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Candesartan Induces a Prolonged Proangiogenic Effect and Augments Endothelium-mediated Neuroprotection after Oxygen and Glucose Deprivation: Role of VEGF-A and B.

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Abstract

Angiogenesis is a key component of recovery after stroke. Angiotensin II receptor blocker (ARB) treatment improves neurobehavioral outcome and is associated with enhanced angiogenesis after stroke. The purpose of this study is to investigate the temporal pattern of the ARB proangiogenic effect in the ischemic brain and its association with vascular endothelial growth factors A (VEGF-A) and B (VEGF-B). Wistar rats were exposed to 90-minute middle cerebral artery occlusion (MCAO) and treated with candesartan (1 mg/kg) at reperfusion. The proangiogenic potential of the cerebrospinal fluid (CSF) was determined at 8, 24, 48 and 72 hours using *in vitro* Matrigel™ tube formation assay. In addition, the expression of VEGF-A and B was measured in brain homogenates using Western blotting at the same time points. A single candesartan dose induced a prolonged proangiogenic effect and a prolonged upregulation of VEGF-A and B *in vivo.* In the ischemic hemisphere, candesartan treatment was associated with stabilization of hypoxia inducible factor-1alpha (HIF-1a) and preservation of angiopoietin-1 (Ang-1). The effect of ARB treatment on endothelial cells was studied *in vitro.* Our results identified brain endothelial cells as one target for the action of ARBs and a source of the upregulated VEGF-A and B, which exerted an autocrine angiogenic response, in addition to a paracrine neuroprotective effect. Taken together, this study highlights the potential usefulness of augmenting the endogenous restorative capacity of the brain through the administration of ARBs.

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Introduction

Angiogenesis has been linked to a better recovery after stroke (Krupinski et al., 1994; Manoonkitiwongsa et al., 2001; Navaratna et al., 2009). The role of angiogenesis, however, is not limited to creating a "conduit" to restore oxygen and nutrient delivery. It is now believed that angiogenesis is coupled to neurorestorative processes including neurogenesis and synaptogenesis (Teng et al., 2008; Beck and Plate, 2009; Xiong et al., 2010). It has been demonstrated that these vessels provide neurotrophic support to the newly formed neurons, thereby, improving functional recovery following an ischemic stroke (Navaratna et al., 2009).

There is extensive preclinical evidence that ARB treatment after ischemic stroke improves neurobehavioral outcome and enhances recovery (Engelhorn et al., 2004; Hosomi et al., 2005; Kozak et al., 2008; Guan et al., 2011a; Guan et al., 2011b; Guan et al., 2011c; Ishrat et al., 2013). In contrast to reports from other vascular beds where inhibition of angiogenesis has been documented (Willis et al., 2011), ARB treatment has been associated with increased vascular density in the brain (Munzenmaier and Greene, 2006). Chronic pretreatment with losartan (Forder et al., 2005) or valsartan (Li et al., 2008a) increased vascular density and reduced infarct volume after ischemic injury.

The prototypical angiogenic molecule, VEGF-A, has a well-established role in physiological as well as pathological angiogenesis, mediated mostly by VEGF receptor-2 (VEGFR2) (Ferrara, 1995; Zhang et al., 2000; Shibuya, 2013). In addition to its angiogenic potential, VEGF-A is involved in learning and memory, stimulates neurogenesis and exerts a neuroprotective effect (Jin et al., 2000; Matsuzaki et al., 2001; Jin et al., 2002; Cao et al., 2004). Stabilization of HIF-1 under ischemic conditions induces VEGF-A expression, possibly by binding to its promoter region (Levy et al., 1995; Huang et al., 1996; Wenger, 2002). Less is known, however, about the

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relatively new VEGF family member, VEGF-B. Although its proangiogenic effect is controversial, VEGF-B exerts vascular and neuroprotective effects against a wide range of apoptotic stimuli via VEGF receptor-1 (VEGFR1) (Sun et al., 2006; Li et al., 2008b; Li et al., 2009; Zhang et al., 2009). In a rodent model of focal cerebral ischemia, VEGF-B knockout animals showed 50% larger infarcts as compared to their wild type counterparts (Li et al., 2008b). Angiopoietins, another group of vascular-specific growth factors, play a prominent role in angiogenesis as well as in vessel maturation and function (Davis and Yancopoulos, 1999; Thurston et al., 1999; Thurston et al., 2000). Ang-1 expression falls dramatically after focal cerebral ischemia, resulting in a leaky blood brain barrier. Increases in Ang-1 levels, however, preserve vascular integrity after focal ischemia and/or VEGF administration (Thurston et al., 2000; Zhang and Chopp, 2002; Zhang et al., 2002; Tao et al., 2011). The use of the aforementioned angiogenic and vascular protective factors might seem to be a promising treatment for acute ischemic stroke. However, the challenging route of administration limits the benefit of such a treatment. An attempt to augment the endogenous production of these growth factors represents an attractive alternative.

The present study was designed to investigate the temporal pattern of candesartan's proangiogenic effect as well as VEGF-A and B upregulation in response to a single treatment. In addition, we show for the first time the involvement of the vascular endothelium in candesartan's neuroprotective effect.

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Materials and Methods

All experimental protocols were approved by the Care of Experimental Animal Committee of Georgia Regents University/ Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Medical Center.

Experimental cerebral ischemia: A total of 30 adult male Wistar rats (Charles River Breeding Company, Wilmington, MA), weighing between 280–300 grams, were subjected to 90-minute (MCAO) using intraluminal suture model, as described previously (Kozak et al., 2009). Successful MCAO was confirmed by the presence of hemiparesis prior to reperfusion. Animals with Bederson score < 3 were excluded from the study together with animals that are obtunded or unable to move. At reperfusion, animals received either saline or 1 mg/ kg of candesartan (a gift from AstraZeneca) via tail vein injection and were randomized into 4 different groups (8, 24, 48 and 72-hour groups). All animals were singly housed before and after surgery, with free access to food and water. At the aforementioned time points, CSF and brain tissues were collected and snap-frozen.

Cell culture and treatments: *The human cerebral microvascular endothelial cell line (hCMEC/D3)* was a kind gift from Dr. Jason Zastre (University of Georgia, Athens, GA). Endothelial cells were grown in MCDB-131 complete medium (VEC Technologies, Rensselaer, NY). Cells were serum starved overnight prior to treatment with candesartan (0.1, 1, 10 µg/ml), or losartan potassium (0.05 µg/ml, Sigma-Aldrich, St. Louis, MO) in serum-free Eagle's Minimum Essential Medium (EMEM- ATCC, Manassas, VA) and compared to untreated controls. Clinically relevant concentrations of candesartan (0.1 µg/ml) and losartan (0.05 µg/ml) were determined according to the published pharmacokinetic data (Schulz and Schmoldt, 2003). For neutralization experiments, VEGF-A neutralizing antibody, VEGF-B neutralizing antibody

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or both (2 µg/ml- R&D systems, Minneapolis, MN) were added to the conditioned media collected from endothelial cells 30 minutes prior to neuronal treatment. Normal goat IgG (2 µg/ml- R&D systems, Minneapolis, MN) was used as a control. *Mouse primary cerebral cortical neuronal cultures* were isolated from embryonic day 17 fetuses (E17) of CD1 mice (Charles River Laboratories, Wilmington, MA) as described previously (Paxinos and Franklin, 2001; Pillai et al., 2008). The experimental protocol is approved by Georgia Regents University, Committee on Animal Use for Research (Pillai). Isolated neurons were cultured in Neurobasal medium (Life Technologies, Grand Island, NY), supplemented with B27 (Life Technologies, Grand Island, NY), 2 mM L-glutamine (Life Technologies, Grand Island, NY) and antibiotics (Cellgro Manassas, VA). Neurons were used for experiments between days 5 and 7 *in vitro.*

Oxygen and glucose deprivation (OGD): To mimic ischemic conditions that occur during stroke, neuronal and endothelial cells were incubated in a hypoxia chamber (Biospherix Proox Model C21, Lacona, NY) at O_2 concentration <1 % and 5% CO_2 at 37°C. Culture medium was changed to the glucose-free Neurobasal-A medium (Life Technologies, Grand Island, NY). After 2 hours of OGD, cells were reoxygenated and received serum-free EMEM with or without treatment.

Tube formation: The ability of endothelial cells to align into tube-like structures was measured using Matrigel™ tube formation assay as described previously (Kozak et al., 2009). *To measure the proangiogenic potential of CSF,* confluent cells were serum starved overnight, harvested and suspended in a mixture of serum-free EMEM and growth factor-reduced Matrigel™ (BD Biosciences, San Jose, CA) in a ratio of 70: 30. The mixture was quickly transferred to a 96-well plate (100µl/well). Approximately 5 $x10^4$ cells in a volume of 100 µl were seeded into each quadruplicate well. CSF (50 µl) was added and the mixture is allowed to solidify. Images were

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captured for the center of each well after 24 hours using Zeiss Axiovert microscope at an objective lens magnification of 10x. Tube-like structures were counted in a blinded fashion. Tubes were defined as endothelial cells that had aligned to form > 90% closed structures. *To measure the proangiogenic effect of candesartan treatment*, confluent endothelial cells were exposed to either normoxic or 2-hour OGD conditions as explained previously. The cells were then treated with candesartan or losartan. At 24 hours, cells were harvested and the same procedure was followed. Additional images were captured and analyzed at 24, 48 and 72 hours.

