

Inhibition of aldose reductase prevents angiogenesis in vitro and in vivo

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Abstract We have recently shown that aldose reductase (AR, EC 1.1.1.21) a nicotinamide adenine dinucleotide phosphate-dependent aldo–keto reductase, known to be involved in oxidative stress-signaling, prevents human colon cancer cell growth in culture as well as in nude mice xenografts. Inhibition of AR also prevents azoxymethane-induced aberrant crypt foci formation in mice. In order to understand the chemopreventive mechanism(s) of AR inhibition in colon cancer, we have investigated the role of AR in the mediation of angiogenic signals in vitro and in vivo models. Our results show that inhibition of AR significantly prevented the VEGF- and FGF -induced proliferation and expression of proliferative marker Ki67 in the human umbilical vein endothelial cells (HUVEC). Further, AR inhibition or ablation with siRNA prevented the VEGF- and FGF -induced invasion and migration in HUVEC. AR inhibition also prevented the VEGF- and FGF- induced secretion/expression of IL-6, MMP2, MMP9, ICAM, and VCAM. The anti-angiogenic feature of AR inhibition in HUVEC was associated with inactivation of PI3 K/AKT and NF- κ B (p65) and suppression of VEGF receptor 2 protein levels. Most importantly, matrigel plug model of angiogenesis in rats showed that inhibition of AR prevented infiltration of blood cells, invasion, migration and formation of capillary like structures, and expression of blood vessels markers CD31 and vWF. Thus, our results demonstrate that AR inhibitors could be novel agents to prevent angiogenesis.

Keywords Aldose reductase · Angiogenesis · Endothelial cells · Cancer and inflammation

Abbreviations

AP1	Activator protein
AR	Aldose reductase
DHN	1,4-dihydroxynonene
FGF	Basic fibroblast growth factor
Fid	Fidarestat
GSH	Glutathione
GS-HNE	Glutathionyl-4-hydroxynonenal
GS-DHN	Glutathionyl-1,4-dihydroxynonene
HNE	4-hydroxy-trans-2-nonenal
HUVEC	Human umbilical vascular endothelial cells
MMP	Matrix metalloproteinases
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF- κ B	Nuclear factor kappa B
ROS	Reactive oxygen species
siRNA	Small interfering RNA
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cells
vWF	Von-Willebrand factor

Introduction

Angiogenesis, formation of new blood vessels by endothelial cells, is a highly complex and tightly regulated physiological process, implicated in various pathological diseases such as wound healing, tissue remodeling, thrombosis, rheumatoid arthritis, diabetic retinopathy and tumor growth [1–3]. Angiogenesis in tumors allows the growth and invasiveness of tumor cells leading to metastasis to distant organs [2–4]. Angiogenesis, especially in a solid

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tumor progression plays a critical role because when tumor mass increases beyond 2–3 mm, formation of new blood vessels is presumably essential in supplying nutrients and oxygen and removing waste products [1–3]. In angiogenesis the interaction of tumor cells with vascular endothelial cells leads to the release of various growth factors, cytokines, chemokines, etc. [4, 5]. These molecules in turn by autocrine and paracrine fashion activate inflammatory signaling leading to differentiation of vascular endothelial cells. In addition, the important concept of tumor angiogenesis is that in the tumor microenvironment tumor-associated endothelial cells are genetically unstable and show enhanced responsiveness to growth factors such as VEGF, FGF and EGF [4–6]. Therefore, antiangiogenic therapy represents one of the most promising approaches to regulate tumor growth and metastasis.

Various reports suggest that induction of angiogenic factors is triggered by various stresses, including oxidative stress [7]. For instance, reactive oxygen species (ROS) such as superoxide anions ($O_2^{\bullet-}$), hydroxyl radical (OH^{\bullet}), and H_2O_2 are generated via activated phagocytes under various pathological conditions, including inflammation, ischemia and reperfusion, sepsis and UV radiation [7–11]. The vascular endothelium is one of the prime targets of these inflammatory oxidants. Further, it has been shown that activation of redox sensitive transcription factors such as NF- κ B and AP1 involved in transcribing various inflammatory genes that synthesize growth factors, cytokines, chemokines and matrix metalloproteases which cause endothelial cell growth, adhesion, invasion, migration and angiogenesis [7, 12–14]. NF- κ B is the first eukaryotic transcription factor shown to respond directly to oxidative stress-mediated tumorigenesis and angiogenesis [12, 13]. Antioxidants such as N-acetyl cysteine, resveratrol, lycopene, green tea and epigallocatechin-3-gallate have been shown to attenuate the angiogenesis in various tumor microenvironments via inhibition of NF- κ B [14–19]. Therefore, anti-angiogenic therapy by using anti-oxidants represents one of the most promising approaches to control tumor growth, invasion and angiogenesis. In fact, several antioxidants are under drug development programme and clinical trials to identify novel anti-angiogenic and anti-cancer agents. Our recent studies with human vascular umbilical endothelial cells (HUVEC) suggest that the polyol pathway enzyme- aldose reductase (AR; AKR1B1), a member of aldo-keto reductase super family, is a regulator of activation of NF- κ B and expression of adhesion molecules, ICAM, VCAM induced by cytokines such as TNF- α [20, 21].

We have previously shown that AR besides reducing aldo-sugars efficiently catalyzes the reduction of lipid aldehydes such as 4-hydroxy-trans-2-nonenol (HNE) and their glutathione conjugates such as GS-HNE to 1,

4-dihydroxynonene (DHN) and GS-DHN, respectively with low (μ M) Km compared to glucose with Km in mM range [22–24]. Further, we have demonstrated that inhibition of AR could prevent protein kinase C, NF- κ B, and AP-1 activation and the increase in cell growth caused by HNE and GS-HNE, but not by GS-DHN [22–27]. These studies suggested that the already reduced form of glutathione-lipid aldehyde, GS-DHN is insensitive to AR inhibition and could be the main mediator of oxidative stress-induced NF- κ B activation. Using human colon cancer Caco-2 cells we have demonstrated that AR inhibition as well as ablation by siRNA prevents the growth factors such as FGF- and PDGF-induced activation of NF- κ B, expression of Cox-2 and production of PGE2 [22, 28]. Most remarkably, in the nude mice xenograft model, we have shown that inhibition of AR by ARsiRNA as well as by AR inhibitor, fidarestat completely prevented growth of human colon adenocarcinoma cells (SW480 and HT29) implanted subcutaneously [22, 28]. Further, recent results with azoxymethane (AOM) model in male BALB/c mice showed that the inhibition of AR by a pharmacological inhibitor as well as AR gene knockout in mice significantly prevented aberrant crypt foci formation and AOM-induced expression of inflammatory markers, iNOS and Cox-2 and preneoplastic marker proteins, cyclin D1 and beta-catenin and activation of NF- κ B in mice colons [29]. However, the involvement of AR in the angiogenesis is unknown. Since NF- κ B plays a key role in angiogenesis by regulating transcription of angiogenic growth factors (such as VEGF and FGF), we hypothesized that inhibition of AR could inhibit angiogenesis. We now show that inhibition of AR effectively prevents in vitro angiogenesis (capillary-like tube structure and spheroid formation, invasion and migration) of HUVEC by causing suppression of pro-angiogenic growth factor secretion and MMPs, and adhesion molecules' expression and NF- κ B activation. Further, our results show that inhibition of AR could prevent in vivo angiogenesis in a rat matrigel-plug model. These findings for the first time indicate that AR is an excellent novel therapeutic target for the prevention of angiogenesis.

