ARNO regulates VEGF-dependent tissue responses by stabilizing endothelial VEGFR-2 surface expression

Hanna K. Mannell1,2, Joachim Pircher2, Daniel I. Chaudhry2, Stefan K.C. Alig2, Elisabeth G. Koch2, Ramona Mettler1,2, Ulrich Pohl1, and Florian Krötz2*

1Walter-Brendel Centre for Experimental Medicine, Schillerstrasse 44, Munich 80336, Germany; and 2Cardiology, Medical Policlinic, Ludwig-Maximilians-University, Ziemssenstrasse 1, Munich 80336, Germany

Received 7 January 2011; revised 30 September 2011; accepted 6 October 2011; online publish-ahead-of-print 13 October 2011

Aims

The vascular endothelial growth factor (VEGF) stimulates angiogenesis by induction of vessel permeability, proliferation, and migration of endothelial cells, an important process in ischaemic diseases. ADP-ribosylation factor (ARF) nucleotide-binding site opener (ARNO) (cytohesin-2) is a guanine exchange factor important for cellular signalling through ARF GTPases. However, a role for ARNO in VEGF-dependent endothelial processes has so far not been documented. Therefore, we investigated whether ARNO has a role in VEGF-dependent activation of endothelial cells and thus vessel permeability.

Methods and results

ARNO expression was observed in endothelial cells in vitro by RT–PCR, western blotting, and immunofluorescence as well as ex vivo by immunohistochemical staining of mouse aorta. Treatment with the cytohesin inhibitor SecinH3 or with an ARNO siRNA prevented VEGF-dependent Akt activation, assessed by detection of phosphorylated Akt, and proliferation of endothelial cells in vitro, measured by methylthiazoletetrazolium (MTT) reduction. In addition, ARNO suppression reduced VEGF-induced permeability in vessels of the mouse (C57BL/6) cremaster muscle in vivo, as measured by extravasation of fluorescein isothiocyanate (FITC)-dextran. Moreover, ARNO knock-down accelerated ligand-induced reduction in vascular endothelial growth factor receptor-2 (VEGFR-2) surface expression, internalization, and degradation, as assessed by flow cytometry and western blotting, respectively.

Conclusion

Our findings indicate an important and novel role for endothelial ARNO in VEGF-dependent initiation of angiogenesis by regulation of VEGFR-2 internalization in endothelial cells, resulting in the activation of the Akt pathway, vessel permeability, and ultimately endothelial proliferation. Thus, ARNO may be a new essential player in endothelial signalling and angiogenesis.

Keywords

ARNO†, Cytohesin-2†, VEGFR-2†, Angiogenesis†, Endothelial cells

1. Introduction

The vascular endothelial growth factor (VEGF) is one of the most prominent inducers of angiogenesis in vitro and in vivo. Its angiogenic effects are mediated through induction of endothelial cell hyperpermeability, cell proliferation, and migration to prepare the endothelium for tube formation.1,2 By binding to its endothelium-specific receptors, VEGF activates signalling cascades, such as the phosphatidylinositol 3 kinase (PI3-K) pathway.3–5 This pathway is crucial for VEGF-mediated endothelial cell survival, proliferation, migration, and tube formation, and thus has become the target for therapeutic angiogenesis.6,7 Following PI3-K-dependent phosphatidylinositol (3,4,5)-triphosphate (PIP3) generation, several signalling mediators and adaptor proteins containing a pleckstrin homology domain bind to PIP3 to become activated.8

ARNO [ADP-ribosylation factor (ARF) nucleotide-binding site opener/cytohesin-2] is one out of four cytohesin isoforms, which can bind to PI3-K generated PIP3.9 ARNO serves as a guanine exchange factor primarily for family members of the Ras-related GTPases ARFs, it activates these factors by facilitating the exchange
of GDP to GTP. ARF proteins regulate vesicular transport, where they control the assembly of vesicles, Golgi transport, and trafficking between endosomal compartments and the plasma membrane.\(^\text{10,11}\)

Interestingly, one of the ARF proteins, ARF6, was previously shown to play a role in VEGF-dependent processes.\(^\text{12}\) The cytohesin homologue in Drosophila, Steppke, was shown to inhibit the action of the forhead transcription factors FOXO.\(^\text{13}\) FOXO negatively regulate VEGF-dependent endothelial cell cycle progression and proliferation of cardiomyocytes, thereby playing a critical role in the cardiovascular system.\(^\text{14}\) FOXO transcription factors are also under the control of PI3-K-dependent Akt activation.\(^\text{5}\) However, an involvement of ARNO in VEGF-dependent signalling and angiogenesis in endothelial cells has so far not been reported.