Cell proliferation: Endothelial cell proliferation was assessed by BrdU colorimetric assay kit (Roche Indianapolis, IN) according to the manufacturer's protocol. Cells were plated at a density of 5 $x10^3$ cells/well in 96-well plate and left overnight to attach. Cells were serum starved, exposed to normoxic or 2-hour OGD followed by treatment with different doses of candesartan. BrdU labeling solution was then added. At 24 hours, absorbance was measured at 450 nm.

Cell migration: Endothelial cell migration was assessed by the *in vitro* wound healing assay as described previously (Kochuparambil et al.). Images were taken at 0, 18 and 24 hours using phase contrast microscopy on an inverted microscope at an objective lens magnification of 5x. The width of the scratch was measured at 20 fixed points in each well and the average was calculated. The percent migration was presented as fold-increase relative to the control.

Western blotting: Protein expression was measured by western blotting as described previously (Guan et al., 2011c). Non-specific binding was blocked by incubating the membranes in 5% milk in TBST for 60 minutes prior to overnight incubation with primary antibodies against VEGF-A, phospho-VEGFR-1 (Tyr 1213) (Millipore, Billerica, MA), VEGF-B (Abcam, Cambridge, MA), HIF-1α, Angiopoietin-1 (SantaCruz, Dallas, TX), total VEGFR-1, phospho-VEGFR-2 (Tyr 996), total VEGFR-2, GAPDH and cleaved caspase-3 (Cell Signaling, Danvers, MA). β-Actin (Sigma-

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Aldrich, St. Louis, MO) were used as an endogenous loading control. Densitometric measurements were done using ImageJ software and results were represented as fold-increase relative to the control group.

Slot blot: Detection of nitrotyrosine (NY) and 4-hydorxynonenal (4-HNE) was done using slot blot technique as described previously (Abdelsaid et al., 2010). Briefly, tissue or cell lysate were immobilized on nitrocellulose membrane using Whatman Minifold slot blot system (GE Healthcare Bio-Sciences, Pittsburgh, PA). Membranes were blocked then incubated with primary anti-nitrotyrosine antibody (Millipore, Billerica, MA) or anti- 4-HNE (Alpha Diagnostic Intl, San Antonio, TX), followed by peroxidase labeled goat anti-mouse IgG (EMD Chemicals, San Diego, CA). Densitometric measurements were done using ImageJ software and results were represented as fold-increase relative to the control group.

Angiotensin II determination: Angiotensin II release into the cell culture media was quantified using angiotensin II enzyme immunoassay kit (SPI-BIO). The cell supernatant was collected 24 hours after treatment and centrifuged at 13,000 rpm for 5 minutes to get rid of any cells. The supernatant was then used for the assay according to the manufacturer's instructions.

Statistical Analysis: All statistics were carried out using NCSS 8 software. Results were expressed as mean ± standard error of the mean (±SEM). Data were statistically analyzed using two-sample unpaired Student's t-test for single comparisons. One Way Analysis of Variance (ANOVA) was used for multiple comparisons and followed by Dunnett's two-sided multiple comparison test. Neutralizing antibody experiments were analyzed by two-way ANOVA. Data from CSF proangiogenic effect experiment were analyzed using linear regression. Results were considered statistically significant at $P< 0.05$.

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Results

Candesartan treatment induces a prolonged proangiogenic state. CSF collected from candesartan- treated animals induced a prolonged proangiogenic response as evident by profound increases in tube formation in brain endothelial cells. Candesartan treatment enhanced the proangiogenic potential of CSF as compared to saline treatment, an effect that was maintained throughout the measured time points. The mean slope for the candesartan group was 10- fold higher than the saline group (0.24+/-0.03 vs. 0.02+/-0.01; $P<0.05$) **(Fig 1A- C)**.

A single candesartan dose induces a prolonged upregulation of VEGF-A and B expression in vivo. In the contralateral hemisphere, candesartan enhanced VEGF-A and B expression at all the studied time points. In comparison to saline treatment, VEGF-A expression increased by 40-50% as early as 8 hours and continued till 48 hours. At 72 hours, Candesartan increased VEGF-A expression 2-fold. In the same hemisphere, VEGF-B expression was elevated by 20-40% in the first 48 hours and by over 50% at 72 hours, as compared to the saline group **(Fig 2A, C)**. In the ipsilateral hemisphere, however, candesartan treatment induced an early rise in the expression of both VEGF-A and B, an effect that was blunted at later time points. At 8 and 24 hours, VEGF-A expression was ~ 20% higher in the candesartan than the saline group. VEGF-B elevation, however, was more dramatic. Candesartan resulted in over 85% increase in VEGF-B levels at 8 hours that came down to 30% at 24 hours, as compared to the saline levels. **(Fig 2B, D)**.

Candesartan treatment stabilizes HIF-1a, exerts an antinitrative effect and preserves Ang-1 expression in the ischemic hemisphere. In saline-treated animals, HIF-1a stabilization was observed as early as 8 hours after focal cerebral ischemia. Ipsilateral hemisphere had 60% higher HIF-1a levels than contralateral hemisphere. Such stabilization was blunted at 24 hours after stroke. Candesartan treatment enhanced both the extent and duration of HIF-1a stabilization.

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HIF-1a levels were 2.3-fold higher in ipsilateral as compared to the contralateral hemisphere at 8 hours. This stabilization continued to be observed at 24 hours, where HIF-1a levels were 30% higher in ipsilateral versus contralateral hemispheres **(Fig 3A)**.

In saline-treated animals, a 67% elevation in the nitrative stress marker nitrotyrosine (NY) was detected in the ipsilateral as compared to the contralateral side. Candesartan treatment reduced NY levels back to control levels **(Fig 3B)**. Similarly, direct treatment of endothelial cells with peroxynitrite (ONOO-) increased NY level by 84%. Treatment with Candesartan reduced the NY level to 25% **(Supplemental Figure 1A)**. Moreover, candesartan exerted an antioxidant effect following treatment with either hydrogen peroxide (H_2O_2) or ONOO-. Either treatment increased the level of 4-hydroxynonenal (4-HNE), an oxidative stress marker, by 40%. Candesartan treatment reduced its level back to control levels. **((Supplemental Figure 1B, C)**.

We further investigated if candesartan treatment affects Ang-1 levels after stroke. In saline-treated group, we observed a 40% reduction of Ang-1 levels in ipsilateral as compared to the contralateral hemisphere. Candesartan preserved Ang-1 levels in ipsilateral hemisphere at 24 hours, resulting in only 8% reduction in Ang-1, as compared to the contralateral hemisphere **(Fig 3C)**.

Candesartan enhances endothelial VEGF-A and B expression as well as their receptor activation in vitro. Under normoxic conditions, VEGF-A expression was enhanced by 31, 36 and 25% in groups treated with 0.1, 1 and 10 µg/ml, respectively **(Fig 4A)**. The induction of VEGF-A expression was more pronounced under OGD conditions, resulting in 36, 57 and 60% increase in the same treatment groups, respectively **(Fig 4B)**. VEGF-B followed the same pattern with an increase of 30-35% under normoxic conditions in all treatment groups **(Fig 4C)**. However, in OGD, VEGF-B increased by 30, 85 and 90% in the same treatment groups, respectively **(Fig**

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4D). We studied whether the upregulated VEGF-A and B could exert an autocrine effect on endothelial cells. Therefore, we measured the phosphorylation of VEGFR1 and 2 as an indicator of VEGF-B and A functions, respectively. Candesartan enhanced phosphorylation of VEGFR1 by 25 and 65% and VEGFR2 by 30 and 65% in normoxic and OGD conditions, respectively **(Fig 4E- H)**. Taken together, candesartan enhances expression of endothelial VEGF-A and B as well as their receptor phosphorylation irrespective of the oxygenation status of the cell.

Candesartan enhances endothelial cell proliferation, migration and alignment into tube-like structures in a dose-dependent manner. At 24 hours, candesartan increased tube formation by 3, 3.5 and 4.5- fold in groups treated with 0.1, 1 and 10 µg/ml of candesartan, respectively, displaying a dose-dependent effect **(Fig 5A)**. We conducted a 3-day time course under both normoxic and OGD conditions, using one candesartan dose (1 µg/ml). Candesartan treatment resulted in a 5 to11- fold and a 3 to 4- fold increase in tube-like structures under normoxic and OGD conditions, respectively (**Fig 5B- E**). Similarly, endothelial cell proliferation increased under normoxic conditions by 13, 37 and 46% in the groups treated with 0.1, 1 and 10 µg/ml of candesartan, respectively, displaying a dose-response relationship **(Fig 5F)**. Candesartan treatment under OGD conditions resulted in 27, 57 and 55% increase in BrdU incorporation in the aforementioned candesartan concentrations, respectively **(Fig 5G)**. In agreement with the proliferation and tube formation data, 0.1 and 1 µg/ml of candesartan enhanced cell migration by 58% and 82%, respectively at 18 hours and by 63 and 73%, respectively at 24 hours under normoxic conditions **(Fig 5H- L).** Under OGD conditions, candesartan enhanced cell migration by 33 and 37% at 18 hours and by 38 and 57% at 24 hours in the tested concentrations, respectively **(Fig 5M)**.

