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The ADMA/DDAH Pathway Regulates VEGF-mediated Angiogenesis

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Short title: ADMA/DDAH regulates VEGF-mediated Angiogenesis

Word count of body: 5460

Word count of abstract: 191

Number of figures: 6

Objectives – Asymmetric dimethylarginine (ADMA) is a nitric oxide synthase (NOS) inhibitor and cardiovascular risk factor associated with angiogenic disorders. Enzymes metabolising ADMA, dimethylarginine dimethylaminohydrolases (DDAH) promote angiogenesis, but the mechanisms are not clear. We hypothesized that ADMA/DDAH modifies endothelial responses to vascular endothelial growth factor (VEGF) by affecting activity of Rho GTPases, regulators of actin polymerization and focal adhesion dynamics. *Methods and Results* – The effects of ADMA on VEGF-induced endothelial cell motility, focal adhesion turnover and angiogenesis were studied in human umbilical vein endothelial cells (HUVECs) and DDAH I heterozygous knockout mice. ADMA inhibited VEGF-induced chemotaxis in vitro and angiogenesis in vitro and in vivo in a NO-dependent way. ADMA effects were prevented by overexpression of DDAH, but were not associated with decreased proliferation, increased apoptosis or changes in VEGFR-2 activity or expression. ADMA inhibited endothelial cell polarisation, protrusion formation and decreased focal adhesion dynamics, resulting from Rac1 inhibition following decrease in phosphorylation of vasodilator stimulated phosphoprotein (VASP). Constitutively active Rac1 and, to a lesser extent dominant negative RhoA abrogated ADMA effects in vitro and in vivo. *Conclusion* - The DDAH/ADMA pathway regulates VEGF-induced angiogenesis in a NO- and Rac1 dependent manner.

Key Words: Rho GTPase, ADMA, DDAH I, VEGF, angiogenesis

Condensed Abstract: The endogenous NO synthase inhibitor and cardiovascular risk factor ADMA, inhibits angiogenesis. We show for the first time, that ADMA inhibits VEGF-induced endothelial cell motility and angiogenesis by modulating NO-dependent Rho GTPase activity. Further, normal angiogenic responses to VEGF are restored by over-expression of DDAH or active Rac1.

High plasma levels of ADMA are associated with cardiovascular disorders including atherosclerosis, hypertension, homocysteinemia, diabetes mellitus, insulin resistance and hypercholesterolemia.^{1,2} ADMA and the related methylarginine analogue monomethylarginine (L-NMMA) inhibit NO production by competing with L-arginine for binding to NOS.² Levels of methylarginines are regulated by the enzymes DDAH I and II, which metabolise these factors.² DDAH I heterozygote (HT) knockout mice have raised ADMA levels and a hypertensive phenotype as a consequence of reduced NO signalling.³ Further, dysregulation of the ADMA/DDAH pathway is associated with impaired angiogenesis, which contributes to disease pathology. In a mouse model of hypercholesterolemia, DDAH I activity is inhibited by lipid-induced oxidative stress, leading to elevated ADMA levels, reduced NO production and impaired angiogenesis.⁴ ADMA metabolism through DDAH over-expression promotes angiogenesis *in vitro*,^{5,6} promotes sprouting from aortic rings,^{7,8} and enhances angiogenesis in tumours and following ischemia^{6,9} but the mechanisms are not fully understood.

ADMA/DDAH pathway may influence angiogenesis by affecting expression of vascular endothelial growth factor (VEGF). DDAH over-expression enhances VEGF expression in C6 glioma cells⁶ and in human and murine endothelial cell lines.⁵ ADMA/DDAH may also influence VEGF responses by affecting NO bioavailability. NO signalling is critically involved in VEGF-mediated chemotaxis and angiogenesis.^{10,11} NO production is increased through a mechanism requiring VEGFR-2 activation, which may also involve upregulation of eNOS, increased cytosolic calcium, and/or phosphatidyl inositol 3 kinase (PI3K) activation.^{10,11} Changes in NO levels affect the activity of Rho GTPases, key regulators of actin dynamics.¹² Coordinated activation of Rho GTPases Rac1, RhoA and Cdc42 is required for cell motility.¹² Rac1 and Cdc42 mediate focal contact/complex formation and polymerization of the actin cytoskeleton at the leading edge of the cell, while RhoA controls stability of focal adhesions, stress fibre formation in the main body of the cell, and retraction at the rear.¹² ADMA activates RhoA and inhibits Rac1 and Cdc42 in

pulmonary endothelial cells, leading to inhibition of spontaneous endothelial cell motility and vessel sprouting from aortic rings.⁷ Rho GTPases also play an essential role in regulating motility and angiogenesis in response to growth factors including VEGF, although the requirement for individual GTPases appears to depend on the experimental model of angiogenesis.¹³⁻¹⁶ RhoA is required for VEGF-induced angiogenesis in Matrigel plugs¹³ while Rac1 and Cdc42 but not RhoA are important in phorbol ester- and growth-factor- induced angiogenesis in collagen.¹⁴ VEGF-mediated motility requires Rac1 activity,¹⁵ but the role of RhoA is controversial.^{13,15,16}

We hypothesized that ADMA metabolism alters VEGF signalling via its effects on Rho GTPases. We show for the first time that ADMA inhibits VEGF-induced endothelial chemotaxis and angiogenesis in vitro and in vivo. These effects result from reduced NO signalling and inhibition of pSer239VASP and Rac1, important in cell polarization and focal adhesion turnover.

