Dimethylarginine dimethylaminohydrolase activity modulates ADMA levels, VEGF expression, and cell phenotype.

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DIMETHYLARGININE DIMETHYLAMINOHYDROLASE ACTIVITY MODULATES ADMA LEVELS, VEGF EXPRESSION AND CELL PHENOTYPE.

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ABSTRACT

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthase and is metabolised by dimethylarginine dimethylaminohydrolase (DDAH). Elevated levels of circulating ADMA correlate with various cardiovascular pathologies but there is less known on the cellular effects of altered DDAH activity. We modified DDAH activity in cells and measured the changes in ADMA levels, morphological phenotypes on Matrigel and expression of vascular endothelial growth factor (VEGF). DDAH over-expressing ECV304 cells secreted less ADMA and when grown in Matrigel, had enhanced tube formation compared to untransfected cells. VEGF mRNA levels were 2.1-fold higher in both ECV304 and murine endothelial cells (sEnd.1) over-expressing DDAH. In addition the DDAH inhibitor, S-2-amino-4(3-methylguanidino)butanoic acid (4124W 1mM), markedly reduced human umbilical vein endothelial cell (HUVEC) tube formation *in vitro*. We have found that upregulating DDAH activity lowers ADMA levels, increases the levels of VEGF mRNA in endothelial cells and enhances tube formation in an *in vitro* model, whilst blockade of DDAH reduces tube formation.

Keywords: Dimethylarginine dimethylaminohydrolase (DDAH), Asymmetric dimethylarginine (ADMA), Vascular endothelial growth factor (VEGF), tube formation, angiogenesis, endothelial function; nitric oxide (NO); gene expression; endogenous nitric oxide synthase inhibitors.

INTRODUCTION

All three isoforms of nitric oxide synthase (NOS) are inhibited by the endogenous methylated arginines N^GN^G-dimethylarginine (ADMA) and L-N^G-monomethylarginine (L-NMMA) [1;2]. These methylarginines are synthesised when arginine residues in proteins are methylated by the action of protein arginine methyltransferases (PRMT) [3]. As the proteins are hydrolysed, free ADMA and L-NMMA are released into the cytosol and may compete with L-arginine, the substrate for NOSs, and lead to inhibition of nitric oxide (NO) generation. The major route of clearance of ADMA and L-NMMA is hydrolysis to citrulline and methylamines by dimethylarginine dimethylaminohydrolases (DDAHs) [4;5]. Whilst it is clear that increased plasma concentrations of ADMA correlate with various cardiovascular pathologies [6-8] and that pharmacological administration of ADMA or L-NMMA produces substantial cardiovascular effects [9], there is less known about the effects of the endogenous ADMA/DDAH pathway on the behaviour of cells that generate ADMA. The aim of the present study was to test the hypothesis that manipulation of DDAH activity or expression would alter ADMA levels and affect cell phenotype. The focus was on the ability of cells to form “tube-like” structures when grown in Matrigel.

METHODS

Studies were undertaken in 3 cell types – ECV304 cells, human umbilical vein endothelial cells (HUVEC) and the murine endothelial cell line sEnd.1. Studies to explore the effects of DDAH over-expression were undertaken using ECV304 cells because these cells transfect readily and migrate to form linear "tube-like" structures in Matrigel [10;11]. To determine whether the effects seen with ECV304 cells might be relevant to endothelial cells we studied human umbilical vein endothelial cells (HUVEC) and a murine endothelial cell line sEnd.1. We also used a sEnd.1 cell line that over-expresses DDAH-II that we have described previously [12].

Cell culture

Unless otherwise stated cell culture reagents were purchased from Invitrogen. ECV304 cells were grown in M199 medium (Sigma) supplemented with 10 % (v/v) foetal bovine serum, penicillin 1000U/ml, streptomycin 1000 μg/ml and L-glutamine 2 mM as previously described [13]. HUVEC were cultured as described previously [4] and used prior to passage 6. sEnd.1 cells were cultured as described previously [12].

Construction of expression plasmids

Human kidney poly(A)+ RNA was reverse transcribed using an oligo(dT) primer and DDAH II cDNA was PCR amplified using primers P1-GATCGAATTCAGGATGGGGACGCCGGGG (homologous with nucleotides –2 to 15, encoding an upstream *Eco*R1 site, underlined) and P2-

GATCTCTAGATCAGCTGTGGGGGCGTGTG (homologous with nucleotides 858 to 840, encoding an upstream *Xba*1 site, underlined). The PCR product was gel purified and cloned into the *Eco*R1/*Xba*1 site of the mammalian expression vector pcDNA3 (Invitrogen), to give clones D2X7 and D2X10. A reverse orientation DDAH II plasmid was constructed by PCR amplification of DDAH II cDNA using primers P3-CAGGATCCGATGGGGACGCCGGGGGGGGG (homologous to nucleotides 1 to 21, encoding an upstream *Bam*H1 site, underlined) and P4-CTGAAGCTTCGACCTCTGGCCTACGAGCG (homologous to nucleotides 301 to 282, encoding a downstream *Hind*III site, underlined). The gel purified PCR product was cloned in the reverse orientation into the *Hind*III/*Bam*HI site of pcDNA3 (Invitrogen), to give clones ASD2C and ASD2B.

