peter Bünning (Aventis, Frankfurt, Germany), the antibody against murine ACE was from R&D Systems GmbH (Wiesbaden-Nordenstadt, Germany). The AMPK antibodies and the antibody against phospho-p53 (Ser15) and acetyl-coenzyme A carboxylase (ACC) were from Cell Signaling (MA, USA), the antibody against pACC was from Merck Millipore (Merck KGaA, Darmstadt, Germany), the antibody directed against β-actin was from New England Biolabs (Frankfurt, Germany), the antibody recognizing p53 and PECAM-1 were from Santa Cruz Biotechnology (Heidelberg, Germany), the antibody against eNOS and was from Transduction Laboratories (Heidelberg, Germany), and the anti-KLF2 antibody was from Novus Biologicals (Littleton, CO). 5-Aminoimidazole-4-carboxamide-1-β-d-ribofuranoside (AICAR) and all other substances were from Sigma-Aldrich (Deisenhofen, Germany).

Cell Culture

Human umbilical vein endothelial cells were isolated and cultured as described previously.1 Human coronary artery endothelial cells were obtained from CellSystems Biotechnologie Vertrieb GmbH (Troisdorf, Germany) and human aortic endothelial cells from Lonza (Basel, Switzerland). Murine lung endothelial cells were isolated and cultured as described.2 Confluent cultures of endothelial cells were washed twice in culture medium containing 2% fetal calf serum and were either maintained under static conditions or exposed to shear stress (12 dyn/cm²) in a temperature-controlled cone-plate viscosimeter, as described.3 Unless otherwise stated in the figure legends, shear stress was applied for 48 hours.

Animals

Genetically modified mice lacking either the AMPKα1 (Sv129 background) or the AMPKα2 subunit (C57BL/6 background), their respective wild-type littermates and floxed AMPKα2 mice (C57BL/6 background) were generated as described4 by Benoit Viollet (INSERM, U1016, Paris, France) and bred at the Goethe University Hospital animal facility. Floxed AMPKα2 mice were crossed with animals expressing the Cre-deleter under the control of the VE-cadherin promoter to generate mice lacking the α2 subunit specifically in endothelial cells (AMPKα2EC). Mice in which the miR-143/145–coding genomic region was replaced with a lacZ reporter (miR-LacZ) were generated as described,5 and bred at the animal facility of the Max-Planck-Institut für Herz- und Lungenforschung, Bad Nauheim. Experiments conformed with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23). All experiments were approved by the governmental authorities (Regierungspräsidium Darmstadt: F28/06, F28/23 and F28/38). Age-, gender-, and strain-matched animals (usually littermates) were used throughout. Animals were euthanized, under anesthesia (isoflurane 2.5%), by a transverse cut through the large abdominal vessels, the arteries and organs rinsed with cold phosphate-buffered saline and snap-frozen. Experimental diabetes was induced in C57BL/6 mice (Charles River, Sulzfeld, Germany) by a single intraperitoneal injection of streptozotocin (STZ; 150 mg kg⁻¹ body weight) 8-10 weeks prior to experiments.

LacZ Reporter Gene Assay

The activity of the miR-143/145 promoter (fused to LacZ) was determined by measuring β-galactosidase activity (Tropix, Bedford, Mass) as described.7
Perfused Hindlimb

