Erratum

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Journal of Cell Science 120, 1502 (2007) doi:10.1242/jcs.03445

There was an error published in *J. Cell Sci.* **120**, 929-942.

We apologise for the incorrect publication of a reference, for which the wrong year, title and journal details were given.

The correct reference is shown below.

Leiper, J., Nandi, M., Torondel, B., Murray-Rust, J., Malaki, M., O'Hara, B., Rossiter, S., Anthony, S., Madhani, M., Selwood, D. et al. (2007). Disruption of methylarginine metabolism impairs vascular homeostasis. *Nat. Med.* **13**, 198-203.

The ADMA/DDAH pathway is a critical regulator of endothelial cell motility

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Accepted 15 January 2007
Journal of Cell Science 120, 929-942 Published by The Company of Biologists 2007
doi:10.1242/jcs.002212

Summary

Asymmetric dimethylarginine (ADMA) is an inhibitor of nitric oxide production associated with abnormal blood vessel growth and repair, however, the mechanism of action of ADMA is not well understood. We studied the role of exogenous and endogenous ADMA in the regulation of cell motility and actin cytoskeleton in porcine pulmonary endothelial cells (PAECs) and pulmonary microvascular endothelial cells (PMECs) from knockout mice that lack one of the enzyme metabolising ADMA, dimethylarginine dimethylaminohydrolase I (DDAHI) as well as endothelial cells overexpressing DDAH in vitro.

We show that ADMA induced stress fibre and focal adhesion formation and inhibited cell motility in primary pulmonary endothelial cells. The effects of ADMA depended on the activity of RhoA and Rho kinase and were reversed by overexpression of DDAH, nitric oxide donors and protein kinase G activator, 8-bromo-cGMP. ADMA also inhibited the activities of Rac1 and Cdc42 in cells but these changes had a minor effect on cell motility. Endogenous ADMA increased RhoA activity and inhibited cell motility in PMECs from DDAHI knockout mice and inhibited angiogenesis in vitro. These results are the first demonstration that metabolism of cardiovascular risk factor ADMA regulates endothelial cell motility, an important factor in angiogenesis and vascular repair.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/120/6/929/DC1

Key words: Endothelial, Movement, Rho GTPases, Nitric oxide, Protein kinase G, Cytoskeleton

Introduction

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthases (NOS) (Vallance et al., 1992). Increase in ADMA plasma levels is associated with angiogenic disorders in hypercholesterolemia, insulin resistance, hypertension and homocysteinemia (Cooke, 2003) and a lack of normal endothelial regeneration following balloon injury in vitro (Azuma et al., 1995).

ADMA is released as a result of proteolysis of proteins containing methylarginine residues (Kakimoto and Akazawa, 1970). The major route of clearance of ADMA is hydrolysis to citrulline and methylamines by dimethylarginine dimethylaminohydrolases I and II (DDAHI and II) (Vallance and Leiper, 2004). Studies from this laboratory show that decreasing endogenous ADMA by overexpression of human DDAHII transgene in vitro promotes capillary formation in human and murine endothelial cell lines whereas pharmacological inhibition of DDAH inhibits capillary formation (Smith et al., 2003). Increased ADMA metabolism has also been linked to high vascularity of human tumours (Kostourou et al., 2002).

The mechanisms by which ADMA may affect blood vessel growth are not well understood but one possibility is that the ADMA-DDAH pathway regulates endothelial cell motility. Endothelial cell motility depends on nitric oxide production; some authors have reported activation of motility by nitric oxide (Goligorsky et al., 1999; Kawasaki et al., 2003), and others its inhibition (Lau and Ma, 1996). NO initiates diverse cellular signalling cascades that include serine phosphorylation of proteins by a downstream effector of NO, protein kinase G (PKG) (Yaroslavskiy et al., 2005; Koh and Tidball, 2000), S-nitrosylation of target proteins (Koh and Tidball, 2000) or adenosine 5′-diphosphate (ADP) ribosylation (Clancy et al., 1995). Importantly, NO has been shown to affect the expression and activity of Rho GTPases, key regulators of cell motility and the actin cytoskeleton (Sauzeau et al., 2003; Hou et al., 2004). In smooth muscle and HEK293 cells, PKG inhibits RhoA activity by phosphorylating it on serine 188 (Sawada et al., 2001; Murthy et al., 2003) whereas in HEK293 cells PKG can indirectly activate Rac (Hou et al., 2004). RhoA mRNA and protein expression were positively regulated by NO/PKG in rat aortas in vivo (Sauzeau et al., 2003). In a reciprocal manner, increase in the activity of RhoA and its downstream effector, Rho kinase has been shown to downregulate eNOS expression (Takemoto et al., 2002; Shiga et al., 2005) and activity (Ming et al., 2002) in endothelial cells in vitro. Coordinated actions of Rho GTPases RhoA, Rac1 and Cdc42 are required for a directional movement of cells. Rac1 and Cdc42 mediate the attachment and expansion of the cell front, whereas RhoA is required for the maturation and turnover of cell attachments called focal adhesions, and retraction of the following end of the cell (Ridley, 2001; Nobes and Hall, 1995). Though basal activity of RhoA is important for cell contractility required for cell migration (Ridley, 2001),

in certain cell types RhoA activation promotes stress fibre formation and enlargement of focal adhesions, resulting in strong adhesion to the substratum and inhibition of cell motility (Cox et al., 2001; Webb et al., 2003). Interestingly, NO inhibitor, NG-nitro-L-arginine methyl ester (L-NAME) was shown to induce formation of stress fibres and large focal adhesions and inhibit cell movement in cultured human umbilical vein endothelial cells (Goligorsky et al., 1999), but the role of Rho GTPases in this process has not been studied.

Although nitric oxide is the most likely mediator of ADMA-induced effects, ADMA can also have NO-independent effects on endothelial gene expression (Smith et al., 2005).

In this investigation we aimed to establish the effects of exogenous and endogenous ADMA on endothelial cell motility and determine the role of nitric oxide and Rho GTPases in this process. We show that exogenous and endogenous ADMA as well as the enzymes responsible for its metabolism, DDAHs, play an important role in the regulation of endothelial cell motility and angiogenic responses in vitro. Regulation of endothelial cytoskeleton and motility by ADMA depends on the activity of RhoA and Rho kinase and is mediated by nitric oxide.

Results

ADMA but not SDMA increases stress fibre and focal adhesion formation in endothelial cells

Porcine pulmonary artery endothelial cells (PAECs) treated with ADMA (100 μM, 24 hours) became less polarized morphologically and showed increased formation of stress fibres and focal adhesions (Fig. 1I-K), as compared with untreated controls (Fig. 1A-C). By contrast, symmetric dimethylarginine (SDMA) which does not act as an inhibitor of NOS, had no effect (Fig. 1E,F,G). 100 μM ADMA had maximal effect on cell phenotype in PAECs and therefore we chose this concentration for further studies of cell responses. This concentration of ADMA was previously found to induce significant changes in gene expression in human coronary artery endothelial cells (Smith et al., 2003). The earliest changes in the distribution of actin filaments were observed at 4 hours of incubation with 50-100 μM ADMA (data not shown).

ADMA but not SDMA inhibits endothelial motility

Control, untreated PAECs moved at a speed of 45±10 μm/hour (± s.d.), and showed translocation of 192±49 μm (Fig. 1D,M,N). ADMA (50-100 μM) reduced cell speed to 21±8 μm/hour (P<0.01, comparison with controls) and translocation to 70±40 μm (P<0.01, comparison with controls; Fig. 1L,M,N). The effect of ADMA on cell translocation was greater than its effect on the cell speed suggesting that cells moved less persistently in any one direction. SDMA had no significant effect on cell motility (Fig. 1H,M,N). Assuming that the effects of ADMA may result from NOS inhibition, we starved the cells of NOS substrate, L-arginine 24 hours prior to the addition of the inhibitor. Although low concentrations of ADMA (10 μM) did not affect cell movement in standard culture conditions, starving cells of L-arginine significantly increased the effect of low doses of the inhibitor on cell speed (27±10 μm/hour; P<0.05, comparison with controls) and translocation (116±51 μm; P<0.05, comparison with controls) (Fig. 1M,N). This result suggests a possible role of NOS in ADMA-induced effects.

Overexpression of DDAHI and DDAHII prevents the effects of exogenous ADMA on endothelial cell cytoskeleton and motility

In order to test whether increasing ADMA metabolism in cells would reverse the effects of exogenous ADMA, we overexpressed DDAHI and DDAHII in cells via adenoviral gene transfer. These viruses also express green fluorescent protein (GFP) which allowed an easy identification of infected cells in motility assays and immunofluorescence studies (Fig. 2, bottom). Recombinant DDAHI adenovirus (AdDDAHI) abolished the effect of ADMA on stress fibre and focal adhesion formation in PAECS (Fig. 2F-I), whereas the control adenovirus, AdGFP had no effect (Fig. 2A-D). Similar to the effects of DDAHI, overexpression of DDAHII reduced focal adhesion and stress fibre formation in ADMA-treated PAECs (Fig. 2K-N). Overexpression of AdDDAHI and AdDDAHII in ADMA-treated cells restored cell speed and translocation to control values (Fig. 2J,O,U,W). Interestingly, some untreated cells overexpressing AdDDAHI and AdDDAHII also showed reduction in stress fibre and focal adhesion formation (Fig. 2P-S, and data not shown). AdDDAHI and AdDDAHII did not affect cell speed in untreated cells but increased their translocation to 220±50 μm and 198±45 μm, respectively (P<0.05, comparison with controls; Fig. 2U,W).