Transfection of ECV304 cells

24 h prior to transfection the cells were seeded at 80 % confluence onto a 9 mm petri dish. Transfection was carried out using TransFast transfection reagent (Promega) according to the manufacturer's instructions. 48 h after transfection, cells containing the expression plasmids were selected by incorporating 800 μg/ml G418 sulphate (Sigma) into the cell culture medium. Approximately 10 days after addition of G418 sulphate, individual colonies of resistant cells were transferred to a 24-well plate containing M199 media supplemented with 400 μg/ml G418 sulphate. Cells over-expressing DDAH II mRNA were screened by northern blotting, using a DDAH II cDNA probe.

Northern blotting

Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and probed for β-actin and DDAH II as previously described [14]. A human vascular endothelial growth factor (VEGF1) cDNA probe was generated by PCR. All mRNA levels described were corrected against β-actin mRNA levels. Flk-1 expression was determined by binding to 3' primer (TCTGCAGTGCACCACAAAGACAC), designed from human flk-1 sequence (AF035121), and phosphorylated by T4 polynucleotide kinase (Promega). Transcripts that hybridised to the probes were detected using a phosphoimager (Fugi BAS 1000).

Immunoblotting

Aliquots of cell lysates were resolved by 12% SDS-PAGE, DDAH II was found to migrate at 38-40 kDa and was detected using an antibody raised in rabbit against a peptide from DDAH $II_{241-255}$ as previously described [15].

Cell growth assay

Cells were seeded onto a 96-well microtiter plate at a density of 500 cells per well and grown in ECV304 medium. Cell growth was assayed (in triplicate) over a 4-day period using the cell titer 96 Aqueous One solution Cell Proliferation Assay (Promega).

Matrigel assay

ECV304 or HUVEC were overlaid onto 100 µl Matrigel^{TM} (Becton Dickinson) in 24-well plates. Digital photographs of the cells were collected using an inverted

microscope (Axiovert) coupled to an Improvision Apple Macintosh system, each experiment was performed in duplicate (n refers to number of duplicates) and 5 random pictures were taken of each well at a magnification of x 10. The area of linear "tube" formation in each picture was analysed, by an individual who was blinded to the cell types and treatments, using the Openlab (Improvision) software package and all "tubes" across a fixed field of magnification (x 10) were measured [11]. Where cells were treated with oxindole or VEGF, data were expressed as % of control (untreated ECV304 cells).

ADMA measurements

60 % confluent cells were grown in fresh culture medium for 24h. Methylated arginines in the conditioned medium were quantitated by HPLC as previously described [6].

DDAH activity assay

Cells were grown to confluence in 150 cm^2 flasks, cells were scraped and lysed for 20 min at 4 °C in sodium phosphate pH 6.6 (10 mM) buffer containing 0.1 % Triton X, cleared by centrifugation and assayed for DDAH activity as previously described [4].

Statistics

Results were compared using students unpaired t-test. Values are given as means ± SEM where n=3 in separate experiments unless otherwise stated.

RESULTS AND DISCUSSION

Characterization of DDAH II over-expressing ECV304 cells

To determine whether an increase DDAH activity would alter the behaviour of cells that make ADMA we transfected ECV304 cells with the DDAH II over-expression construct DDAH II/pCDNA3 to create stable over-expression cell lines. In order to achieve results relevant to changes that might occur during physiological transcriptional regulation of DDAH [15], we selected lines that over-expressed DDAH II by about 2-fold. Basal levels of DDAH II mRNA were 2.3 ± 0.9 (n=5) fold higher in D2X10 cells compared to untransfected cells (Fig 1A; $p<0.05$). Immunoblotting of the DDAH II over-expressing ECV304 cells with a polyclonal antibody to DDAH $II_{241-255}$ revealed a distinct band at ~ 40 kDa and confirmed increased expression of DDAH II protein (Fig 1B). This increase in protein expression was demonstrated to have an effect on DDAH activity since DDAH II over-expressing cells converted greater amounts of [^{14}C]L-NMMA to [^{14}C]citrulline than untransfected cells (Fig 1C; $p<0.05$).

