Expression of microRNAs is essential for arterial myogenic tone and pressure-induced activation of the PI3-kinase/Akt pathway

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Received 10 July 2013; revised 20 September 2013; accepted 4 October 2013; online publish-ahead-of-print 13 November 2013

Time for primary review: 25 days

Aims

The myogenic response is the intrinsic ability of small arteries to constrict in response to increased intraluminal pressure. Although microRNAs have been shown to play a role in vascular smooth muscle function, their importance in the regulation of the myogenic response is not known. In this study, we investigate the role of microRNAs in the regulation of myogenic tone by using smooth muscle-specific and tamoxifen-inducible deletion of the endonuclease Dicer in mice.

Methods and results

In order to avoid effects of Dicer deletion on smooth muscle differentiation and growth, we used an early time point (5 weeks) after the tamoxifen-induction of Dicer knockout (KO). At this time point, we found that myogenic tone was completely absent in the mesenteric arteries of Dicer KO mice. This was associated with a reduced pressure-induced Akt-phosphorylation, possibly via increased phosphatase and tensin homologue (PTEN) expression, which was found to be a target of miR-26a. Furthermore, loss of myogenic tone was associated with a decreased depolarization-induced calcium influx, and was restored by the L-type channel agonist Bay K 8644 or by transient stimulation with angiotensin II (Ang II). The effect of Ang II was dependent on AT1-receptors and activation of the PI3-kinase/Akt pathway.

Conclusion

In this study we have identified novel mechanisms that regulate myogenic tone in resistance arteries, which involves microRNA-dependent control of PI3-kinase/Akt signalling and L-type calcium influx. Furthermore, we have demonstrated that transient stimulation by Ang II can have long-lasting effects by potentiating myogenic tone.

Keywords

Calcium † microRNA † Myogenic tone † PI 3-kinase † Angiotensin

1. Introduction

Despite substantial variations in perfusion pressure, organs are able to maintain local control of blood flow and capillary blood pressure as a result of precise regulation of the diameter of small resistance arteries. The diameter of these arteries is regulated by perfusion pressure and shear stress as well as by multiple local factors such as metabolites and gases. The contractile response of resistance arteries following an increase in intraluminal pressure is referred to as the Bayliss effect or myogenic response and the resulting myogenic tone contributes significantly to the regulation of arterial diameter in many vascular beds, including the mesenteric, cerebral, coronary, skeletal muscle, and renal circulation. This is an important mechanism to avoid pressure increases in capillaries, which may result in fluid leakage and organ damage. Dysregulation of the myogenic response can cause local ischaemia or vasogenic oedema while a general increase in myogenic activity results in increased peripheral resistance that could contribute to an elevated systemic blood pressure.

The signalling mechanisms underlying the myogenic response are quite complex and involve stretch-sensitive integrins, receptors, and ion channels. Stretch-induced activation of these mechanosensors leads to a depolarization and calcium influx via voltage-gated calcium channels. Furthermore, when intracellular calcium levels decline, the myogenic response is maintained by calcium sensitization and actin polymerization via the Rho and protein kinase C signalling pathways. In addition to these mechanisms, the PI3-kinase/Akt signalling pathway was recently demonstrated to be involved in regulation of blood pressure and myogenic tone via membrane translocation and activation of L-type calcium channels.
In recent years, microRNAs (miRNAs) have been identified as important regulators of vascular contractility and essential components of stretch-induced contractile smooth muscle cell differentiation in the vascular wall. However, the role of miRNAs in the myogenic response in pressurized arteries has not been investigated previously. MicroRNAs are known to be dysregulated in several cardiovascular disease states and may thus represent novel targets for therapeutic intervention. It is therefore of considerable importance to identify their role in physiological processes such as the regulation of myogenic tone and vascular resistance.

In the present study, we have used small mesenteric arteries from a smooth muscle specific and tamoxifen-inducible knockout (KO) of the miRNA-processing endonuclease Dicer in order to identify the global role of miRNAs in the myogenic response. We found that miRNAs are essential for the myogenic response and that loss of this response in Dicer KO mesenteric arteries is mediated, at least in part, by a reduction of pressure-induced activation of the PI3K/Akt pathway and reduced L-type calcium-channel activity.