ADMA-induced changes are mediated by Rho GTPases

Rho GTPases are regulators of actin dynamics and cell motility (Ridley, 2001). Treatment of PAECs with exogenous ADMA (100 μM, 24 hours) increased the activity of RhoA by 110±28% (P<0.01, comparison with untreated controls) (Fig. 3). By contrast, ADMA reduced the activity of Rac1 by 51±13% and the activity of Cdc42 by 54±12% (P<0.01, comparison with untreated controls; Fig. 3). Changes in Rho GTPases activity were concentration- and time-dependent and showed maximum change at ADMA concentration of 100 μM and after 24 hour incubation (Fig. 3).

In order to determine, whether Rho GTPases are mediators of ADMA-induced changes in PAECs, we expressed selected dominant negative RhoA and constitutively active Rac1 and Cdc42 in cells treated with the inhibitor. The RhoA inhibitor, N19RhoA and the Rho kinase inhibitor, Y-27632 reduced formation of central stress fibres and focal adhesions in ADMA-treated cells (Fig. 4D-H) and restored cell speed and translocation to control values (Fig. 4P,Q). N19RhoA did not significantly alter morphology or motility in untreated cells (supplementary material Fig. S1 and Fig. 4P,Q).

Constitutively activated Cdc42, L61Cdc42 did not significantly affect cell morphology, speed or translocation in ADMA-treated (Fig. 4M-O,P,Q). However, some highly overexpressing cells showed increased formation of stress fibres and peripheral focal contacts and decreased formation of focal adhesions in the cell centre (supplementary material Fig. S1). This was consistent with previously reported effects of the microinjected protein in HUVECs (Wojciak-Stothard et al., 1998).

Overexpression of the constitutively activated Rac1, reduced stress fibre and focal adhesion formation in both untreated (supplementary material Fig. S1) and ADMA-treated cells (Fig. 4J-L), but the cells remained largely immotile (Fig. 4M). This indicates that a simple reduction in stress fibre and focal adhesion formation is not sufficient in preventing the effects of

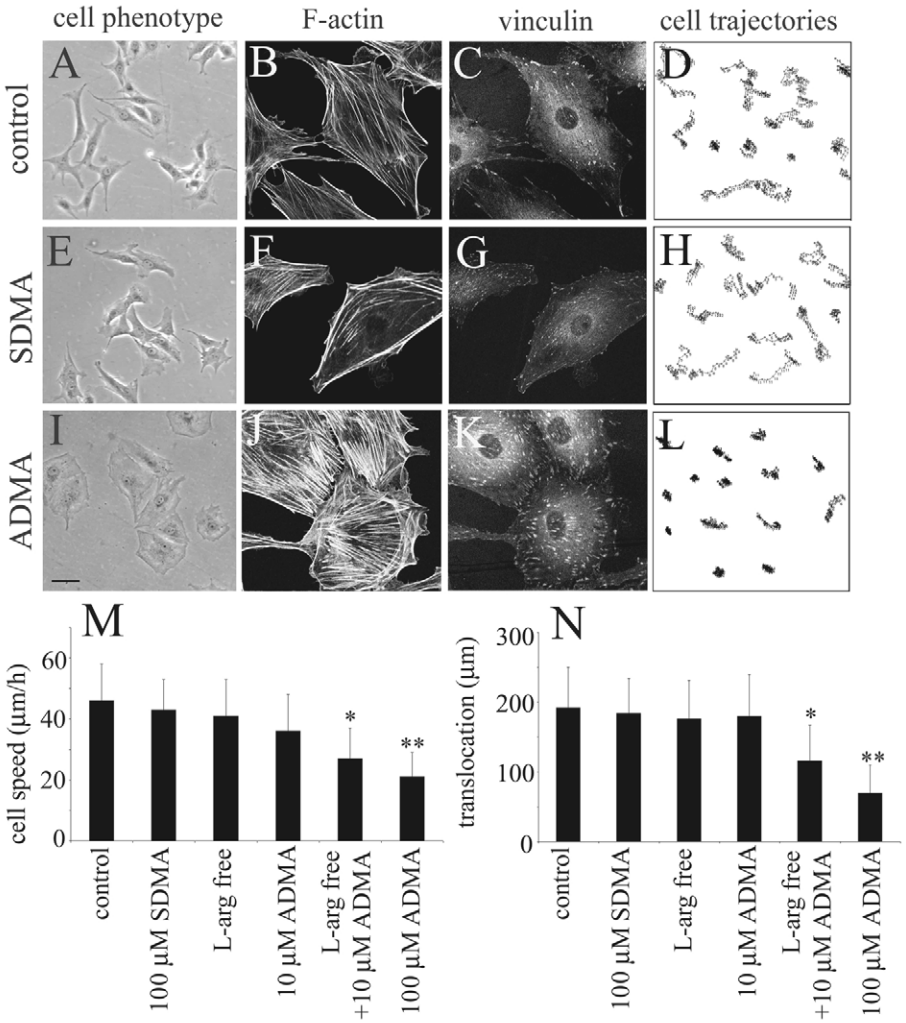


Fig. 1. ADMA induces formation of stress fibres and focal adhesions in PAECs and inhibits cell motility. (A-D) Control untreated cells; (E-H) cells treated with 100 μM SDMA; (I-L) cells treated with 100 μM ADMA for 24 hours. (A,E,I) Phase contrast microscopy images showing sparsely growing PAECs. (B,C,F,G,J,K) Fluorescent images showing staining for F-actin and a focal adhesions component vinculin. (D,H,L) Representative images of cell trajectories. Bar, 100 μm (A,E,I), 10 μm (B,C,F,G,J,K) and 130 μm (D,H,L). (M) Changes in the mean speed of cell movement in untreated cells, cells treated with SDMA (100 μM), ADMA (100 μM) or the cells grown in arginine-free medium with or without ADMA. (N) Total cell translocation defined as a distance between starting point and an end point of each trajectory. *P<0.05; **P<0.01 (comparison with untreated controls).

ADMA on endothelial motility and that the appropriate levels of Rac1 activity may be required for coordinated movement of cells. The actions of activated Rac1 in the regulation of endothelial cell motility will require further study.

Nitric oxide is a mediator of ADMA-induced responses

Nitric oxide has been shown to regulate endothelial cytoskeleton and motility (Kawasaki et al., 2003). In order to verify the role of NO in the observed effects, we added NO donor, *S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) to ADMA-treated cells. In another approach, we cultured cells with a NOS inhibitor, L-NAME and compared its effects to those induced by ADMA. In order to estimate the levels of nitric oxide released by the cells under various conditions, we measured the levels of nitrite, a stable end product of NO degradation.

Cell cytoskeleton and focal adhesions

SNAP (10 μM) decreased basal levels of stress fibres and focal adhesions in control cells, and prevented formation of focal adhesions and stress fibres in ADMA-treated cells (Fig. 5A,B,D,E). Consistently, SNAP restored cell speed and translocation in ADMA-treated cells to control values (Fig. 5F and Fig. 6A,B). However, at higher concentrations SNAP (>100 μM) induced a loss of F-actin in cells, often resulting in cell rounding and inhibition of cell motility (data not shown). Similar to ADMA, L-NAME induced formation of stress fibres and focal adhesions in cells (Fig. 5G,H). SNAP prevented the effects of L-NAME on cell cytoskeleton, focal adhesion formation (not shown) and motility (Fig. 6A,B).

Nitrite levels

ADMA and L-NAME reduced nitrite concentration from 7.3 μM in controls to 3.5 μM and 4.1 μM, respectively (P<0.01, comparison with controls), whereas SDMA had no effect. In cells treated with SNAP, nitrite concentration increased twofold over controls and this high level was maintained irrespective of the presence of ADMA or L-NAME (Fig. 6C). Consistent with its specific effect on ADMA, overexpression of AdDDAHI and AdDDAHII restored nitrite levels to control values in ADMA- but not in L-NAME-treated cells. SDMA, AdGFP, AdDDAHI and AdDDAHII did not have a significant effect on nitrite levels in control cells (Fig. 6C). These results indicate that nitric oxide plays a major part in cells' motile responses to ADMA.

Although DDAHI did not affect cell speed or nitrite levels in L-NAME-treated cells, it increased cell translocation in those cells (Fig. 6B) indicating, that some of the actions of these enzymes may be independent of NO generation.