Changes in DDAH activity affected the concentration of ADMA secreted by cells into culture media over a 24h period, such that this fell from 6.31 ± 0.21 μg/ml (22.9 ± 0.8 μM) in control ECV304 cells to 5.10 ± 0.07 μg/ml (18.6 ± 0.3 μM) in the DDAH II over-expressing lines (Fig 1D; $P<0.01$). Levels of SDMA, which is not a DDAH substrate, were unchanged between the two cell lines. Consequently, the ratio of ADMA: SDMA was 3.23 ± 0.1 and 2.73 ± 0.15 ($p=0.024$) for ECV304 and the over-expressing cells respectively (n=5). These results confirm that DDAH activity is a determinant of ADMA release by cells and suggest that the intracellular concentrations of ADMA/L-NMMA are modulated by this enzyme. The results show clearly that increased DDAH activity decreases ADMA accumulation. This is

important because the Km of DDAH for ADMA is rather high (150μM) and the relevance of DDAH to the control of the low levels of ADMA found in cells and tissues has been questioned on theoretical grounds.

Although altered expression of DDAH clearly affected cell phenotype and behaviour (see below) we were unable to determine directly whether this was due to changes in NO generation. ECV304 cells are human in origin and produce NO that is at the limit of levels of detection. Consequently changes were small, even when cells were stimulated with calcium ionophore. It therefore remains possible that the effects of DDAH over-expression are independent of NO. This intriguing possibility or the possibility of compartmentalised changes in NO should be addressed in future studies.

DDAH II over-expression does not influence cell growth

The growth of cells was unaffected by DDAH over-expression. When grown under standard conditions no differences in cell growth were observed over a 4-day period between the untransfected cells and a DDAH over-expressing cell line (Fig 1E). DDAH over-expressing cells grown in culture flasks exhibited identical morphology to ECV304 cells and the cells transfected with the reverse orientated construct.

DDAH II over-expression enhances tube formation

Growth of ECV304 cells on Matrigel resulted in cell migration and elongation to form "tube-like" structures as described previously [10;16]. Cell lines which over-expressed DDAH II (Fig 2A) had greater tube formation than either untransfected ECV304 cells or cells transfected with a reverse orientated DDAH construct (ASD2B & ASD2C) (Fig 2B). This effect is unlikely to be due to an artefact of clonal selection since it was reproduced in two independently derived over-expressing lines (D2X7 &

D2X10). The area of the "tubes" after 48 h in DDAH over-expressing cells was 233.5 ± 48.0 % (D2X7) and 205.1 ± 30.5 (D2X10) compared to control cells (Fig 2B; for both cell lines $p<0.005$, n=4). There was no significant difference in DDAH activity or tube formation between the untransfected cells and those transfected with a reverse orientated DDAH construct.

Effect of the DDAH inhibitor on tube formation.

HUVEC also form "tube-like" structures on Matrigel similar to those produced by ECV304 cells. Addition of the DDAH inhibitor S-2-amino-4(3-methylguanidino)butanoic acid, 4124W (1 mM, [4]) reduced tube formation by HUVEC from 0.43 ± 0.04 mm^2 to 0.25 ± 0.03 mm^2 ($p<0.001$, n=4; Fig 3A & 3B). This concentration of 4124W inhibits DDAH isoforms by 96.1 ± 2.1 % and does not affect cell viability (Fig 3C;[4]).

VEGF expression is increased in DDAH II over-expressing cells

VEGF stimulates the formation of tube-like structures by ECV304 and other cell types [10;11]. To determine whether VEGF might be involved in the effects of DDAH over-expression we assessed expression of VEGF. Northern blotting of ECV304 cells with a probe for VEGF showed that in DDAH over-expressing cells there was 209 ± 27 % VEGF mRNA compared to wild type cells ($p<0.01$, n=11; Fig 4) or cells transfected with the reverse orientated construct. In contrast, mRNA for the VEGF receptor flk-1 mRNA was unchanged between DDAH II over-expressing cells and untransfected cells (data not shown). Similar results were also observed with DDAH II-over-expressing murine endothelial cells (sEnd.1 – data not shown).

Effect of a VEGF receptor antagonist on tube formation assays

DDAH over-expression increases VEGF expression and is associated with the enhanced formation of "tube-like" structures. Treatment of ECV304 with VEGF (10 $\mu g\ l^{-1}$) increased tube formation by 161.2 ± 18.6 %. To determine whether the increased tube formation observed by DDAH II over-expression could be reduced by interfering with VEGF signalling we used oxindole-1**,** which competes for the ATP binding site on flk-1 [17]. In this series of experiments tube formation in DDAH over-expressing cells was 193.3 ± 24.0% greater than in untransfected ECV304 cells. Addition of oxindole-1 (10 µM) reduced the area of tubes formed by over-expressing and wild type cells by 57.7 ± 11.3% and 60.8 ± 7.8 % respectively (Fig 5 A & B). In the presence of oxindole-1, tube formation did not differ between ECV304 wild type and DDAH over-expressing cells.