We therefore investigated whether ARNO is necessary for VEGF-dependent signalling and cell proliferation in human microvascular endothelial cells (HMEC) and if so, whether this is a process affecting vascular endothelial growth factor receptor-2 (VEGFR-2)-dependent vessel permeability in vivo.

### 2. Methods

#### 2.1 Chemicals

Rabbit phospho Akt Ser 473 antibody (\#9272), rabbit Akt antibody (\#2920), rabbit VEGFR-2 (\#2479), phospho-VEGFR2 antibody (\#2478), and rabbit VE-Cadherin antibody (\#2158) were purchased from Cell Signalling (Frankfurt am Main, Germany). Goat ARNO antibody (\#sc-9727), mouse His antibody (\#sc-53073), and goat Lamin A/C antibody (\#sc-6215) were purchased from Santa Cruz Biotechnology (Heidelberg, Germany), and mouse ARNO antibody (clone 6H5) was from Abnova (Taipei, Taiwan). Mouse monoclonal fluorescein isothiocyanate (FITC)-labelled VEGFR-2 (\#FAB357F) and IgG1 isotype control antibody (\#IC002F) for flow cytometry was from BD Biosciences (Heidelberg, Germany). Biotinylated anti-goat antibody for immunohistochemistry recently labelled secondary antibodies were from Invitrogen (Darmstadt, Germany). Horseradish peroxidase-conjugated (HRP) for flow cytometry was from BD Biosciences (Heidelberg, Germany). Chemicon (Hampshire, England). Horseradish peroxidase-conjugated antibodies were from Calbiochem (Darmstadt, Germany) and fluorescently labelled secondary antibodies were from Invitrogen (Darmstadt, Germany). Biotinylated anti-goat antibody for immunohistochemistry was from Vector Laboratories (Burlingame, CA, USA). VEGF-A\(_{165}\) was purchased from Tebu-bio (Offenbach, Germany). His-tagged VEGF was from Abcam (Cambridge, UK) and VEGF-2 kinase inhibitor II was purchased from Merck (Darmstadt, Germany). All other chemicals were from Sigma-Aldrich (Taufkirchen, Germany).

#### 2.2 Cell lines and cell culture

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as previously described\(^\text{15}\) according to the Declaration of Helsinki. The umbilical cord was rinsed with warm phosphate-buffered saline containing 0.7 mM CaCl\(_2\) (PBS\(^+\)) and incubated with 1 mg/mL collagenase A solution in a humidified incubator for 10 min. Endothelial cells were then washed out of the vessel with DMEM containing 20% newborn calf serum, followed by 5 min centrifugation at 178 g and subsequent wash with DMEM. Purified endothelial cells were obtained in culture flasks in endothelial growth medium and left to grow at 37°C in a humidified incubator with 5% CO\(_2\).

The procedure was approved by a university ethic review board. HMEC were provided by Ades et al.\(^\text{16}\) and cultured as described previously.\(^\text{17}\)

#### 2.3 siRNA transfections

HMEC were transfected with 30 nM siRNA (siRNA 1) or 50 nM siRNA (siRNA 2) using the magnetofection method in combination with the Effectene kit from Qiagen (Hilden, Germany) as previously described.\(^\text{18}\) Validated siRNAs for human ARNO and negative control siRNA were purchased from Qiagen (siRNA 1, Hilden, Germany) and Invitrogen (siRNA 2, Darmstadt, Germany). Transfected cells were left 48 h to allow for siRNA-mediated knock-down as described by the suppliers before submitted to assays. For siRNA sequences, see Supplementary material online, Methods.