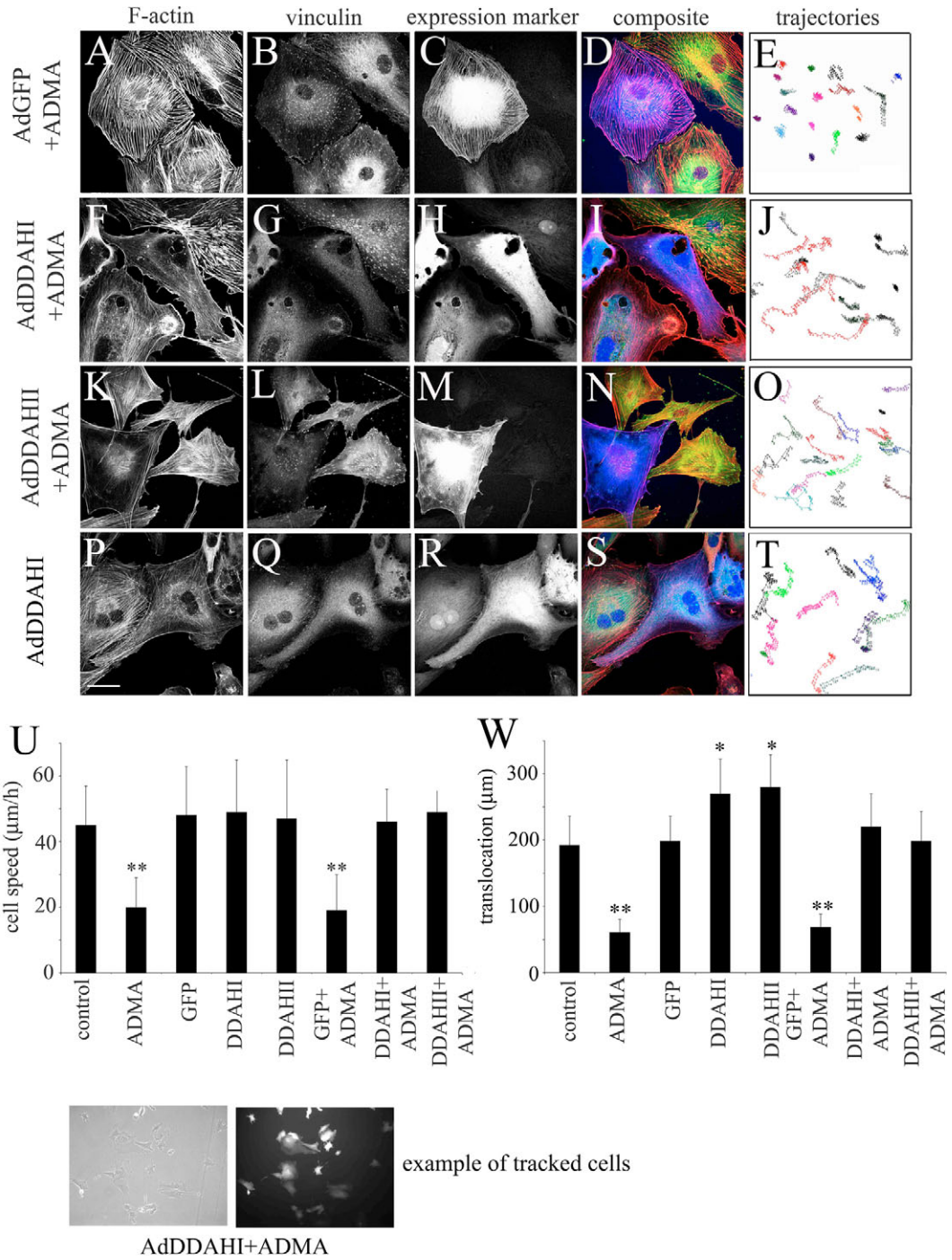


Fig. 2. Overexpression of DDAHI and DDAHII prevents ADMA-induced increase in stress fibre and focal adhesion formation and restores normal motility in ADMA-treated cells. (A-E) Cells incubated with ADMA and infected with control adenoviruses to express AdGFP. (F-O) Cells treated with ADMA and overexpressed AdDDAHI (F-J) and AdDDAHII (K-O). (P-T) Cells overexpressing AdDDAHI without the addition of ADMA. In A-T the columns from the left to right show F-actin distribution, vinculin distribution, the cells overexpressing recombinant proteins, composite images and cell trajectories, as indicated. In the composite images F-actin is red, vinculin is green and the cells overexpressing GFP are blue (pseudocolour). Bar, 10 μm (180 μm in E, J, O, T). (U) Changes in the cell speed; (W) changes in total cell translocation in non-treated cells and cells overexpressing AdGFP, AdDDAHI, AdDDAHII. The cells were left untreated or were treated with 100 μM of ADMA or SDMA, as indicated. *P<0.05; **P<0.01, comparison with untreated controls. (Bottom left) The two images are an example of fluorescently labelled cells expressing recombinant DDAHII that were used for cell tracking. Only overexpressing cells (96% of total cell population) were tracked.

Endogenous DDAHI was diffusely distributed in the cytoplasm of PAECs, although some of the protein showed colocalisation with F-actin in membrane ruffles and lamellipodia (Fig. 6D). Cultured PAECs appeared to express predominantly DDAHI, although expression of DDAHII was detectable by western blotting (Fig. 6E).

The effects of SNAP and L-NAME on RhoA activity

We showed that inhibition of RhoA and Rho kinase activity completely prevented the effects of ADMA, suggesting that RhoA is the main mediator of cell responses. As NO has been shown to modify the activity and expression of Rho GTPases (Ellerbroek et al., 2003; Sauzeau et al., 2003), we studied the activity of RhoA in cells cultured in the presence of NOS inhibitor L-NAME or in the presence of NO donor, SNAP.

RhoA was activated by L-NAME at the concentrations of 0.5-1.0 mM (Fig. 7A). Similar to ADMA, a maximal effect of L-NAME was observed after 24 hour incubation with the inhibitor (Fig. 7B).

SNAP (10 μM) significantly decreased the activity of

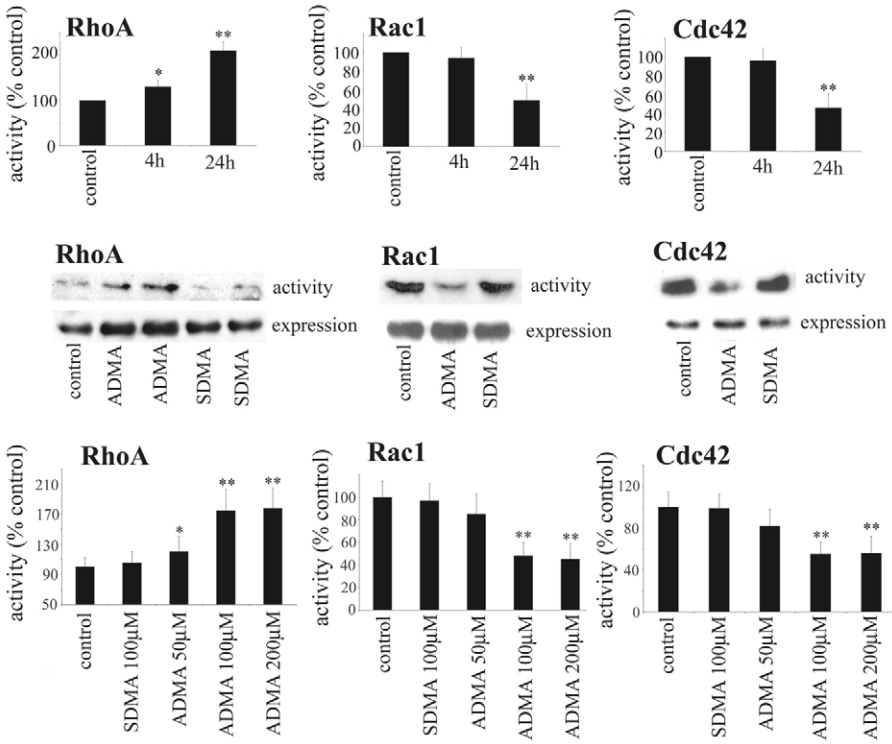


Fig. 3. ADMA but not SDMA activates RhoA and inhibits Rac1 and Cdc42 in PAECs. The three graphs at the top show changes in the activity of RhoA, Rac1 and Cdc42 following 4 or 24 hours incubation with the drugs. Representative examples of western blots illustrating changes in Rho GTPases activity upon treatment with 100 μM ADMA or SDMA are shown below. The two graphs at the bottom show concentration-dependent changes in RhoA, Rac1 and Cdc42 activities in cells treated with SDMA or ADMA for 24 hours. **P*<0.05; ***P*<0.01 (comparison with untreated controls).

RhoA with a maximal effect (36±19% of control levels) at 4 hours of incubation. Low levels of RhoA activity were maintained throughout 24 hours of the study, although at later timepoints the activity of RhoA started to rise (Fig. 7C), presumably due to the temporary effect of SNAP on NO levels. SNAP or L-NAME did not change RhoA expression levels in PAECs.

NO has been reported to inhibit RhoA activity by increasing its phosphorylation on Ser188 which prevents RhoA localisation to the membrane (Ellerbroek et al., 2003). We immunoprecipitated P-Ser188RhoA from cell lysates of the untreated cells and the cells treated with ADMA, SDMA and SNAP as well as the cells treated with both SNAP and ADMA. We observed a 20% decrease in the levels of P-Ser188RhoA in ADMA-treated cells and a 50% increase in SNAP-treated cells. SNAP also restored the levels of P-Ser188RhoA in ADMA-treated cells to the levels seen in control, untreated cells. This pattern of changes was consistent with the observed ADMA effects on nitrite levels in PAECs.

In order to verify the results obtained with anti-Ser188RhoA antibody, we expressed a haemagglutinin-tagged non-phosphorylatable RhoA mutant, Ala188RhoA-HA (Sauzeau et al., 2000) in PAECs. Overexpression of Ala188RhoA increased formation of stress fibres and enlarged focal adhesions in PAECs. Although the pattern of cell response was consistent with the effects of NO inhibition, Ala188RhoA-overexpressing cells were less flattened than ADMA-treated cells and often had an elongated morphology with bundles of parallel stress fibres spanning the cells and focal adhesions localised to the cell periphery (Fig. 8A-F), which indicates that other signalling pathways may also be important in the regulation of cell morphology. These changes were not observed in the cells transfected with an empty vector (data not shown). The expressing cells were identified by immunofluorescence (Fig. 8C,F) and the levels of expression were examined by western blotting (Fig. 8G). Ala188RhoA inhibited cell motility and translocation to the same extent as ADMA (Fig. 8H,I).

The role of protein kinase G in the effects of ADMA

In vascular smooth muscle cells PKG mediates NO-induced phosphorylation of RhoA on Ser188 (Sauzeau et al., 2000). In order to examine the role of PKG in ADMA-induced effects, we incubated the untreated and ADMA-treated PAECs with the PKG activator, Br-cGMP (8-bromo-cGMP, sodium salt; 500 μM). In another approach, we incubated PAECs with a specific PKG inhibitor, Rp-8-pCPT-cGMPS (100 nM). The concentrations of Br-cGMP and Rp-8-pCPT-cGMPS were based on studies in vascular endothelial and smooth muscle cells (Luedders et al., 2006; Sauzeau et al., 2003). Br-cGMP decreased F-actin levels in untreated cells (Fig. 9C), consistent with its effects on cultured vascular smooth muscle cells (Sauzeau et al., 2000), and decreased focal adhesion and stress fibre formation in ADMA-treated cells (Fig. 9G,H). By contrast, Rp-8-pCPT-cGMPS increased stress fibre and focal contact formation in untreated cells (Fig. 9E,F).