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Losartan treatment promotes a proangiogenic state and increases VEGF-A and B expression in vitro. We conducted *in vitro* experiments to test whether candesartan proangiogenic effect can be exerted by other ARBs. Losartan treatment increased VEGF-A and B expression by 1.8 and 2.5- fold, 24 hours after treatment **(Fig 6A)**. Phosphorylation of VEGFR1 and 2 were increased by 2.5 and 3- fold at the same time point **(Fig 6B, C)**. We quantified tube formation and migration after losartan treatment. Losartan enhanced endothelial tube formation by 2.5- fold **(Fig 6D- F)**. Similarly, endothelial cell migration increased by 45 and 70% at 18 and 24 hours following treatment **(Fig 6G)**.

Both VEGF-A and B are required for the proangiogenic effect of candesartan. Consistent with the controversial angiogenic role of VEGF-B, we observed a modest reduction in endothelial cell migration following its neutralization. A more pronounced reduction was observed following VEGF neutralization, consistent with its well-documented angiogenic role. Neutralization of both growth factors exerted a synergistic –rather than an additive- inhibitory effect on endothelial cell migration at 24 hours **(Fig 7A- G)**.

Candesartan induces a paracrine neuroprotective effect via endothelial VEGF-A and B. Conditioned media collected from candesartan- treated endothelial cells decreased neuronal death by 40%, as documented by decreased cleaved caspase-3 expression. There was a trend of increasing levels of cleaved caspase-3 with the blockade of either growth factor. The increase in cell death was not significant, however, until both growth factors were blocked simultaneously **(Fig 8A)**.

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Discussion

The main findings of this study include a prolonged angiogenic effect, associated with enhanced VEGF-A and B expression *in vivo* and *in vitro*, in response to a single dose of candesartan treatment. These effects were associated with stabilization of HIF-1α, preservation of Ang-1 and reduction in tyrosine nitration at 24 hours. In addition, we identified endothelial cells as one of the cellular sources of the enhanced production of VEGF-A and B that induced an autocrine angiogenic response as well as a paracrine neuroprotective effect.

While several studies have demonstrated the proangiogenic effect of ARBs, the temporal pattern and the molecular mechanisms involved in the angiogenic response remain to be fully elucidated. Here, we show that the CSF collected after a single post-stroke candesartan administration stimulated a proangiogenic response as early as 8 hours and lasted for up to 72 hours. This effect was evident by the ability of the CSF, collected at different time points, to transform brain endothelial cells into tube-like structures resembling blood vessels *in vitro*. Our previous work has linked candesartan's beneficial effects to enhanced production of VEGF-A, the prototypical angiogenic molecule, as well as the relatively new VEGF isoform, VEGF-B (Guan et al., 2011c). The exact function of VEGF-B is still controversial. Although its neuroprotective and antiapoptotic functions are proven in several models (Li et al., 2008b), there is conflicting evidence regarding its angiogenic potential (Wright, 2002; Bhardwaj et al., 2003; Rissanen et al., 2003; Silvestre et al., 2003; Mould et al., 2005). In a recent study, VEGF-B was found to be upregulated in the ischemic border zone, following temporary MCAO (Xie et al., 2013). We sought to examine the temporal pattern of VEGF-A and B upregulation in both hemispheres after a single candesartan administration. Our findings show an increased expression of both isoforms in the ipsilateral as well as the contralateral hemispheres. Nevertheless, VEGF-A upregulation

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was more pronounced and lasted longer in the contralateral hemisphere, suggesting a role of this hemisphere in the process of recovery (Guan et al., 2011c). The increase in VEGF-B, on the other hand, was most pronounced at 8 hours in the ipsilateral hemisphere, consistent with its main role as a prosurvival factor.

We sought to investigate the upstream signaling molecules that lead to the upregulation of VEGF-A in both hemispheres. HIF-1 regulates the expression of multiple genes involved in hypoxia-related adaptations, including VEGF-A (Wenger, 2002; Youn et al., 2011). We report an enhanced HIF-1a stabilization by the treatment which might explain, at least partly, the upregulation of VEGF-A expression. In the contralateral hemisphere, however, other mechanisms could be involved in VEGF-A upregulation in response to treatment. In a retinopathy of prematurity model, candesartan treatment upregulated the expression of hemeoxygenase-1 (HO-1) and VEGF-A under normoxic conditions (El-Remessy et al., 2013). Further studies are warranted, however, to extrapolate these findings to the contralateral hemisphere in our stroke model.

Previous studies have demonstrated the inactivation of VEGF-A signaling by oxidative stress via PI3K tyrosine nitration (el-Remessy et al., 2005; Abdelsaid et al., 2010). In this study, we show an antioxidant and antinitrative effect of candesartan treatment. *In vitro*, candesartan decreased 4-HNE level, a marker of lipid peroxidation, after exposure to oxidizing conditions. Similarly, candesartan reduced NY levels in response to ONOO- treatment in the same model. We, thereby, postulate that candesartan, by the virtue of its antioxidant and antinitrative effects, reduces peroxynitrite production and protein nitration, respectively. *In vivo*, we report decreased protein nitration in the ipsilateral hemisphere 24 hours after ischemia/ reperfusion, a condition characterized by massive production of oxidizing and nitrating species. This finding suggests the

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possibility of improved VEGF-A signaling due to reduced protein nitration.

VEGF-A is a known vascular permeability factor and high serum VEGF levels have been associated with cerebral microbleeds after acute ischemic stroke (Dassan et al., 2012). We have previously shown a preservation of barrier function by candesartan treatment following an ischemic insult. We sought to explain the dilemma of the improved barrier function in spite of the elevated VEGF-A levels. In our current study, we report a preservation of Ang-1 expression in the stroke hemisphere 24 hours after treatment. Ang-1 exerts a barrier protective function as well as a synergistic angiogenic effect with VEGF-A after stroke (Valable et al., 2005). A diagram that depicts the proposed mechanisms of action of candesartan is shown in **Fig 8B**.

To study the contribution of endothelial cells to the observed effect of candesartan, we measured VEGF-A and B expression in human brain microvascular endothelial cells. Our results have shown increased VEGF-A and B production in a dose- dependent fashion irrespective of the oxygenation status. Yet, the increase was more pronounced under OGD than normoxic conditions, suggesting a role of hypoxia-induced adaptations in the function of candesartan. Indeed, candesartan treatment enhanced different steps of angiogenesis *in vitro* in microvascular endothelial cells, including cell proliferation, migration as well as tube formation in a dose-dependent manner. Interestingly, losartan showed a proangiogenic action *in vitro* similar to that of candesartan treatment (**Fig 6A-G)**, in spite of the different functional inhibitory characteristics as well as different lipophilicities, among other differences between the two ARBs. However, studies using other ARBs are needed to confirm the existence of a drug class effect.

In order to assess the contribution of VEGF-A and B to the proangiogenic effects of candesartan, we neutralized either or both growth factors *in vitro.* Neutralization of either VEGF-A or B

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reduced the angiogenic potential of candesartan as evident by reduced endothelial cell migration. Nevertheless, a sharp reduction of candesartan angiogenic effect was observed following neutralization of both growth factors simultaneously. This supports the role of VEGF-B as an angiogenic factor, either directly or indirectly via increasing cell survival. We have previously shown an enhanced proangiogenic state *in vitro* by candesartan treatment with or without exogenous angiotensin II treatment. Our recent work identified BDNF as a mediator of this effect secondary to unopposed AT2 receptor stimulation (Alhusban et al., 2013). The current study further supports our previous findings, since VEGF-A and BDNF expression is interrelated (Chen et al., 2005; Li et al., 2006). In this study, since no exogenous angiotensin II was added, we tested whether brain endothelial cells can produce and secrete angiotensin II locally. Angiotensin II was detectable in cell culture supernatant under normoxic and OGD conditions **(Supplemental Figure 2A)**. There was a trend of increasing angiotensin II with OGD conditions that did not reach significance in the tested sample size. Candesartan treatment enhanced endothelial cell secretion of angiotensin II in a dose-dependent fashion **(Supplemental Figure 2B)**, possibly due to the interrupted negative feedback system secondary to angiotensin receptor blockade. It has been previously shown that locally produced angiotensin II is regulated by an autocrine negative feedback mechanism, operating independently of the systemic renin angiotensin system (Gigante et al., 1997) , lending further support to our findings.

While *in vivo* studies are limited to be correlative and hard to prove the causal relationship between VEGF-A and B upregulation and neurovascular protection, we attempted to examine that concept *in vitro*. We neutralized either or both growth factors in the conditioned media collected from endothelial cells. Neutralization of either isoform reduced the protective effect of

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the conditioned media on primary neurons, as evident by the higher expression of cleaved caspase-3, a marker of apoptosis. However, neuroprotection was significantly minimized when both isoforms were blocked simultaneously. This key figure demonstrates, for the first time, that neuroprotection due to candesartan is mediated, at least partly, through augmenting the endothelial cell-secreted growth factors, VEGF-A and B. The failure of direct neuroprotection in stroke therapy has promoted a more holistic approach to treatment, taking into consideration the communication between different brain cell types and especially considering endothelial cells as a “neuroprotective organ” (Guo et al., 2008). In agreement, a recent study has demonstrated the involvement of VEGF-A in endothelium- mediated neuroprotection after stroke (Ishikawa et al., 2013).