#### 2.4 Cell extracts for western blot analysis and cell fractioning

Protein lysates were prepared and protein content quantified as described previously.\(^\text{19}\) Lysates were then subjected to western blot analysis as described elsewhere.\(^\text{19}\) For inhibition of endocytosis cells were treated with hypertonic medium (HTM) (0.45 mol/L sucrose and 1% BSA in Dulbecco’s DMEM) for 30 min or with 20 mM methyl-β-cyclodextrin (MβCD) for 20 min before VEGF stimulation (50 ng/mL, 10 min) as previously described.\(^\text{20}\)

#### 2.5 RT–PCR and RNA isolation

Total RNA was isolated using the PeqGOLD total RNA kit from Peqlab (Erlangen, Germany). A total of 2 µg RNA was subjected to RT–PCR using the Titan One tube RT–PCR system (Roche, Mannheim, Germany) according to the supplier’s protocol (Promega, Mannheim, Germany). Following primer sequences were used: ARNO forward primer 5′agggaggccgctcttag3′, ARNO reverse primer 5′aggggtgctgcgtcttc3′, β-actin forward primer 5′tgccactttccgactaggt3′, β-actin reverse primer 5′agtctcgccagttggaac3′ (all produced by Biomers.net, Ulm, Germany).

#### 2.6 Immunohistochemical and fluorescent staining

For immunohistochemical staining of ARNO, ex vivo mouse aorta was isolated as previously described\(^\text{21}\) and embedded in OCT Tissue Tek (Sakura Finetek Europe Zoeterwonde, NL) and immediately frozen at −20°C. The aorta was cut into 10 µm thick slices with a Cryostat (MICROM International GmbH, Walldorf, Germany). For staining the ABC-technique was used.\(^\text{21}\) Pictures were taken with a Leica DMRD microscope connected to a Leica DC 300 F camera (Leica microsystems, Wetzlar, Germany) using the software Leica QWin Standard version 2.5. For immunofluorescent microscopy of ARNO, cells were grown to confluence on 8-well microscope slides from IBIDI ( Martinsried, Germany) and stained and visualized as previously described.\(^\text{22}\)

#### 2.7 MTT assay

Cell proliferation of HMEC was measured by the reduction in methylthiazol tetrazolium (MTT, 0.5 mg/mL, 2 h) as described elsewhere.\(^\text{22}\) After siRNA transfection, cells were immediately put on starvation media (1% serum), in which the cells were kept throughout the experiment. A total of 50 ng/mL VEGF was added to the starvation media after the t = 0 h measurement (directly after siRNA transfection or 24 h after changing to starvation media by cells treated with SecinH3). Data were set in relation to cells treated with DMSO or control siRNA and measured according to the supplier’s protocol.

#### 2.8 Measurement of sVEGFR-2

The concentration of VEGFR-2 in the supernatant of cells transfected with ARNO siRNA and control siRNA was measured by Quantikine\(^\text{16}\) human soluble VEGF R2 Immunoassay (R&D Systems, Abingdon, UK) according to the supplier’s protocol.