SUMMARY

The results of this study show that DDAH over-expression increases formation of tube-like structures and that pharmacological inhibition of DDAH reduces the formation of tube-like structures in HUVEC cells. DDAH over-expression is associated with an increase in VEGF expression and is blocked by a VEGF Flk-1 receptor antagonist. These findings clearly show that DDAH is an important modulator of the behaviour of cells that make ADMA and that the ADMA/DDAH pathway has important paracrine/autocrine functions, probably mediated in part by VEGF. It is not known whether the results we have seen are mediated by effects of ADMA on NOS pathways (as the cells we have used are of human origin and produce very low levels of NO) or whether the effects of ADMA on other arginine handling pathways may also be important. The relevance of this *in vitro* data to *in vivo*

processes such as angiogenesis remains to be determined, however ADMA affects angiogenesis *in vivo* [18], DDAH over-expression in tumour cells leads to the formation of more bloody, angiogenic tumours [19] and DDAH II is highly expressed in the placenta, a tissue in which angiogenesis is prominent. Furthermore, retinoic acid, an angiogenic factor important for embryonic development of the cardiovascular system, increases DDAH II expression and NO production [15]. The current study strongly suggests that modulation of VEGF and certain basic properties of cell phenotype are important actions of the ADMA/DDAH pathway.

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FIGURE LEGENDS

Figure 1: Characterisation of DDAHII over-expressing cells

A] DDAH II mRNA levels are greater in D2X10 than ECV304 cells.

ECV304 cells transfected with pcDNA3 / DDAH II plasmid (D2X10) contain 2.31 ± 0.89 fold higher levels of DDAH II mRNA than untransfected cells ECV304 (*p=0.017, where n=5) as shown by northern blotting where DDAH II mRNA was corrected for differences in β-actin mRNA expression.

B] DDAH II protein is over-expressed in D2X10 cells as determined by immunoblotting using a polyclonal rabbit antibody raised against DDAH $II_{241-255}$. Equal amounts of protein were loaded into each lane.

C] DDAH activity, measured by [^{14}C]L-NMMA conversion to citrulline, is significantly enhanced in the DDAH II over-expressing cells D2X10 compared to wild type ECV304 cells (*$p<0.05$, n=3).

D] Levels of ADMA secreted by 60 % confluent cells into fresh culture media after 24 h, concentration of ADMA decreased from 22.93 ± 0.75 μM in untransfected ECV304 cells to 18.55 ± 0.26 μM in the D2X10 cells (*$p<0.01$, where n=3).

E] DDAH II over-expression does not effect the growth of ECV304 cells.

Determined by cell proliferation assay (Promega); growth curves of ECV304 and D2X10 cells were superimposable.

Figure 2: DDAH activity appears to correspond to Matrigel tube formation

A] DDAH activity, measured by [^{14}C]L-NMMA conversion to citrulline, is significantly enhanced in the DDAH II over-expressing cells D2X7 and D2X10

compared to control cells (the wild type ECV304 cells and cells transfected with the reverse orientated DDAH construct: ASD2B and ASD2C)

B] DDAH II over-expression effects the pattern of cells growing on Matrigel. Area of tube formation on Matrigel is significantly enhanced in the DDAH II over-expressing cells D2X7 ($p= 0.004$) and D2X10 ($p= 0.002$) compared to untransfected ECV304 cells. There was not a significant difference between the wild type ECV304 cells and either ASD2B or ASD2C; neither was there a significant difference between the D2X7 and D2X10 cell lines, where n=10 from at least 2 independent experiments. All analyses of tube area were undertaken by an individual blind to cell type and protocol

Figure 3:

A] The DDAH inhibitor 4124W (1mM) inhibits Matrigel tube formation. HUVEC were plated onto Matrigel and treated ± 4124W (1 mM). Pictures are representative of one experiment where n=4 performed in duplicate.

B] Tube area of HUVEC after 24h fell from 0.43 ± 0.04 mm^2 in untreated cells to 0.25 ± 0.03 mm^2 in cells treated with 4124W (*$p<0.001$, n=4).

C] DDAH activity of HUVEC lysates, measured by [14C]L-NMMA conversion to citrulline, is significantly reduced by 4124W (1mM; **$p<0.01$, n=3)

Figure 4: DDAH over-expression increases VEGF mRNA levels.