In summary, our findings provide new insights on the benefits of candesartan and that they are mediated through orchestrated actions of multiple players, including endothelial VEGF-A and B. The study further points to the potential usefulness of augmenting the endogenous reparative capacity of the brain for the management of acute ischemic stroke.

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Authorship Contributions

Participated in research design: Soliman, El-Remessy, Pillai, Somanath, Ergul, and Fagan.

Conducted experiments: Soliman and Ishrat.

Contributed new reagent or analytic tools: Pillai.

Performed data analysis: Soliman.

Wrote or contributed to the writing of the manuscript: Soliman, El-Remessy, and Fagan.

Disclosures

The authors have nothing to disclose. The contents do not represent the views of the funding agencies Department of Veterans Affairs or the United States government.

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Figure Legends

Fig. (1): CSF from candesartan-treated animals induces a prolonged proangiogenic effect. **A, B:** Representative micrographs of tubes formed after treatment with CSF collected at 72 hours from saline and candesartan (1 mg/kg)-treated animals, respectively. CSF was collected 72 hours after reperfusion. **C:** Quantification of tubes after treatment with CSF collected from saline and candesartan (1 mg/kg)-treated animals 8, 24, 48 and 72 hours after reperfusion (n=2-3/ group, *P<0.05).

Fig. (2): A single candesartan dose induces a prolonged enhancement of VEGF-A and B expression in both hemispheres. **A, B:** Quantification of VEGF-A expression in the contralateral and ipsilateral hemispheres respectively at 8, 24, 48 and 72 hours after reperfusion with saline or candesartan (1 mg/kg). **C, D:** Quantification of VEGF-B expression in the contralateral and ipsilateral hemispheres at the same time points (n=3/ group, * Significantly different from the saline group at the same time point, P<0.05).

Fig. (3): Candesartan treatment stabilizes HIF-1a, exerts an antinitrative effect and preserves Ang-1 in the ischemic hemisphere. **A:** Quantification of HIF-1a in both ipsi- and contralateral hemispheres at 8 and 24 hours after reperfusion with saline or 1 mg/kg of candesartan (n=3/ group for the 8-hour time point, n= 5-6/ group for the 24-hour time point, *P<0.05). **B, C:** Quantification of NY and Ang-1, respectively, in both hemispheres 24 hours after reperfusion with either saline or 1 mg/kg of candesartan (n=4/ group, *P<0.05).

Fig. (4): Candesartan enhances the expression of VEGF-A and B as well the phosphorylation of their receptors in vitro. **A, C:** Quantification of VEGF-A and B, respectively, in endothelial cell lysate after 24-hour treatment with candesartan 0.1, 1 and 10 μg/ml under normoxic conditions (n= 4-6/ group, * P<0.05). **B, D:** Quantification of VEGF-A and B in endothelial cell

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lysate, respectively, after 2-hour OGD and 24-hour treatment with candesartan 0.1, 1 and 10 µg/ml respectively (n= 4/ group, * P<0.05). **E, G:** Determination of VEGFR1 and 2 activities, as measured by their phosphorylation levels, after 24 hours of treatment with candesartan (1 ug/ml). **F, H:** Determination of VEGFR1 and 2 phosphorylation after 2 hours of OGD and 24-hour treatment with 1 ug/ml of candesartan (n=3-4/ group, *P<0.05).

Fig. (5): Candesartan enhances endothelial cell tube formation, proliferation and migration in a dose- dependent fashion. **A:** Quantification of the number of tubes formed after 2-hour OGD and 24-hour treatment with 0.1, 1 and 10 µg/ml of candesartan (n=4-10/ group, *P<0.05). **B, C:** Representative micrographs of tubes formed at 72 hours under normoxic conditions in control and candesartan (1 µg/ml)-treated groups, respectively. **D, E:** Quantification of the tubes formed under normoxic and OGD conditions, respectively, 24, 48 and 72 hours after treatment with 1 µg/ml of candesartan (n= 4-9/ group, *P<0.05). **F, G:** Quantification of endothelial cell proliferation 24 hours after treatment with 0.1, 1 and 10 µg/ml of candesartan under normoxic conditions and OGD conditions respectively (n= 4/ group, *P<0.05). **H- K:** Representative micrographs of endothelial cell migration in wound healing assay at 0 hours, untreated control- 18 hours**,** candesartan 0.1 µg/ml- 18 hours and candesartan 1 µg/ml- 18 hours. **L, M:** Quantification of endothelial cell migration after 18 and 24 hours of treatment under normoxic and OGD conditions, respectively (n= 5-8/ group, * significantly different from control at the same time point, P< 0.05).

Fig. (6): Losartan treatment promotes a proangiogenic state and increases VEGF-A and B expression in vitro. **A:** Determination of VEGF-A and B expression in the endothelial cell lysate following 2 hours of OGD and 24 hours of treatment with 0.05 µg/ml of losartan. (*Ctrl, Los:* untreated control and losartan-treated cells respectively, n=3-5, *P<0.05). **B, C:** Determination

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of VEGFR1 and 2 phosphorylation in endothelial cell lysate after 2 hours of OGD and 24 hours of treatment with 0.05 µg/ml of losartan (n=3, *****P<0.05). **D, E:** Representative micrographs of tubes formed after 2-hour OGD and 24-hour losartan (0.05 µg/ml) treatment. **F:** Quantification of tubes formed after 2- hour OGD and 24-hour treatment with the same losartan concentration (n=5 and 4 for control and losartan groups respectively, *P<0.05). **G:** Quantification of endothelial cell migration after 18 and 24 hours of treatment with the same concentration of losartan (n= 4 and 3 for control and losartan groups respectively, * significantly different from control at the same time point, P<0.05).

Fig (7): Candesartan-induced proangiogenic effect is mediated through VEGF-A and B. **A-F:** Representative micrographs of endothelial cell migration in wound healing assay with or without candesartan treatment and VEGF-A and B neutralizing antibodies. **G:** Quantification of endothelial cell migration after 18 and 24 hours of treatment with candesartan with or without neutralizing antibodies. (n= 3-6/ group, *significantly different from control at the corresponding time point, #significantly different from cand 0.1- at the corresponding time point, P<0.05).

Fig (8A): Candesartan-induced VEGF-A and B upregulation exerts a paracrine neurportective effect. Determination of cleaved caspase-3 in mouse primary cortical neurons after 2 hours of hypoxia and 24 hours of treatment with conditioned media. Conditioned media was collected from endothelial cells after 2 hours of OGD and 24 hours of reoxygenation with or without candesartan (0.1µg/ml) treatment. VEGF-A and B neutralizing antibodies were added to the collected media prior to neuronal reoxygenation (*E-Ctrl, E-Cand*: conditioned media collected from untreated and candesartan-treated endothelial cells respectively. *E-Cand+VAnAb, E-Cand+VBnAb, E-Cand+both:* Conditioned media from candesartan- treated endothelial cells+

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VEGF-A neutralizing antibody or VEGF-B neutralizing antibody or both respectively. (n= 3-6/ group, *significantly different from control, #significantly different from E-Cand 0.1, $P<0.05$).

B: Schematic representation of the mechanisms involved in candesartan proangiogenic, vascular, and neuroprotective effects. Candesartan treatment induces neuroprotective and proangiogenic effects via an integrated action of VEGF-A and B. In addition, candesartan's antioxidant and antinitrative actions suggest an improved VEGF-A signaling. Preservation of Ang-1 might contribute to the synergistic angiogenic response, while exerting a simultaneous anti-permeabilizing effect, hence the preservation of barrier function after candesartan treatment.

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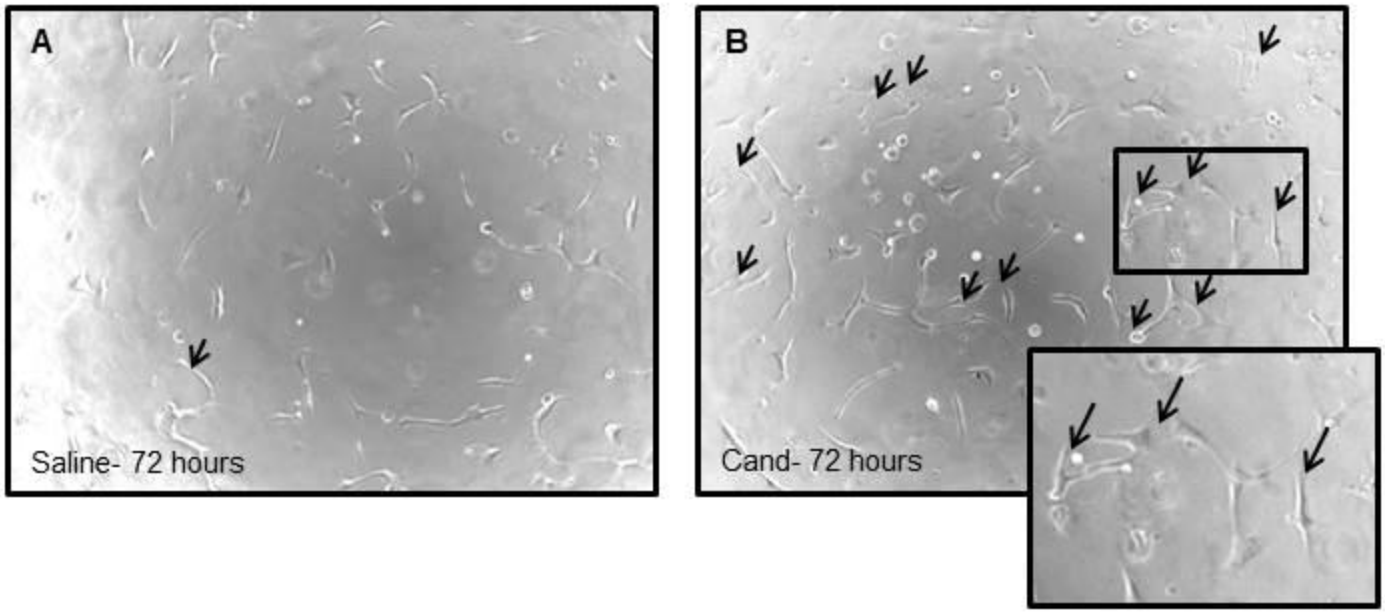
Figure 1