Methods

Cell culture and treatments

Primary human umbilical vein endothelial cells (HUVEC, PromoCell) were cultured in endothelial growth medium containing 2 % foetal calf serum (FCS) and growth supplements (C-22010, Promocell). For experiments, the cells were incubated in starvation media containing 1% FCS and no growth supplements, or where stated, in L-arginine-free media (PromoCell). ADMA (100 μ M, Calbiochem, Merck Biosciences, Germany) or N^G-Nitro-L-arginine Methyl Ester (L-NAME, 1 mM, Calbiochem) were added to the medium for 16 hours prior to measurement of cell responses. S-nitroso-N-acetylpenicillamine (SNAP, 10 μ M, Calbiochem) and human recombinant VEGF-165 (25 ng/ml, R&D Systems, Abingdon, UK) were added for the duration of experiments. cGMP in cell lysates was measured after treatment with VEGF for 5 minutes, using a commercial kit (Biotrak EIA system, RPN226, GE Healthcare, for details please see www.ahajournals.org).

Adenovirus Construction and Transfection

Adenoviral gene transfer was used to over-express DDAH I, RhoAN19, Rac1V12 or Cdc42V12 or GFP (transfection control) as described at a multiplicity of infection (MOI) of 200.⁷

Analysis of VEGF, VEGFR-2 and cleaved caspase-3

Soluble human VEGF secretion was quantified using an ELISA kit (QIA51, Calbiochem).

VEGFR-2, phospho(Tyr1175)-VEGFR-2 and cleaved caspase-3 (Asp175) were measured using sandwich ELISA kits (Cell Signaling, New England Biolabs, UK) (for details please see

www.ahajournals.org).

RhoA, Rac1 and Cdc42 GTP-binding

Active RhoA, Rac1 and Cdc42 was measured by affinity precipitation and detected by Western blotting as previously described.⁷

Cell proliferation and migration

Migration assays were performed in Transwell dishes and the optical density of 6 random fields per filter (triplicate samples) was analysed in Adobe Photoshop. Proliferation was evaluated in HUVECs cultured for 2 days in serum-reduced media (1 % FCS) by colorimetric analysis using CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, Madison, WI).

Both assays were validated by manual counting of cells (for details please see www.ahajournals.org).

Focal adhesion analysis

Focal adhesions were visualised in HUVECs by immunofluorescence using anti-vinculin antibodies. Alternatively, live HUVECs were transfected with pDsRed2-N1-Zyxin and seeded in optical bottom 96-well plates (for details please see www.ahajournals.org). Images were recorded using confocal microscopy and processed using LaserPix software. Cell adhesions in the first frame (t = 0) were labelled red, and in the last, were labelled green (pseudocolours, t = 16 minutes). Protrusions and cell elongation (circularity of a fitted ellipse) were analysed using Image

J software (National Institute of Health). Focal adhesion turnover was calculated as the proportion of remodelling adhesions (green and red) to constant adhesions (yellow).

Collagen I in vitro angiogenesis assay

Cells were seeded onto 24-well plates (5×10^4 per well) pre-coated with PureCol Collagen I solution (Nutacon BV, Leimuiden, Netherlands, 2 mg/ml, 300 μ l/well). After 24 hours at 37°C, the cells were fixed (4 % formaldehyde/PBS) and images from 4 random fields were analysed. Total tubule length was calculated using Image J software.

Basement membrane plug angiogenesis assay

All experiments were carried out under a Home Office Licence and conducted according to the Animal Scientific Procedures Act 1986. Eight week-old wildtype (WT) or DDAHI heterozygous (HT) mice³ were injected subcutaneously in both flanks with Cultrex Matrigel basement membrane extract (R&D Systems) and 500 ng/ml VEGF,¹⁷ and with adenoviruses where indicated (4×10^8 plaque forming units / ml Matrigel). After 7 days, mice (n=5) were sacrificed and plugs recovered. Histological sections were stained with toluidine blue or antibodies against PECAM-1, with DAPI nuclear counter-stain. Angiogenesis was quantified by measuring haemoglobin (Hb) content in plugs (for details please see www.ahajournals.org).¹⁸

Retrovirus Construction and Transfection of VASP mutants

Retroviral constructs encoding human VASP mutants (S239A-VASP and S239D-VASP), were inserted into the retroviral vector LXS.N. Retroviral stocks were generated by co-transfection with pVSVG (Clontech) and pGag-pol (Clontech) into HEK 293T cells and used to infect HUVEC. HUVECs stably expressing transgenes were selected with G418 (0.6mg/ml, Sigma). Expression of vesicular stomatitis virus glycoprotein (VSVG)-tagged VASP were assessed by Western blotting and distribution of pSer239-VASP in HUVECs by immunostaining with mouse monoclonal anti-pSer239VASP antibody (1:100, Upstate Biotechnology) (for details please see www.ahajournals.org).

Statistical analysis

Data are expressed as means \pm SEM. Differences among groups were assessed by ANOVA followed by post-hoc tests. A p value <0.05 was taken as statistically significant, n=3, unless stated otherwise.

Results

ADMA inhibits VEGF-induced chemotaxis and capillary network formation in vitro.

Cell migration across Transwell filters in basal conditions (no VEGF) was 4.5 ± 0.5 cells/200 μm^2 in 4-hour assay and increased 2-fold upon the addition of VEGF ($p < 0.001$, Figure 1A and 1B). Exogenous ADMA (100 μM) did not influence basal cell migration, but reduced VEGF response to control levels ($p < 0.001$, Figure 1A and 1B). Lower dose of ADMA (10 μM) had no effect on VEGF-mediated migration in basal culture medium, but had a significant inhibitory effect in arginine-free culture medium (please see Supplemental Figure I in www.ahajournals.org). Over-expression of DDAH I restored the chemotactic response towards VEGF in the presence of ADMA (Figure 1A and 1B).