Similar to the effects of SNAP, Br-cGMP restored cell speed and translocation to control levels and reduced RhoA activity in ADMA-treated cells (Fig. 9I,J,K). In control cells, Br-cGMP did not significantly affect cell speed or translocation (Fig. 9I,J) but reduced the activity of RhoA (Fig. 9K). PKG inhibitor, Rp-8-pCPT-cGMPS significantly reduced cell speed to 30±9 μm/hour and translocation to 130±45 μm, respectively (*P*<0.01, comparison with controls) (Fig. 9I,J) and increased the activity of RhoA in PAECs (Fig. 9K).

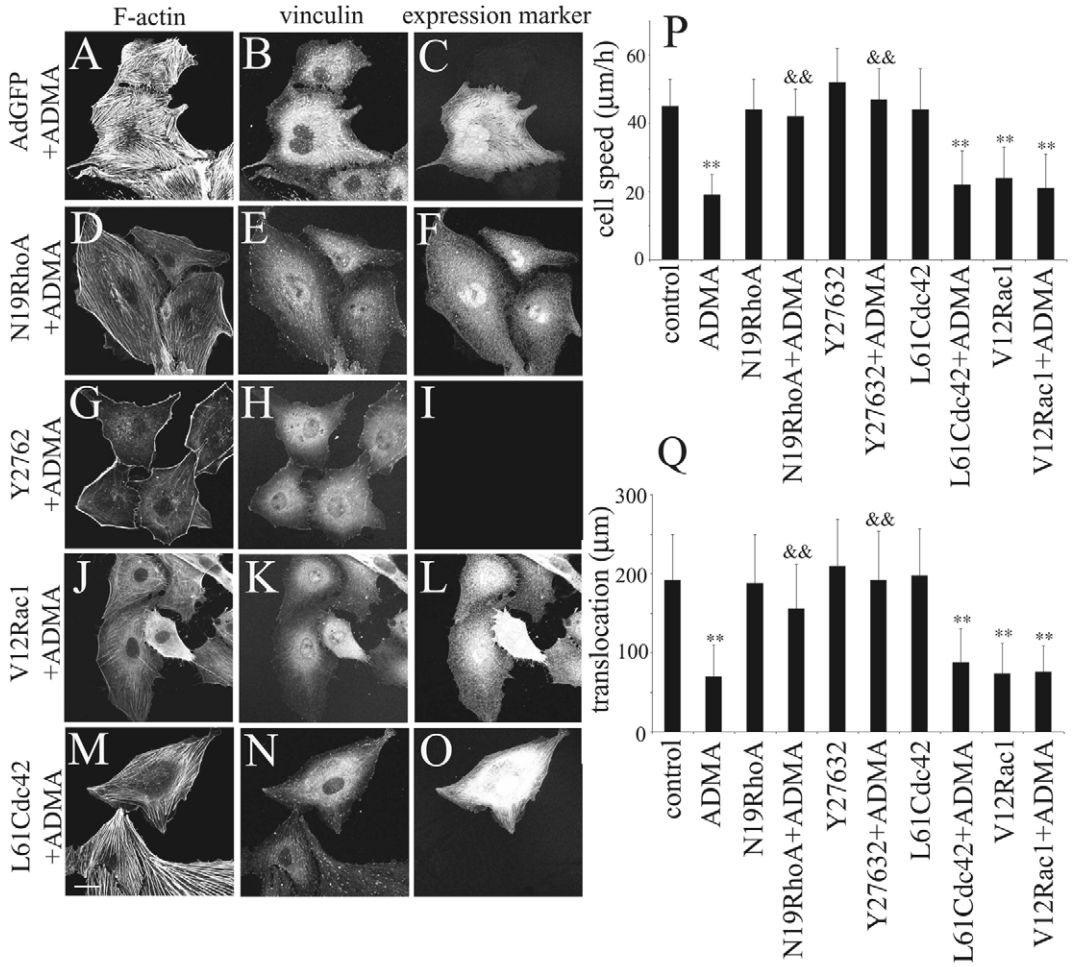


Fig. 4. Inhibition of RhoA and Rho kinase prevents the effects of ADMA on the actin cytoskeleton and focal adhesions, and restores cell speed and translocation to control values. Adenoviral gene transfer was used to induce expression of GFP (A-C), inhibitory mutant of RhoA, N19RhoA (D-F), activated Rac1, V12Rac1 (J-L) GFP-tagged activated Cdc42, L61Cdc42 was introduced by transfection (M-O). The cells in G-I were treated with the Rho kinase inhibitor, Y-27632 (10 μM) for 2 hours. The cells were stained for F-actin (left column), vinculin (middle column) and Myc (right column) to identify overexpressing cells. Bar, 15 μm. (P) Cell speed in ADMA-treated PAECs. (Q) The effect of mutant Rho GTPases on cell translocation. Cell cytoskeleton and motility were studied 24 hours after adenoviral infection. $^{**}P<0.01$ (comparison with untreated controls); $^{\&\&}P<0.01$ and $^{\&}P<0.05$ (comparison with ADMA-treated cells).

Increase in endogenous ADMA in transgenic mice inhibits endothelial motility in vitro and increases the activity of RhoA

In order to investigate whether manipulation of endogenous ADMA levels in endothelial cells would have similar effects to adding exogenous ADMA, we compared cell phenotype and motility of cultured pulmonary microvascular endothelial cells (PMVECs) from wild-type and heterozygous DDAHI knockout (HT) mice. HT animals have approximately twofold higher ADMA plasma levels compared to wild-type littermates and thus provide a good model to test the effects of raised ADMA levels (Smith et al., 2005). PMVECs from HT mice cultured in vitro were well spread and showed decreased ruffling and increased formation of stress fibres as compared to the cells from wild-type littermates (Fig. 10A-D). They also showed a significant reduction in cell speed and translocation. HT cells moved at a speed of 12±7 μm/hour with translocation of 170±65 μm whereas wild-type controls moved at the speed of 20±10 μm/hour with translocation of 240±71 μm, $P<0.05$ (Fig. 8E,F,H,I). PMVECs from HT mice also showed reduced nitrite levels in culture medium (4.9±3 μM in HT compared to 10 μM ±4 in controls, $P<0.01$) (Fig. 10G) and a higher activity of RhoA (135±15% of control levels; $P<0.05$) (Fig. 10J), changes consistent with the effects of exogenous ADMA on porcine pulmonary endothelial cells. The activities of Rac1 and Cdc42 in PMVECs from DDAH heterozygous knockout animals were not significantly different, though a tendency to decreased activity was seen (Fig. 10J).

Mouse aortic ring assay: the effects of ADMA on angiogenesis

In order to assess the effect of increased endogenous ADMA on angiogenic potential of endothelial cells, we compared the number of microvessels sprouting from aortic rings taken from thoracic aortas of DDAHI HT knockout mice with the number of microvessels from aortas taken from wild-type littermates.

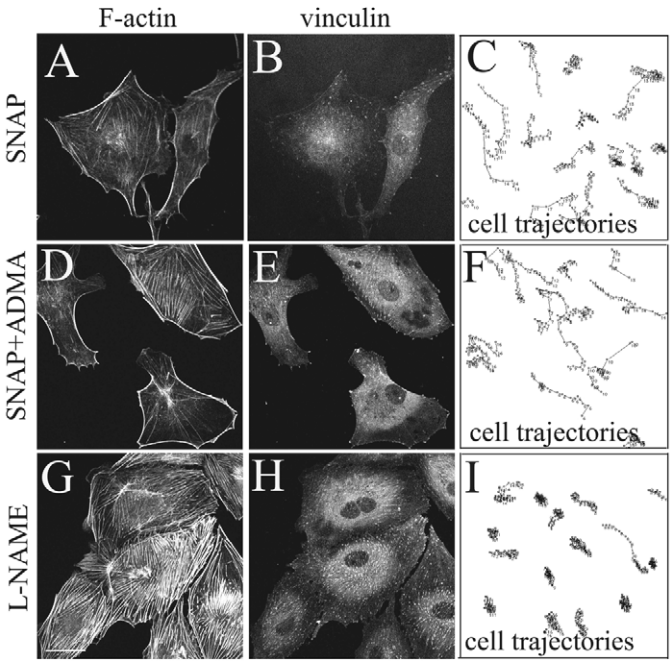


Fig. 5. The effects SNAP and L-NAME on the actin cytoskeleton, focal adhesion formation and cell movement in PAECs. (A-C) Cells incubated with 10 μM SNAP for 4 hours; (D-F) cells incubated with ADMA (100 μM) and SNAP (10 μM) for 24 hours; (G-I) cells incubated with L-NAME for 24 hours. The columns from left to right show F-actin distribution in cells, vinculin distribution and cell trajectories, as indicated. Bars, 15 μm (A,B,D,E,G,H) and 170 μm (C,F,I).

The number of microvessels sprouting from the aortic rings of HT mice (9±5) was significantly lower than the number of microvessels sprouting from aortic rings of wild-type mice (15±6, $P<0.05$) (Fig. 11). The addition of exogenous ADMA to aortic rings from wild-type mice reduced the number of vessels to 6±5 ($P<0.01$, comparison with wild-type controls), while the addition of SDMA had no effect (Fig. 11).

Discussion

We show for the first time that metabolism of ADMA plays an important role in the regulation of endothelial cell motility and angiogenic responses in primary endothelial cells in vitro. We observed that exogenous as well as endogenous ADMA induced stress fibre formation and inhibited cell motility as a result of nitric oxide-mediated activation of RhoA and Rho kinase. Overexpressing enzymes responsible for ADMA clearance restored normal endothelial cell phenotype and motility (Fig. 12).