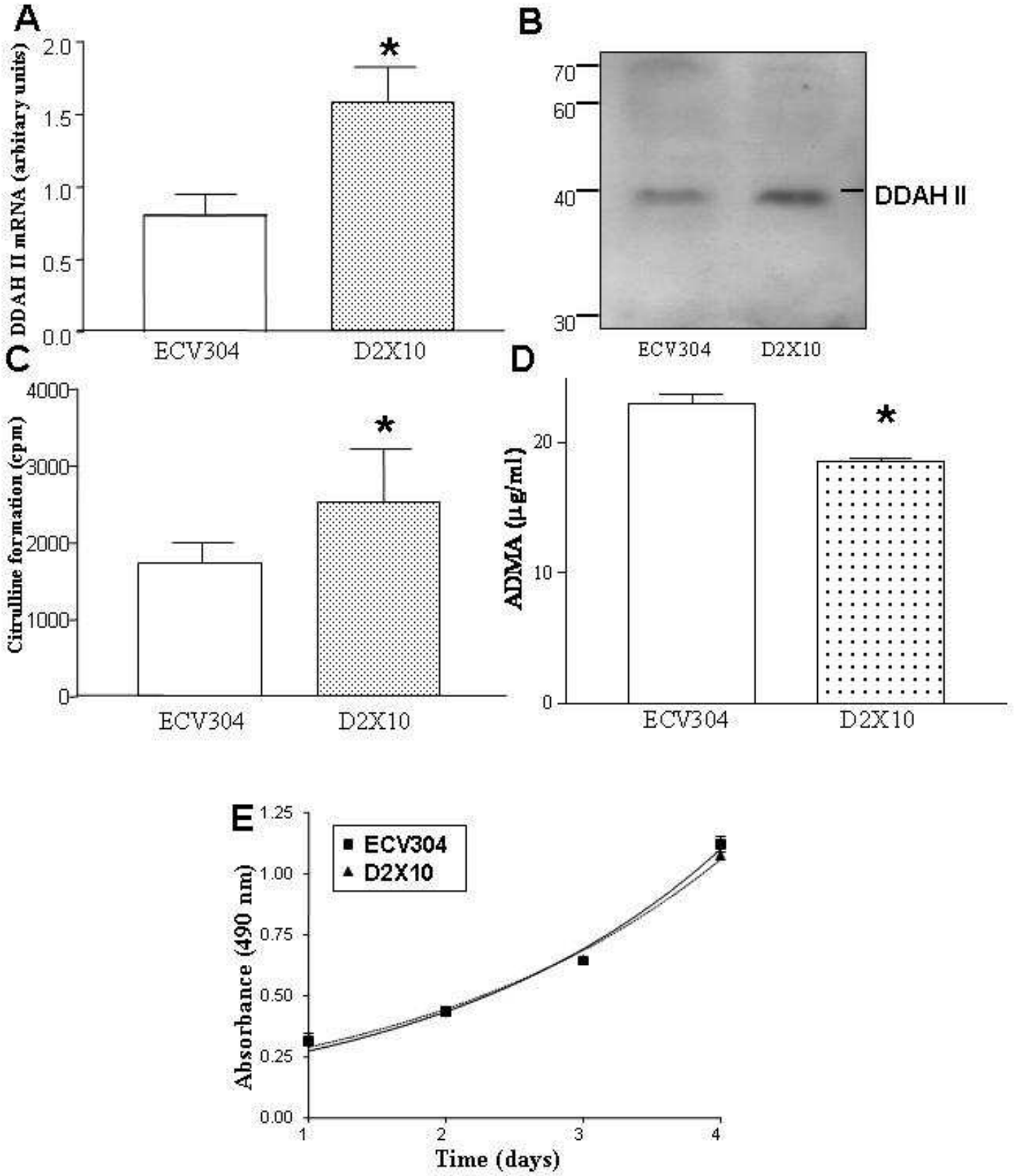
Northern blotting revealed levels of VEGF mRNA were significantly higher in the DDAH II over-expressing cells compared to the untransfected ECV304 cells, where n=3, levels of mRNA were corrected against differences in β-actin mRNA expression.

Figure 5: Effect of DDAH II over-expression and oxindole-1 on tube formation

A] DDAH II over-expressing and wild type ECV304 cells were plated onto Matrigel, the over-expressing cells had significantly higher tube areas than the wild type cells ($p < 0.05$). Over-expressing and untransfected cells were treated with oxindole-1 (10 μM) shown after 48 h, this Flk-1 receptor antagonist reduced tube formation. Pictures are representative of one experiment where n=4 performed in duplicate.

B] Effect of Oxindole-1 on tube area.