ADMA impairs VEGF-induced angiogenesis in vitro and in vivo.

To study the effects of ADMA on early stages of VEGF-induced angiogenesis, we used a collagen I tube formation assay. Within 24 hours after plating, endothelial cells began to align and form a network of tubular structures. While untreated cells showed little evidence of tube formation, VEGF induced formation of extensive cell networks, increasing tubulogenesis by 150 %, compared to controls ($p < 0.001$) (Figure 2A, B). ADMA (100 μM) had no effect in basal conditions, but reduced VEGF effects by 40 % ($p < 0.01$, Figure 2A and 2B).

To investigate the relevance of these observations in vivo, we analysed blood vessel formation in Matrigel plugs co-injected with VEGF in DDAH I heterozygous (HT) knockout mice. These mice

have raised ADMA levels and show abnormal cardiovascular responses as a result of decreased NO signalling.³ VEGF-mediated angiogenesis was severely impaired in DDAH I HT mice compared to their wildtype littermates (Figure 2C-E). The presence of blood vessels was confirmed by immunofluorescent staining of platelet endothelial cell adhesion molecule-1 (PECAM-1) (Supplemental Figure II in www.ahajournals.org). In the absence of VEGF, angiogenesis was minimal (Figure 2E). The analysis of haemoglobin levels confirmed that VEGF increased blood supply into the plug, and that this was significantly impaired in DDAH I HT mice compared to WT (83 % decrease, $p < 0.05$, Figure 2E and Supplemental Figure II in www.ahajournals.org). When VEGF was co-injected with DDAH I adenoviruses into HT mice, angiogenesis was enhanced by 197 % ($p < 0.05$, HT compared to HT + DDAH I, Figure 2E). Sustained adenovirus-mediated overexpression of DDAH I was confirmed by Western blotting (Supplemental Figure II in www.ahajournals.org).

ADMA effects are mediated by inhibition of NO signalling, and are independent of proliferation and apoptosis.

Since VEGF-mediated motility and angiogenesis require NO, we hypothesized that ADMA effects on VEGF-induced angiogenesis may result from inhibition of NO signalling. L-NAME, a NOS inhibitor structurally unrelated to methylarginines, inhibited VEGF-mediated chemotaxis to the same extent as ADMA ($p < 0.001$ compared to VEGF), while the NO donor, SNAP significantly reduced the effects of ADMA, confirming previously reported role NO in ADMA signalling (please see Supplemental Figure III in www.ahajournals.org).⁷ Intracellular levels of cGMP, a downstream effector of NO, were reduced by ADMA in control and VEGF-treated group. DDAH did not significantly change cGMP levels in basal conditions but prevented ADMA effects in VEGF-treated group (Supplemental Figure III). Changes in cGMP were consistent with previously reported changes in nitrite levels in culture media induced by ADMA/DDAH.^{7,19}

Endothelial cell proliferation and apoptosis are important in the regulation of angiogenic responses. VEGF enhanced endothelial cell proliferation by 34 % ($p < 0.001$, compared to no treatment) but ADMA had no effect on VEGF-mediated proliferation within 48 hours of treatment (Supplemental Figures III and IV). It has recently been reported that ADMA is pro-apoptotic.²⁰ However, while VEGF inhibited cell apoptosis by 27 %, ($p < 0.05$ compared to no treatment), ADMA had no significant effect on apoptosis regardless of the presence of serum, growth factor supplements or VEGF (Supplemental Figure III).

ADMA prevents VEGF-induced protrusion formation, focal adhesion turnover and reduces cell polarity.

Inhibition of basal NO production by endothelial cells is associated with decreased motility and enlarged focal adhesions, that may result from imbalance in the assembly and disassembly of cell attachments.^{7,21} We hypothesized that ADMA would affect motility by altering focal adhesion turnover and distribution. Within one hour after seeding, VEGF promoted formation of lamellipodia and peripheral localisation of the focal adhesion protein, vinculin (Figure 3A). ADMA reduced peripheral vinculin localisation, while over-expression of DDAH I restored the VEGF-induced phenotype (Figure 3A). At later stages of cell spreading, ADMA-treated cells showed accumulation of enlarged central focal adhesions, the effect prevented by DDAH (Figure 3B). To study focal adhesion turnover and protrusion formation in live endothelial cells, the cells were transfected to express fluorescently-labelled zyxin, a marker of intermediate/late focal adhesions.²² Image analysis of time-lapse confocal images was used to quantify the numbers of remodelled focal adhesions and extension/retraction of the cell periphery (please see Supplemental Figure IV in www.ahajournals.org). VEGF promoted protrusion formation and focal contact remodelling and these effects were significantly inhibited by ADMA and rescued by over-expression of DDAH I (Figure 3C). Migratory endothelial cells exhibit an elongated, polarized

morphology. ADMA prevented cell elongation in both control and VEGF-treated groups (Figure 3D).

ADMA alters Rho GTPase activation independently of VEGFR-2 activation.

DDAH II was reported to increase VEGF mRNA in endothelial cell lines.⁵ However we did not observe any measurable changes in VEGF protein expression in HUVECs over-expressing DDAH I (data not shown). Since VEGFR-2 mediated signalling is involved in NO dependent endothelial cell responses to VEGF,¹⁰ we tested whether ADMA could affect VEGFR-2 expression or activation. However, neither activity nor expression of VEGFR-2 was affected by either ADMA or DDAH I (please see Supplemental Figure V in www.ahajournals.org).