The effects of ADMA are likely to result from an inhibition of NOS and a decrease in NO bioavailability. We observed that the effects of ADMA were accompanied by a decrease in NO production and were reversed by the NO donor, SNAP. Depriving cells of NOS substrate, L-arginine enhanced the effects of ADMA. In addition, another NOS inhibitor, L-NAME mimicked the actions of ADMA on cells. Consistent with our findings, L-NAME has been shown to induce formation of stress fibres and focal adhesions and increase tracking forces in cultured HUVECs (Goligorsky et al., 1999).

We show that exogenous and endogenous ADMA increased RhoA activity in pulmonary endothelial cells, which resulted in the formation of stress fibres and enlarged focal adhesions. This is consistent with the previously reported effects of recombinant RhoA microinjected into adherent cells (Machesky and Hall, 1996). Activation of RhoA and Rho kinase has been shown to inhibit or stimulate cell motility, depending on the cell type and culture conditions (Webb et al., 2003; Itoh et al., 1999; Zhou and Kramer, 2005). Basal activity of RhoA is important for cell body contraction, a necessary step in cell migration (Ridley, 2001) but its activation may also lead to the high level of substrate adhesion through stress-fibre-associated focal adhesions and inhibition of cell movement (Zhou and Kramer, 2005). RhoA/Rho kinase may also restrict membrane protrusions to the leading edge (Worthylake and Burridge, 2003). Our data suggests that the activation of RhoA/Rho kinase by ADMA in mouse and porcine pulmonary endothelial cells leads to the inhibition of cell motility as a result of an enhanced cell-substratum adhesion rather than inhibition of cell protrusions. In support of this hypothesis, we observed an increase in cell-substratum adhesion in medium containing ADMA but not SDMA (supplementary material Fig. S2). We did not observe increased protrusive activity in PAECs treated with Y-27632, as opposed to the previously reported effects of the inhibitor in HUVECs (Wojciak-Stothard and Ridley, 2003), indicating that cell responses to the inhibitor are cell-type specific.

Activation of RhoA by ADMA in PAECs was accompanied by a decrease in serine 188 phosphorylation of the protein, both changes prevented by NO donor, SNAP. NO-mediated phosphorylation of RhoA on Ser188 in vascular smooth muscle cells was shown to inhibit RhoA and decrease cell contractility (Sauzeau et al., 2000). Serine phosphorylation of RhoA protects the protein from ubiquitin-mediated proteasomal degradation (Rolli-Derkinderen et al., 2005) and enhances its interaction with Rho guanine-dissociation inhibitor, thus decreasing its activity (Ellerbroek et al., 2003). We did not observe NO-mediated changes in RhoA expression in pulmonary endothelial cells. Consistently, HeLa cells and NIH3T3 fibroblasts overexpressing constitutively active cyclic GMP-dependent kinase showed a decrease in RhoA activity but no changes in RhoA protein expression (Sawada et al., 2001). However, activation of NO/PKG in rat and human artery smooth muscle cells increased RhoA mRNA and protein levels (Sauzeau et al., 2003), indicating that cell responses are cell type specific.

We found that changes in the levels of Ser188-phosphorylated RhoA in PAECs correlated with changes in RhoA activity measured in a GST-rhotekin binding assay. Similar correlation was reported in bovine aortic and rat muscle cells (Gudi et al., 2002; Krepinsky et al., 2003). However, in certain cell types, such as PC12 cells, this assay may not reflect changes in RhoA activity induced by PKG-mediated serine 188 phosphorylation because in those cells phosphorylated RhoA can still interact with rhotekin while losing its ability to interact with other downstream effectors (Nusser et al., 2006).

The role of RhoA phosphorylation in ADMA effects was confirmed by the overexpression of a non-phosphorylatable RhoA mutant, Ala188RhoA. Ala188RhoA inhibited cell motility and translocation, and induced stress fibre and focal

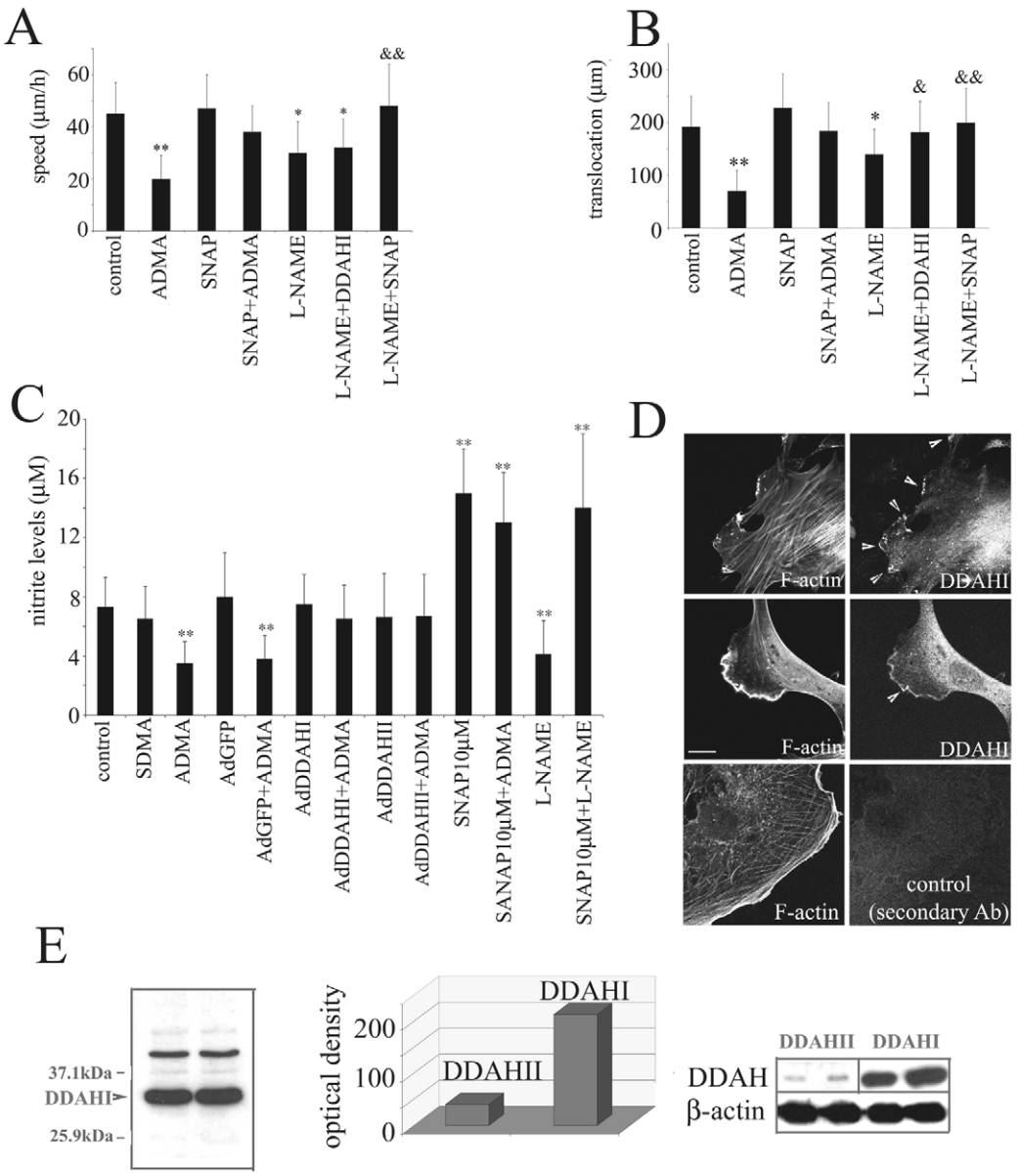


Fig. 6. The effects of SNAP and L-NAME on cell speed (A), translocation (B) and nitrite levels (C). SNAP (10 μM) and L-NAME (1 mM) were added to untreated cells or cells treated with ADMA and incubated for 24 hours. Nitrite levels in culture medium were measured using a standard Griess assay. In (D) two sets of corresponding images show localisation of endogenous DDAHI in normal PAECs. The cells were stained for F-actin (left) and DDAHI (right). White arrows point to colocalisation of F-actin and DDAHI in membrane ruffles and lamellipodium. Bar, 8 μm. (E) shows expression of DDAHI in untreated PAECs (western blot on the left). Differences in expression levels between DDAHI and DDAHII in PAECs are also shown in a graph and a corresponding western blot on the right.

contact formation, consistent with the effects of ADMA and L-NAME. We show that the effects of ADMA-induced NO deprivation are at least in part, mediated by PKG. The PKG activator, Br-cGMP completely reversed the effects of ADMA on the actin cytoskeleton, cell motility and RhoA activity in PAECs. The response pattern to the specific PKG inhibitor, Rp-8-pCPT, generally mimicked the actions of ADMA, but its effects on cell motility and RhoA activation were only partial, which suggests that other kinases, such as protein kinase A may also be involved. Br-cGMP preferentially activates PKG but may also affect the activity of PKA as a result of a crosstalk between cGMP and cAMP signalling pathways (Algara-Suarez and Espinosa-Tanguma, 2004). Interestingly, both PKA and PKG were shown to phosphorylate RhoA on Ser188 in cultured rabbit smooth muscle cells (Murthy et al., 2003).