2. Methods
A detailed description of all methods can be found in Supplementary material online, Methods.

2.1 Animals
Intraperitoneal tamoxifen and vehicle injections of SMMHC-CreERT2/Dicerfl/fl10,11 were performed for five consecutive days at the age of 4 weeks as described previously. Tamoxifen- and vehicle-treated mice are herein referred to as Dicer KO and WT, respectively. Unless stated otherwise, all experiments were performed at 5 weeks post-tamoxifen treatment. At this time point, the mice were euthanized by cervical dislocation. In agreement with previous reports,11 we did not find any significant effect of tamoxifen injection alone on the contractile function of Cre-negative mesenteric arteries (data not shown). All experiments were approved by the Malmö/Lund animal ethics committee (M167-09 and M213-12). This investigation conforms to Directive 2010/63/EU of the European Parliament.

2.2 Pressure myography
Pressure myograph experiments were performed on second-order mesenteric arteries as described previously. Distensibility was analysed by comparing the passive vessel diameter at 45–120 mmHg to the passive vessel diameter at 20 mmHg.

2.3 Pressurization of small mesenteric arteries
Mesenteric arterial trees from WT and Dicer KO mice were divided in two equal halves and mounted on glass cannulas in a pressure myograph chamber (Living Systems Instrumentation). All the branches on the pressurized half were ligated and the vessels were equilibrated for 3 h in HEPES-buffered saline solution in 0 mmHg. Pressure was then either increased to 95 mmHg for 10 min or maintained at 0 mmHg. Vessels were snap-frozen in acetone–TCA (10%)–DTT (10 mM) on dry ice.

2.4 Wire myography
Wire myograph experiments for the evaluation of contractile force in mesenteric arteries were performed as previously described.13 Distensibility was analysed by comparing the passive vessel diameter at 45–120 mmHg to the passive vessel diameter at 20 mmHg.

2.5 Calcium measurement
Second-order mesenteric arteries were mounted in a heated myograph chamber (Living Systems Instrumentation) and incubated with the calcium indicator Fluo-4 AM (10 µM; Invitrogen) at room temperature for 40 min at 45 mmHg. The chamber was then placed on the stage of an inverted Zeiss Axiovert 200M microscope, heated to 37°C, and allowed to accommodate for another 40 min at 45 mmHg to allow hydrolysis of the Fluo-4 AM. The Fluo-4 fluorescence signal in response to 60 mM KCl was monitored by a Zeiss Pascal LSM 5 confocal system with a Zeiss Plan-Neofluar 40× (NA. 1.3) oil immersion lens and normalized to basal fluorescence.

2.6 Cell culture and transfection
Vascular smooth muscle cells were isolated from mouse aorta by enzymatic digestion as described previously. Cells were transfected with commercially available synthetic microRNA mimics for miR-26a or negative control (MIS-SION® microRNA, Sigma-Aldrich) using Oligofectamine transfection reagent (Invitrogen).

2.7 Quantitative real-time PCR (qRT-PCR)
Total RNA isolation and qPCR analysis was performed using Qiagen miRNeasy mini kit and Qiagen primers as described previously.18 Array analysis of miRNAs was performed using mouse cardiovascular disease miRNA PCR array (Qiagen).

2.8 Protein extraction and western blotting
Protein extraction of preparations frozen in liquid nitrogen and western blotting were performed as described previously. For analysis of LC2 phosphorylation, preparations frozen in acetone-dry ice were thawed to room temperature, repeatedly washed in aceton–DTT (10 mM), freeze-dried and extracted in SDS-sample buffer overnight at room temperature.

2.9 Statistics
Values are presented as mean ± SEM. P-values were calculated by Student’s t-test for single comparisons, by one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc testing for multiple comparisons or by two-way ANOVA for comparison of pressure–diameter relationships. Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, Inc.), P < 0.05 was considered statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001.

3. Results
3.1 Loss of myogenic tone represents an early phenotype in the inducible and smooth muscle-specific Dicer KO mice
Intraperitoneal tamoxifen injections of inducible Dicer KO mice resulted in a time-dependent decrease of Dicer and miR-145 expression in vascular tissues (Supplementary material online, Figure S1A–C). To further evaluate the effect of Dicer KO in smooth muscle, we performed an array analysis of 48 different miRNAs in Dicer KO aorta at 2–4 weeks post tamoxifen treatment. This analysis revealed that, on average, ~40% of the microRNA expression was still present in the smooth muscle at 2 and 3 weeks post tamoxifen treatment (Figure 1A). At 4 weeks post tamoxifen treatment, the average miRNA expression was reduced to 14%, suggesting that it is primarily during the last week of the 5-week period following Dicer deletion that loss of miRNAs affects smooth muscle protein expression and function. We thus consider the 5-week time point to represent early effects of miRNA down-regulation in smooth muscle.