ADMA impairs focal adhesion remodelling and angiogenesis via Rac1.

The effects of ADMA on VEGF-induced cell motility are likely to be mediated by Rho GTPases, important regulators of actin dynamics. ADMA reduced the activity of Rac1 and Cdc42 and increased the activity of RhoA in both control and VEGF-treated cells, consistent with its effects on pulmonary endothelial cells under basal conditions (Figure 4A, B).⁷ To further investigate the role of Rho GTPases in the effects of ADMA, mutant Rho GTPases were overexpressed by adenoviral gene transfer. Constitutively active Rac1 (Rac1V12) prevented inhibition of focal adhesion remodelling by ADMA, restoring it to similar levels as VEGF, while constitutively active Cdc42 (Cdc42V12) or dominant negative RhoA (RhoAN19) had no effect (Figure 4C). To investigate how these observations might relate to angiogenesis *in vivo*, Matrigel plugs implanted in DDAHI HT mice were co-injected with VEGF and Rho GTPase mutants. Rac1V12 significantly enhanced angiogenesis in DDAH I HT mice, increasing haemoglobin (Hb) content in plugs from 32 +/- 26 to 559 +/- 97 (arbitrary units, $p < 0.01$, Figure 4D), comparable to Hb levels in wildtype mice either without (513 +/- 138) or with Rac1V12 (554 +/- 110, Figure 4D). Over-

expression of RhoAN19 in wildtype mice did not affect VEGF-induced angiogenesis (Fig. 4D), while in DDAH1 HT knockout mice, some noticeable, though statistically not significant increase was observed ($p = 0.081$, Figure 4D). Over-expression of Cdc42V12 did not influence VEGF-induced angiogenesis in either wildtype or DDAH I HT knockout mice (Figure 4D).

ADMA reduces Rac1 activity in pulmonary endothelial cells by decreasing NO/PKG-mediated phosphorylation of VASP on Ser239.¹⁹ Consistently, ADMA decreased VASP phosphorylation on Ser239 in HUVECs treated with VEGF (Figure 5A-D). The upper and lower band of the VASP doublet, which is detected by anti-pSer239 antibody is Ser157-phosphorylated and -non-phosphorylated protein, respectively (Figure 5C).²³ Overexpression of mutant of VASP (S239A-VASP), in which the preferred PKG site is blocked from phosphorylation, inhibited tube formation to the same extent as ADMA (Figure 5E). Conversely, a phosphomimetic VASP mutant (S239D-VASP) increased tube formation in control cells and cells treated with VEGF and ADMA, while nonphosphorylatable VASP mutant (S239A-VASP) decreased tube formation, mimicking the effects of ADMA (Fig.5E). The small reduction in tube formation in VEGF-treated cells by S239D-VASP compared to untreated vector control, may indicate that dynamic changes in VASP phosphorylation are important in this response. Expression of VSVG-VASP mutants was confirmed by Western blotting (please see Supplemental Figure V in www.ahajournals.org). Overexpression of S239D-VASP increased Rac1 activity in HUVECs (Supplemental Figure V). Rac1 can act as an upstream regulator of RhoA activity²⁴ and therefore we verified its role in ADMA-induced effects on RhoA. Overexpression of constitutively activated Rac1 (V12Rac1) inhibited RhoA activity in control (untreated) HUVECs but did not abolish RhoA activation by ADMA (Supplemental Figure VI in www.ahajournals.org).

Discussion

We show for the first time that a functional DDAH I pathway is required for the angiogenic response to VEGF, by regulating Rho GTPase activity. Our in vitro studies indicate that ADMA, a substrate for DDAH, impairs endothelial cell responses to VEGF by reducing NO dependent chemotaxis and focal adhesion turnover, and abrogating protrusion formation, cell polarisation and alignment into capillary networks. ADMA does not affect VEGFR-2 activation or expression, but inhibits VEGF-mediated phosphorylation of VASP and activation of Rac1. Focal adhesion turnover and angiogenesis in vivo are restored by increasing Rac1 activity, indicating a key role for this GTPase as a target in ADMA-induced angiogenic pathologies.

Angiogenic morphogenesis requires tightly controlled regulation of apoptotic and proliferative pathways. In our studies, ADMA did not modulate the effects of VEGF on cell proliferation or apoptosis, in contrast to other reports showing anti-proliferative and pro-apoptotic effects.^{20,25} While these discrepancies may result from the use of different cell types and culture conditions, it is unlikely that pro-apoptotic effects of ADMA play a role in our system; we observed significant short-term effects of ADMA on chemotaxis in vitro, and in vivo substantial numbers of endothelial cells accumulated in Matrigel plugs in DDAH I HT mice in spite of high tissue and plasma levels of ADMA. DDAH I over-expression also did not induce secretion of soluble VEGF, nor did it affect VEGFR-2 activation or expression. These differences are likely to be isoform-specific as DDAH II but not DDAH I over-expression induces VEGF mRNA and secretion in endothelial cells.^{5,26}

The dose of ADMA used in our in vitro studies was relatively high (100 $\mu\text{mol/L}$) while lower doses were only effective in media deprived of NOS substrate, L-arginine, confirming the involvement of NO signalling. In vivo, heterozygous DDAH I gene deletion in mice increases plasma concentration of ADMA 0.5–0.7 $\mu\text{mol/L}$ similar to the levels reported in patients with multiple cardiovascular risk factors.³ We found that these mice showed decreased angiogenic

responses to VEGF. It is likely that local changes in ADMA concentrations in cells and tissues are higher than estimated. Plasma levels of ADMA vary from 0.2-1 $\mu\text{mol/L}$ but tissue levels may reach 15 $\mu\text{mol/L}$.²⁷ Another likely mechanism to explain the effect of low concentrations of ADMA in vivo may involve endothelial progenitor cells (EPCs). EPCs are important in neoangiogenesis and their mobilization, differentiation, and function are inhibited by plasma concentrations of ADMA.²⁵