A
Saline- 72 hours
B
Cand- 72 hours

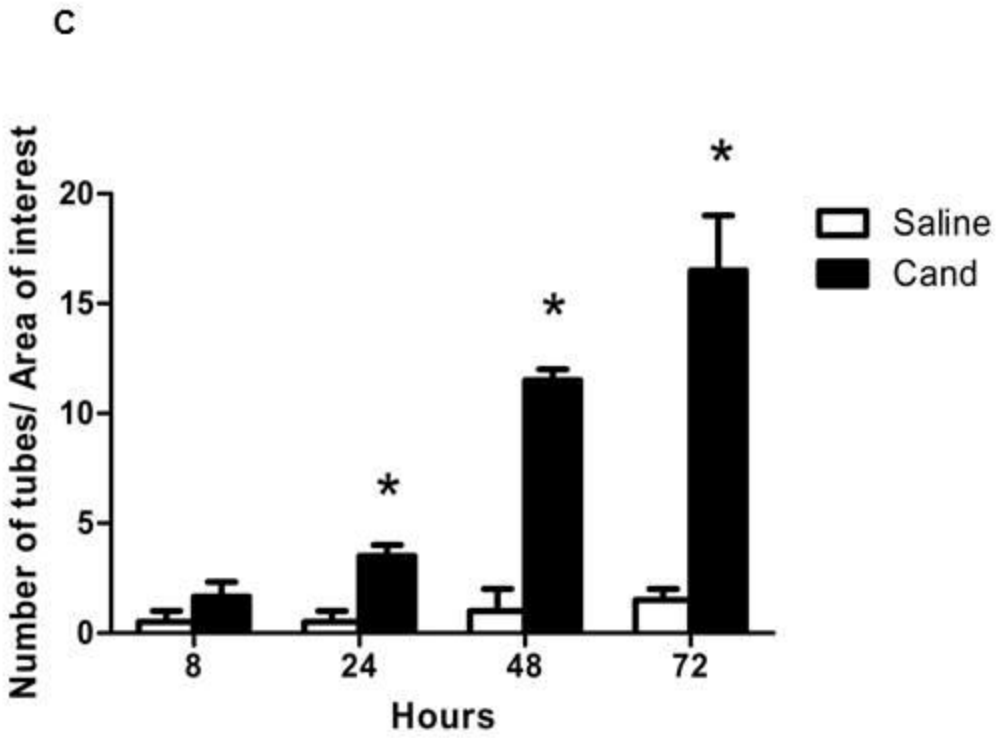

C
Number of tubes/ Area of interest
20
15
10
5
0
8
24
48
72
Hours
Saline
Cand
*
*
*

Figure 2

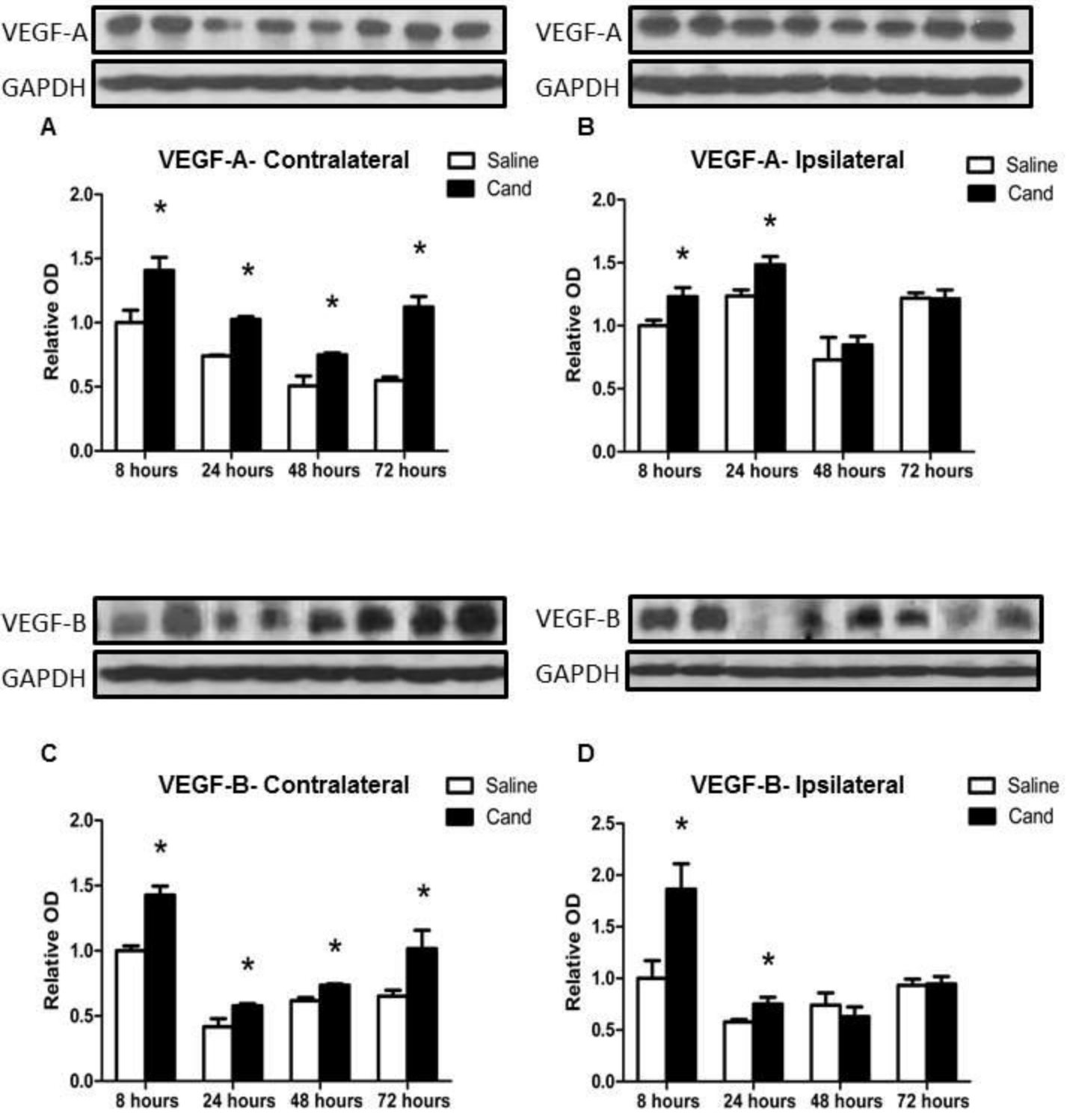
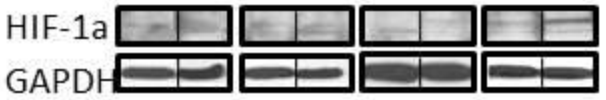
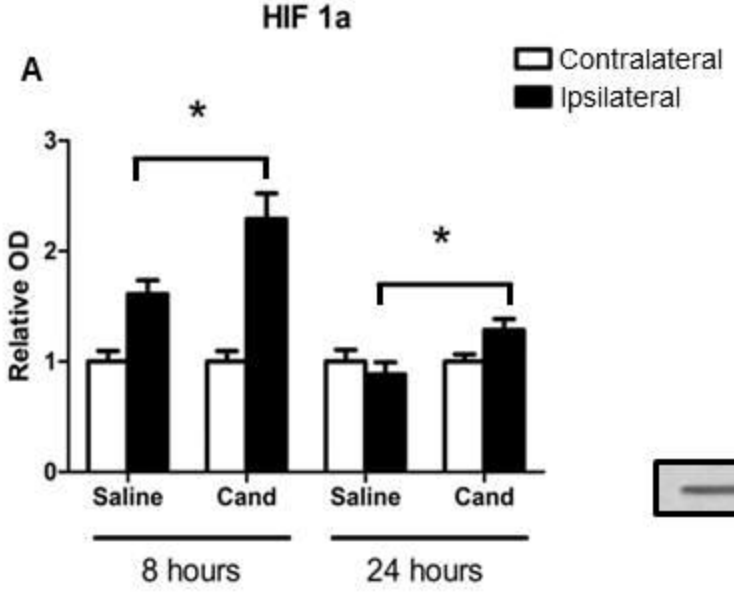
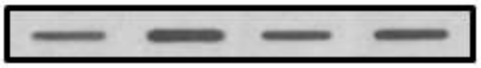