In earlier studies, we have shown that smooth muscle-specific deletion of Dicer results in significant reduction of miRNAs in the aorta, portal vein, and urinary bladder. To confirm the effect of Dicer KO at 5 weeks post tamoxifen treatment in small mesenteric arteries, we performed qPCR analysis of selected, highly expressed, miRNAs. As shown in Figure 1B, deletion of Dicer in smooth muscle caused a significant reduction of these miRNAs. While the miR-143/145 cluster is...
relative specific for smooth muscle, miR-26a may also be expressed in other cell types present in the mesenteric artery.

The myogenic response was studied in Dicer KO and WT mesenteric arteries using pressure myographs. In this setup, mesenteric arteries were cannulated on glass pipettes and subjected to stepwise increases in intraluminal pressure. In WT vessels, this resulted in a myogenic response and reduced diameter at pressure levels in intraluminal pressure. In WT and Dicer KO vessels, suggesting that altered smooth muscle mass is not a contributing factor to the lack of myogenic tone in Dicer KO arteries.

Over time, loss of Dicer-dependent miRNAs has a negative effect on the expression of smooth muscle differentiation markers. However, the effect of Dicer KO on smooth muscle differentiation may vary between tissue types and time after tamoxifen treatment. Quantitative PCR (qPCR) of mesenteric arteries at 5 weeks post tamoxifen treatment showed that some contractile markers were reduced at the mRNA level (Figure 2D). However, by western blot analysis, we found only a modest reduction of one contractile marker (tropomyosin-1) at the protein level (Figure 2E). Other markers such as α-actin, desmin, calponin, and SM22α were not affected by loss of miRNAs. Thus, reduced contractile differentiation is likely not the cause of the abolished myogenic tone in mesenteric arteries within 5 weeks of Dicer deletion.

3.2 The loss of myogenic tone in Dicer KO arteries is not due to vascular remodelling or reduced expression of contractile proteins

To evaluate whether deletion of Dicer results in vascular remodelling at 5 weeks post tamoxifen, mesenteric arteries were perfusion fixed under 95 mmHg pressure in calcium-free conditions and then sectioned and stained with haematoxylin and eosin (Figure 2A). Analysis of the vessel morphometry revealed no significant difference in lumen diameter (Figure 2B) or wall thickness (Figure 2C) between WT and Dicer KO vessels, suggesting that altered smooth muscle mass is not a contributing factor to the lack of myogenic tone in Dicer KO arteries.

We have previously demonstrated that long-term deletion of Dicer can affect the polymerization of actin filaments and subsequently the contractile machinery of the smooth muscle cells.7 To test the integrity of the contractile filament system, we analysed calcium-independent contractile function in WT and Dicer KO mesenteric arteries using Calyculin A to inhibit myosin light-chain phosphatase activity in nominally calcium-free conditions. Calyculin A-induced contractility was unchanged in Dicer KO mesenteric arteries, suggesting that loss of myogenic tone is not due to altered function of the contractile machinery in Dicer KO vessels at this time point (Figure 3A). However, the contractile response to depolarization by 60 mM KCl was significantly reduced in Dicer KO arteries (Figure 3B), indicating that voltage-gated calcium influx may be affected. To test this possibility, we loaded WT and Dicer KO mesenteric arteries with the calcium indicator fluo-4 and evaluated the relative calcium increase in response to KCl in pressurized arteries. In accordance with the reduced contractile response to KCl, we found a significant decrease in fluo-4 signal intensity after KCl stimulation in Dicer KO arteries (Figure 3C). Furthermore, pressure-induced phosphorylation of myosin light chains, which is known to be calcium dependent,15 was completely abolished in Dicer KO mesenteric arteries (Figure 3D).