Under basal conditions, ADMA has been shown to activate RhoA and inhibit Rac1 in a NO/protein kinase G (PKG)-dependent manner,^{7,19} and we observed similar effects in the presence of VEGF. Inhibition of Rac1 by ADMA appears to be particularly deleterious to VEGF-induced angiogenic responses. This may reflect a more critical role played by Rac1 in angiogenesis. Endothelial-specific deletion of Rac1 is lethal at embryonic day 9.5 due to aberrant angiogenesis, and Rac1-deficient endothelial cells fail to form lamellipodia, migrate and undergo angiogenic morphogenesis in response to VEGF.²⁸ Further, and reminiscent of the DDAH I HT phenotype,³ endothelial-specific downregulation of Rac1 results in mild hypertension and impaired angiogenesis as a result of decreased NO signalling.²⁹ In support of the role of NO/Rac1 in ADMA effects, VEGF-mediated chemotaxis was restored by NO donors and over-expression of constitutively activated Rac1 restored focal adhesion remodelling and angiogenesis in DDAH I HT knockout mice. The mechanism by which Rac1 regulates angiogenesis is likely to involve control of both cell-cell and cell-matrix interactions. Cell-matrix interaction induces dynamic cellular protrusions followed by the assembly of endothelial cell aggregates and rudimentary cords, requiring Rac1-dependent integration of both actin remodelling and microtubule dynamics.³⁰ These dynamic protrusions are thought to be critical in supporting proper cell-cell contact and alignment in capillary morphogenesis.³⁰ In line with a Rac1 dependent mechanism therefore, we observed that ADMA abrogated lamellipodia formation, cell polarisation and

capillary morphogenesis *in vitro*, while *in vivo*, endothelial cells were able to invade Matrigel plugs in DDAH I HT mice, but failed to align and coalesce into capillary vessels.

Although Rac1 appears more important in control of VEGF-induced angiogenesis, a role of RhoA can not be completely excluded. RhoA inhibition increased remodelling of focal adhesions in basal conditions, possibly as an effect of decreased adhesion to the substratum,⁷ and increased blood supply in Matrigel plugs in DDAH I HT knockout mice. RhoA may contribute to inhibition of VEGF-mediated angiogenesis by regulating mechanical remodelling of extracellular matrix (ECM) and matrix invasion.¹³ Rac1 reduced RhoA activity in basal conditions but did not abolish RhoA activation by ADMA, which suggests that RhoA activity is controlled by two different mechanisms. Rac1 is thought to inhibit RhoA by activation of p190 Rho GTPase activating protein³¹ while NO/PKG inhibit RhoA by increasing phosphorylation on Ser188, which enhances its interaction with Rho guanine-dissociation inhibitor.³²

VEGFR-2 mediates the effects of VEGF on endothelial motility.¹¹ Interestingly, while ADMA did not prevent VEGFR-2 autophosphorylation, it may affect downstream signalling pathways in a spatially dependent manner, by preventing VEGFR-2 localisation to lamellipodia.^{33,34} Rac1 regulates lamellipodia formation,^{12,35,36} and a decrease in its activity may prevent proper localisation of activated VEGFR-2. In support of this, we observed reduced localisation of VEGFR-2 in lamellipodia in HUVECs treated with ADMA following monolayer wounding (Supplemental Figure VII in www.ahajournals.org).

ADMA inhibits Rac1 activity in pulmonary endothelial cells by decreasing NO/cGMP-dependent phosphorylation of vasodilator activated phosphoprotein (VASP) on Ser239.¹⁹ VASP regulates actin dynamics and its localisation and activity is associated with Rac1 activation.^{23,37} In control and VEGF-treated HUVECs, ADMA decreased VASP Ser239 phosphorylation, while non-phosphorylatable mutant of VASP mimicked the effects of ADMA on endothelial tube formation *in vitro*, supporting a role upstream of Rac1 in ADMA-induced effects on angiogenesis.

In further support of our data, VASP phosphorylation by PKG has recently been shown to enhance angiogenesis³⁸ and increase vascular smooth muscle cell matrix invasion (Thomas Renné, unpublished observation). Full characterization of the effects of Rac1/RhoA activity imbalance induced by ADMA on angiogenesis will require further studies.

In summary, our observations indicate that the DDAH I/ADMA pathway plays a critical role in regulating VEGF-mediated endothelial cell motility and angiogenesis, and that altered NO signalling and Rho GTPases activity are central to angiogenic regulation by DDAH I/ADMA.

Acknowledgments: We thank Dr Anna Huttenlocher (University of Wisconsin-Madison, Madison, Wisconsin) for the gift of pDsRed2-N1-Zyxin and Dr Paul Frankel (Rayne Institute UCL) for the gift of pVSVG and pGag-pol.

Sources of Funding: British Heart Foundation grant PG/07/004

Disclosures: None

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Figure 1. ADMA inhibits VEGF-induced chemotaxis (A, B). Representative images (A) and quantitation (B) of transmigrated cells are shown in the absence (white bars) or presence of VEGF (black bars), expressed as % of untreated controls. ** p<0.01, *** p<0.001, n=3.