ECV304 and D2X10 cells were treated as descried above. Oxindole-1 (10 μM) significantly reduces the area of tube formation of DDAH II over-expressing cells by 60.1 ± 9.7 % ($^{\$}P = 0.021$) and untransfected cells by 36.3 ± 8.1 % ($^{*}P < 0.001$) compared to untreated cells. Values are the total area of tubes formed on Matrigel after 48 h, n>4.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1.5
1.0
0.5
0.0
*
ECV304
D2X10
B
70
60
40
30
DDAH II
ECV304
D2X10
C
Citrulline formation (cpm)
4000
3000
2000
1000
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D2X10
D
ADMA (µg/ml)
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D2X10
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2
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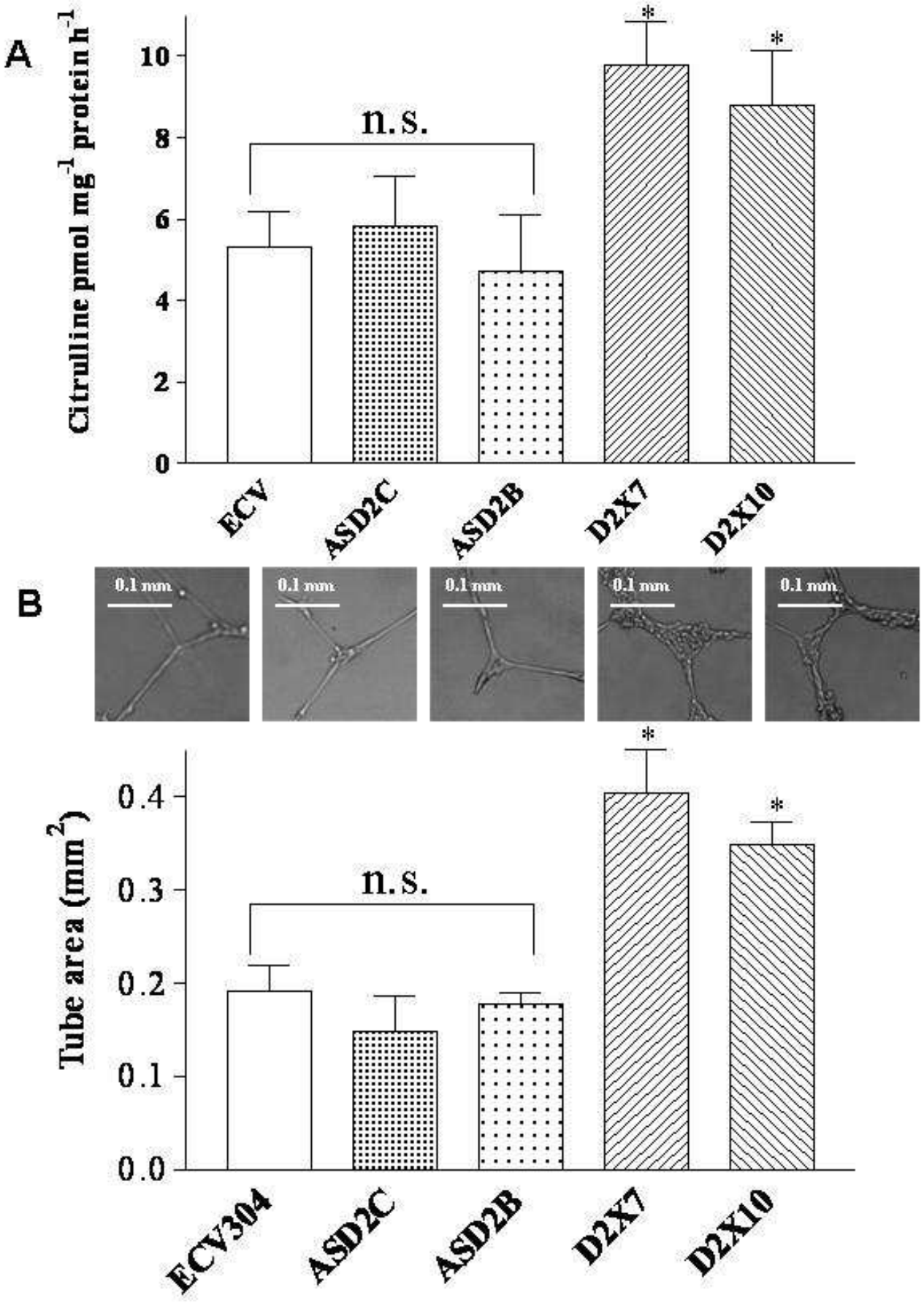
Time (days)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6
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*
*
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ASD2B
D2X7
D2X10
B
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0.1 mm
0.1 mm
0.1 mm
0.1 mm
Tube area (mm2)
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0.3
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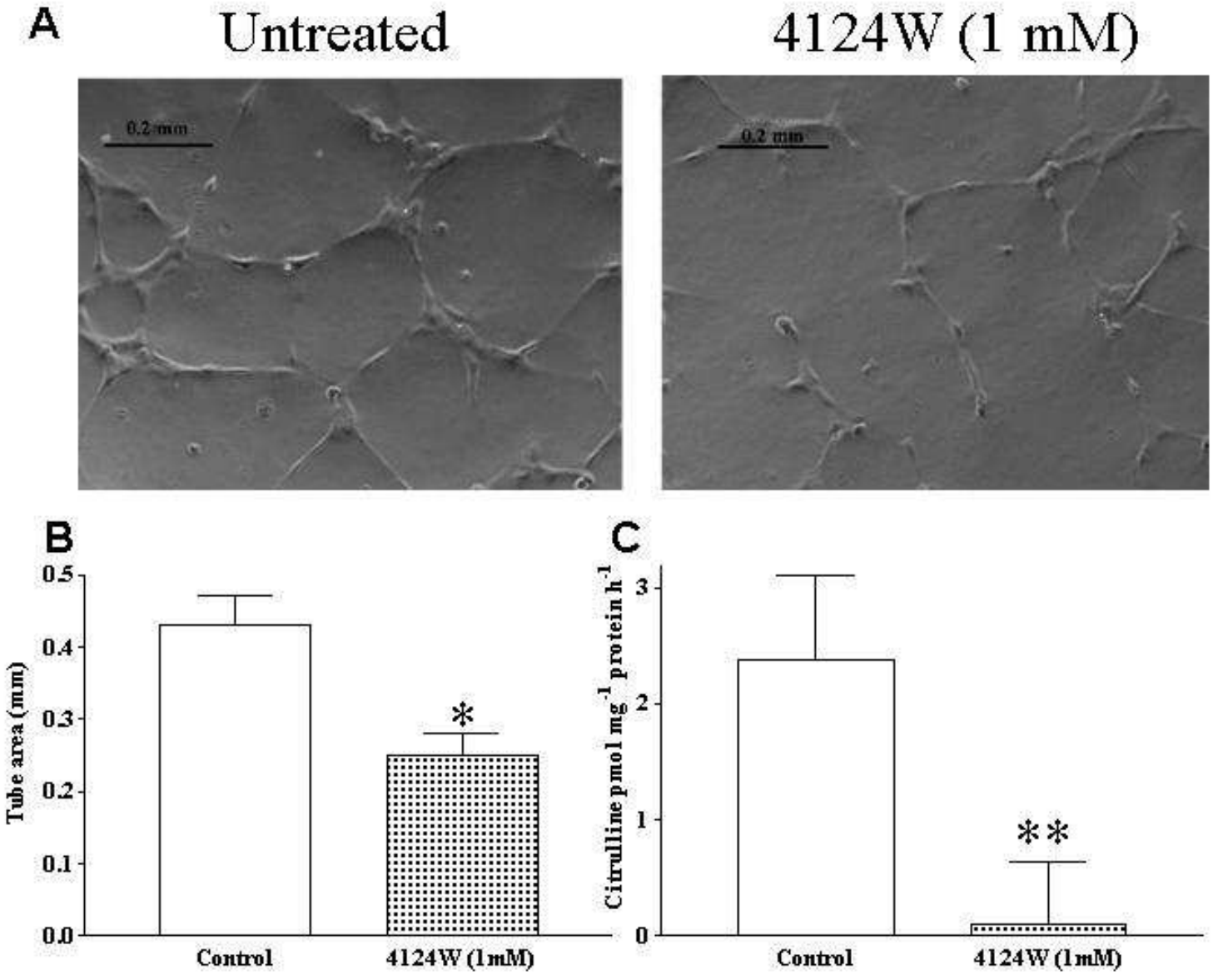