VEGF-A
GAPDH
VEGF-A
GAPDH
A
VEGF-A- Contralateral
Saline
Cand
Relative OD
2.0
1.5
1.0
0.5
0.0
*
*
*
*
8 hours
24 hours
48 hours
72 hours
B
VEGF-A- Ipsilateral
Saline
Cand
Relative OD
2.0
1.5
1.0
0.5
0.0
*
*
8 hours
24 hours
48 hours
72 hours
VEGF-B
GAPDH
VEGF-B
GAPDH
C
VEGF-B- Contralateral
Saline
Cand
Relative OD
2.0
1.5
1.0
0.5
0.0
*
*
*
*
8 hours
24 hours
48 hours
72 hours
D
VEGF-B- Ipsilateral
Saline
Cand
Relative OD
2.5
2.0
1.5
1.0
0.5
0.0
*
*
8 hours
24 hours
48 hours
72 hours

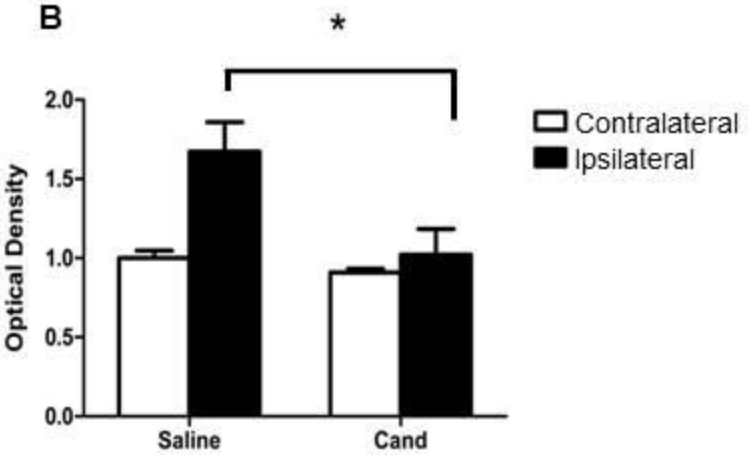
Figure 3

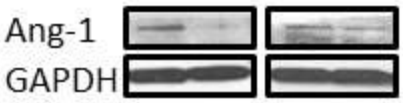

HIF-1a
GAPDH


HIF 1a
A
Contralateral
Ipsilateral
*
*
Relative OD
3
2
1
0
Saline
Cand
Saline
Cand
8 hours
24 hours



NY


B
*
Optical Density
2.0
1.5
1.0
0.5
0.0
Saline
Cand
Contralateral
Ipsilateral


Ang-1
GAPDH

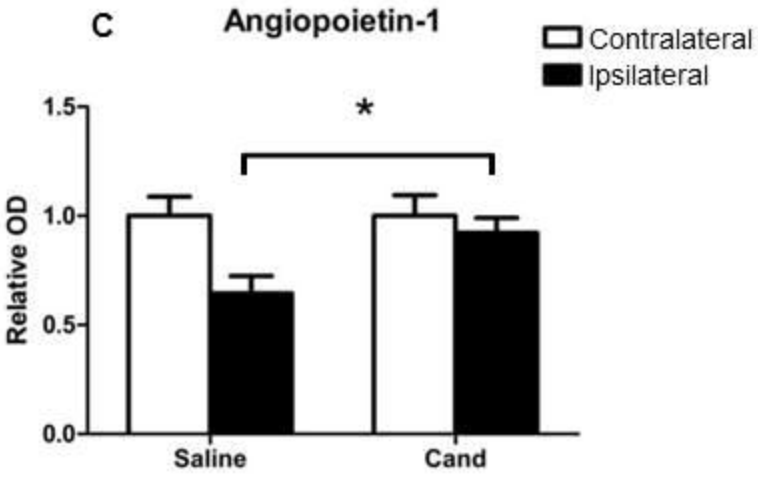

C
Angiopoietin-1
Contralateral
Ipsilateral
*
Relative OD
1.5
1.0
0.5
0.0
Saline
Cand

Figure 4

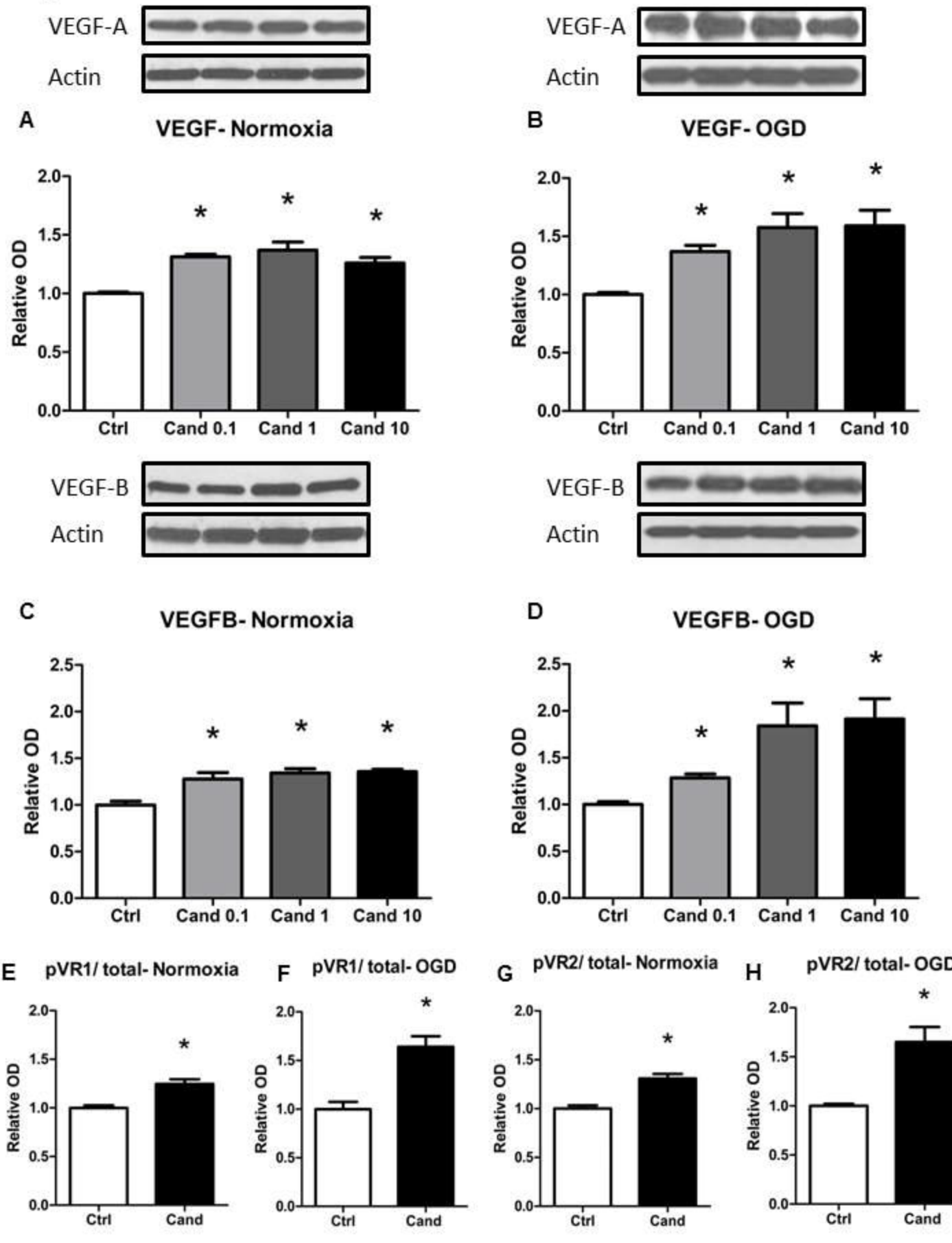
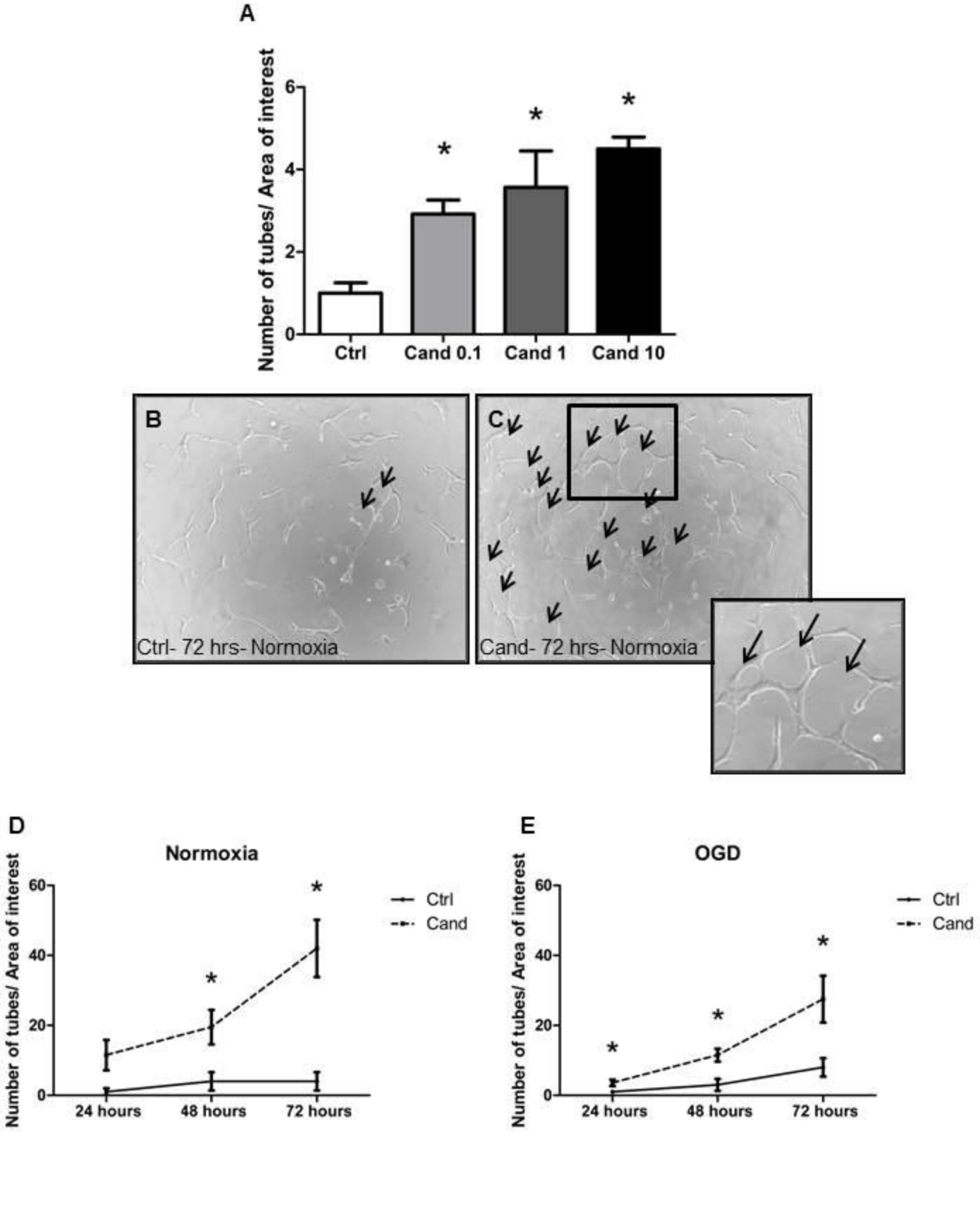