Materials and methods

Chemicals and reagents

Ham's F12 K, PBS, penicillin/streptomycin, trypsin, and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA). Antibodies against AKT, p65, MMP2, MMP9, VEGFR-2 and GAPDH were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Phospho-VEGFR-2 was purchased from Cell applications Inc (San Diego, CA). Anti-NO₂-Tyr was purchased from EMD

Biosciences, Gibbstown, NJ. Fidarestat was obtained as a gift from Sanwa Kagaku Kenkyusho Co. Ltd. (Japan). Cell invasion assay kit was obtained from Chemicon International Inc. (Billerica, MA). FGF and VEGF other reagents used in Western blot analysis were obtained from Sigma (St. Louis, MO). All other reagents used were of analytical grade.

Cell culture

Human umbilical vascular endothelial cells (HUVEC) were obtained from Cell Application Inc and grown in Ham's F-12 K medium containing 10% FBS and cultured at 37°C under an atmosphere containing 5% CO₂.

Measurement of cytotoxicity

HUVEC were plated in a 96-well plate at 2,500 per well and growth arrested in 0.1% FBS with or without AR inhibitor fidarestat (5 μM), or transfected with AR-siRNA or control siRNA using RNAiFect reagent (Qiagen). After 24 h, VEGF or FGF (10 ng/ml) was added to the medium and the cells were incubated for another 24 h. Cells incubated with the AR inhibitor alone served as control. Cell viability was determined by MTT assay as described earlier [22].

Tube formation assay

The endothelial cell tube-like formation assay was performed using HUVEC as described elsewhere [30, 31]. Briefly, fifty micro liters of reduced growth factors basement membrane extract (BME) solution was added to each well of 96 well plate and incubated at 37°C for 30 min to allow gel formation. HUVEC (7,500 cells/well) in Ham's F12 K basal medium with or without VEGF/FGF (10 ng/ml) and/or AR inhibitor, fidarestat with different concentrations plated on BME gel. For AR siRNA and scrambled siRNA group, cells were transfected and plated on BME gel. After an overnight incubation, the network growth area was examined using an inverted microscope (50×).

Spheroid formation

Spheroid formation assay in HUVEC was performed as described elsewhere [30, 31]. Briefly, HUVEC (4,000/ml) were suspended in Ham's F12 K containing 20% (v/v) methocel, seeded into non-adherent round-bottom 96-well plates and incubated overnight. The methocel used was prepared by dissolving 6 g of carboxymethylcellulose (Sigma–Aldrich) in 500 ml of Ham's F-12 K. The spheroids were harvested by gently pipetting, centrifuged at 500 rpm for 5 min, and embedded into neutralized collagen gels with 1:1 ratio. The spheroids in collagen solution

were rapidly transferred into 96-well plate and incubated at 37°C for 24 h with or without VEGF (10 ng/ml) and/or AR inhibitor, fiderestat (5 μM). The spheroid images were captured using a camera linked to an inverted microscope (50×).

Determination of Ki67 expression in HUVEC

HUVEC grown 70–80% confluent in T-25 flasks were pre-incubated for 24 h with and without fidarestat (5 μM) followed by stimulation with VEGF or FGF (10 ng/ml) for another 24 h. Expression of cell proliferation marker Ki-67 was determined by FACS analysis as per manufacturer's instructions (BD Biosciences, San Jose, CA). HUVEC (1 × 10⁶/ml) were fixed in 70–80% ethanol by incubating at –20°C for 2 h. Cells were washed with buffer (PBS with 1% FBS, pH7.2) and incubated with PE-conjugated ki67 mAb for 30 min. After washing HUVEC were diluted in BD Via-Probe™ cell viability solution and analyzed with a minimum of 10,000 events using Cell Quest version 3.3 software for data analysis (Becton–Dickinson, San Jose, CA).

Invasion and migration assays

Invasive assay was performed using Basement Membrane Extract. Fifty micro liters of BME solution was added on top of 8 micron polyethylene terephthalate membrane to each well of 96 well plate and incubated at 37°C for 4 h to allow gel formation. HUVEC (20,000 cells/well) in basal medium with or without VEGF (10 ng/ml) and/or AR inhibitor, fidarestat (5 μM) plated on Matrigel. After 24 h of incubation invasion of cells towards bottom side of the well was measured using Calcein-AM fluorescent dye. Migration of HUVEC was performed by scratch wound assay as described elsewhere [32].

Matrigel plug model in rats (in vivo angiogenesis)

Five hundred micro liters of matrigel (growth factor free) with or without VEGF (10 ng/ml) and/or fidarestat (20 μM) was injected subcutaneously (s.c.) into the dorsal tissue of Fischer 344 rats (*n* = 4/group) and AR inhibitor, fidarestat (16 mg/kg body weight) was given through drinking water [33]. After 1 week, rats were killed and plugs were excised and perfusion-fixed with 4% paraformaldehyde and stored in 70% ethanol. The fixed matrigels were stained with hematoxylin and eosin. Immunohistochemical analysis was performed by using monoclonal antibodies against CD31/PECAM-1 and von-Willebrand factor (vWF) using Dako-Cytomation LSAB + System-HRP kit. Stained sections were observed under Nikon epi-fluorescence microscope (200×).

Immunoprecipitations and Western blot analysis

To examine the expression/phosphorylation of TIMP-1, p-VEGFR2, VEGFR2, MMP2, MMP9, AKT, p-65 and GAPDH proteins, Western blot analysis was carried out. Equal amounts of protein from liver tissue extracts were subjected to 12% SDS–PAGE followed by transfer of proteins to nitrocellulose filters and probing with the indicated antibodies. The antigen–antibody complex was detected by enhanced chemiluminescence (Pierce, Piscataway, NJ). For immunoprecipitations, 50 mg of cell lysate protein was incubated overnight at 4°C with anti-nitrotyrosine antibody and the covalently coupled antigen–antibody complex were separated using Pierce classic IP kit from Thermo Scientific USA, by following the manufacturer’s instructions. The separated immune complexes were denatured at 95°C followed by SDS–PAGE and Western blotting using antibodies against AKT and PI3 K.

Determination of angiogenic factors-induced adhesion and matrix metalloproteinases in HUVEC by ELISA

HUVEC were grown confluent in 6 well plates and pre incubated with and without fidarestat for 24 h. Cells were treated with VEGF and FGF (10 ng/ml) for another 24 h and cell culture medium was subjected to ELISA analysis of VCAM, ICAM, IL-6, MMP2, MMP9 and IFN- γ using Ray Biotech ELISA kits as per the manufacturer’s instructions. Values were plotted against the standards obtained in the assay along with the experimental.