Figure 2. ADMA impairs VEGF-induced angiogenesis in vitro and in vivo. (A) shows representative images of tube formation on collagen I and (B) shows quantitation of tube formation. (C) shows Matrigel plugs from wildtype (WT) and DDAH I heterozygous knockout (HT) mice and (D) plug sections stained with toluidine blue (inset: magnified cross-section of a vessel). Arrows point to vessel formation. (E) shows haemoglobin content in plugs (n=5, * p<0.05). All plugs contained VEGF except for WT control group shown in (E).

Figure 3. ADMA inhibits polarisation and focal adhesion turnover. Cells were fixed one (A) or sixteen hours (B) after plating and focal complexes/adhesions were visualised by immunofluorescence. Remodelling index of focal adhesions marked with zyxin-red is shown in (C), while changes in cell elongation are shown in (D) (n = 10). * p<0.05, ** p<0.01, comparisons with VEGF+ADMA group.

Figure 4. ADMA impairs focal adhesion turnover and angiogenesis by altering Rho GTPase activity. (A, B) are representative images of Western blots showing activity changes of RhoA, Rac1 and Cdc42 in untreated cells or cells treated with ADMA and VEGF (1 hour), as indicated. Densitometric analysis of the blots is shown below (n=3), * p<0.05. (C) and (D) show the effects of mutant Rho GTPases on focal adhesion remodelling and the in vivo angiogenesis, respectively.

Mutant RhoGTPases were overexpressed via adenoviral gene transfer as described in Materials and Methods (n=5). In (D) white bars show measurements in wildtype (WT) mice, and black bars in DDAH heterozygous knockout (HT) mice. * p<0.05, ** p<0.01

Figure 5. ADMA inhibits angiogenesis by inhibiting VASP activation. After treatment with VEGF (10 minutes), pSer239VASP was assessed by immunofluorescent staining (A), with fluorescence intensity shown in (B) * p< 0.05, ** p< 0.01, (n = 5). Changes in VASP phosphorylation on Ser239 were confirmed by Western blotting (C), with densitometric analysis shown in (D), (n=2). In (E), tube formation was assessed in cells expressing S239D-VASP and S239A-VASP (black bars). Uninfected (white bars) and empty vector controls (grey bars) are also shown. ** p< 0.01, comparisons between empty vector group and VASP mutants groups.

