Annexin A2 Promotes Angiogenesis After Ischemic Stroke Via Annexin A2 Receptor - AKT/ERK Pathways

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Abstract

Promoting angiogenesis to restore circulation to the ischemic tissue is still an important therapeutic target in stroke. Here, we ask whether the Ca^{2+} -regulated, phospholipid-and membrane-binding protein-Annexin A2 (ANXA2) may regulate angiogenesis after stroke. Compared with wild type (WT) mice, the density of microvessels in brain and the number of new vessels sprouting from aortic ring were significantly increased in *Anxa2* knock-in ($\text{ANXA2}^{+/+}$) mice. After focal cerebral ischemia, proliferation of brain endothelial cells in $\text{ANXA2}^{+/+}$ mice was significantly elevated at 7 days post-stroke, which further improved behavioral recovery. To assess the pro-angiogenic mechanisms of ANXA2, we used brain endothelial cells cultures to investigate its effects on cell tube-formation and migration. Recombinant ANXA2 increased tube-formation and migration of brain endothelial cells either under normal condition or after OGD injury. These protective effects of recombinant ANXA2 were regulated by interaction with ANXA2 receptor (A2R), and the ability of ANXA2-A2R to activate AKT/ERK pathways. Taken together, our study indicates that ANXA2 might be involved in angiogenesis after ischemic stroke. Further investigation of ANXA2-A2R will provide a new therapeutic target for stroke.

Introduction

Stroke, as a devastating central neuronal system disease, affects over 10 million people all over the world and is one of the most common cause in cardiovascular diseases-induced death [1,2]. Prevent neuronal death and improve recovery was the priority in the past decades. However, clinically effective therapies remain elusive. Thus, to restore neurological deficits, developing novel therapies after stroke beyond the hyper-acute phase ischemia is extremely important.

As the first responder to ischemia and reperfusion injury, vasculature is the basis for any neuroprotective therapy to increase blood flow for brain energy demand, which further emphasizes the significant effect of angiogenesis in stroke recovery. Therapeutic angiogenesis is a process that pharmacologically stimulates of the regrowth of new blood vessels so as to increase cerebral blood flow to the ischemic tissue [3]. By binding to their membrane receptors on brain endothelial cells, angiogenic growth factors could regulate angiogenesis via promoting endothelial cell migration, proliferation and survival in the ischemic brain area [4].

The Ca^{2+} -regulated, phospholipid-and membrane-binding protein, Annexin A2 (ANXA2) has been found in endothelial cells [5], tumor cells [6], monocytes, macrophages and dendritic cells [7-9]. In the oxygen-induced retinopathy (OIR), ANXA2 might promote retinal neoangiogenesis by increasing perivascular fibrin clearance through stabilizing its binding partner, p11[10]. More importantly, low dose tPA plus recombinant ANXA2 therapy significantly augmented microvessel density, synaptophysin and VEGF expression in peri-infarct areas in rat focal stroke [11]. Thus, ANXA2 is emerging as a potential regulator of angiogenesis in ischemic stroke.

Methods

Animals

Anxa2 knock-in mice (*ANXA2*^{+/+}, C57BL/6 background), which overexpress *ANXA2*, were commercially generated by standard homologous recombination at Cyagen (Suzhou, China). Male adult C57BL/6 mice (8-10 weeks) (from Shanghai Slac Laboratory Animal) were used. The environmental conditions which maintain the mouse were: 12-hour dark/12-hour light cycle, humidity range of 30–50%, temperature range of 20–25 °C. All behavior experiments were performed between 9:00 and 19:00. All animal experiments were conducted in accordance with the ethical guidelines of the Zhejiang University Animal Experimentation Committee and Institutional Animal Care. Every Effort has been made to minimize animal suffering, and a minimum number of animals were used.