Determination of PI3 K activity

PI3 K activity in HUVEC was determined using a competitive ELISA kit (Echelon Biosciences, Inc., Salt Lake City, UT) as described previously [28]. Briefly, HUVEC cells were serum-starved for overnight with or without fidarestat and stimulated with VEGF (10 ng/ml) for 20 min. The cells were washed and total cell lysate was made using lysis buffer. The supernatant was incubated with anti-PI3 K p85- α antibody (5 μ l antibody/sample) for 1 h at 4°C followed by protein A-agarose chromatography. The PI3 K activity in the immunoprecipitated beads was measured by incubating with phosphatidylinositol (4, 5)-biphosphate [PI(4, 5)P₂] substrate (100 pmol) in kinase reaction buffer for 2 h at room temperature with shaking. The supernatant was incubated with PI-[3, 4, 5]P₃ detector protein for 1 h at room temperature followed by transfer to a PI-[3, 4, 5]P₃-coated detection plate for 1 h at room temperature for competitive binding. After washing with Tris buffer a peroxidase-linked secondary detector was added and developed with 3,3',5,5'-tetramethylbenzidine (TMB) solution. The PI-[3, 4, 5]P₃ detector protein binding

to the plate was determined by measuring the absorbance at 450 nm. The colorimetric signal is inversely proportional to the amount of PI-[3,4,5]P₃ produced by PI3 K.

Statistical analysis

Data are presented as mean \pm S.E and the *P* values were determined using the unpaired Student’s *t* test.

Results

Inhibition of AR prevents angiogenic factors-induced proliferation of HUVEC

To test the hypothesis that AR inhibition can prevent angiogenesis, initially we determined the effects of AR inhibition on angiogenic factors-induced proliferation of HUVEC. As shown in the Fig. 1a, treatment of HUVEC with VEGF or FGF for 24 h significantly stimulated the growth as determined by MTT assay. The increase in growth was attenuated in the presence of AR inhibitor, fidarestat. In the absence of angiogenic factors, fidarestat alone did not affect the HUVEC growth. To exclude non-specific effects of a pharmacological inhibitor, we ablated the AR message by transient transfection of HUVEC with ARsiRNA. Transfection of HUVEC with ARsiRNA but not scrambled ARsiRNA caused >95% ablation of AR protein (data not shown). Consistent with our previous data, transfection of HUVEC with ARsiRNA significantly (~75%) inhibited VEGF- and FGF-induced cell growth (Fig. 1b). The inhibitory activity of fidarestat on VEGF- and FGF-induced HUVEC proliferation was also assessed by flow cytometry using PE-labeled Ki67, a proliferation marker (Fig. 1c). Treatment of HUVEC with VEGF or FGF increased the Ki67 expression, whereas AR inhibition significantly prevented it. These results suggest that AR mediates VEGF- or FGF-induced HUVEC growth.

Inhibition of AR prevents angiogenic factors-induced capillary-like tube structure network and spheroid formation

We next examined the effect of AR inhibition on angiogenic factors-induced tube formation in HUVEC. The HUVEC were plated in a 96 well plate containing growth factor –reduced matrigel and determined the effect of AR inhibitor on VEGF- and FGF-induced capillary—like structure formation (in vitro angiogenesis). As shown in the Fig. 2a inhibition of AR by fidarestat prevented angiogenic factors such as VEGF- and FGF-induced capillary—like tube structure formation in a concentration dependent manner. The capillary-like tube structure formation by

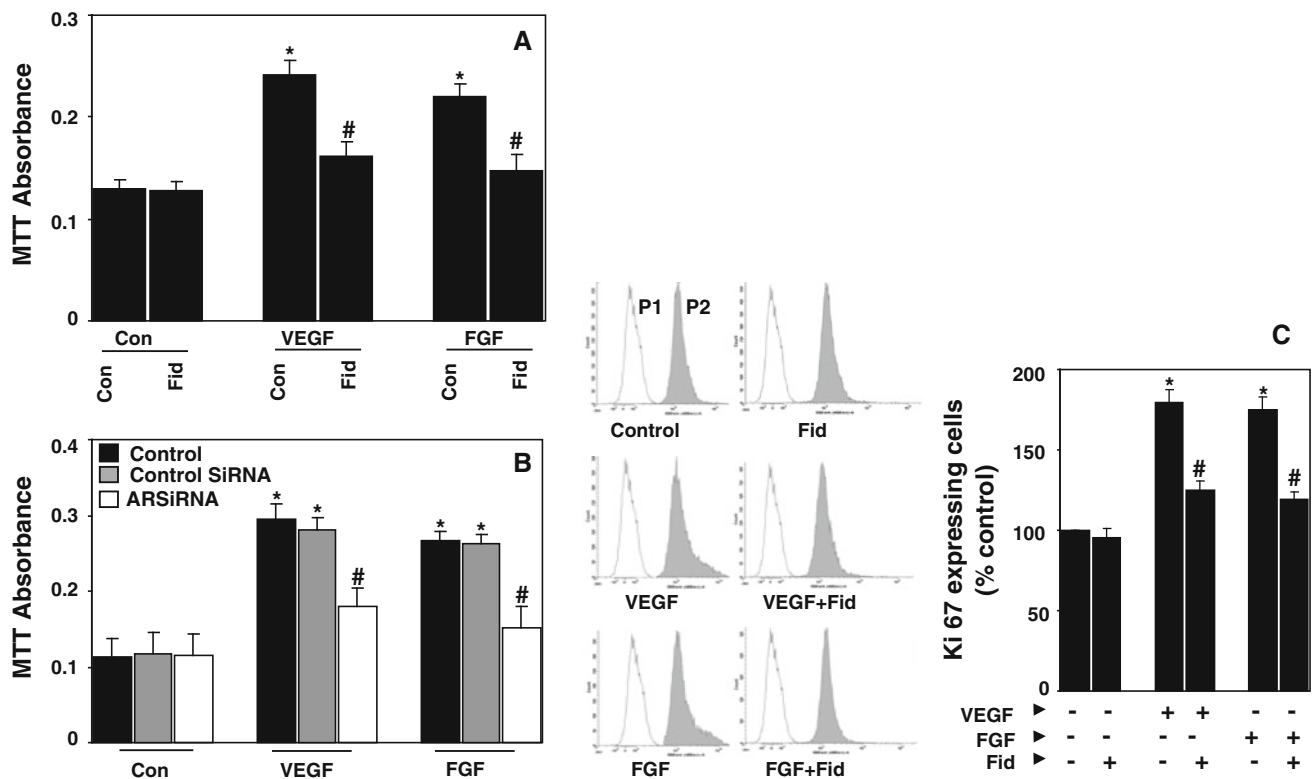


Fig. 1 Inhibition of AR prevents pro-angiogenic factors-induced proliferation of HUVEC. **a** and **b** Growth-arrested HUVEC were pre-incubated with fidarestat (5 μM) or transfected with ARsiRNA or scrambled ARsiRNA for 24 h followed by stimulation with VEGF or FGF (10 ng/ml) for 24 h. Cell viability was determined by MTT assay. **c** Expression of Ki-67 in HUVEC was determined by flow cytometry using PE-labeled Ki-67 antibodies (P2: filled histogram)

and isotype control antibodies as negative control (P1: empty histogram). The data was analyzed by using Cell Quest version 3.3 software. Bar graph represents percentage of Ki 67 expressing cells compared to control. Bars represent mean ± S.E. (n = 6; two independent experiments with triplicates); * P < 0.001 vs. control and #, P < 0.001 vs. growth factor-treated cells