Bradykinin- and acetylcholine-induced vasodilator responses of the mouse hindlimb were measured as changes in perfusion pressure as described\(^8\) with slight modifications. Briefly, mice were euthanized, under anaesthesia (isoflurane 2.5%), by a transverse cut through the large abdominal vessels, a before a Teflon i.v. catheter (0.67 mm, 24 gauge, brand Durflo; Terumo, Leuven, Belgium) was introduced into the aorta, advanced to one of the iliac arteries and tied. Venous congestion was avoided by cutting the inferior caval vein. The hindlimb was permanently perfused with modified filtered Krebs–Henseleit solution (mmol/L: NaCl 118.3; KCl 4.7; CaCl\(_2\) 1.8, MgSO\(_4\) 1.2, KH\(_2\)PO\(_4\) 1.2, NaHCO\(_3\) 25, EDTA 0.026, glucose 11.1; pH 7.40 aerated with 95% O\(_2\)/5% CO\(_2\)) using a roller pump, whereas a pressure transducer and a compliance chamber were connected to a side port of the system. Flow rate was gradually increased up to a corresponding perfusion pressure of around 100 mmHg, where a considerable amount of spontaneous myogenic tone is present and further preconstruction can be avoided. However, when a substance produced a pronounced change in resistance, the flow rate was adjusted to reduce perfusion pressure or phenylephrine was used to increase pressure. When a stable pressure plateau was reached, endothelium-dependent (acetylcholine, bradykinin) or -independent vasodilators (sodium nitroprusside) were applied in increasing concentrations as a bolus (100 μl) in glucose solution (50 g/L). To avoid artifacts attributable to the tachyphylaxis/desensitization of the endothelial B\(_2\) kinin receptor, studies in the presence or absence of the ACE inhibitor ramiprilat (30 μmol/L) were performed in different mice. Agonist-induced vasodilatations were calculated as changes in perfusion pressure or normalized to the effects elicited by SNP.

Immunoblotting

Cells or organ homogenates were lysed in nonidet lysis buffer left on ice for 10 minutes and centrifuged at 10000g for 10 minutes. SDS-PAGE was performed and proteins were detected using their respective antibodies, as described.\(^9\)

Real-time qPCR

Total RNA from cells or organ homogenates was extracted using TriReagent (Sigma). Equal amounts of RNA were reverse-transcribed using SuperScript III reverse transcriptase (Invitrogen, Karlsruhe, Germany). Quantitative real-time PCR was performed using the Mx4000 Multiplex Quantitative PCR System (Stratagene, La Jolla, CA) and SYBR Green Master Mix (Thermo Fisher Scientific GmbH, Karlsruhe, Germany) for detection of the products. Primer sequences previously designed and tested elsewhere\(^10,11\) were used to detect pri- and pre-miRs and effectiveness was determined following stimulation with TGF-β in human coronary artery smooth muscle cells. Primers were from Biospring (Frankfurt, Germany) and the sequences as follows:

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACE</td>
<td>5’-GCCACCTGGGAACCTACAACACCAAC-3’</td>
<td>5’-GCAACTGGTTCACATCAAACCTCC-3’</td>
</tr>
<tr>
<td>AMPKα1</td>
<td>5’-CWGWWYGWGRCTGTAGTCCCTTTGGAAGGT-3’</td>
<td>5’-TCAAYCCGACAGAGGCTCCGAGCC-3’</td>
</tr>
<tr>
<td>AMPKα2</td>
<td>5’-ARYTGGRRWTGAATGGAAGGT-3’</td>
<td>5’-CTGGRGGTGGTGGARGAACCAGA-3’</td>
</tr>
<tr>
<td>p53</td>
<td>5’-CTGCCCTCAACAGATGGTTTCTG-3’</td>
<td>5’-CTATCTGAGCAGCCTCATGG -3’</td>
</tr>
</tbody>
</table>
KLF2
forward: 5´- ATTCCAGTGCCATCTGTGCGAT-3'
reverse: 5´-CCCAGGTCTCTGCTGGTCCAATAA-3'

pri-miR143
forward: 5´-CAAGGGTTTGCTGTGCTCAGTCAA-3'
reverse: 5´-TGTCGCTCAGTCTGCTCAGTCAA-3'

pre-miR143
forward: 5´-TGAGGTGCCTGTGCATC-3'
reverse: 5´-GCTACAGTGCTTTCATCTCAGACTC-3'

pri-miR145
forward: 5´-TGAGTTTCCTCCTTCCTTCA-3'
reverse: 5´-TTGAACCCTCATCCTGTGAGCC-3'

pre-miR145
forward: 5´-GGATGCAGAAGAGAACTCCA-3'
reverse: 5´-CCTCATCCTGTGAGCCAG-3'