#### 2.9 Animals and surgical procedure for the cremaster muscle model

All animal experiments were performed in wild-type Black6-mice that had been purchased from Charles River (Sulzfeld, Germany). Animal surgical...
procedures were performed following anaesthesia as described elsewhere, \textsuperscript{23} using intraperitoneal injection of 8 mg/L Midazolam (3 mg/kg body weight) (CuraMED Pharma, Karlsruhe, Germany), and 200 mg/L medetomidinhydrochloride (0.3 mg/kg body weight) (Pfizer, Berlin, Germany; produced by Orion Pharma, Espoo, Finland) in 0.9% NaCl. The anaesthesia was monitored by checking response to pain. During the experiment, the anaesthesia was sustained by continuous injection of the solution by an injection pump via a catheter in the V. jugularis (0.1–0.2 mL/min/kg body weight). Cremaster muscle preparation was performed as previously described with minor modifications. \textsuperscript{24} Upon anaesthesia, a catheter in the left carotid artery was inserted. Cremaster muscle preparation was performed on a heated plexi-glass stage with cover slips for microscopy. After incision of the scrotal sack, the cremaster muscle was hoisted from the scrotal sack by surgical thread, and the surrounding connective tissue was removed. The muscle was then incised on the ventral side and fixed to the cover slip. During the length of the experiment, the tissue was continuously perfused with pre-warmed (35°C) Krebs–Henseleit solution pH 7.4 (118 mM NaCl, 4.8 mM KCl, 2.5 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 25 mM NaHCO\(_3\), 1.2 mM KH\(_2\)PO\(_4\)). After every experiment, mice were euthanized through intravenous injection of 320 mg/kg body weight sodium pentobarbital (Bayer, Leverkusen, Germany). All experiments were conducted in full accordance with the German animal protection law and approved by the district government of upper Bavaria (Regierung von Oberbayern, approval reference number AZ55.2-1-54-2531-162-08). The investigation conforms to the \textit{Guide for the Care and Use of Laboratory Animals} published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

2.10 \textit{In vivo} permeability assay

To analyse vascular permeability, \textit{in vivo} extravasation of FITC-dextran was assessed in mouse cremaster muscle vessels by measuring FITC-dextran extravasation using intravital microscopy as previously described. \textsuperscript{25} For a detailed description of this assay, see Supplementary material online, Methods.

2.11 VEGFR-2 surface expression

Cells were washed and detached non-enzymatically from cell culture dishes using 5 mM EDTA, pelleted, rinsed in phosphate-buffered saline containing Ca\(^{2+}\) (PBS\(^{+}\)) and fixed with 3% formalin for 10 min. Cells were then incubated with FITC-labelled VEGFR-2 antibody (1:40 in PBS\(^{+}\)), rinsed and suspended in PBS\(^{+}\) for subsequent measurement by flow cytometry using a FACS Canto II (BD Biosciences,

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{Endothelial ARNO expression. (A) HUVEC and HMEC ARNO mRNA were detected with RT–PCR (n = 3) and ARNO protein was visualized with western blotting (n = 3). β-Actin and GAPDH, respectively, were used as loading controls. (B) Treatment with ARNO siRNA 1 (30 nM) or ARNO siRNA 2 (50 nM) reduced ARNO protein expression (both n = 4, HMEC). GAPDH or Lamin A/C was used as loading controls. (C) Immunofluorescent staining of ARNO (green) was performed during non-stimulated conditions as well as after different times of VEGF stimulation (50 ng/mL) (n = 3, HMEC). Treatment with VEGFR-2 tyrosine kinase inhibitor II (200 nM) was used to confirm the specificity of VEGF on ARNO activity (n = 3, HMEC). Additional stainings include VE-Cadherin to visualize the membrane and DAPI to visualize nuclei. (D) Immunohistochemical staining of sections of mouse aorta shows endothelial expression of ARNO \textit{in vivo} (brown) and additional staining of nuclei (dark blue). As control for non-specific antibody-binding mouse aorta sections were stained with only secondary antibody. (E) The ARNO expression level was not affected by different VEGF exposure (50 ng/mL) times, as measured by western blotting (n = 5, HMEC).}
\end{figure}
Heidelberg, Germany). To control for background staining, control cells were stained with a FITC-labelled isotype IgG1 control antibody. For a detailed protocol, see Supplementary material online, Methods.

2.12 Trypan blue exclusion
Treated HMEC were washed with PBS+ followed by accutase (PAA, Germany) incubation to detach cells. Cells were collected and spun down. Cell pellets were diluted in 0.1% trypan blue solution, and cells were counted after 2 min incubation at RT.

2.13 VEGFR internalization
Cells were starved for 24 h followed by treatment with a His-tagged VEGF (50 ng/mL). Removal of remaining VEGFRs on the cell surface was performed as described elsewhere.° Cells were lysed and subjected to western blotting, and VEGF-His was detected using an anti-His antibody.

2.14 Statistical analysis
Data were analysed using Student’s t-test or one-way ANOVA as appropriate. All data are presented as means ± SEM. Results were considered significant at an error probability level of P < 0.05.