HUVEC was completely abolished at a higher concentration (10 M) of fidarestat. Consistent with our data obtained with AR inhibitor, transfection of HUVEC with AR-siRNA significantly (~85%) inhibited VEGF- and FGF-induced tube formation (Fig. 2a). We next determined the effect of fidarestat on VEGF-induced three-dimensional endothelial cell organization and morphogenesis using HUVEC spheroids, i.e., cell aggregates, embedded in a collagen gel. In the presence of VEGF, HUVEC spheroid sprouted and invaded the surrounding collagen gel forming capillary-like structures (Fig. 2b). AR inhibition prevented the VEGF-induced sprout formation in a significant manner. Collectively, these results suggest that AR inhibition prevents VEGF- and FGF-induced angiogenesis in HUVEC.

Inhibition of AR prevents angiogenic factors-induced invasion and migration of HUVEC

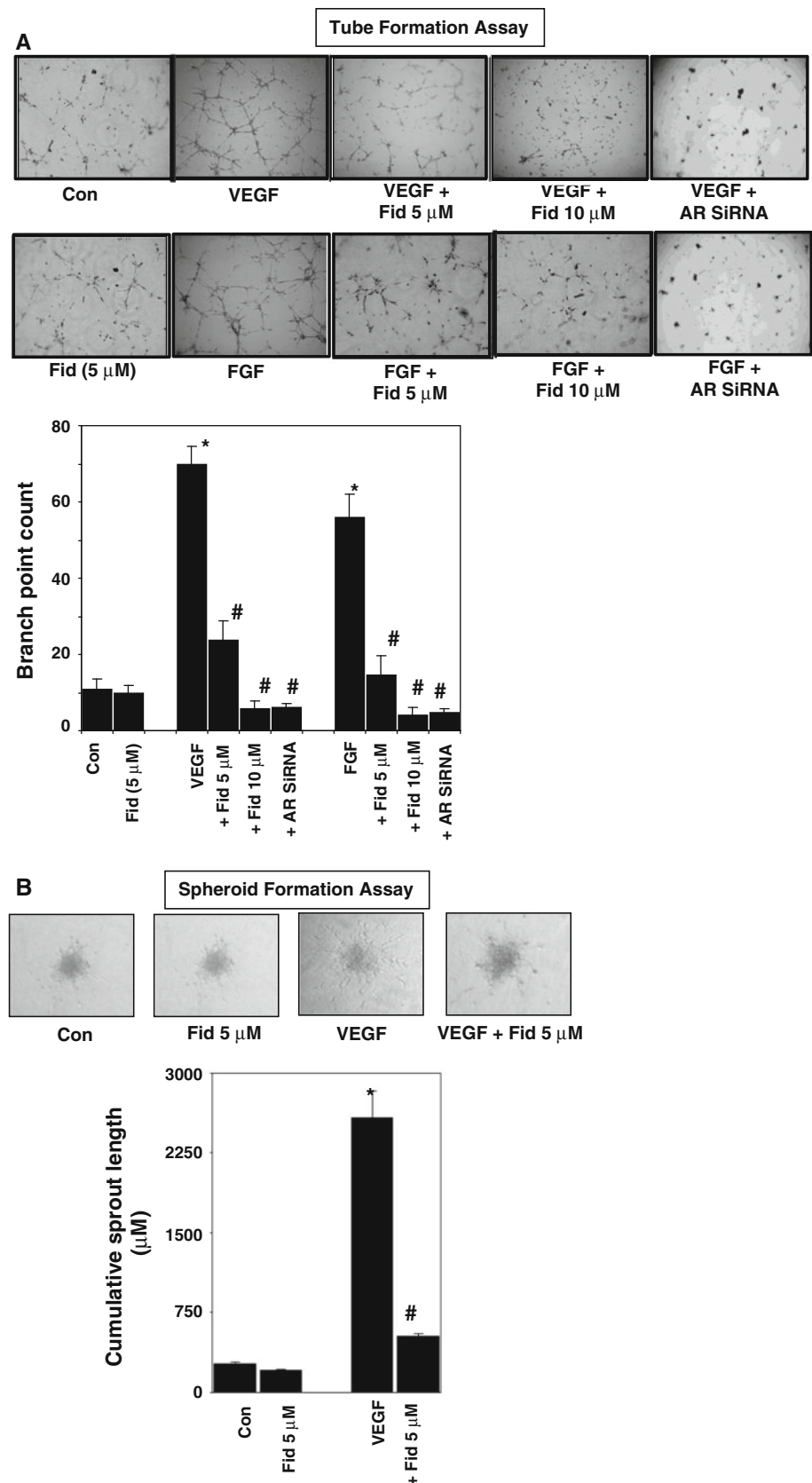
Since secretion of growth factors and cytokines by tumor cells is known to cause invasion and migration of endothelial cells which cause neovascularization, we determined the effect of AR inhibition on growth factors –induced invasion

of HUVEC. As shown in the Fig. 3a, treatment of HUVEC with VEGF or FGF caused pronounced invasion of endothelial cells, whereas inhibition of AR by fidarestat significantly prevented it. Next, we determined the effect of AR inhibition on VEGF- or FGF-induced migration of HUVEC using wound scratch model assay. Our Results indicate that inhibition of AR by pharmacological inhibitor or ablation of AR by using specific siRNA significantly prevented the migration of HUVEC (Fig. 3b). These results indicate that AR inhibition prevents invasion and migration of endothelial cells.