VEGF-A
Actin
VEGF-A
Actin
A
VEGF- Normoxia
B
VEGF- OGD
Relative OD
2.0
1.5
1.0
0.5
0.0
Ctrl
Cand 0.1
Cand 1
Cand 10
VEGF-B
Actin
VEGF-B
Actin
C
VEGFB- Normoxia
D
VEGFB- OGD
2.5
E
pVR1/ total- Normoxia
F
pVR1/ total- OGD
G
pVR2/ total- Normoxia
H
pVR2/ total- OGD
Cand

Figure 5


A
Number of tubes/ Area of interest
0
2
4
6
Ctrl
Cand 0.1
Cand 1
Cand 10
B
Ctrl- 72 hrs- Normoxia
C
Cand- 72 hrs- Normoxia
D
Normoxia
Number of tubes/ Area of interest
0
20
40
60
24 hours
48 hours
72 hours
Ctrl
Cand
E
OGD
Number of tubes/ Area of interest
0
20
40
60
24 hours
48 hours
72 hours
Ctrl
Cand

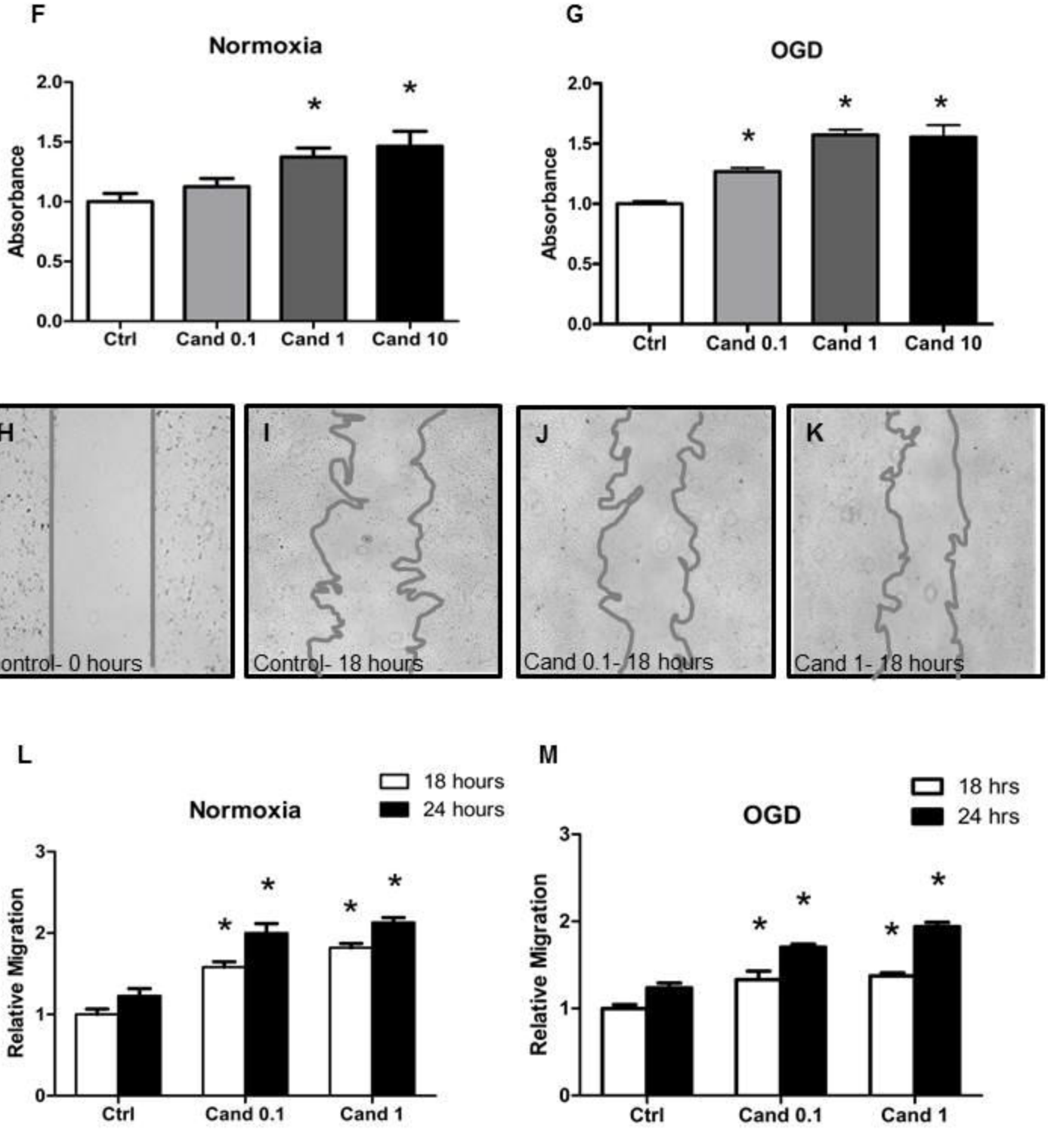

F
Normoxia
Absorbance
2.0
1.5
1.0
0.5
0.0
Ctrl
Cand 0.1
Cand 1
Cand 10
G
OGD
H
Control- 0 hours
I
Control- 18 hours
J
Cand 0.1- 18 hours
K
Cand 1- 18 hours
L
Normoxia
18 hours
24 hours
Relative Migration
3
2
1
0
M
OGD
18 hrs
24 hrs