There are three special regions on *ANXA2*^{+/+} mice knock-in allele: region 1 is located at the border between homology arm and CAG promoter sequences; region 2 is located at the border between CAG promoter and *Anxa2*-cDNA sequences; region 3 is located at the border between *Anxa2*-cDNA and homology arm sequences (**Supplementary Fig. 1A**). For genotyping of *ANXA2*^{+/+} mice, four pairs of primers were applied. The primers sequences are listed in *Supplementary Table*. For primer pair of *ANXA2*-region 1F and *ANXA2*-region 1R, the PCR products were 463 bp (region 1 of the knock-in allele). For primer pair of *ANXA2*-region 2F and *ANXA2*-region 2R, the PCR products were 383 bp (region 2 of the knock-in allele). For primer pair of *ANXA2*-region 3F and *ANXA2*-region 3R, the PCR products were 437 bp (region 3 of the knock-in allele). For the pair of Internal-control F and Internal-control R the PCR products were 689 bp (Internal control) (**Supplementary Fig. 1B**). The PCR program was 94 °C for 2 mins (1×); 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 30 s (30×); and 72 °C for 1 min (1×).

Reagents

Growth Factor Reduced Matrigel was purchased from Corning (356230, USA). Recombinant human *ANXA2* protein was purchased from R&D System (D9409-AN-050, USA). Anti-BrdU antibody was purchased from Abcam (ab1893, USA). Anti-Lectin was purchased from Vectorlabs, (B-1415-2, USA). Anti-Tubulin monoclonal antibody was purchased from Abcam (ab210797, USA). Anti-A2R polyclonal antibody was purchased from Novus Biologicals (NBP-53077, USA). Anti-AKT polyclonal antibody was purchased from Cell Signaling Technology (9272s, USA). Anti-p-AKT polyclonal antibody was purchased from Cell Signaling Technology (4060s, USA). Anti-ERK monoclonal antibody was purchased from Santa Cruz Biotechnology (sc-81457, USA). Anti-phospho-ERK monoclonal antibody was purchased from R&D System (DYC1483-5, USA). Alexa Fluor® 488 AffiniPure donkey anti-goat IgG were purchased from Jackson ImmunoResearch Laboratories (705-545-147, USA). Alexa Fluor® 488 IgG fraction monoclonal mouse anti-Biotin were purchased from Jackson ImmunoResearch Laboratories (200-542-211, USA). Alexa Fluor® 594 IgG fraction monoclonal mouse anti-Biotin were purchased from Jackson ImmunoResearch Laboratories (200-582-211, USA).

Immunohistochemistry

Brains from mice were sliced in 20 µm thickness using a Thermo Fisher® freezing microtome. After being washed 3 times with PBS for 5 mins each, the brain slices were placed into the glass Petri dish with the 10 mM citric acid (pH=6) antigen retrieval buffer which was preheated to 90-100°C. The glass Petri dish was heated in a microwave oven for 10 mins. Then it was cooled to room temperature. Next, the brain slices were permeabilized with 0.3% (w/v) Triton X-100 in PBS at room temperature for 30 mins. After blocking in PBS containing 5% normal donkey serum at room temperature for 2 h, the brain slices were first incubated with anti-BrdU antibody and anti-Lectin antibody at 4 °C overnight and then with Alexa Fluor-conjugated secondary antibodies at room temperature. Fluoroshield™ with DAPI (Sigma-Aldrich, USA) was used to label the nuclei. Before permeabilizing with 0.3% (w/v) Triton X-100 in PBS, the sections used for BrdU staining were pretreated with 1 M hydrochloric acid at 37 °C for 30 mins before the above steps, washed with 0.1 M sodium tetraborate for 3 times, and neutralized for 5 mins each time. Then the primary and secondary antibodies were incubated according to the above process. Images were taken by a laser confocal microscope (Leica SP8, Germany). All the image quantification analyses were done by imageJ-win64. All the data were collected and analyzed by the rater who was blind to experiments design.

Aortic ring angiogenesis assay

The assay developed by Nicosia and Ottinetti was modified [12]. Aortas of 2 weeks WT control or *ANXA2^{+/+}* littermate mice were sliced into 1mm diameter rings. Aortic rings were then added in DMEM with 2.5% fetal bovine serum (FBS) to a 24-well plate that were pre-coated with a rat tail collagen I gel (BD Biosciences, UK). Medium was changed every 2 days. At the sixth day of culture, the explants were stained by anti-Lectin (B-1415-2, Vectorlabs, USA) and visualized under a Leica SP8 laser confocal microscope.