To investigate whether a decrease in calcium channels is involved in the reduced calcium influx and contractile response to KCl, we performed qPCR and western blot analysis of calcium-channel expression in WT and Dicer KO mesenteric arteries. In contrast to the effect in portal vein at later time points,8 the expression of the L-type calcium-channel pore-forming subunit (Cacna1c/Ca,1,2)
was unchanged at 5 weeks post tamoxifen in Dicer KO mesenteric arteries (Figure 3E and F). Furthermore, the expression of the auxiliary \( \alpha_2 \delta_1 \)-subunit of the L-type calcium channel, which is known to play a role in the myogenic response, was unchanged (Figure 3G). These results suggest that it may be the activity rather than the expression level of L-type calcium channels that is perturbed in Dicer KO arteries at this time point. We thus treated the Dicer KO mesenteric arteries with an L-type calcium-channel activator (Bay K 8644) and found that this restored pressure-induced myogenic tone in Dicer KO vessels without affecting contractile tone at lower pressure levels (20–45 mmHg) (Figure 3H). This result suggests that a reduced pressure-induced L-type calcium-channel activity is the main cause of the abolished myogenic tone in Dicer KO arteries.

### 3.4 Pressure-induced activation of the PI3K/Akt pathway is abolished in Dicer KO mesenteric arteries and essential for myogenic tone

In vascular smooth muscle, inhibition or deletion of PI3K has been shown to result in loss of myogenic tone and reduced blood pressure via inhibition of L-type calcium influx. To investigate whether the reduced calcium-channel activity in Dicer KO mice is associated with a decrease in Akt activation, we performed western blot analysis on pressurized mesenteric arteries using a phospho-specific (Ser473) Akt antibody. As shown in Figure 4A, pressurization caused a significant increase in Akt-phosphorylation in WT but not in Dicer KO arteries. Furthermore, an essential role of the PI3K/Akt pathway for myogenic tone was confirmed using the PI3K inhibitor LY-294002 (Figure 4B).

Earlier studies have shown that miRNAs are potent regulators of Akt by targeting PTEN, which antagonizes the PI3K-mediated phosphorylation of Akt. We thus tested the expression of PTEN in control and Dicer KO mesenteric arteries and found that deletion of miRNAs significantly increased the level of PTEN (Figure 4C). By using the miRNA target prediction database, TargetScan, we found that multiple miRNAs are predicted to target PTEN. However, miR-26a is one of few with as many as three conserved target sites in the PTEN 3′ UTR. Furthermore, miR-26a is highly expressed in WT smooth muscle and rapidly down-regulated in Dicer KO by ~70% already at 2 weeks post tamoxifen treatment (data not shown). Using a miR-26a mimic, we over-expressed this miRNA in cultured vascular smooth muscle cells isolated from Dicer KO mouse aorta. In accordance with the bioinformatic prediction, we found that the protein expression of PTEN was significantly reduced by miR-26a overexpression (Figure 4D).

### 3.5 Angiotensin II-mediated activation of PI3K/Akt pathway restores myogenic tone in Dicer KO mesenteric arteries

Previous reports have demonstrated that angiotensin II (Ang II) can promote activation of L-type calcium channels via the PI3K/Akt pathway. To investigate whether Ang II-induced contraction was
affected by Dicer KO in smooth muscle, we stimulated mesenteric arteries mounted in a pressure myograph with 100 nM Ang II. In accordance with a reduced L-type calcium-channel activity in Dicer KO arteries, Ang II-induced contraction was significantly reduced in Dicer KO mesenteric arteries (Figure 5A). The remaining Ang II-induced contractile response in Dicer KO was further inhibited by LY-294002, suggesting that Ang II (in contrast to elevated pressure) is able to activate the PI3K/Akt pathway in Dicer KO arteries, despite an increased PTEN expression. This was also confirmed by analysis of Ang II-induced Akt phosphorylation in WT and Dicer KO mesenteric arteries (Figure 5B).

The effect of PI3K on L-type channel activation is partly mediated via recruitment of the α1c-subunit of the channel to the plasma membrane,22 which suggests that the effect of Ang II-induced PI3K activation should be more sustained than the transient contraction observed upon acute stimulation.5,23 To examine whether Ang II-induced activation of L-type calcium channels could potentiate myogenic tone in Dicer KO mesenteric arteries, we transiently stimulated vessels with 100 nM Ang II under low pressure and then evaluated the myogenic response. Although the vessels were only transiently exposed to Ang II, myogenic tone was completely restored in Dicer KO mesenteric arteries after Ang II stimulation (Figure 5C and D). The effect was inhibited by the AT1-receptor antagonist candesartan and by transient inhibition of PI3K using LY-294002 during the Ang II stimulation (Figure 5D).