Inhibition of AR prevents angiogenic factors-induced secretion/expression of ICAM, VCAM, MMP2, MMP9 and IL-6 in HUVEC

VEGF and FGF are known to play an important role in the angiogenesis by inducing secretion of adhesion molecules, matrix metallo proteinases, and cytokines, which are known to be involved in endothelial cell proliferation, migration, and differentiation. Therefore, to gain insights into the mechanism(s) by which AR inhibition prevents

Fig. 2 Inhibition of AR prevents angiogenic factors-induced capillary-like tube structure and spheroid formation in HUVEC. Growth-arrested HUVEC (**a**) or HUVEC spheroids (**b**) were incubated in 96 well plate with or without VEGF or FGF (10 ng/ml) and/or AR inhibitor, fidarestat. After an overnight incubation, the network growth area was examined using an inverted microscope 50 \times . Angiogenic effect was measured by counting the relative branch points. Spheroids were digitally quantified by measuring the cumulative length of the sprouts. Bars represent mean \pm S.E. ($n = 6$); * $P < 0.001$ vs. control and #, $P < 0.001$ vs. growth factor-treated cells



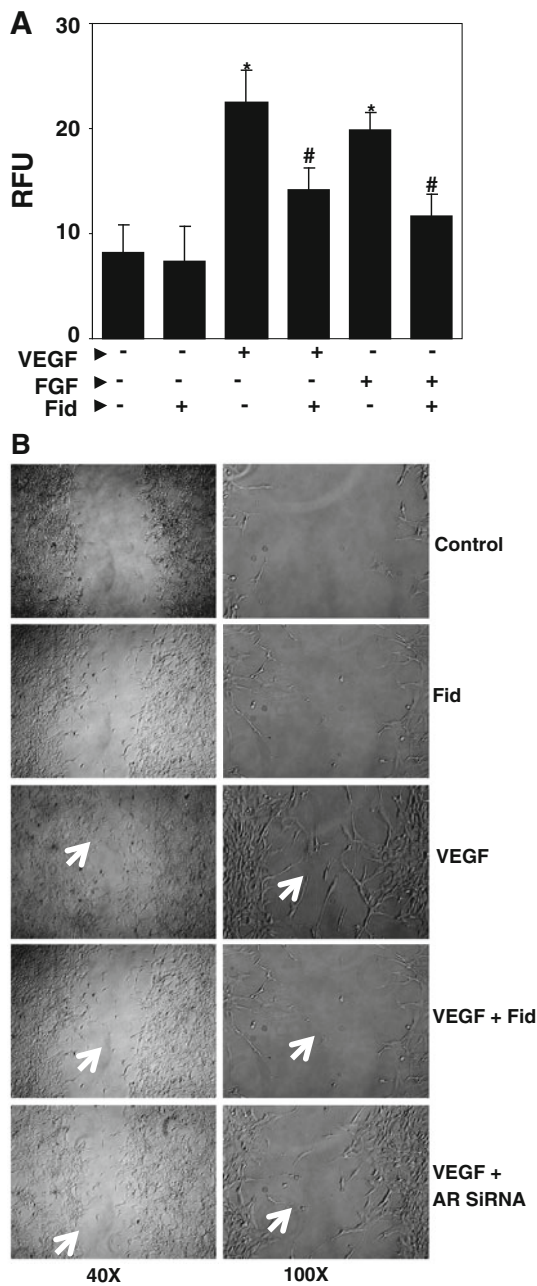


Fig. 3 Inhibition of AR prevents angiogenic factors-induced invasion and migration of HUVEC. **a** Invasion assay in HUVEC was performed in 96 well plate using basement membrane extract. Invasion of cells towards bottom side of the well was measured using Calcein-AM fluorescent dye at 485/520 nm excitation/emission. **b** Inhibition of AR prevents growth factors-induced migration of HUVEC in a scratch wound assay. Wound scratches were made with a sterile pipette tip in the wells at the sub-confluent stage. After 24 h of incubation with and without AR inhibitor, the migration of cells was observed and photographs were taken using Nikon camera fixed to inverted microscope (40 × and 100 ×). The white arrow indicates the area of scratch and HUVEC migration. Bars represent mean ± S.E. (n = 6); * P < 0.001 vs. control and #, P < 0.001 vs. growth factor-treated cells

angiogenesis, we determined the effect of AR inhibition on VEGF- and FGF-induced secretion/expression of ICAM, VCAM, MMP2, MMP9 and IL-6 in HUVEC (Fig. 4a). Treatment of HUVEC with VEGF or FGF caused pronounced increase in the secretion of ICAM, VCAM, MMP2, MMP9 and IL-6 and inhibition of significantly prevented the release of these proteins into the culture media. Similarly, AR inhibition also prevented the expression of ICAM, VCAM, MMP2 and MMP9 proteins (Fig. 4b), suggesting AR inhibition prevents invasion and migration of HUVEC by inhibiting the expression of MMPs. Since the activities of MMPs are regulated by the physiological inhibitor proteins, TIMPs, we next examined the effect of AR inhibition on growth factor-induced expression of TIMP-1, a specific regulator of MMP-9 and a well-established prognostic marker for colon cancer, in HUVEC cells. The levels of TIMP-1 are not detectable by Western blot analysis (data not shown). Similarly, inhibition of AR increased the secretion of IFN- γ (Fig. 4a), an anti-proliferative and anti-angiogenic cytokine, which decreased in the presence of VEGF or FGF suggesting that AR inhibition prevents proliferation as well as angiogenesis of endothelial cells. The VEGF mediated pro-survival signal transduction to the nucleus is caused by its interaction with receptors such as VEGF-R1, VEGF-R2, and VEGF-R3. Among the three VEGF receptors, VEGF-R2 is known to play an important role in angiogenesis [36]. Next, we tested whether AR inhibition mediated prevention of HUVEC migration and tube formation was caused by a decrease in the protein level of VEGF-R2. As shown in the Fig. 4b, inhibition of AR significantly prevented the VEGF-induced expression its receptor VEGF-R2 at 24 h. We next measured the effect of AR inhibition on VEGF-induced phosphorylation of VEGF-R2 in a time dependent manner. Our results shown in Fig. 4c indicate that VEGF caused phosphorylation of VEGF-R2 and inhibition of AR prevented the VEGF-induced phosphorylation. The total levels of VEGF-R2 were not affected up to 2 h of VEGF-treatment.

Inhibition of AR prevents VEGF-induced formation of capillary-like tube structure network in matrigel plugs in vivo

The effect of AR inhibition on endothelial cell invasion and organization into capillary-like structures was investigated using the in vivo matrigel plugs assay, performed in rats. Rats were subcutaneously injected in the dorsal midline with growth factor-reduced matrigel containing VEGF and/or fidarestat. After 1 week, plugs were excised and analyzed to assess the presence of endothelial cells and