The relatively slow timecourse of Rho GTPases activity changes induced by ADMA suggests that changes in gene expression may also be involved. This will require further studies. We have recently shown in gene array studies that ADMA significantly changes expression of many proteins regulating actin cytoskeleton and Rho-like GTPases activity including Rho guanine nucleotide exchange factor 15,

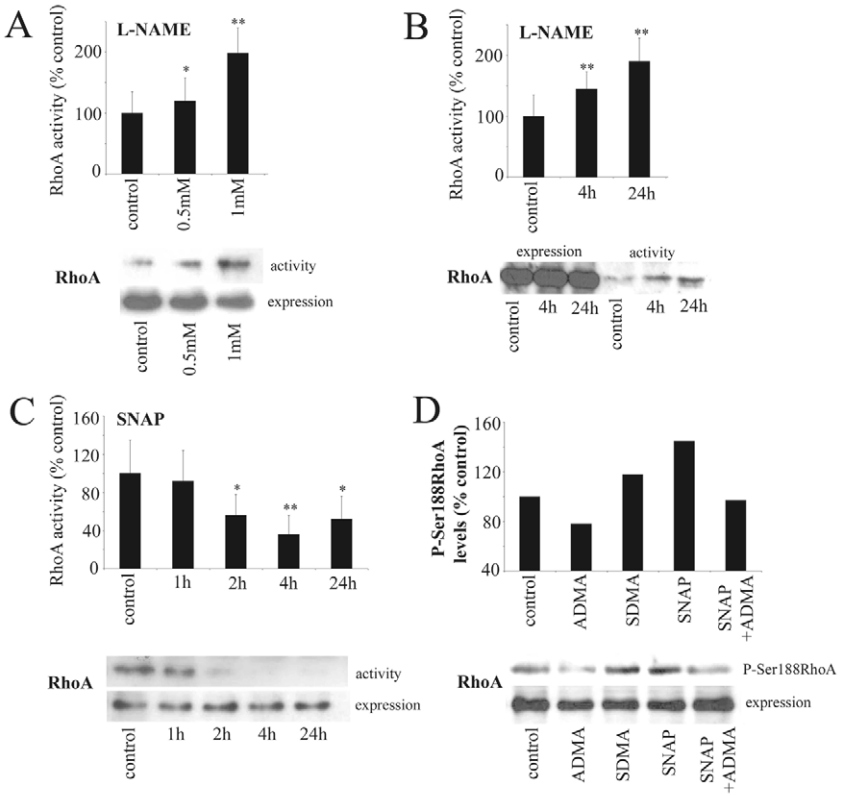


Fig. 7. The effect of L-NAME and SNAP on the activity of RhoA. (A,B) Graphs show RhoA activity in cells treated with (A) L-NAME (0.5 mM and 1 mM) for 24 hours, or (B) treated with L-NAME (1 mM) for 4 and 24 hours. (C) Graph shows time-dependent changes in RhoA activity in cells treated with SNAP (10 μM). (D) Graph shows changes in Ser188 phosphorylation of RhoA in cells treated with ADMA (100 μM), SDMA (100 μM), SNAP (10 μM) or SNAP and ADMA. ADMA and SDMA were added to the cells for 24 hours whereas SNAP was added for 4 hours. Representative examples of western blots are shown below the graphs. Values in A and C are means of three experiments whereas values in B are means of two experiments. *P<0.05, **P<0.01 (comparison with untreated controls).

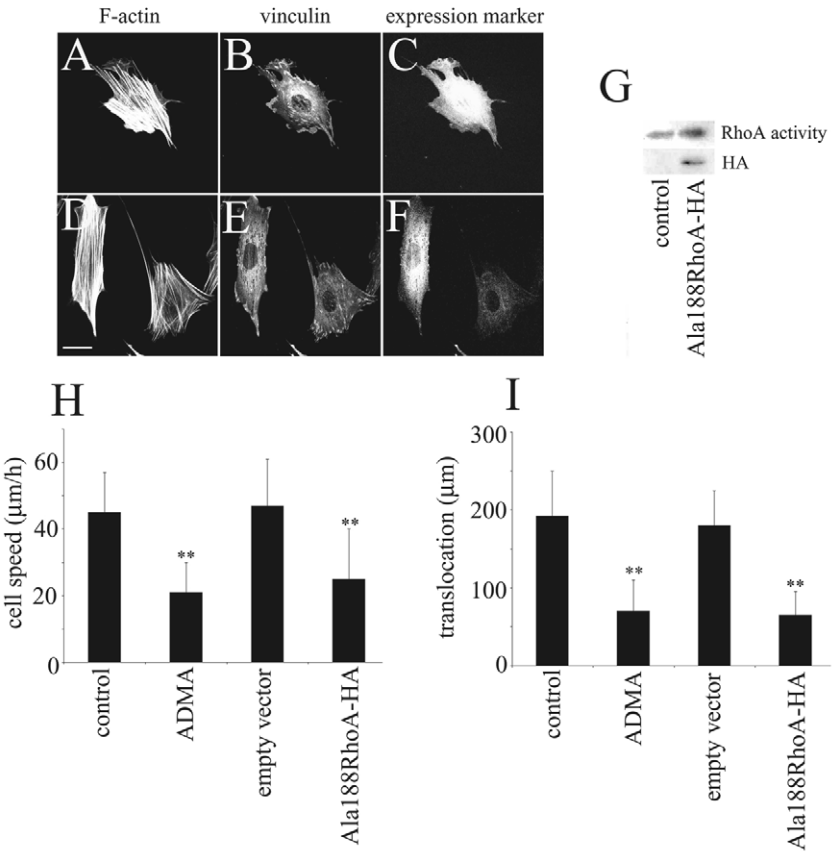


Fig. 8. The effects of overexpression of non-phosphorylatable mutant of RhoA, Ala188RhoA in PAECs. The cells were stained for F-actin (A,D), vinculin (B,E) and haemagglutinin (C,F) 24 hours after transfection. Bar, 10 μm. (G) Western blot of control cells and cells overexpressing Ala188RhoA-HA. The blot was probed for RhoA and haemagglutinin (HA), as indicated. (H,I) Cell speed and translocation in control, untreated cells, cells treated with 100 μM ADMA, cells transfected with an empty vector (transfection control) and cells transfected with Ala188RhoA. **P<0.01 (comparison with untreated controls).

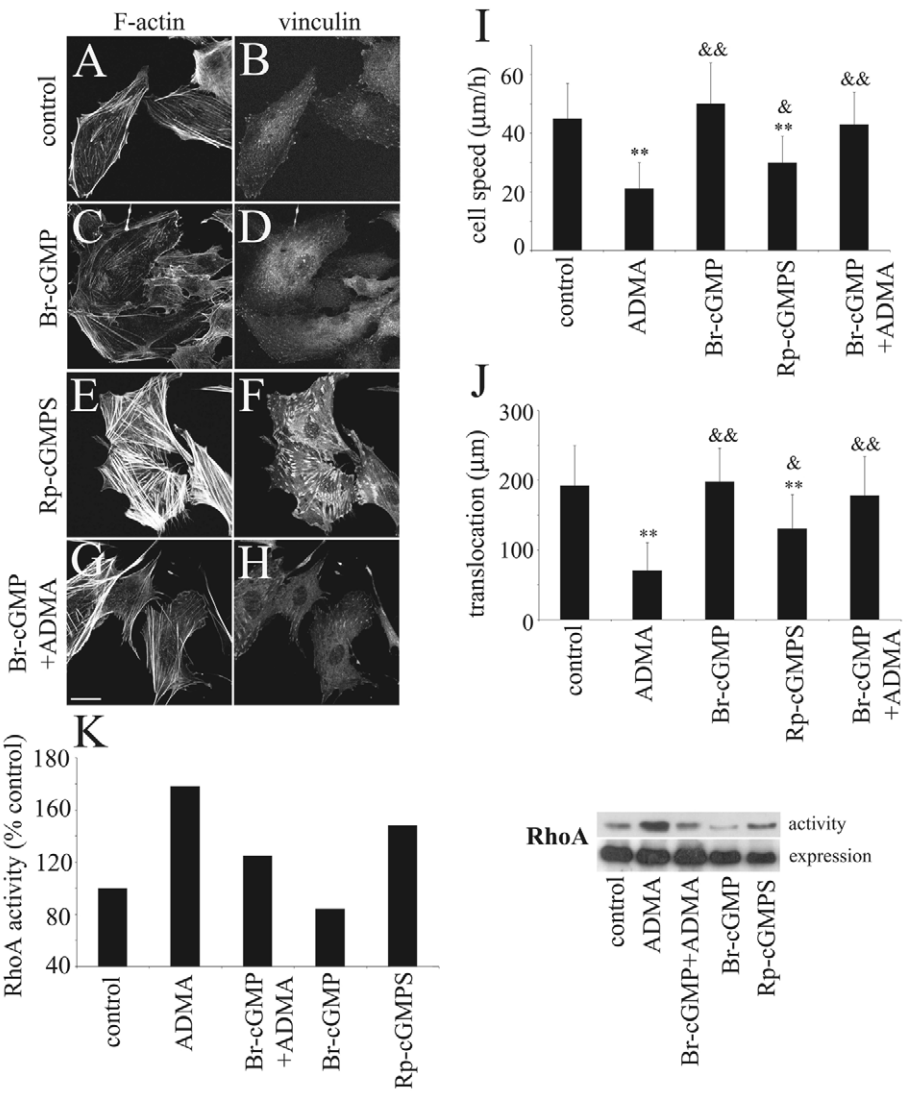


Fig. 9. The effects of the PKG activator, Br-cGMP, and PKG inhibitor, Rp-cGMPS, on untreated and ADMA-treated PAECs. (A-H) The effects of Br-cGMP and Rp-cGMPS on F-actin and vinculin distribution, in untreated cells and ADMA-treated cells, as indicated. Bar, 10 μm. (I,J) The effects of the drugs on cell speed and translocation, respectively. (K) Changes in RhoA activity in cells treated with ADMA (100 μM), ADMA (100 μM) and Br-cGMP (500 μM), Br-cGMP (500 μM) alone or Rp-cGMPS (100 nM). A representative example of a western blot is shown beside the graph. In A-H and K, ADMA was added to the cells for 20 hours while Br-cGMP and the Rp-cGMPS were added for 2 hours. In motility assays (I,J) all drugs were added to the cells at the same time and incubated for 24 hours. Values in I,J are means of three experiments whereas values in K are means of two experiments. $^{**}P<0.01$ (comparison with untreated controls). $^{\&}P<0.05$ and $^{\&\&}P<0.01$ (comparison with ADMA-treated cells).

myotonic dystrophy kinase-related Cdc42-binding kinase (Cdc42-MRCK), Wiscott-Aldrich syndrome interacting protein (WIP), alpha actinin, filamin, gelsolin-like protein, ezrin and myosin 1C (Smith et al., 2005).