3. Results

3.1 ARNO expression in human endothelial cells
ARNO mRNA and protein were detected in both HUVEC and HMEC by RT−PCR and western blotting, respectively (both n = 3, Figure 1A). The ARNO protein expression was reduced 48 h following transfection with two different ARNO siRNAs (n = 4, HMEC, Figure 1B). Immunofluorescent staining of ARNO showed a cytosolic and membranous localization under non-stimulated conditions (1% starvation media), whereas 2 min of VEGF stimulation promoted an enhanced translocation to the membrane, as seen by co-staining with VE-Cadherin, which was still maintained after 10 min of VEGF stimulation (n = 3, HMEC). This membrane translocation was prevented by treatment with a VEGFR-2 inhibitor (VEGFR-2 tyrosine kinase inhibitor II, n = 3, HMEC, Figure 1C). The expression of ARNO in endothelial cells in vitro was confirmed by immunohistochemical staining of ARNO in sections of the mouse aorta ex vivo (Figure 1D), where ARNO was detected primarily at the cell membrane on the luminal side but also in the cytosol. The in vitro expression level of ARNO was not changed upon different times of VEGF exposure (n = 5, HMEC, Figure 1E).

3.2 Suppression of ARNO activity abrogates VEGF-dependent Akt phosphorylation
We next investigated the influence of ARNO activity on VEGF-dependent endothelial Akt activation. While VEGF treatment resulted in an increase of Akt phosphorylation (P < 0.001, n = 11, HMEC), treatment with the cytohesin inhibitor SecinH3 (15 μM, 60 min) completely abolished this effect (P < 0.001, n = 12, HMEC, Figure 2A). To confirm the specific role of ARNO in this process, we next performed this assay using ARNO siRNA. ARNO siRNA 1 treatment (30 nM) prevented VEGF-dependent Akt phosphorylation to a similar extent (P < 0.05, n = 3, HMEC, Figure 2B). Knock-down using ARNO siRNA 2 (50 nM) confirmed the reduction in VEGF-dependent Akt activation (P < 0.05, n = 4, HMEC).

3.3 ARNO inhibition impairs VEGF-dependent endothelial cell proliferation
We next investigated whether ARNO inhibition would also affect endothelial cell proliferation, a process relying on Akt activation. Whereas treatment with VEGF (50 ng/mL) induced endothelial cell proliferation (P < 0.05, n = 12, HMEC) treatment with the cytohesin inhibitor SecinH3 (10 μM) significantly impaired the VEGF induced response (P < 0.05, n = 12, HMEC, 48 h following treatment, Figure 3A). To confirm the influence of ARNO in this process, cells

![Figure 2](https://example.com/figure2.png)

**Figure 2** ARNO suppression abrogates VEGF-dependent Akt phosphorylation. (A) VEGF stimulation (50 ng/mL, 10 min) induced phosphorylation (Ser 473) of Akt (**P < 0.001, n = 11, HMEC). Treatment with a pharmacological cytohesin inhibitor (SecinH3, 15 μM, 60 min) abolished this effect (**P < 0.001, n = 12, HMEC). **P < 0.05, n = 10; ***P < 0.01, n = 12. (B) Transfection with ARNO siRNA 1 (30 nM) or ARNO siRNA 2 (50 nM) also prevented the VEGF-dependent Akt phosphorylation (**P > 0.05, **P < 0.01, n = 3–6, HMEC). Total Akt and GAPDH were detected as equal loading controls. To calculate statistical significance, the density of the protein bands was measured and the ratio between pAkt/Akt was calculated.
were next treated with ARNO siRNA before treatment with VEGF. Indeed, ARNO siRNA 1 treatment (30 nM) as well as ARNO siRNA 2 treatment (50 nM) also significantly impaired the VEGF-dependent endothelial cell proliferation in comparison to cells treated with control siRNA ($P < 0.001$, $n = 24–30$, HMEC, 48 h after transfection, Figure 3B and C).