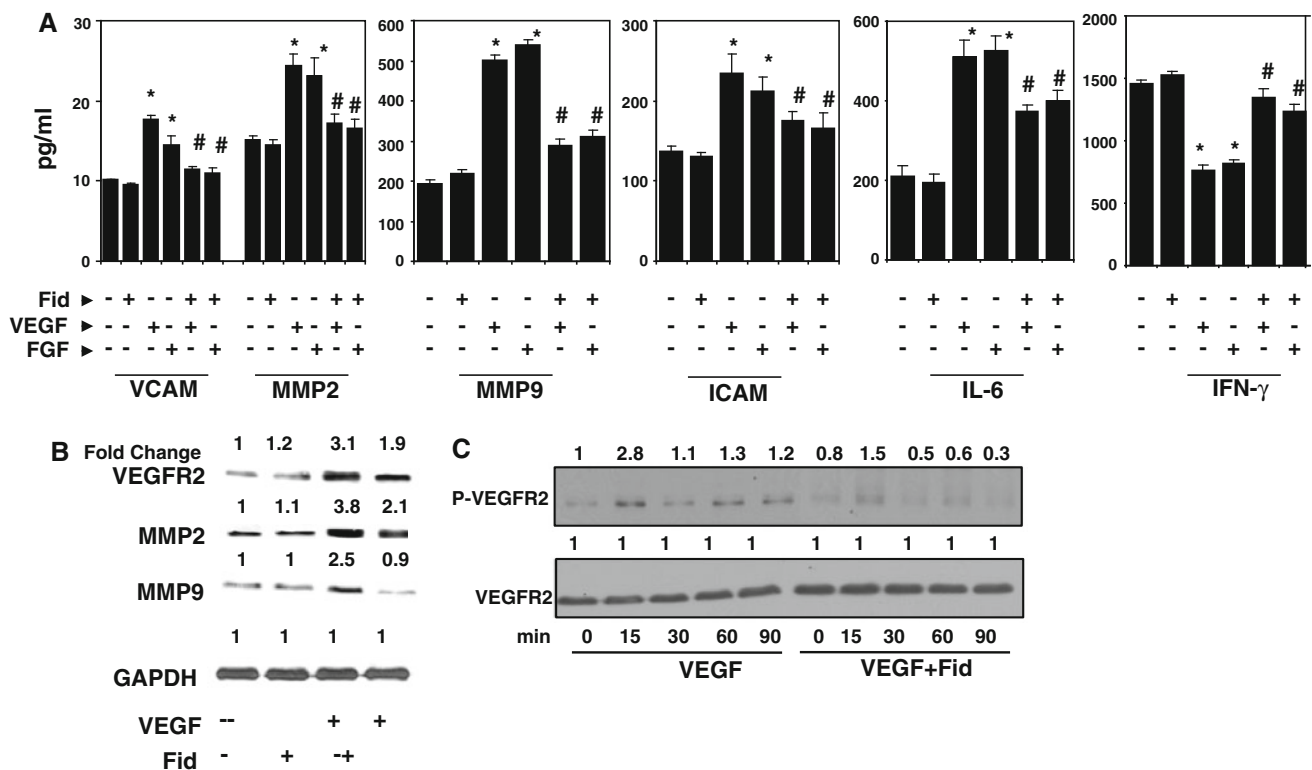


Fig. 4 Inhibition of AR prevents angiogenic factors-induced secretion of ICAM, VCAM, MMP2, MMP9 and IL-6 in HUVEC. **a** Growth arrested HUVEC were treated with VEGF or FGF (10 ng/ml) and/or fidarestat (5 μ M) for 24 h and cell culture medium was subjected to ELISA analysis using RayBiotech ELISA kits as per manufacturer's instructions. **b** After 24 h stimulation of HUVEC with VEGF, Western blot analysis performed using antibodies against

VEGF-R2, MMP-2, MMP-9, and GAPDH. **c** Western blot analysis of VEGF-R2 phosphorylation was determined using Phospho specific VEGF-R2 antibodies. Fold change was quantified by using Kodak Image station 2000R loaded with Kodak 1-dimensional image-analysis software (Kodak, Rochester, NY). Bars represent mean \pm S.E. ($n = 4$); * $P < 0.001$ vs. control and #, $P < 0.001$ vs. growth factor-treated cells

neovascularization. As shown in the Fig. 5a, H&E staining the maximal localization of cells organized in capillary-like structures occurred in plugs containing VEGF-treated group. Immunohistochemistry with anti-CD31 and anti-vWF antibodies showed that infiltrating cells were endothelial cells and that VEGF enhanced the angiogenesis with respect to control (Fig. 5a). Presence of red blood cells in some of these capillaries indicates that they were functional. We have also quantitated the newly vascularized vessels in the entire plug counted under a light microscope. The average of counts from four plugs per group was plotted in the graph shown in Fig. 5b. The VEGF-induced increase in the number of capillary structures was significantly reduced in rats treated with fidarestat. Our results thus indicate that VEGF-induced increase of capillary-like structures formation, infiltration of endothelial cells and red blood cells was significantly inhibited by co-treatment with VEGF and fidarestat which was not observed in specimens treated with fidarestat alone. These findings suggest that AR inhibition could prevent VEGF activity on endothelial cells migration and invasion in vivo also.

Inhibition of AR prevents activation of PI3 K/AKT and NF- κ B in HUVEC cells

Since activation of PI3 K/AKT is known to be involved in endothelial cell survival and VEGF-induced migration and differentiation [34, 35], we next measured the effect of AR inhibition on the VEGF-induced activation of PI3 K and AKT. As shown in the Fig. 6a, VEGF stimulated PI3 K activity approximately 2.0 folds as compared to control and inhibition of AR significantly ($\sim 65\%$) prevented it. Similarly, inhibition of AR significantly prevented the VEGF-induced phosphorylation of AKT (Fig. 6b). To examine if AR inhibition prevented the AKT and PI3 K activity by regulating the nitrosation of AKT and PI3 K, we have determined nitro-AKT and nitro-PI3 K levels by using immunoprecipitation studies. Our results shown in Fig. 6c demonstrate that AR inhibition prevents nitrosation of both AKT and PI3 K suggesting that AR inhibition prevents nitrosation of these kinases. Collectively, these results suggest that inhibition of AR could prevent VEGF-induced activation of PI3 K/AKT pathway. Further, to

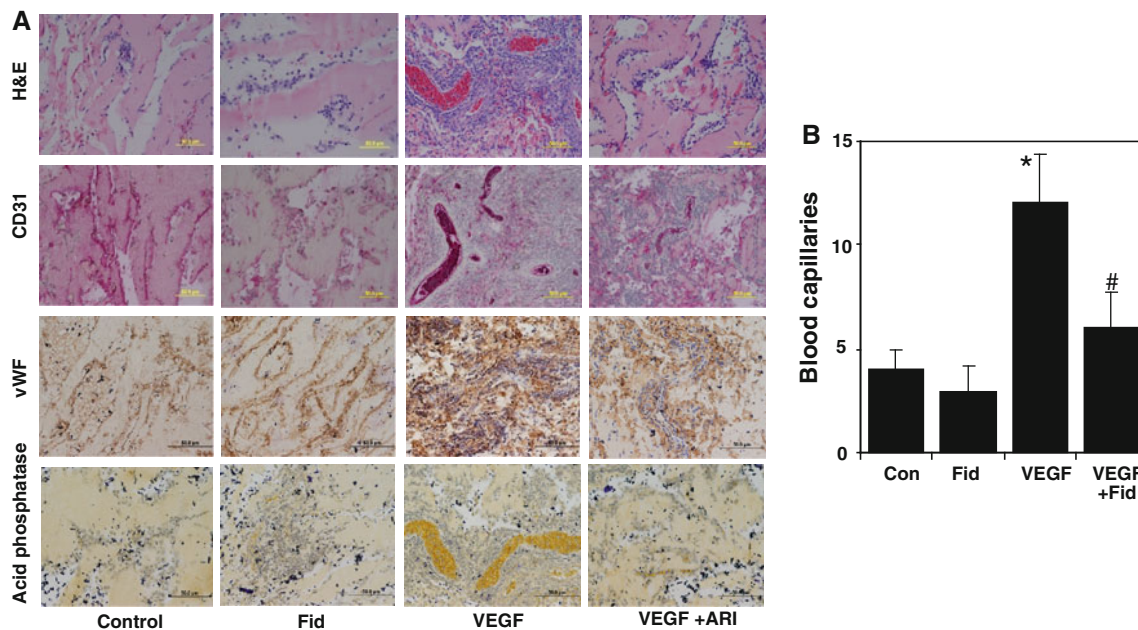


Fig. 5 Inhibition of AR prevents VEGF-induced angiogenesis in rat matrigel plug model. **a** Five hundred μ l of growth factor-free matrigel with or without VEGF (10 ng/ml) and/or fidarestat (20 μ M) was injected subcutaneously (s.c.) into the dorsal tissue of Fischer 344 rats ($n = 4$) and AR inhibitor, fidarestat (16 mg/kg body weight) was given through drinking water. After 1 week, rats were killed and plugs were excised and fixed with 4% paraformaldehyde. Serial