Photothrombotic model of stroke

Photothrombotic model was performed as previously described to induce focal cerebral ischemia [13-15]. In brief, mice were anesthetized with isoflurane and the heads were fixed on a stereotaxic apparatus. A cold light source (17000 lux; diameter 2.0 mm) was placed on the mouse skull surface (1.5 mm lateral to bregma). Rose Bengal solution (Sigma, USA) was dissolved in saline and injected into mice intraperitoneally, at 100 mg/kg. The head of the mouse was illuminated for 10 mins through the intact skull 5 mins after drug injections. Sham mouse was injected with Rose Bengal solution at same dosage and did not subject to illumination.

Grid-walking test

The grid-walking test was performed on a metal grid area of 20 × 32 × 50 cm of width, length and height, with a 12 mm square wire mesh, as previously described [14,15]. A video camera was placed under the apparatus to record the gait of mouse. Mouse was allowed to freely move on the apparatus for 5 mins. The number of foot errors and the normal steps of left forelimb were counted (the left forelimb was counted for 100 steps per video). The ratio of forelimb errors was calculated as follows: total number of

foot errors / (total number of foot errors + total number of the normal steps) *100. Foot errors were considered to occur when the forelimb was not providing support and the foot went through the grid hole, or animal was resting with the grid at the level of the wrist. The video analysis was performed offline by the rater who was blind to experiments design.

Cylinder test

The cylinder test was performed as previously described [16,15]. In short, mouse was allowed to freely explore for 5 min in a Plexiglas cylinder (15 cm in height with a diameter of 10 cm). A camera was positioned above the apparatus to record the exploratory rear of mouse. Exploratory rear was considered to occur when the mouse spontaneously reared by touching the wall of cylinder with its forelimbs. The time (in seconds) spent by the both forelimbs or each forelimb of mouse pressing the wall of cylinder was counted. The percentage of time spent on each forelimb were used to derive an asymmetry index which was calculated as follows: (the time spent on ipsilateral forelimb - the time spent on contralateral forelimb) / total time spend on forelimb. The video was analyzed by the rater who was blind to experiments design.

Human brain microvascular endothelial cell (HBMEC) culture

HBMECs were obtained from Cell Systems Corporation (Kirkland, WA, USA). The method of HBMECs culture has been described previously [17]. In brief, the cells of passage 6th to 12th were grown with EBM-2 Basal Medium (Lonza, USA) containing Endothelial Cell Growth Medium-2 (Lonza, USA). HBMECs were cultured with RPMI-1640 containing 10% FBS. All cells were kept in a 37 °C humidified incubator with 5% CO₂. HBMEC was treated with indicated concentrations (from 0.1 to 1 µg/ml) of recombinant ANXA2. OGD model was used to mimic ischemic stroke in vitro. The cells were cultured with glucose-free RPMI-1640 in a sealed chamber (Billups-Rothenberg, USA) loaded with mixed gas containing 95% N₂ and 5% CO₂ for 3 hrs. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate cell viability. Cell viability was expressed as a percentage relative to the normal cells. The data represented 6-12 separate wells assayed per data point, with about 500–1000 cells counted per well.

In Vitro Angiogenesis Assays

In vitro, angiogenesis was assessed by scratch migration and tube formation assays. The ability of endothelial cells to form capillary-like structures was evaluated by the Matrigel tube formation assay. In short, HBMECs (4×10⁴ cells/well) were inoculated in 48-well culture plates which were pre-coated with growth factor-reduced Matrigel. Then cells were maintained with different treatments at 37 °C for 24 hrs. The number of the branch point and tubes in 3 random fields from each well were counted.

To measure the migration abilities of HBMECs, scratch migration assay was performed. After being seeded in 12-well plates, HBMECs were scratched by a 200 µl pipette tip. Then, the cells were incubated with different treatments for 2 days. HBMEC migration was monitored every day. Three different visual

fields were randomly selected per well for observation and photograph under a phase contrast microscopy. The percentage of areas occupied by migrated cells were used to estimate the migration ability of HBMECs.

Western blotting

Cell or tissue samples were homogenized in RIPA buffer and the protein concentration was measured using a BCA assay. Samples of the same weight (50 µg per sample) were loaded on 12% SDS-PAGE gel, followed by transfer to PVDF membrane (Millipore, USA). The PVDF membranes were probed with the following primary antibodies: Tubulin (1: 5000), A2R (1: 1000), AKT (1: 1000), p-AKT (1: 1000), ERK (1: 1000) and p-ERK (1: 1000) at 4 °C overnight. HRP-linked secondary antibodies were then applied accordingly at room temperature for 2 hrs. Chemiluminescence was used to detect the signals of protein.