3.4 Suppression of ARNO activity reduces the VEGF-induced Dextran tissue accumulation in vivo

Having observed a significant effect of ARNO inhibition on VEGF-dependent endothelial signalling and cellular proliferation, we hypothesized that it would affect the tissue response to VEGF in vivo. Therefore, we next measured the extravascular tracer (FITC-Dextran) accumulation of vessels in mouse cremaster muscle. In

Figure 4 Cytohesin inhibition impairs endothelial permeability in response to VEGF in vivo. (A) Representative photos of endothelial permeability assessed in the mouse cremaster muscle model by injection of FITC-dextran (5% solution, MW 150 kDa) after 0 min (left panel) and 25 min (right panel) of VEGF (10 μg/mL) stimulation. Pictures were taken with identical contrast and brightness settings. (B) VEGF application induced endothelial permeability in vessels of the mouse cremaster muscle in vivo ($P < 0.05$ in comparison to $t = 0$ min, $n = 3$), whereas treatment with Secin H3 (15 μM) impaired this process ($P < 0.001$ compared with the respective DMSO control, $n = 4$). Evaluation of permeability was measured by fluorescence intensity of 5 ROIs/picture.
these experiments, application of VEGF (10 μg/mL) resulted in a strong extravasation of FITC-dextran already after 5 min and increased even further with time (P < 0.05 at all time points, n = 3). However, after SecinH3 (15 μM) application, the VEGF-dependent extravasation of FITC-Dextran was significantly impaired at every time point measured (P < 0.001; n = 4, Figure 4A and B).

3.5 ARNO knock-down reduces the VEGFR-2 surface levels

As the GTPase ARF6 is important for tyrosine kinase receptor endocytosis and recycling and is one of the direct targets of ARNO, we next hypothesized that the abrogated VEGF signalling upon ARNO inhibition could be due to impairment of VEGFR internalization. To elucidate if inhibition of endocytosis results in reduced VEGF-dependent signal transduction and thus may be a plausible route of ARNO action, clathrin-dependent and independent endocytosis was prevented by treatment with HTM destroying the endosomes and with the cholesterol-depleting agent MβCD, respectively. Similar to the effect seen after ARNO suppression, no Akt phosphorylation following VEGF stimulation could be detected after endocytosis inhibition (P < 0.05, n = 3, HMEC, Figure 5A). Importantly, this treatment did not markedly reduce cell viability (93 and 90% cells still viable respectively, n = 8–6, HMEC, Figure 5B). To elucidate whether ARNO inhibition influences the internalization of the VEGFR-2, we next measured the surface expression of VEGFR-2 with flow cytometry on intact cells treated with ARNO siRNA (30 nM) using an antibody recognizing only the external part of VEGFR-2. On cells transfected with control siRNA (30 nM), the VEGFR-2 surface expression declined, as expected, upon increasing time of VEGF incubation and reached a minimum after 30 min. Surprisingly, ARNO siRNA treatment significantly accelerated this process (P < 0.05, n = 6, HMEC, Figure 5C), reaching base line levels already after 2 min of VEGF stimulation. To rule out the possibility of increased shedding of VEGFR-2 from the membrane upon ARNO siRNA treatment, VEGFR-2 amounts were measured in the supernatant following different times of VEGF stimulation. As seen in Figure 5D no increase in VEGFR-2 amounts could be detected in the supernatant of these cells compared with control siRNA treatment (n = 4, HMEC). In turn, when treating cells with a His-tagged VEGF (50 ng/mL) for the time points, where lower surface VEGFR-2 levels were observed, followed by trypsination of existing VEGFR-2 on the cell surface and subsequent detection of internalized VEGF-His, more VEGF-His was detected intracellularly upon ARNO siRNA treatment compared with control siRNA treatment (n = 3, HMEC, Figure 5E).