sections (5 μ m thick) of matrigel plugs were stained with hematoxylin/eosin, anti-CD31/PECAM-1 and anti-Von Willebrandt factor and observed under the microscope (400 \times). **b** The graph shows the number of newly vascularized vessels in the entire plug counted under light microscope. Bars represent mean \pm S.E. ($n = 4$); * $P < 0.001$ vs. control and #, $P < 0.001$ vs. growth factor-treated cells

determine the downstream signals of PI3 K/AKT pathway leading to activation of NF- κ B, we next determined the effect of AR inhibition on the phosphorylation of p65, a hetero-dimer subunit of NF- κ B. As shown in the Fig. 6b, inhibition of AR by fidarestat prevented the phosphorylation of p65 suggesting that inhibition of AR could prevent NF- κ B activation by inhibiting the activation of PI3 K/AKT in HUVEC. Since NF- κ B is a redox sensitive transcription factor and increased oxidative stress is known to activate the NF- κ B [12], we next measured the efficacy of AR in preventing the generation of protein-HNE adducts, as a marker for oxidative stress, in HUVEC cells. Our results indicate that AR inhibition significantly prevented the formation of protein-HNE adducts in growth-factor treated HUVEC (Fig. 6c).

Discussion

Angiogenesis involves endothelial cell survival, invasion, proliferation, migration and differentiation [1–4]. Potent pro-angiogenic growth factors such as VEGF, FGF and some extracellular matrix proteins via NF- κ B can trigger signaling pathways involved in the differentiation of endothelial cells [37–39]. Since AR mediates oxidative stress-induced signals that increase transcription of

inflammatory markers [22–26], which play a significant role in diseases such as cardiomyopathy, hypertension, wound healing, tissue remodeling, diabetic retinopathy and cancer which require increased blood supply, we determined the possible role of AR in angiogenesis. The present study for the first time reveals that AR mediates angiogenic features of HUVEC in vitro as well as in vivo, and suggest novel anti-angiogenic efficacy of AR inhibitors.

Various reports show that oxidative stress plays an important role in the initiation of inflammatory signaling in the vasculature of organs [7, 10]. For example, ROS have also been implicated in ischemic and reperfusion injury to a large variety of tissue [40]. Yasuda et al. [41] showed that exposure of endothelial cells to lower concentrations of H_2O_2 stimulates angiogenesis. Further, they demonstrated that cancer cells produce ROS which could be one of the triggers of the angiogenic process in endothelial cells. The ROS cause up-regulation of proangiogenic growth factors such as VEGF, FGF via transcriptional activation of NF- κ B, AP-1, Sp1 and Sp3 [14, 42]. The NOX family of NAD(P)H oxidases is a good source of ROS that could influence tumor cell proliferation via the redox-regulated transcription factor NF- κ B which in turn regulates numerous genes involved in apoptosis, cell proliferation, metastasis and angiogenesis [8, 42]. We have shown

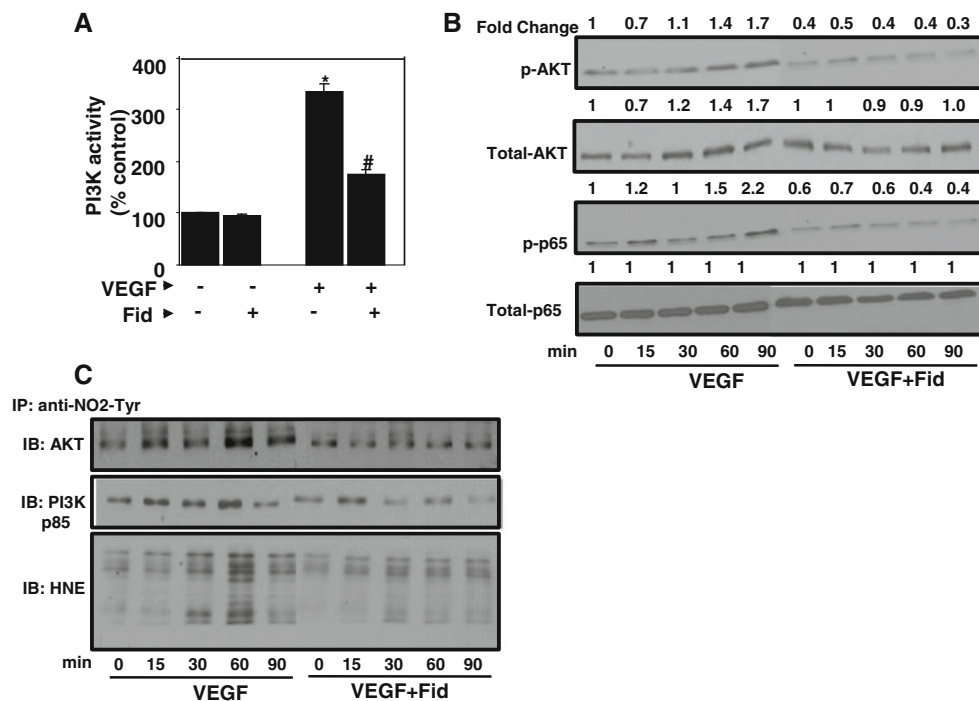


Fig. 6 Inhibition of AR prevents VEGF-induced activation of PI3K/AKT, NF- κ B and expression of VEGF-R2 and matrix metalloproteinases in HUVEC. Growth-arrested HUVEC were pre-incubated with fidarestat (5 μ M) or carrier for 24 h followed by stimulation with VEGF (10 ng/ml) for (a) 20 min to determine PI3K activity as described in materials and methods and (b) Western blots were developed using antibodies against p-AKT, p-p65 and GAPDH. c Equal amount of time dependent VEGF induced HUVEC cell lysates were immunoprecipitated with anti-NO₂-tyr antibodies and

immunocomplexes were separated using Pierce classic IP kit as described in methods. The immune complexes were subjected to Western blot analysis and probed with anti-AKT, anti-PI3K p85 and protein-HNE antibodies. Fold change was quantified by using Kodak Image station 2000R loaded with Kodak 1-dimensional image-analysis software (Kodak, Rochester, NY). Bars represent mean \pm SE ($n = 4$); * $P < 0.001$ compared with control and #, $P < 0.001$ compared with cells treated with growth factor