4. Discussion

Myogenic tone, which is known to be important for auto-regulation of blood flow and blood pressure, is initiated by stretch-induced depolarization, followed by activation of voltage dependent L-type calcium channels.1,2 However, despite intense investigation in this field, the mechanisms underlying the regulation of myogenic tone are not fully understood. Several factors including stretch-sensitive activation of ion channels, ion transporters, and signalling pathways have been suggested to be involved and it is likely that a combination of these factors regulate myogenic tone.

Herein, we have identified an additional mechanism in the control of myogenic tone involving miRNAs. Over time, deletion of Dicer-dependent miRNAs in smooth muscle results in severe effects on smooth muscle differentiation and contractile function, demonstrating the essential role of these small noncoding RNAs. The use of inducible smooth muscle-specific Dicer KO has enabled us to decipher processes that are especially sensitive to miRNA regulation by using early time...
points following tamoxifen treatment. In this work, we show that the myogenic response is abolished at a time point (5 weeks) where vessel dimensions and calcium-independent contraction are still unaffected by the loss of miRNAs. The loss of myogenic tone in Dicer KO vessels was found to be associated with an increased PTEN expression, abolished stretch-sensitive Akt phosphorylation and reduced calcium influx (Figure 6). Accordingly, the pressure-induced and calcium-dependent phosphorylation of myosin light chains was abolished in Dicer KO vessels. Bay K 8644, which is known to promote the opening probability of L-type calcium channels, moreover normalized myogenic tone in Dicer KO mesenteric arteries. L-type calcium channels are known to play an essential role in the regulation of blood pressure and conditional deletion of these channels in smooth muscle results in loss of myogenic tone.24 We have previously shown that long-term (10 weeks) deletion of miRNAs results in reduced L-type calcium channel expression in the portal vein, likely via up-regulation of CamKII.8 By analysing the time-course of miRNA knock-down after Dicer deletion, we found that most miRNAs were still significantly expressed for up to 3 weeks after tamoxifen treatment. Thus, the phenotype observed in mesenteric arteries at 5 weeks after Dicer KO represents the initial effects of miRNA knockdown in smooth muscle and cannot be attributed to the long-term effects of Dicer KO such as reduced smooth muscle mass6 or reduced expression of contractile proteins and L-type calcium channels.25

Figure 4 Loss of pressure-induced activation of Akt in Dicer KO arteries is associated with an increased expression of PTEN, which is inhibited by miR-26a. (A) Akt phosphorylation was analysed by western blot in WT and Dicer KO mesenteric arteries following 10 min pressurization at 95 mmHg (n = 3–9). (B) The role of PI3K/Akt signalling for the development of myogenic tone was evaluated by pressure myography using WT mesenteric arteries treated with the PI3K inhibitor LY-294002 (10 μM) (n = 4). The regulation of PTEN by miRNAs was analysed by western blot in WT and Dicer KO mesenteric arteries (C) and in isolated Dicer KO smooth muscle cells transfected with either negative control or miR-26a mimic (n = 6, respectively). For (A), statistical significance was determined by one-way ANOVA and for (B) using repeated-measures two-way ANOVA. Multiple comparisons were corrected by Bonferroni’s t-test. Statistical significance of (C and D) were determined using two-tailed Student’s t-test. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 5 Angiotensin II-mediated activation of PI3K/Akt pathway can restore myogenic tone in Dicer KO mesenteric arteries. (A) Contractile responses of mesenteric arteries to angiotensin II (Ang II; 100 nM) were evaluated by pressure myography in WT and Dicer KO vessels with or without the PI3K-inhibitor LY-294002 (LY; 10 μM) (n = 6–8). (B) Western blot analysis of Akt phosphorylation in mesenteric arteries following 5 min stimulation with Ang II (100 nM) (n = 5). (C) Original recordings from pressure myography experiments showing the effect of Dicer KO and Ang II pre-treatment on the myogenic response. (D) Summarized data from pressure myography experiments showing the effect of Ang II pre-treatment on myogenic tone in Dicer KO mesenteric arteries. In some experiments, the vessels were incubated with either the PI3K inhibitor LY-294002 (LY; 10 μM) or the Ang II receptor blocker, candesartan (1 μM), during the temporary Ang II stimulation (n = 8). For (A and B), statistical significance was determined using one-way ANOVA and multiple comparisons were corrected by Bonferroni’s t-test. *P < 0.05, **P < 0.01.
that inhibition of this pathway lowers blood pressure by reducing L-type calcium-channel activity partly by promoting vasoconstriction. However, Ang II stimulation of pressurization of myogenically active arteries could promote Akt-phosphorylation despite an increase in PTEN expression. This may simply be due to the fact that 100 nM Ang II is a much stronger stimulus than an increase in pressure. However, it is notable that Ang II has recently been demonstrated to inhibit PTEN activity, which would counteract the increased PTEN expression in Dicer KO arteries.