3.6 ARNO suppression reduces VEGFR-2 protein levels

Next we assessed the tyrosine phosphorylation of VEGFR-2 by western blotting upon ARNO siRNA treatment to elucidate if the

**Figure 5** VEGFR-2 surface expression is impaired upon suppression of ARNO. (A) Inhibition of clathrin-dependent endocytosis by treatment with hypertonic medium (HTM) as well as inhibition of clathrin independent endocytosis by treatment with methyl-β-cyclodextrin (MβCD, 20 mM) prohibited VEGF-dependent Akt phosphorylation (both *P < 0.05, n = 3, HMEC). (B) Effect of treatment with DMSO (v/v), SecinH3, hypertonic medium (HTM) or methyl-β-cyclodextrin (MβCD, 20 mM) on cell viability was measured by trypan blue exclusion (*P < 0.05 vs. non-treated cells, n = 6–8, HMEC). (C) FACS analysis of the extracellular part of VEGFR-2 on intact cells revealed a time-dependent decline in the surface expression of VEGFR-2 upon VEGF stimulation (50 ng/mL). Treatment with ARNO siRNA 1 (30 nM) significantly reduced the surface expression of VEGFR-2 after VEGF stimulation for 2 and 10 min (*P < 0.05, n = 6, HMEC) in comparison with cells treated with control siRNA. (D) The reduction in VEGFR-2 surface levels was not due to shedding of VEGFR-2, as no difference in VEGFR-2 amounts in the supernatant of cells treated with ARNO siRNA 1 (30 nM) or control siRNA could be detected. As a positive control, VEGFR-2 from whole cell lysates was assayed. (E) Treatment with ARNO siRNA 1 (30 nM) resulted in increased intracellular His-tagged VEGF (50 ng/mL) after trypsination of surface VEGFRs (n = 3, HMEC), indicating enhanced internalized VEGFR-2 after 2 and 10 min.
accelerated internalization of the receptor influences the VEGFR-2 activation. As seen in Figure 6A, VEGFR-2 is tyrosine phosphorylated within 2 min of VEGF stimulation (50 ng/mL) and the phosphorylation level declines within 30 min \( (n = 6, \text{HMEC}) \). ARNO siRNA treatment significantly impaired the VEGFR-2 phosphorylation at 2 and 10 min of VEGF treatment in comparison with control siRNA \( (P < 0.05, n = 6, \text{HMEC}) \). However, ARNO siRNA treatment revealed a lower total protein level of VEGFR-2 upon 2 and 10 min of VEGF stimulation (50 ng/mL) in comparison with control siRNA \( (P < 0.05, n = 6 \text{ and } n = 7 \text{ respectively, HMEC}) \). Similarly, treatment with SecinH3 also reduced the total VEGFR-2 levels reaching significance after 10 min of VEGF treatment \( (P < 0.05, n = 6, \text{HMEC}, \text{Figure 6B}) \). Upon endocytosis, inhibition by application of HTM or the cholesterol depleting agent MβCD, the total VEGFR-2 protein level was also reduced \( (P < 0.01 \text{ and } P < 0.05 \text{ respectively, } n = 5–6, \text{HMEC}) \).

### 4. Discussion

ARNO is directly involved in PI3-K-dependent angiogenic signalling and thus of potential importance for malignancy or ischaemia as these conditions are associated with either pathological or compensatory angiogenesis. Interestingly, ARNO has recently gained recognition as being important for the response to insulin\(^{27}\) or for cell adhesion and migration.\(^ {28–30}\) A direct role for ARNO in endothelial proliferatory signals, however, has not yet been demonstrated. In this study, we for the first time detect ARNO in human endothelial cells and demonstrate its importance for VEGF-dependent angiogenic signalling resulting in endothelial permeability and cell proliferation.
ARNO was shown to be expressed in the cytosol and membrane under non-stimulated (basal) conditions, and VEGF stimulation seemed to enhance the translation of ARNO to the membrane in HMEC in vitro, indicating enhanced ARNO activity. Immunohistochemical staining of explanted arteries confirmed the previous findings, as ARNO was shown to be expressed in the endothelium in vivo, primarily but not exclusively, on the luminal side. We demonstrate that ARNO is involved in VEGF signalling in endothelial cells, as the VEGF-dependent Akt activation was prohibited by both treatment with the pharmacological cytohesin inhibitor SecinH3 and by two different ARNO siRNAs. Indeed, application of SecinH3 as well as ARNO siRNA transfection significantly reduced VEGF-induced endothelial cell proliferation, a process highly dependent on Akt activation. Moreover, application of SecinH3 impaired the tissue response to VEGF by reducing extravascular tracer accumulation in vivo, indicating a role in VEGF-dependent permeability, a process that initializes angiogenesis. The SecinH3 action to reduce extravascular accumulation of Dextran tracer can be attributed in part to reduction in vascular permeability but a significant reduction as the result of reduced net filtration (reduced VEGF-induced vasodilation) cannot be excluded. Nevertheless, these effects demonstrate that ARNO may be of potential importance for VEGF-induced angiogenesis.