To quantify mature miR-143 and 145, RNA was isolated using a miRNeasy Mini Kit (QIAGEN GmbH, Hilden, Germany). Specific stem-loop primers were used for reverse transcription and specific primers and FAM-labeled oligonucleotides (universal probe library probe #13, Roche Diagnostics, Mannheim, Germany) were used for the subsequent real-time PCR12 (miR-145 UPL RT primer: 5`-GTCTCTGCGCTGTGCTGCTGAGGATTCGCAAGGCAGAGACTGAGGAT-3`, miR-145 forward: 5`-GTGGCGGTCCCTTTCAGGAA-3`; miR-143 UPL RT primer: 5`-GTCTCTGCGCTGTGCTGCTGAGGATTCGCAAGGCAGAGACTGAGGAT-3`, miR-143 forward: 5´-TGAGATGAAGCACTGT-3'). The relative expression levels of the mature miRNAs and other genes were calculated using the 2^-ΔΔCt method with 18S mRNA (forward: 5'-CTTTGTCGCTCGCTCCTC-3' and reverse: 5'-CTGACCAGGGTTGTTTGTG-3') as a reference.

**Plasmid, Transfection and RNA Interference**

Human umbilical vein endothelial cells were grown to 80–90% confluence and then transfected with either 100 nmol/L Pre-miRNA (Pre-miRTM miRNA Precursor from Ambion, Life Technologies GmbH, Darmstadt, Germany), 100 nmol/L antagomiR (Anti-miRTM miRNA Inhibitor from Ambion) or 30-50 nmol/L specific small interfering RNAs (siRNAs) using the LipofectaminTM RNAiMax (Invitrogen) according to the manual instructions. Transfection was performed 24 hours before the application of shear stress for additional 48 hours. siRNAs were synthesized by Eurogentec (Eurogentec S.A., Seraing, BELGIUM) to the following human target sequences: AMPKα1 (5´-CCAAGUGGAUAGUAGAACU-3´), AMPKα2 (5´-CCAUCUUCGUGCAGAAGAA-3´), KLF2 (5´-AACCUGACUGUAUUAUUTT-3´). For p53 suppression, a mixture of two different siRNAs (Santa Cruz Biotechnology and QIAGEN) was used. A pre-miRNA negative control (Ambion) or a nonrelated, scrambled siRNA without any other match in the human genomic sequence was used as a control.

**In vitro phosphorylation of p53**

Human recombinant p53 (100 ng per sample; Enzo Life Science, Lörrach, Germany) was incubated with human recombinant AMPKα2 (150 μg per sample, Calbiochem from Merck KGaA, Darmstadt, Germany) were incubated in the presence of ATP for 30 minutes at 37°C as described.13 Samples without p53 or AMPKα2 addition were used as controls. The kinase reaction was stopped and phosphorylation assessed by SDS–PAGE.
Statistical Analysis

Data are expressed as mean ± SEM. Statistical evaluation was performed on the absolute values or on log transformed data with Student’s t test for unpaired data, one-way ANOVA followed by a Bonferroni t test or ANOVA for repeated measures where appropriate. Values of P<0.05 were considered statistically significant.

References

1 Busse R, Lamontagne D. Endothelium-derived bradykinin is responsible for the increase in calcium produced by angiotensin-converting enzyme inhibitors in human endothelial cells. *Naunyn Schmiedebergs Arch Pharmacol* 1991;344:126-129.


Online Figure I. Effect of shear stress on ACE expression in human coronary endothelial cells and effect of pharmacological AMPK activation in human umbilical vein endothelial cells. (A) Time-dependent effect of fluid shear stress (0-24 hours) on ACE mRNA expression in cultured human coronary artery endothelial cells. (B) Effect of AICAR (1 mmol/L, 12 hours), thiopental (1 mmol/L, 12 hours) and metformin (10 mmol/L, 24 hours) on ACE mRNA expression in endothelial cells. The bar graphs summarize data obtained in 5-9 independent experiments; *P<0.05, **P<0.01, ***P<0.001 versus the appropriate cells under static conditions (Static, Sol).