In addition to its effect on RhoA activity, exogenous ADMA also decreased the activities of Rac1 and Cdc42 in PAECs but these changes were not essential for the effects of ADMA. Consistently, PMVECs from heterozygous DDAH knockout mice showed impaired motility and an increase in RhoA activity but no significant changes in the activities of Rac1 or Cdc42. DDAHI heterozygous knockout mice show abnormal vascular remodelling and reactivity characteristic of pulmonary hypertension (Leiper et al., 2007) whereas DDAHI homozygous knockouts are not viable. We show in an in vitro angiogenesis assay that that the number of microvessels sprouting from aortic rings taken from HT mice was lower than the number of vessels sprouting from the rings taken from their wild-type littermates; the response comparable to the rings that were treated with ADMA. This confirms the importance of ADMA metabolism in angiogenic responses of endothelial cells.

The role of DDAH will require further studies. Overexpression of DDAH not only prevented the effects of ADMA but also reduced the levels of stress fibres and increased persistence of the cell movement in untreated cells apparently without affecting NO production. This suggests that some of the actions of DDAH are independent of their effects on ADMA metabolism or that overexpression of DDAH in control cells increases the intracellular production of NO that was not detected using Griess assay. Interestingly, neoplastic cells transfected with DDAH and injected in vivo induced better vascularised tumours than vehicle-treated animals (Kostourou et al., 2002). Colocalisation of DDAHI with F-actin in lamellipodia and membrane ruffles in PAECs may indicate a role in protrusion formation. Localisation to lamellipodia has been reported for other proteins important in the regulation of cell motility, including Rho GTPases, protein kinases (Small et al., 2002; Amagasaki et al., 2006) as well as eNOS (Noiri et al., 1996; Bulotta et al., 2006) and tyrosine phosphorylated caveolin-1 (Beardsley et al., 2005). Interestingly, the in vivo analysis of DDAH distribution shows that this enzyme colocalises with NO-generating systems at several anatomical sites (Tojo et al., 1997).

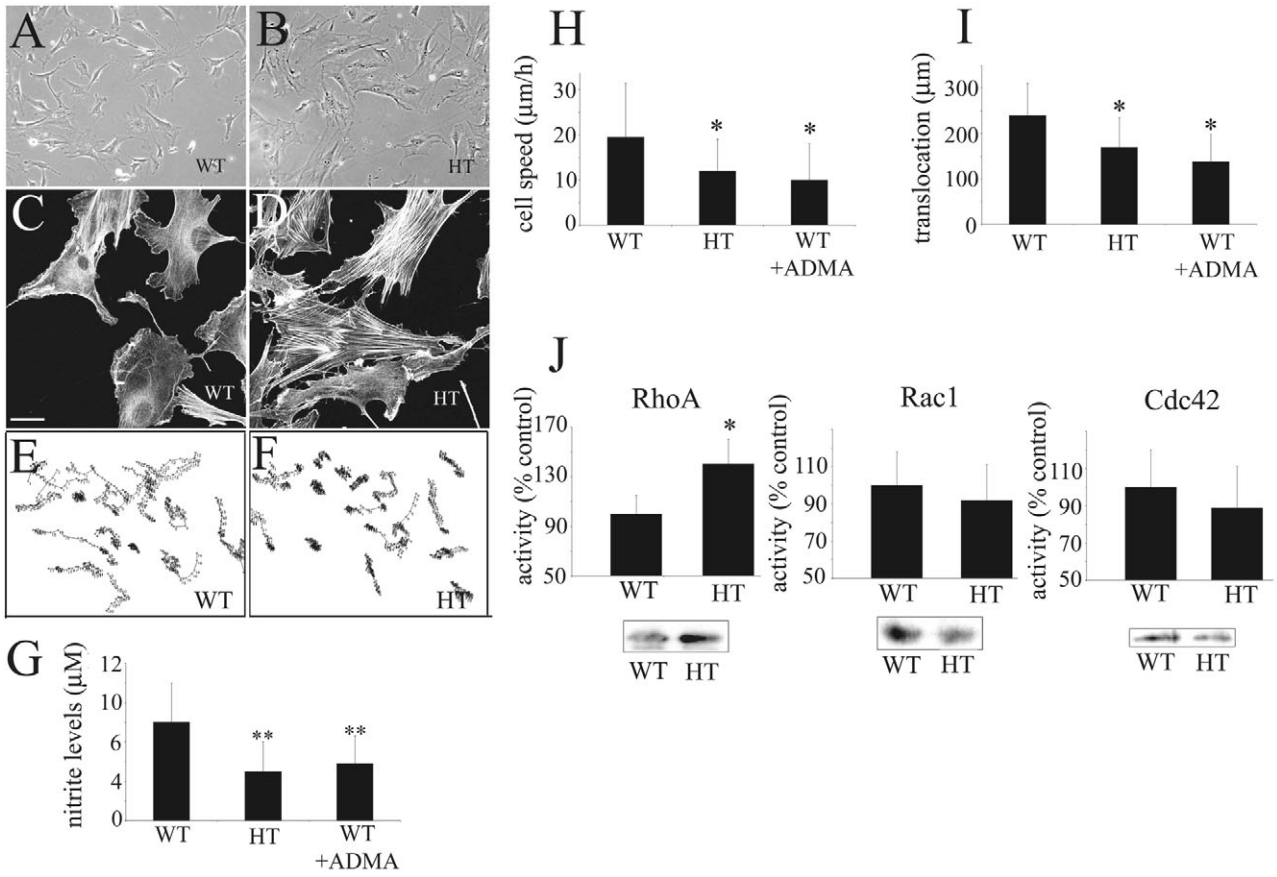


Fig. 10. Pulmonary microvascular endothelial cells from DDAHI heterozygous knockout mice (HT) show phenotypical and functional differences from cells taken from their wild-type (WT) littermates. (A,B) Phase contrast microscopy images; (C,D) fluorescent confocal images of F-actin in WT and HT cells, as indicated. (E,F) Cell trajectories, 24 hour recording. Bar, 50 μm (A,B), 20 μm (C,D) and 120 μm (E,F). (G) Changes in nitrite levels in culture medium of cells from wild-type (WT) mice, heterozygous DDAHI knockout (HT) mice, and cells from WT mice treated with ADMA (100 μM); (H,I) changes in speed and translocation of these cells. (J) Graphs and western blots showing activities of RhoA, Rac1 and Cdc42 in cultured PMVECs taken from WT and HT mice.**P<0.01; *P<0.05 (comparison with WT controls).

In summary, we demonstrated that exogenous and endogenous ADMA is an important regulator of endothelial cell motility. Manipulation of ADMA metabolism may represent a new approach to therapeutically modulating processes involving endothelial motility such as blood vessel growth and repair.

Materials and Methods

Cell culture

Porcine pulmonary artery endothelial cells (PAECs) were purchased from Cell Applications (San Diego, CA) and cultured as described previously (Wojciak-Stothard et al., 2005). The cells were plated in flasks covered with 10 μg/ml bovine fibronectin (Sigma) at the density of 1×10^4 cells/ml and used between three and four passages. In some experiments, the cells were cultured in L-arginine-free DMEM (Gibco) supplemented with 10% foetal bovine serum (FBS) for 24 hours prior to studies of cell motility.

Mouse pulmonary microvascular cells (PMVECs) were isolated from peripheral parts of the lung of DDAHI heterozygous knockout (HT) mice, purified and cultured as described previously (Kuhlencordt et al., 2004). PMVECs from normal wild-type littermates were used as controls. Heterozygous DDAHI knockout mice are to be described elsewhere.

Animals

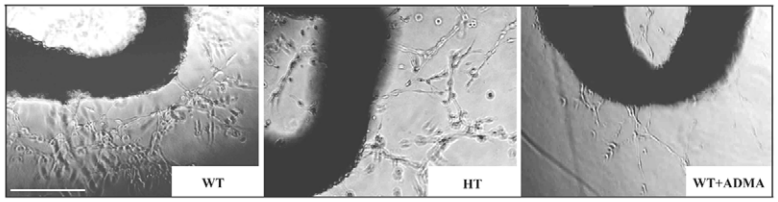
All the experiments were carried out under a Home Office Licence and conducted according to the Animal Scientific Procedures Act 1986.

Inhibitors

Asymmetric dimethylarginine (ADMA, 10-100 μM) (Calbiochem) was added to cell cultures for 1-24 hours. Symmetric dimethylarginine (SDMA, 100 μM; Calbiochem) which does not act as a NOS inhibitor was used as a negative control. In order to study the role of nitric oxide in ADMA-induced effects, NO donor S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP; 10-100 μM) was added to the cells cultured with or without ADMA. Alternatively the cells were cultured with another NOS inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME; 10 μM-1 mM; Calbiochem). In order to study the involvement of protein kinase G (PKG), the PKG activator, guanosine 3′,5′-cyclic monophosphate, 8-bromo-, sodium salt (Br-cGMP,Na; 500 μM; Calbiochem) was added to untreated or ADMA-treated cells. Alternatively, PKG inhibitor, guanosine, 3′,5′-cyclic monophosphorothioate, 8-(4-chlorophenylthio)-, Rp-isomer, triethylammonium (Rp-8-pCPT-cGMPS,TEA; 100 nM; Calbiochem) was added to untreated cells. Following the treatment with the drugs, cell movement, distribution of F-actin and focal adhesions as well as the activity of Rho GTPases RhoA, Rac1 and Cdc42 were analysed in treated and non-treated cells.