To investigate the mechanisms underlying this phenomenon, we hypothesized that the effects on VEGF signalling seen after ARNO inhibition in our experiments could have been due to impaired VEGFR-2 internalization, which occurs following ligand binding. Ligand-dependent internalization of VEGFR-2 seems to rely on clathrin-dependent endocytosis but the receptor is enriched in plasma membrane caveolae and its dissociation from these compartments seems to be crucial for its signalling. Therefore, we first inhibited these processes to investigate if any of these are responsible for VEGF-dependent Akt activation. Indeed treatment of endothelial cells with the cholesterol-depleting agent MβCD or with HTM to destroy the endosomes prohibited VEGF-induced phosphorylation and thus activation of Akt. This demonstrates that an impairment of this process is of crucial importance for proper VEGF signalling. Surprisingly, when assessing surface expression of VEGFR-2 by flow cytometry after VEGF stimulation ARNO siRNA significantly reduced VEGFR-2 surface levels in comparison with control siRNA. The lowest level of VEGFR-2 surface expression after ARNO siRNA was reached already after 2 min of VEGF stimulation, whereas this level was reached only after 30 min of VEGF stimulation in cells treated with control siRNA. This reduction was not due to shedding of extracellular VEGFR-2 from the membrane, as no changes in VEGFR-2 concentrations in the supernatant upon ARNO knock-down and different times of VEGF stimulation were detected. The decrease in surface VEGFR-2 was rather shown to be caused by increased internalization of the receptor, as stimulation with a Histagged VEGF resulted in more intracellular VEGF-His after 2 and 10 min of stimulation in cells suppressed of ARNO compared with control cells. This strongly indicates that ARNO is important for receptor internalization.

Furthermore, the VEGF-induced tyrosine phosphorylation of the VEGFR-2 was also diminished upon ARNO siRNA treatment and significantly less total VEGFR-2 could be detected upon ARNO siRNA or SecinH3 treatment in VEGF-treated cells in comparison with VEGF treatment alone. The latter indicates a possible degradation of the VEGFR-2, which would also explain the loss of VEGFR-2 tyrosine phosphorylation and thus activation. The decline in VEGFR-2 protein levels was also observed after interfering with endosomal pathways using HTM and MβCD, respectively, suggesting a possible route of ARNO function. Thus, the abrogated Akt signalling, the diminished endothelial proliferation, and the impairment in VEGF-dependent endothelial permeability observed upon inhibition or knock-down of ARNO in this study are most likely explained by an acceleration of VEGFR-2 internalization resulting in its enhanced degradation. It is likely, yet beyond the scope of this study, that ARNO governs these processes through activation of its target ARF6, because of its role in endocytic trafficking. However, other substrates for ARNO, such as the GTPase ARL4D, have been identified responsible for remodelling of the actin cytoskeleton and which is also important for the process of endocytosis. Thus, how exactly this process is regulated by ARNO needs further investigation.

In summary, we here show a significant and so far unrecognized involvement of ARNO in VEGF-dependent endothelial cell signalling and angiogenesis initiation. These data may contribute to the further understanding of the regulation of VEGFR-2 during angiogenesis.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements
The authors would like to thank the members of the lab of Prof. Dr Welsch at the Neuroanatomical Department of Ludwig-Maximilians-University, Munich, Germany for valuable help with Immunohistochemistry.

Conflict of interest: none declared.

Funding
Part of this work was supported by the ‘Münchener Medizinische Wochenschrift e. V’ and by the grant KR2298/2-1 given to F.K. and H.K.M. by the Deutsche Forschungsgemeinschaft (DFG).

References