Real-time PCR

Real-time PCR was performed with MonAmp™ SYBR® Green qPCR Mix (MQ10101S, Monad, Shanghai), using a CFX-96 real-time PCR Detection system (Bio-Rad, USA). The relative quantities of total cDNA of ANXA2^{+/+} mice and wild-type mice were quantified using the $\Delta\Delta CT$ method. The primer sequences used were as follows: mouse *Anxa2* (Fw: 5'- ATGTCTACTGTCCACGAAATCCT -3'; Rv: 5'- CGAAGTTGGTGTAGGGTTTGACT -3'), mouse *Actb* (Fw: 5'- GGCTGTATTCCTCCATCG -3'; Rv: 5'- CCAGTTGGTAACAATGCCATGT -3').

Statistical Analysis

All statistical data are represented as mean±s.e. All experiments were reproduced three – four independent times. For experiments with only two groups, unpaired two-tailed t-tests were used. For multiple comparisons, one-way or two-way ANOVA (with repeated measures when appropriate) followed by Tukey and Bonferroni corrections were used. Data were considered statistically significant when the *p* values were less than 0.05.

Result

Increased angiogenesis in ANXA2^{+/+} mice

Several in-vivo studies have demonstrated ANXA2-deficient mice displayed impaired neovascularization [18,19]. To determine the role of ANXA2 in angiogenesis, *Anxa2* knock-in (ANXA2^{+/+}) mice were used. *Anxa2* expression was increased in the cerebral cortex of ANXA2^{+/+} mice (Supplementary Fig. 1C). And western blotting showed that ANXA2 level was upregulated in the cerebral cortex of ANXA2^{+/+} mice (Supplementary Fig. 1D, 1E). Next, to assess neovascularization in brain of ANXA2^{+/+} mice, we dissected cortex from WT mice and ANXA2^{+/+} mice, and measured density of lectin (Fig. 1A). Our data confirmed that ANXA2^{+/+} mice appeared to have increased density of microvessels (Fig. 1B). We therefore investigated whether ANXA2 also directly affect endothelial cell proliferation.

Aortic ring explants from WT mice and *ANXA2*^{+/+} mice formed robust vessel structures (**Fig. 1C**). Compared with that of WT explants, the number of vessels emanating from *ANXA2*^{+/+} mice explants was increased by about 3 folds (**Fig. 1D**). Taken together, these data indicated an increased angiogenesis in *ANXA2*^{+/+} mice.

ANXA2 promotes long-term neurological recovery after ischemic stroke

To assess whether increased angiogenesis in *ANXA2*^{+/+} mice could affect long-term neurological outcome after stroke, we carried out experiments using a Photothrombotic model of stroke. Mice were treated with BrdU before the onset of stroke. As expected, the number of BrdU positive and lectin positive cells was significantly increased in *ANXA2*^{+/+} mice compared to WT mice (**Fig. 2A, 2B**). Early recanalization of blood vessels is correlated with better outcome by restoration of blood supply in ischemic zone, and reduction of cerebral infarction area [20-22]. We next applied Grid-walking and cylinder tests to comprehensively evaluate the sensorimotor functions after stroke. Compared with WT mice, *ANXA2*^{+/+} mice exhibited significant sensorimotor functional improvements in all the behavioral tests, and these sensorimotor improvements were most prominent in the day 7 and day 14 after stroke (**Fig. 2C, 2D**). Taken together, these data suggest that ANXA2 enhances the overall long-term neurological recovery after stroke.

Recombinant ANXA2 promotes in vitro angiogenesis

We next asked whether recombinant ANXA2 promotes in vitro angiogenesis in culture of human brain microvascular endothelial cells (HBMEC) using scratch assay and capillary tube formation assay. Treatments of recombinant ANXA2 did not affect cell viability (**Fig. 3A**). In vitro scratch assay demonstrated that recombinant ANXA2 significantly increased HBMEC migration at 48 hours (**Fig. 3B, 3C**). Moreover, capillary tube formation assay revealed that recombinant ANXA2 significantly increased the tube formation (**Fig. 3D, 3E**), showing elevated branch points (**Fig. 3F**). Thus, recombinant ANXA2 promotes in vitro angiogenesis.