previously that inhibition of AR can prevent growth factors-, cytokines-, chemokines- and hyperglycemia-induced ROS signals via inhibition of redox-sensitive transcription factors, NF- κ B and AP-1 activation [22–27]. Several experimental data suggest that antioxidants such as green tea, quercetin, resveratrol and lycopene consumption significantly inhibit angiogenesis [15–19]. For example, epigallocatechin gallate (EGCG), a putative chemopreventive agent and an important component of green tea, was found to inhibit tumor invasion and the angiogenesis processes that are essential for tumor growth [19]. Evidence suggests that increased ROS and lipid peroxidation derived products such as HNE, induce transcription of fibrogenic cytokines, chemokines and growth factors, etc. which in autocrine/paracrine fashion play a pivotal role in many biological processes, including immune response, recruitment of inflammatory cells, cytotoxicity, antiviral activity, wound repair, angiogenesis, and apoptosis [14, 43].

The entire sequence of events of angiogenesis or neovascularization is mediated by various types of cells, including macrophages, lymphocytes, mast cells, platelets, and polymorphonuclear leukocyte [9, 14]. In RAW264.7

murine macrophages, we have shown that inhibition of AR prevents the lipopolysaccharide-induced release of inflammatory cytokines, chemokines, nitric oxide and ROS [43]. AR inhibition has also shown to prevent hyperglycemia-induced oxidative stress in retinal endothelial cells and expression of VEGF in diabetic rats [44]. These results suggest that AR plays an important role in oxidative stress-mediated inflammatory pathologies, including angiogenesis. Endothelial migration and wound healing are commonly observed under hyperglycemic conditions and may contribute to vascular dysfunction in diabetics [45]. A recent study of D'Souza et al. [46] suggest that inhibition of AR by alrestatin and ablation by AR-siRNA improved wound healing in endothelial cells treated with high glucose. These studies indicate that inhibition of AR could normalize dysfunctional vessels and improve vascular remodeling and angiogenesis under hyperglycemic conditions.

The biological functions of VEGF, and FGF, triggered by external stimuli, are initiated through the activation of intracellular signal cascades involving specific kinases [47]. Formation of a receptor complex between VEGF and its tyrosine kinase receptor VEGF-R2 plays an important

role in coordinating the effects of VEGF on cell adhesion and cell motility [36]. Among the VEGF receptors (VEGF-R1, R2, and R3), VEGF-R2 is believed to play an important role in angiogenesis. VEGF-R2 is a receptor tyrosine kinase, which, on activation by ligand binding, phosphorylates and activates secondary messengers, including AKT, to regulate endothelial cell proliferation and migration [36]. In this context, our results show that exposure of HUVEC to AR inhibitor, fidarestat results in suppression of PI3 K/AKT/NF- κ B activation as well as down-regulation of VEGF-R2 protein levels.

AKT, a serine-threonine kinase, a homologue of a retroviral oncoprotein ν -Akt, is recruited during signal transduction from growth factor receptors and intracellular pathways [28, 34, 35]. Vascular homeostasis and angiogenesis by different stimuli, including VEGF, and angiopoietin-1, is regulated by the activation of AKT [34, 35]. Various reports show that activation of AKT and its upstream kinase phosphatidylinositol 3-kinase (PI3 K) induce angiogenesis in vitro and in vivo. The expression of VEGF in endothelial cells is also regulated by the PI3 K-AKT signaling axis. AKT activation not only promotes endothelial cell survival but also regulates vasomotor responses via phosphorylation of endothelial nitric oxide synthase and VEGF-stimulated endothelial cell migration and differentiation [34, 35]. Our results show that the antiangiogenic effect of AR inhibition is closely associated with inhibition of AKT kinase. Activation of AKT is mediated by receptor tyrosine kinases that, on ligand binding, are autophosphorylated and cause activation of PI3 K. Activated PI3 K generates lipid second messengers, which facilitate recruitment of AKT to the plasma membrane and activation [35]. In HUVEC it is possible that AR inhibition causes inhibition of PI3 K to inhibit Ser473 phosphorylation (activation) of AKT. The AR inhibition-caused suppression of AKT/PI3 K/NF- κ B activation is not restricted to HUVEC because similar effects are evident in various cell lines including VSMC, HLEC, human colon cancer SW480, and HT29 cells [22–27]. Further, we have also examined the effect of AR inhibition in regulation of nitrosation of AKT and PI3 K which is generally observed under stress conditions. Our results indicate that AR inhibition prevents AKT and PI3 K activities probably by preventing their nitrosations.

Recent studies have implicated that multiple growth factors and cytokines, chemokines, adhesion factors, matrix metalloproteinases are involved in various stages of angiogenesis [26, 30, 35]. For instance, IL-17 increases angiogenic activity and in vivo growth of human non-small lung cancer cells in severe combined immuno-deficient mice [48]. Similarly, G-CSF promotes tumor angiogenesis by increasing circulating endothelial progenitor cells [49]. Degradation of the extracellular matrix is a critical component of tumor

metastasis, which is orchestrated by zinc-dependent proteolytic enzymes such as MMPs [12, 13]. Of these, MMP2 and 9, in particular, are believed to have an important role in the initial steps of tumor invasion and metastasis [30]. Expression of MMPs is associated with the expression of physiological inhibitor proteins TIMPs. However, the expression level of TIMP-1 in HUVEC is not detectable in our studies. Similarly, Liu et al. [50] have shown that TIMP-1 levels are not detectable in HUVEC but co-culture of fibroblasts with HUVEC leads to increased TIMP-1 expression. Further, our results also show that AR inhibition significantly prevents the expression of adhesion molecules in HUVEC cells. This is inconsistent with our previous studies in the context to the vascular inflammation and atherosclerosis which show that the inhibition of AR prevents cytokines such as TNF- α -induced ICAM, VCAM expression and apoptosis in HUVEC via inhibiting the activation of NF- κ B. NF- κ B is a redox sensitive transcription factor, whose expression is elevated under oxidative stress conditions [51]. Activation of NF- κ B has been shown to increase the expression of various angiogenic factors including VEGF. Several studies indicate that inhibition of NF- κ B prevents angiogenesis [52]. Thus our current studies indicate that AR inhibition prevents angiogenesis by preventing growth factors-induced oxidative stress, activation of AKT/PI3 K and NF- κ B and expression of angiogenic proteins.

In conclusion, the present study shows that inhibition of AR prevents angiogenesis in vitro and in vivo. The AR inhibition-caused suppression of endothelial cell differentiation, invasion, and migration is associated with inactivation of AKT, suppression of pro-angiogenic growth factors, and down-regulation of VEGF-R2 protein expression. Thus, our studies indicate a novel role of AR in regulation of angiogenic process and therefore, inhibition of AR could prevent cancer growth and metastasis by ameliorating the angiogenesis.

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