Cell migration

Spontaneous migration of endothelial cells was digitally recorded by microscopy using a time-lapse interval of 1 hour over the period of 20 hours. For migration assays the cells were plated in 3 cm Petri dishes covered with 10 μg/ml bovine fibronectin in serum-containing medium. The cells were left untreated or were treated with the drugs added to the medium 4 hours before the recording. The cells were tracked with Openlab 2 software to measure cell speed and translocation (Wojciak-Stothard and Ridley, 2003). Cell translocation was defined as a straight-line distance between the starting point and the end point of each trajectory. Movement of 60 cells was analysed for each condition in three separate experiments.



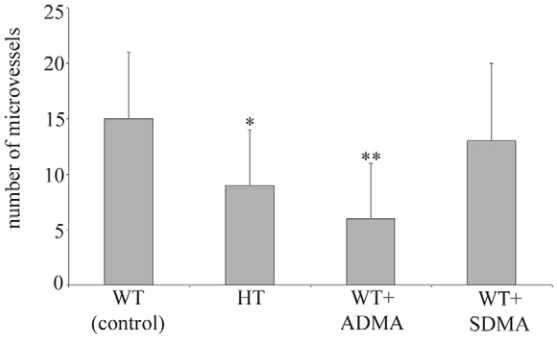


Fig. 11. Microvessel outgrowth from aortic rings from wild-type (WT) and heterozygous DDAHI knockout (HT) mice. The rings were left untreated or were treated with ADMA (100 μM) or SDMA (100 μM) in culture. Microvessel outgrowth was studied 6-7 days after plating. Bar, 500 μm.

Immunofluorescence and localization of F-actin

F-actin, focal adhesions and Myc-tagged proteins were visualised by immuno- and affinity-fluorescence methods and studied by confocal microscopy as described previously (Wojciak-Stothard et al., 2005). Anti-DDAHII and anti-DDAHI antibodies were raised in rabbits against peptides $DDAHII_{241-255}$ and $DDAHI_{238-252}$, respectively as previously described (Achan et al., 2003). The antibodies were used at a dilution 1:100 in phosphate-buffered saline (PBS).

Rho, Rac and Cdc42 GTP-binding assays

RhoA activity was measured with recombinant GST-RBD bound to glutathione beads (Upstate Biotechnology), Rac1 activity with GST-PAK1 PBD (Upstate Biotechnology) and Cdc42 activity with GST-WASP-PBD (Wojciak-Stothard et al., 2005). Affinity-precipitated RhoA, Rac1 and Cdc42 proteins were resolved by SDS-PAGE and detected by western blotting.

Construction of recombinant adenoviruses

Adenoviral gene transfer was used to express DDAHI, DDAHII, constitutively activated Rac1 (V12Rac1) and dominant negative RhoA (N19RhoA) in non-treated cells as well as in cells treated with ADMA. Adenoviral vectors for Rho GTPases were prepared and used as described previously (Wojciak-Stothard et al., 2001).

Recombinant DDAHI and DDAHII adenoviruses (AdDDAHI and AdDDAHII) were generated using the protocol from Stratagene AdEasy System (http://www.coloncancer.org/adeasy/protocol2.htm). Briefly, cDNA encoding DDAHI or DDAHII were subcloned into the transfer vector pAdTrack-CMV. The transfer vector was then cotransformed with pAdEasy-1 DNA plasmid into *E. coli* BJ5183 strain. Transformants were selected for kanamycin resistance, identified by restriction digest, purified, linearised and transfected into 293 human embryonic kidney cells (Microbix Biosystems Ltd) that express E1 genes, to allow purification of adenoviral particles.

Adenoviral vectors inducing expression of green fluorescent protein (GFP) were taken as controls. The cells were infected with adenoviruses at a multiplicity of infection (MOI) of 1000 in medium containing 2% serum for 1 hour, then the medium was replaced with culture medium containing 10% FCS. The cells were used for experiments 18-20 hours after infection.

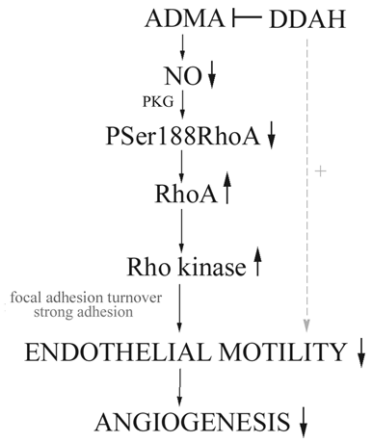


Fig. 12. Proposed signalling pathway of asymmetric methylarginine (ADMA). ADMA inhibits NO production in PAECs. This contributes to a decrease in RhoA phosphorylation on Ser188 which facilitates it membrane localisation and activation. Phosphorylation of RhoA on Ser188 is likely to be carried out by protein kinase G (PKG) but other kinases, such as protein kinase A (PKA) may also be involved. Activation of RhoA and its downstream effector, Rho kinase inhibits endothelial motility, possibly by increasing cell adhesion to the substratum or slowing down turnover of focal adhesions. Impairment of endothelial motility inhibits angiogenic responses of endothelial cells. Enzymes metabolising ADMA, dimethylarginine dimethylaminohydrolases (DDAH) improve persistence of cell movement and prevent the effects of ADMA on cells.

Expression plasmids and cell transfection

GFP-tagged expression plasmid of constitutively activated Cdc42 (L61Cdc42) and pCDNA3-GFP were a kind gift from A. Ridley (Ludwig Institute for Cancer Research, UCL, London, UK); the HA-tagged expression plasmid of Ala188RhoA (Sauzeau et al., 2000) was a kind gift from Gervaise Loirand (Universite de Nantes, Institut du Thorax, Faculte des Sciences, France). The plasmids were introduced into the cells by transfection with Metafectene™ Pro (Biontex, Martinsried, Germany) in serum-containing medium according to the manufacturer's protocol. Transfection efficiency was 20-30% and the experiments were carried out 18-20 hours following transfection. For easy identification of Ala188RhoA-HA-expressing cells during motility assays, the cells were cotransfected with pCDNA3-GFP. Expression of Ala188RhoA-HA was confirmed by western blotting with the use of a rat monoclonal anti-haemagglutinin antibody (Roche Molecular Biochemicals) (1:2000) and a HRP-labelled goat anti-rat antibody (Jackson ImmunoResearch Laboratories, Inc.) (1:3000).

Nitrite determination

The cells were grown in 24-well plates until confluence. The culture medium was then replaced with the Phenol Red-free culture medium Cellgro (Mediatech Inc., Herndon, VA) supplemented with 10% foetal calf serum and the cells were incubated with or without the inhibitors for 24 hours. To measure nitrite, a stable end product of NO degradation, an aliquot of medium (100 μl) from each culture well was mixed with 100 μl of the Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine dihydrochloride in 2% phosphoric acid). The mixture was incubated for 10 minutes at room temperature to allow the colour to develop, and the absorbance at 540 nm was measured in a microplate reader. Concentrations were determined by comparison with a sodium nitrite standard curve.

Determination of P-Ser188RhoA

The cells were left untreated or were treated with ADMA (100 μM), SDMA (100 μM), SNAP (10 μM) or both ADMA and SNAP. The cells were treated with ADMA and SDMA for 24 hours and SNAP was added 4 hours before the end of the experiment. For each condition, cells growing on three 6 cm Petri dishes were lysed and the lysates were precleared with 40 μl of protein A-Sepharose beads (Sigma) per 1 ml of the lysate. Phosphorylated RhoA was immunoprecipitated with a phospho-specific rabbit polyclonal anti-p-RhoA(Ser188)-R antibody (Santa Cruz Biotechnology) pre-adsorbed on protein A-Sepharose beads (Sigma). The protein A-Sepharose-bound immune complexes were then washed. Pellets from the immunoprecipitations were heated at 95°C for 5 minutes in 70 μl of Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting for RhoA.

Mouse aortic ring assay

Angiogenesis was studied by culturing aortic explants taken from 12-week-old heterozygous DDAHI knockout mice and their wild-type littermates as described previously (Masson et al., 2002) with some modifications. Briefly, thoracic aortas

were removed from mice, cleaned from peri-aortic fibroadipose tissue and sectioned into 1-mm long aortic rings. The rings were washed in PBS and placed in 96-well plates covered with three-dimensional Culturex Basement Membrane Extract (R&D Systems) prepared in advance according to the manufacturer's instructions. The rings were left untreated or were incubated for 2 days in RPMI medium supplemented with 10% BCS before ADMA (100 μM) or SDMA (100 μM) were added to the culture medium. The number of sprouting microvessels was analysed 6 days later using a phase contrast microscope. On average, 15 rings were obtained from one aorta and we used eight rings for each condition in three separate experiments.

Statistical analysis

All the experiments were performed in triplicate. Data are presented as means ± standard deviation (s.d.). Comparisons between two groups were carried out with two-tailed Student *t*-tests. When more than two conditions were being compared, a one-way ANOVA test followed by Dunnett's post-test was used. Statistical significance was accepted for $P<0.05$ and all tests were performed with GraphPad Prism version 3.0.

The authors wish to thank Anne Ridley (Ludwig Institute for Cancer Research, University College London) for adenoviral constructs of mutant Rho GTPases, and Gervaise Loirand (Universite de Nantes, Institut du Thorax, Faculte des Sciences, Nantes, France) for expression plasmid for A188RhoA-HA. The work was supported by the British Heart Foundation grants PG/03/081/15732 and PG/02/165 as well as European Vascular Genomics Network LSHM-CT-2003-503254.

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