Recombinant ANXA2 rescues OGD-inhibited HBMEC angiogenesis

We further examined the effects of recombinant ANXA2 on proangiogenic activities after OGD injury. To determine whether recombinant ANXA2 protects HBMEC from OGD-induced cytotoxicity, HBMEC were treated with indicated dose of recombinant ANXA2 treatment during OGD injury. Our result showed that 1 µg/ml recombinant ANXA2 lead to statistically significant increase HBMEC viability compared to OGD alone group (**Fig. 4A**). In vitro scratch and capillary tube formation assays showed that OGD injury induced significant decrease endothelial cells migration and tube formation (**Fig. 4B-4F**), whereas recombinant ANXA2 reversed these effects (**Fig. 4B-4F**). Collectively, these results demonstrate that the recombinant ANXA2 rescues OGD-inhibited angiogenic activities in HBMEC.

ANXA2-induced angiogenesis is ANXA2 receptor dependent

ANXA2 receptor (A2R) presenting on the cell surface has been found as a new vascular regulatory molecule [23]. In cancer cells, A2R can interact with ANXA2 to promote cell adhesion, migration and proliferation [24,25]. To elucidate whether A2R is involved ANXA2-induced angiogenesis, we performed Western blotting to check A2R expression after recombinant ANXA2 treatment. Recombinant ANXA2 led to significantly increased expression of A2R (**Fig. 5A**). More importantly, immunoprecipitation experiments from HBMEC cultures demonstrated that A2R was indeed able to bind to ANXA2 (**Fig. 5B**). We next examined the role of A2R in the ANXA2-induced angiogenesis. Endogenous expression of A2R was suppressed by siRNA (**Fig. 5C**). Compared to scrambled siRNA control, suppression of endogenous A2R diminished recombinant ANXA2-induced endothelial cells migration (**Fig. 5D, 5E**). We also observed significant decrease of tube formation indicated by reduced branch points in A2R siRNA group when compared to scrambled siRNA control (**Fig. 5F-5H**). These data suggested that ANXA2-induced angiogenesis is A2R dependent.

ANXA2-induced AKT and ERK activation is ANXA2 receptor dependent

The two major pathways that regulate endothelial cells proliferation and migration are the ERK and AKT pathways [26]. Activation of these pathways in endothelial cells is necessary for angiogenesis [27,28]. To confirm recombinant ANXA2 could active ERK and AKT pathways, Western blotting was performed to detect AKT and ERK phosphorylation (**Fig. 6A**). We found recombinant ANXA2 successfully activated both pathways (**Fig. 6B**). Interestingly, compared with scrambled siRNA control, recombinant ANXA2 failed to activate both ERK and AKT when endogenous A2R was suppressed (**Fig. 6C, 6D**). Taken together, these results suggest that ANXA2-induced AKT and ERK activation is A2R dependent.

Discussion

Acknowledgements

Not applicable.

Author Contributions

WL and HD designed the study. WL and HL performed the experiments. WL and HL analyzed the data. HD, WL, ZY and HL wrote the paper. All authors read and approved the final manuscript.

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Data Availability

All raw data and materials are available upon reasonable request to Haibin Dai.

Compliance with Ethical Standards

Conflict of interest

The author declare that they have no conflict of interest.

Ethical Approval

All protocols were performed in accordance with the Zhejiang University Guide for the Care and Use of Laboratory Animals

Consent to Participate

Not applicable

Consent for Publication

All authors have read the manuscript and agreed for its publication.

Abbreviations

ANXA2 Annexin A2

WT wild type

ANXA2^{+/+} *Anxa2* knock-in

A2R ANXA2 receptor

I/R ischemia/reperfusion

OIR oxygen-induced retinopathy

Declarations

Declarations

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Figures

Figure 1

Figure 1

Increased angiogenesis in ANXA2^{+/+} mice. A, representative lectin and DAPI immunostaining in the cerebral cortex of WT mice and ANXA2^{+/+} mice. Scale bar, 140 μ m. B, number of microvessels in the cerebral cortex of WT mice and ANXA2^{+/+} mice. C, representative lectin and DAPI immunostaining of aortic ring of WT mice and ANXA2^{+/+} mice. Scale bar, 300 μ m (top), 150 μ m (bottom). D, number of sprouts from aortic ring of WT mice and ANXA2^{+/+} mice at day 6 after culture ex vivo. Data are mean $\pm$ s.e. * P < 0.05; *** P < 0.001. Two-tailed t-test.