Ang II is known to be an important mediator of blood pressure regulation partly by promoting vasoconstriction. However, Ang II stimulation of pressurized resistance arteries ex vivo only results in a transient contraction that peaks at 1 min and returns to near baseline after 5 min. Earlier reports show that phosphorylation of Akt in cultured smooth muscle cells peaks at ~2–5 min after Ang II stimulation, and remains about twofold activated after 40 min. Thus, it is likely that PI3K, apart from its role in the acute response to Ang II, is involved in long-term effects on contractile function of smooth muscle. In accordance with this hypothesis, we show that at 15–20 min following a transient Ang II stimulation, after which Ang II was carefully washed out of the myograph chamber, the loss of myogenic tone was completely restored in Dicer KO arteries. Interestingly, this effect was dependent on Ang II-induced PI3K activation since a temporary inhibition of PI3K, during the Ang II-stimulation, prevented the Ang II-mediated effect on myogenic tone. Furthermore, increased PI3K activation of the Akt-pathway, resulting in augmented myogenic tone and long-lasting effects on smooth muscle contractility by potentiating myogenic tone, possibly via an increased activation of L-type calcium channels. However, Ang II can also activate other signalling pathways such as protein kinase C, MAP kinases, and reactive oxygen species, all of which are known to be involved in the regulation of myogenic tone.

Ex vivo pressure myography of small resistance arteries is the most common method to assess myogenic tone. Although it can be considered an advantage that this technique provides an opportunity to study the effects of pressure and flow in the absence of confounding factors, it is also an important limitation that circulating hormones and neural inputs are not present during the analysis. In vivo, these factors can likely affect myogenic responses and it may therefore be more difficult to interpret the results...
be difficult to predict if the effects observed in vivo can be recapitulated "in vivo." However, in previous studies, we have shown that inducible deletion of Dicer in smooth muscle results in reduced blood pressure already at 6 weeks post tamoxifen treatment, suggesting that myogenic tone is also impaired in vivo in Dicer KO mice.\(^6\) The reduced distensibility of the Dicer KO arteries would, at least in theory, protect against a more drastic reduction in systemic blood pressure at the expense of the vessels ability to adjust to alterations in systemic blood pressure.

In conclusion, our data suggest that the myogenic response is specifically sensitive to loss of miRNAs in smooth muscle. The mechanism behind this effect involves a reduced voltage gated calcium influx and loss of pressure-induced Akt phosphorylation, possibly due to an increased expression of PTEN. Our results further emphasize the importance of miRNAs in vascular smooth muscle and demonstrate a novel mechanism participating in the regulation of myogenic tone. Since regulation of myogenic tone is essential for the control of blood pressure and blood flow, the results presented herein can provide a better understanding for the mechanisms underlying vascular diseases, such as hypertension and oedema, and suggest novel targets for therapeutic intervention.

Acknowledgements

We thank Ina Nordström for technical assistance.

Conflict of interest: none declared.

Funding

This work was supported by the Swedish Research Council; the Swedish Heart and Lung Foundation, the Crafoord Foundation; the Royal Physiographic Society; the Åke Wiberg Foundation; the Jeansson Foundation; the Tore Nilsson Foundation; the Greta and Johan Kock Foundation; the Magnus Bergvall Foundation and the Lars Hierta Memorial Foundation. A.B and K.M.T. were supported by the European Union FP7 Marie Curie Initial Training Network Small Artery Remodeling (SmArt).

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