Figure 1

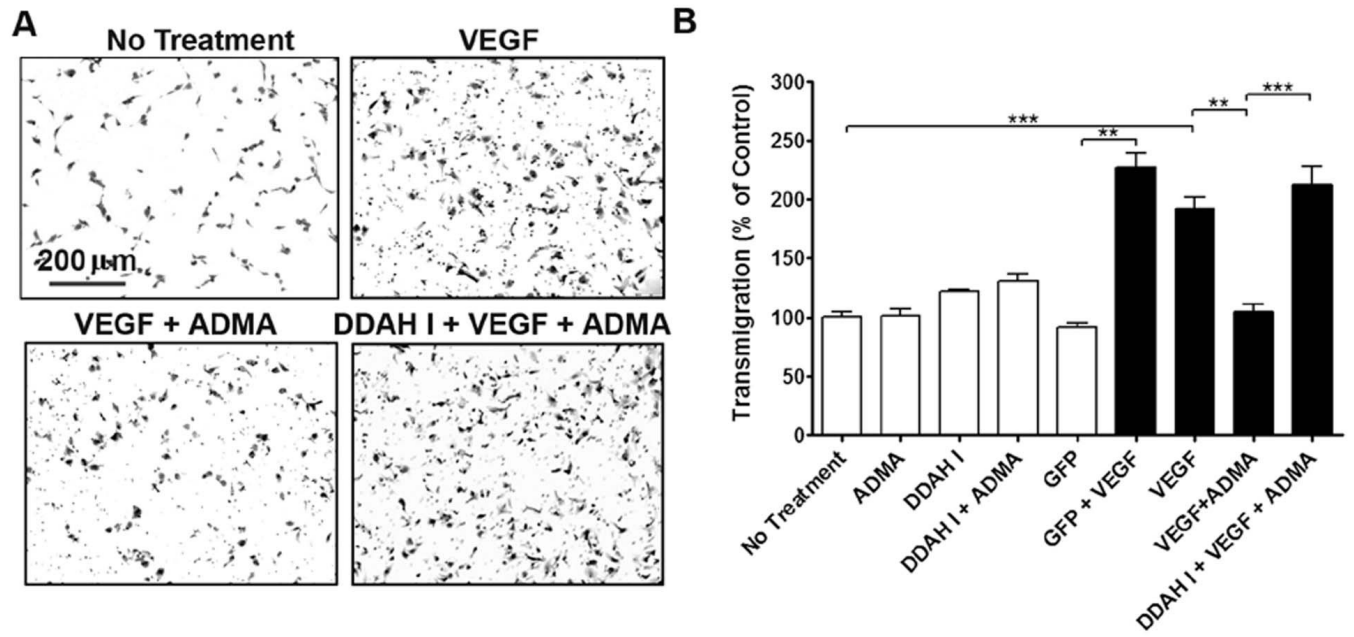


Figure 2

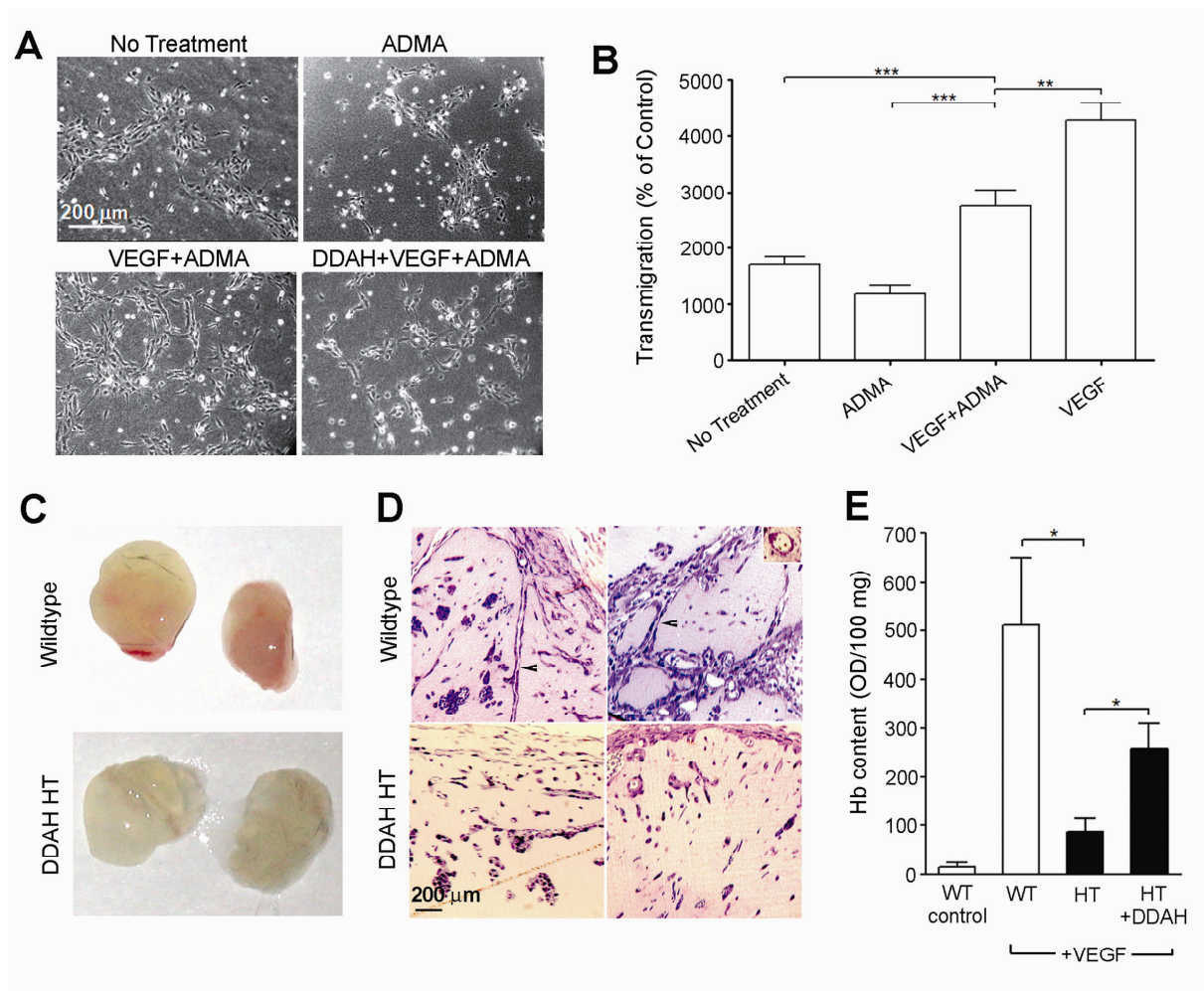