Figure 2

Figure 2

ANXA2 promotes long-term neurological recovery after ischemic stroke. A, representative lectin, BrdU and DAPI immunostaining of WT mice and ANXA2^{+/+} mice at day 7 after stroke. Scale bar, 250 μ m (top), 45 μ m (bottom). B, number of lectin⁺ and BrdU⁺ cells of WT mice and ANXA2^{+/+} mice at day 7 after stroke. C, Foot faults of left forelimb in the grid-walking task. D, Forelimb symmetry in the cylinder task. Data are mean $\pm$ s.e. *P < 0.05; **P < 0.01. Two-tailed t-test (B); two-way ANOVA with Bonferroni adjustment (C, D).

Figure 3

Figure 3

Recombinant ANXA2 promotes in vitro angiogenesis. A, effects of different dose of rA2 (0.1µg/ml -1µg/ml) on the HBMEC viability. B-C, effects of rA2 (1µg/ml) on scratch migration in HBMEC (B) and bar graph of gap closure (C). D-F, effects of rA2 (1µg/ml) on tube formation (D) and bar graph of the number of tubes (E) and the number of branch points (F). Scale bar, 100 µm. n=3 independent experiments. Data are mean±s.e. **P < 0.01; ***P < 0.001. Two-tailed t-test (F); two-way ANOVA with Bonferroni adjustment (C).

Figure 4

Figure 4

Recombinant ANXA2 rescued OGD-inhibited HBMEC angiogenesis. A, effects of different dose of rA2 (0.1µg/ml -1µg/ml) on the viability of HBMEC treated with 3 hrs OGD and 21 hrs reperfusion. B-C, effects of rA2 (1µg/ml) on scratch migration in HBMEC treated with 3 hrs OGD and 21 hrs reperfusion (B) and bar graph of gap closure (C). D-F, effects of rA2 (1µg/ml) on tube formation in HBMEC treated with 3 hrs OGD and 21 hrs reperfusion (D) and bar graph of the number of tubes (E) and the number of branch points (F). Scale bar, 100 µm. n=3 independent experiments. Data are mean±s.e. *P < 0.05; **P < 0.01; ***P < 0.001. One-way ANOVA with post-hoc Tukey adjustment.

Figure 5

Figure 5

ANXA2-induced angiogenesis is ANXA2 receptor dependent. A, expression of A2R in rA2 (0.5µg/ml -1µg/ml) treated HBMEC cultures. B, Immunoprecipitation (IP) of HBMEC with anti-ANXA2 (A2) followed by immunoblotting with anti-A2R. C, A2R RNA expression in A2R and control (Con) short interfering RNA (siRNA) transfected HBMEC cultures. D-E, effects of control siRNA and A2R siRNA on scratch migration in HBMEC treated with 48 hrs rA2 (1µg/ml) (D) and bar graph of gap closure (E). F-H, effects of control

siRNA and A2R siRNA on tube formation in HBMEC treated with 48 hrs rA2 (1µg/ml) (F) and bar graph of the number of tubes (G) and the number of branch points (H). Scale bar, 100 µm. n=3 independent experiments. Data are mean±s.e. *P < 0.05; **P < 0.01; ***P < 0.001. Two-tailed t-test (C); One-way ANOVA with post-hoc Tukey adjustment (E, G, H).

Figure 6

Figure 6

ANXA2-induced AKT and ERK activation is ANXA2 receptor dependent. A-C, pAKT/AKT and pERK/ERK expressions in HBMEC cultures treated with rA2 (1µg/ml) at different time points. D-E, effects of control siRNA and A2R siRNA on pAKT/AKT and pERK/ERK expressions in HBMEC cultures treated with rA2 (1µg/ml) at different time points. n=3 independent experiments. Data are mean±s.e. *P < 0.05; **P < 0.01; ***P < 0.001. One-way ANOVA with post-hoc Tukey adjustment (B, C); two-way ANOVA with Bonferroni adjustment (E, F).

Supplementary Files

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- [SupplementaryData.docx](#)