Online Figure II. ACE expression in aortic arch from wild-type, AMPKα1−/− and α2−/− mice and effect of shear stress on ACE expression in endothelial cells isolated from AMPKα1−/− and AMPKα2−/− mice. (A) Western blots and bar graphs showing ACE expression levels in freshly isolated aortic arch from wild-type (AMPKα1+/+, AMPKα2+/+), AMPKα1−/− or AMPKα2−/− mice. (B) Effect of fluid shear stress on ACE mRNA expression in cultured mouse endothelial cells isolated from lungs of AMPKα2−/− and AMPKα2+/+ mice and their respective littermates. The bar graphs summarize data obtained from 7-9 different animals/independent experiments; *P<0.05, versus the appropriate wild-type.
Online Figure III. Hindlimb vasodilatation in AMPKα2−/−, AMPKα1−/− and AMPKα2ΔEC mice. (A) Acetylcholine (ACh)-induced hindlimb vasodilatation in AMPKα2−/− and AMPKα2+/+ mice. (B) Bradykinin (BK)- and (C) ACh-induced hindlimb vasodilatation in AMPKα1+/+ and AMPKα1−/− mice. (D) ACh-induced hindlimb vasodilatation in endothelial cell specific AMPKα2 deficient mice (α2ΔEC) and respective wild-type mice. Results were calculated as changes in perfusion pressure (ΔPP) and the bar graphs summarize data obtained from 5-8 different animals.
Online Figure IV. Regulation ACE by Pre-miR-143 and 145 and of miR-143 and miR-145 by AMPK. (A) Effect of a control miR (CTL), Pre-miR-143 and Pre-miR-145 on ACE mRNA (48-72 hours) expression in cultured human endothelial cells. (B&C) Effect of either an unrelated siRNA (Scr), siRNA specific for AMPKα2 (si-α2) or AMPKα1 (si-α1) or the antagomiR (AmiR)-143 and -145 on ACE expression in human aortic endothelial cells under static conditions (B&C) and in response to shear stress (C). (D) Time-dependent effect of an unrelated siRNA (si-Scr), AMPKα1 siRNA (si-α1) or AMPKα2 siRNA (si-α2) on miR-143 and miR-145 expression in human umbilical vein endothelial cells. The bar graphs summarize data obtained in 3-10 independent experiments; **P<0.01, ***P<0.001 versus control miR (CTL) or si-Scr treated cells.
Online Figure V. Effectiveness of KLF2 siRNA in human endothelial cells and validation of primers against primary (pri) and precursor (pre) forms of miR-143 and miR-145. (A) Effect of transfection with an unrelated siRNA (si-Scr) or siRNA specific for KLF2 (si-KLF2) on KLF2 mRNA expression under static conditions and in response to shear stress. (B&C) Effect of TGF-β (1 ng/mL, 24 hours) on primary (Pri-) and premature (Pre-) levels of (B) miR-143 (C) and miR-145. The bar graphs summarize data obtained using 3-6 different cell batches; *P<0.05, **P<0.01 versus si-Scr, static or solvent (CTL); #P<0.05 versus si-KLF2, static.

Online Figure VI. Effect of shear stress on ACE levels in miR-143/145 deficient endothelial cells. Effect of shear stress (0-48 hours) on ACE mRNA levels in mouse lung endothelial cells isolated from heterozygous (miR-LacZ+/−) and homozygous (miR-LacZ−/−) mice in which the miR-143/145–coding region was replaced with a LacZ reporter. The graphs summarize data obtained in 7-9 independent experiments; **P<0.01, ***P<0.001 versus cells under static conditions.
Online Figure VII. AMPKα2, p53, miR-145/145 and ACE. (A) Effect of either an unrelated siRNA (Scr), siRNA specific for AMPKα2 (si-α2) or AMPKα1 (si-α1) or the antagomiR (AmiR)-143 and -145 on p53 (Ser15) phosphorylation in human aortic endothelial cells under static conditions. (B) Representative Western blot showing the effect of fluid shear stress (0-72 hours) on Caspase 3 cleavage in HUVEC. Stimulation with Staurosporin (1 μM, 3 hours) was used as a positive control (+ve CTL) for Caspase cleavage. (C) Levels of primary (Pri), precursor (Pre) and mature miR143 and miR145 after pretreatment with an unrelated scrambled siRNA (Scr) or p53 siRNA (si-p53) for 12 to 24 hours (D) ACE mRNA expression in response to shear stress after pre-treatment with si-Scr or si-p53. The bar graphs summarize data obtained using 3-6 different experiments. *P<0.05, **P<0.01 versus respective control, ##P<0.01 versus Shear, si-Scr.