Figure 3

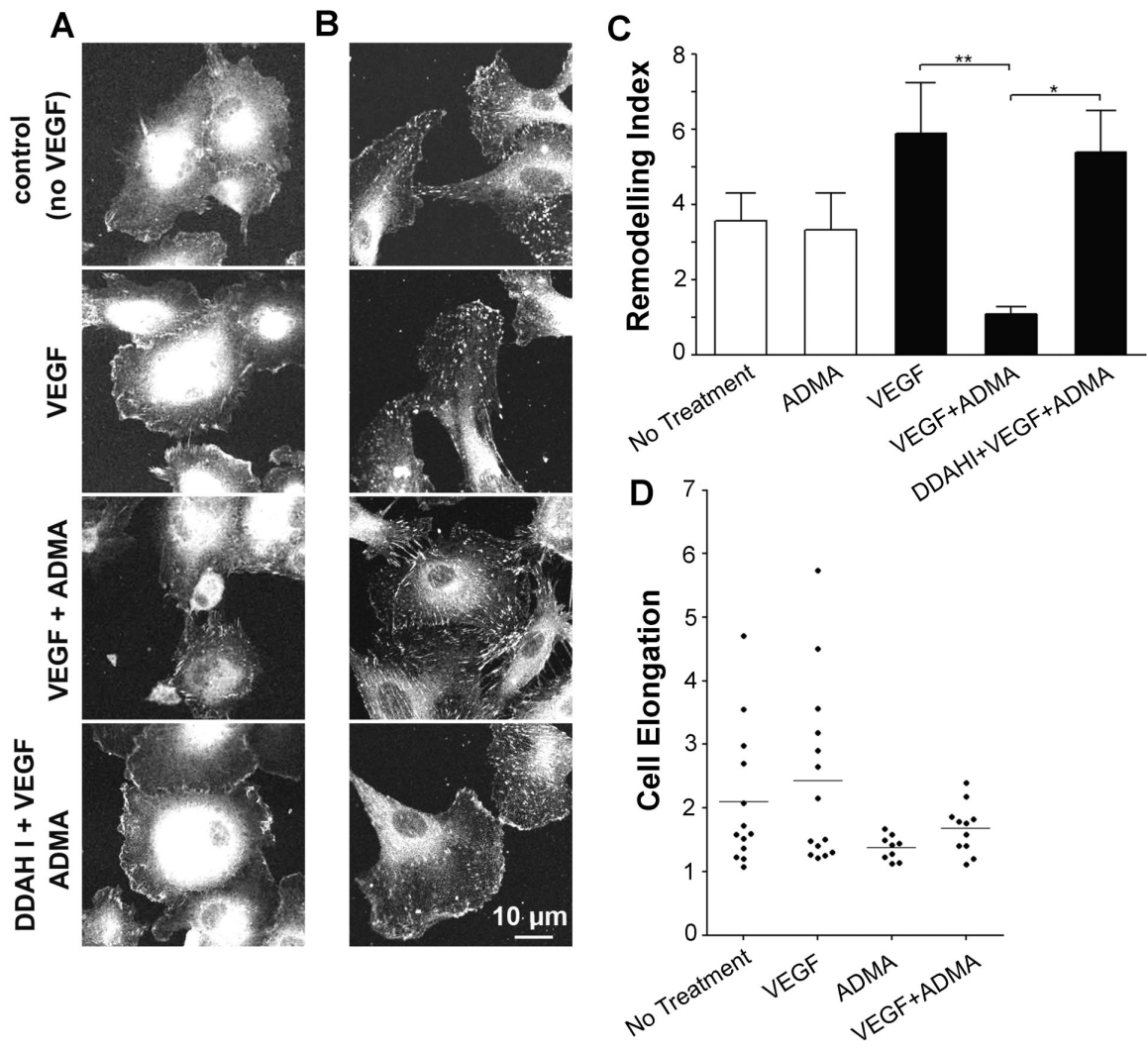


Figure 4

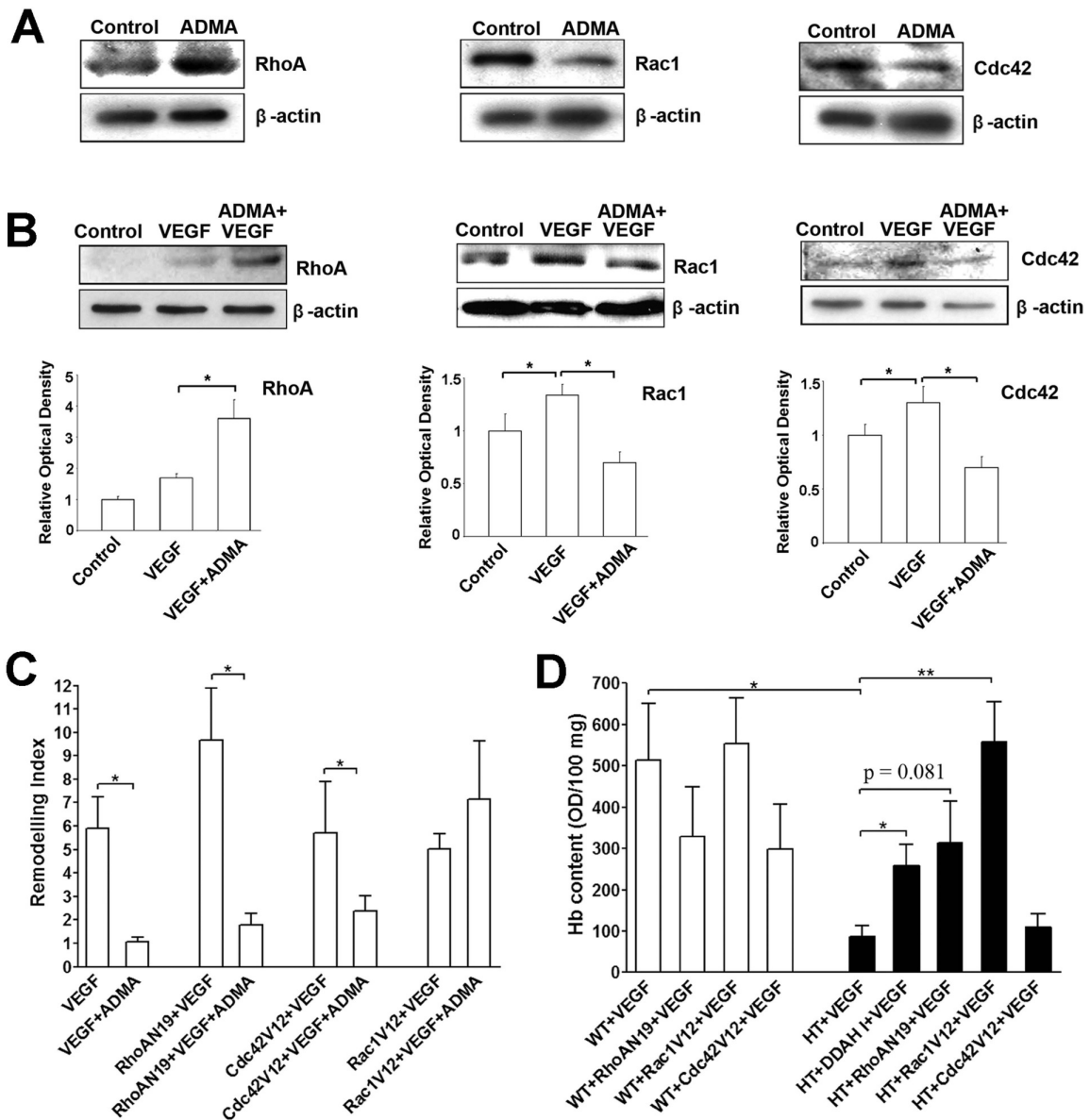


Figure 5