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0.0
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Control
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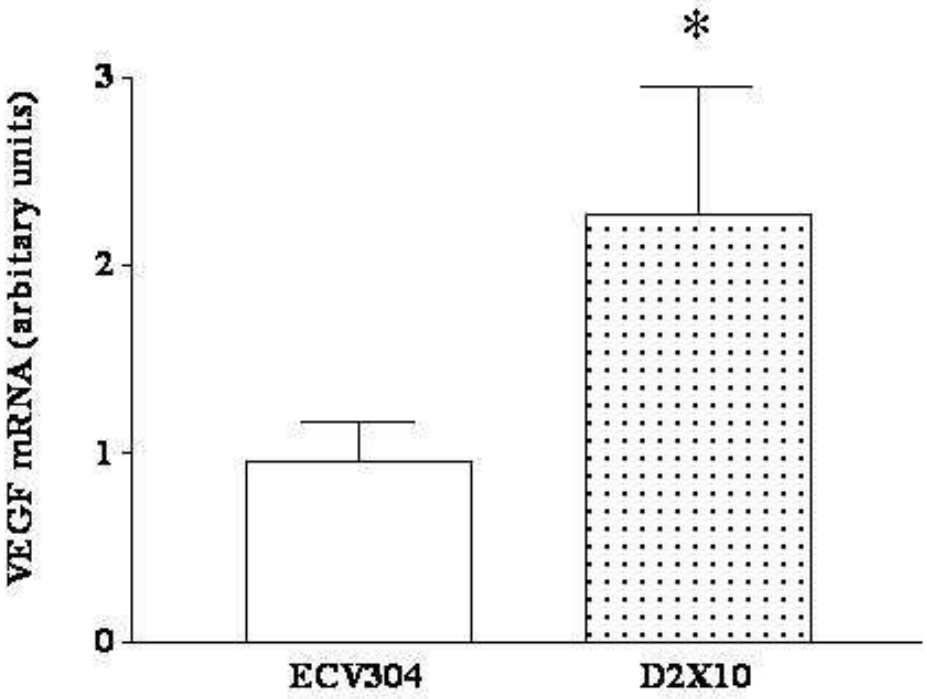
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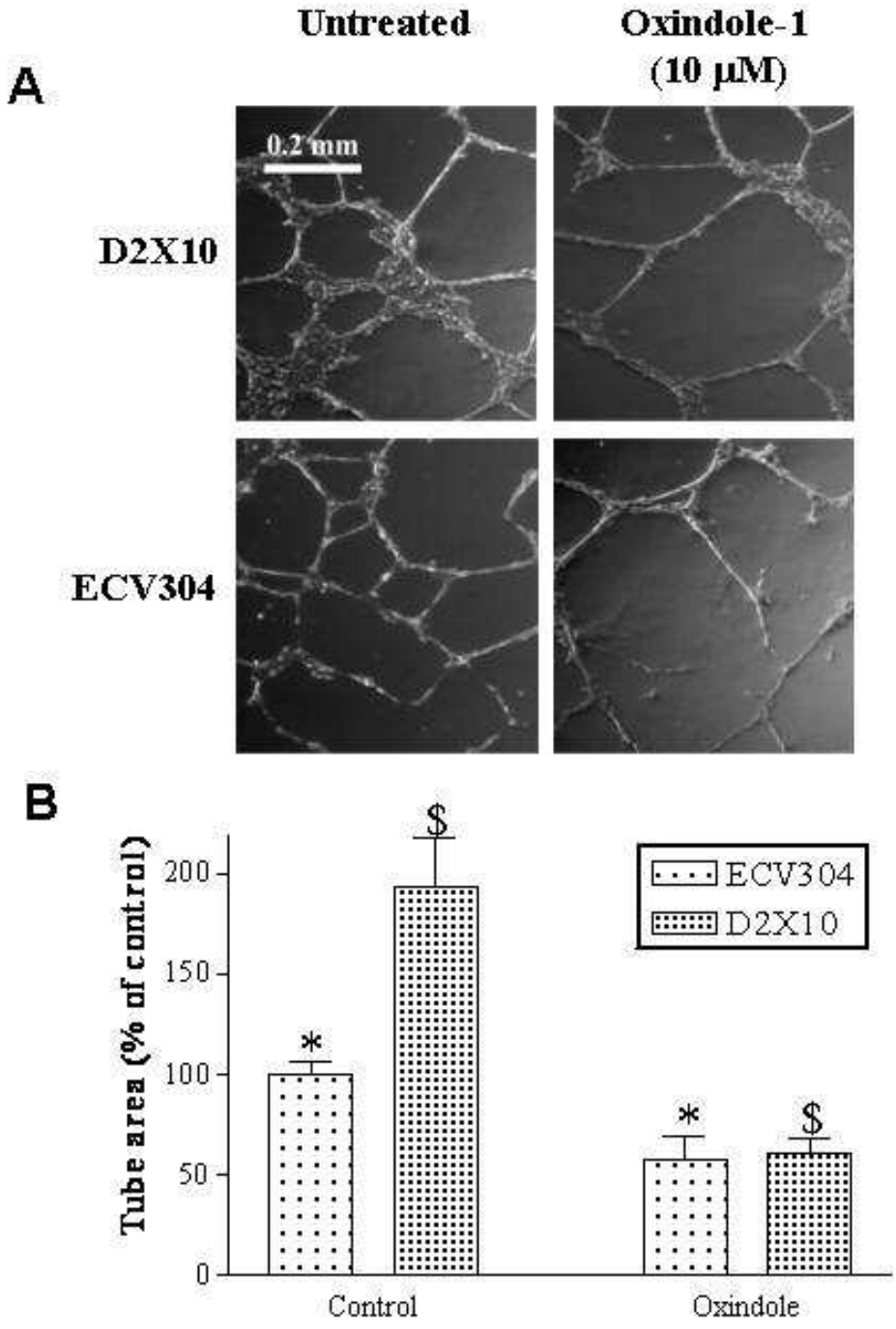
ECV304
D2X10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D2X10
ECV304
B
Tube area (% of control)
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150
100
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0
*
$
*
$
ECV304
D2X10
Control
Oxindole