Figure 6

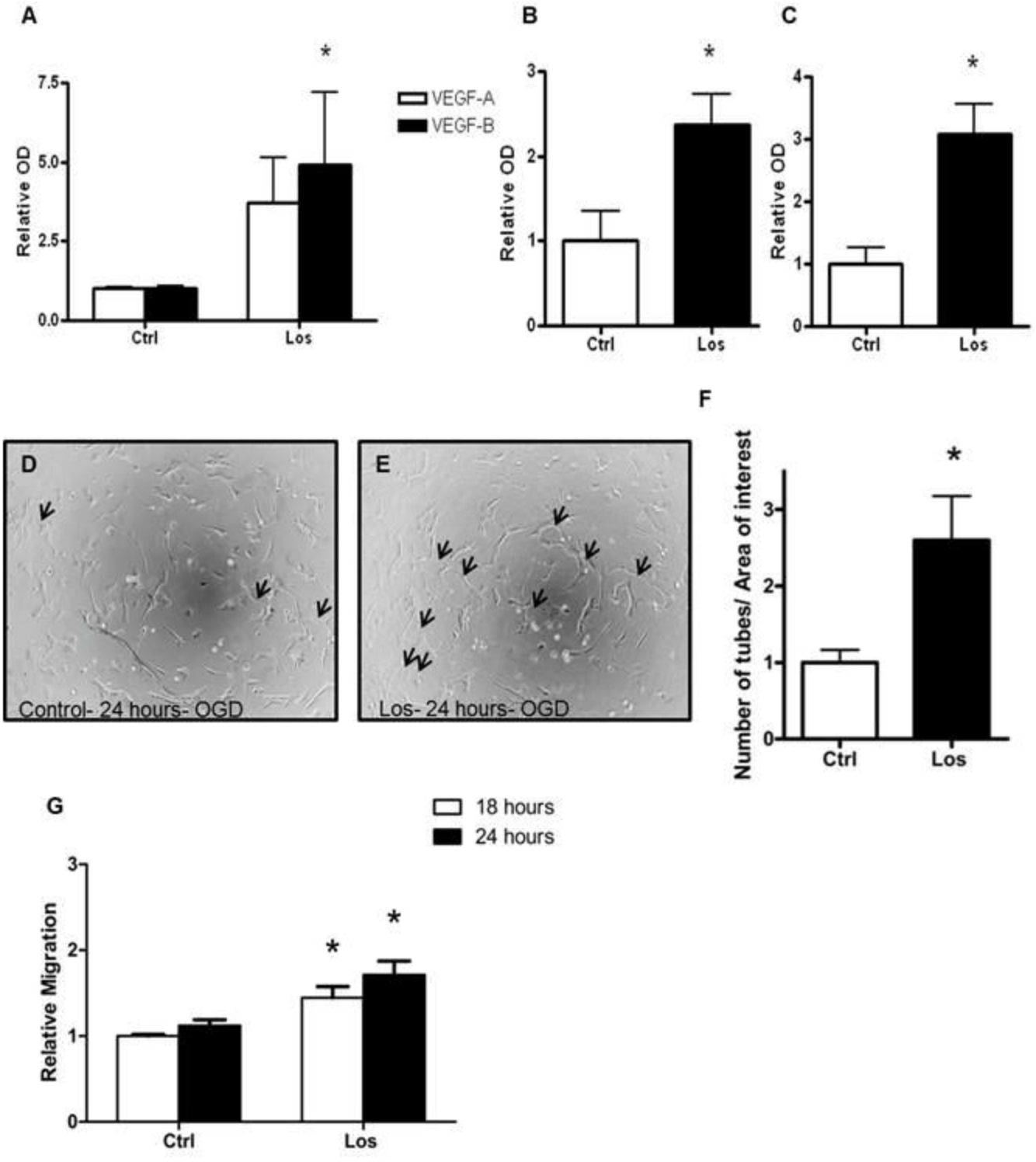
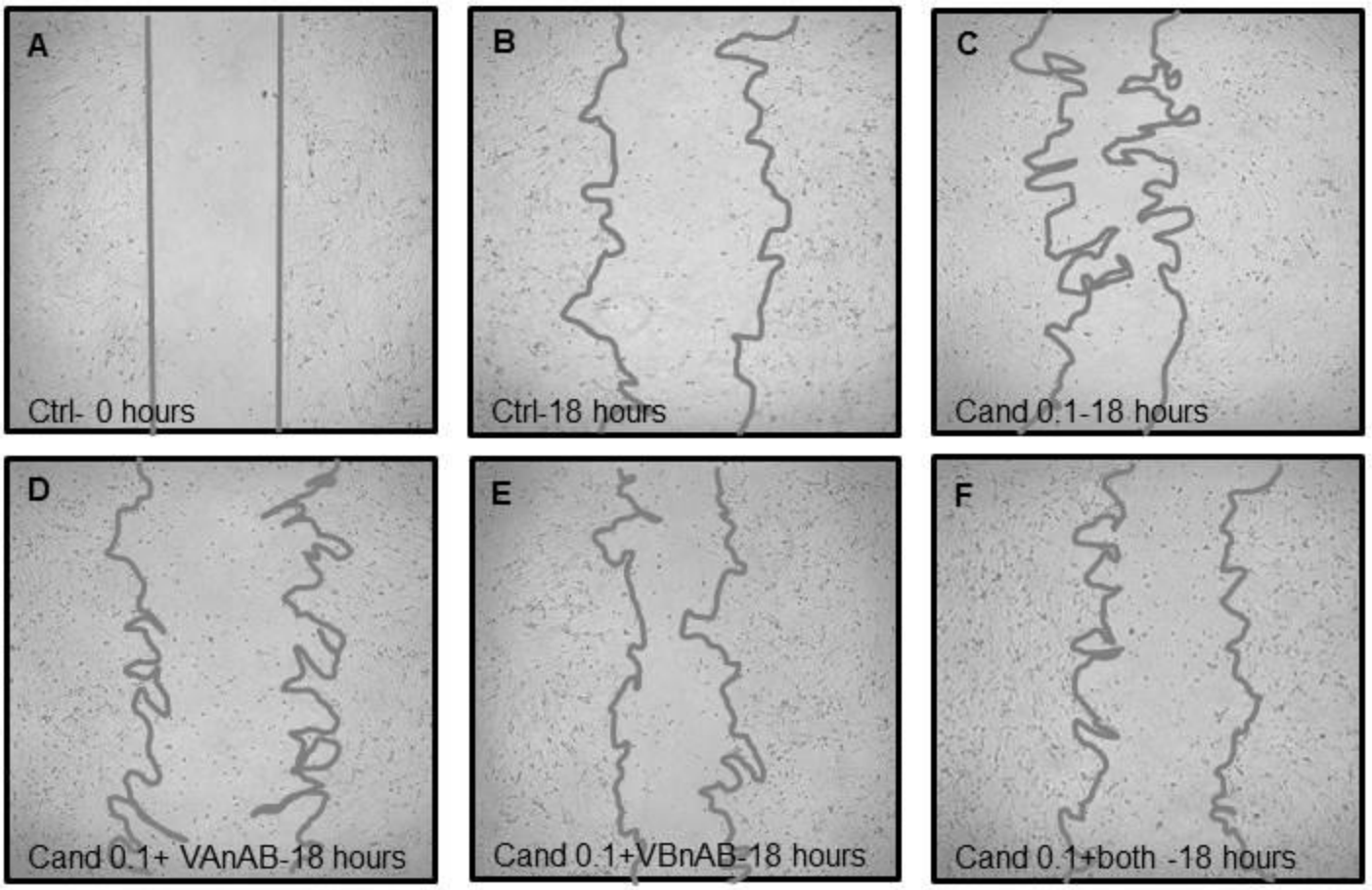

A
B
C
D
E
F
G
VEGF-A
VEGF-B
Relative OD
Ctrl
Los
Control- 24 hours- OGD
Los- 24 hours- OGD
Number of tubes/ Area of interest
18 hours
24 hours
Relative Migration

Figure 7


A
Ctrl- 0 hours
B
Ctrl-18 hours
C
Cand 0.1-18 hours
D
Cand 0.1+ VAnAB-18 hours
E
Cand 0.1+VBnAB-18 hours
F
Cand 0.1+both -18 hours

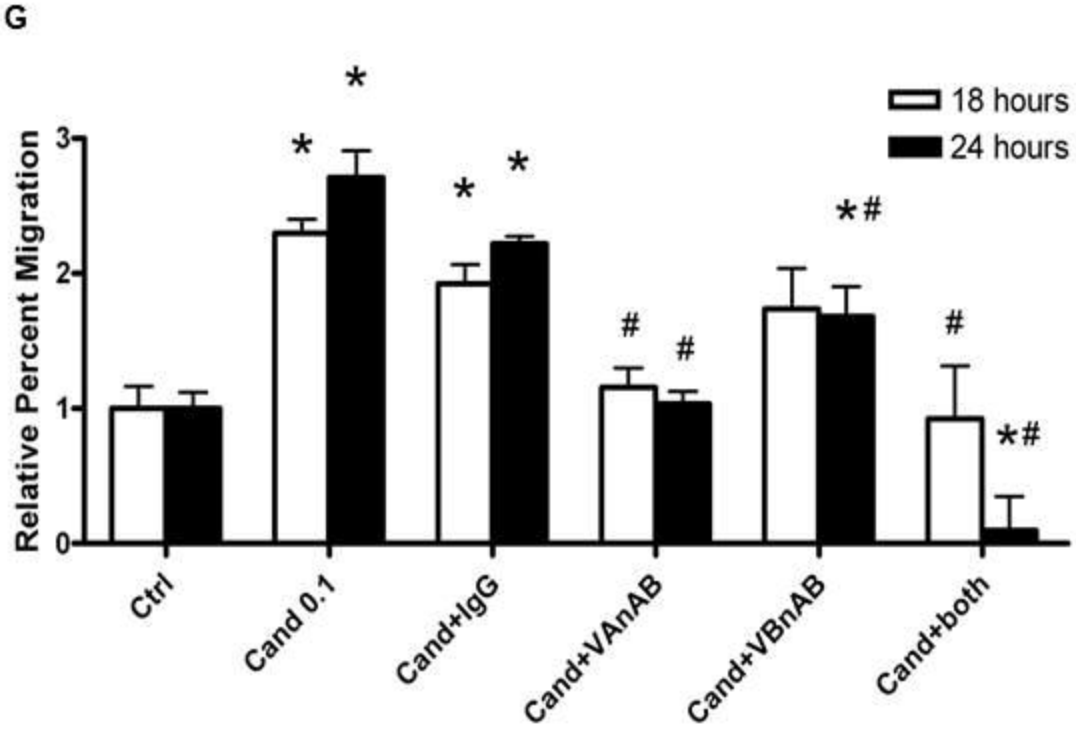
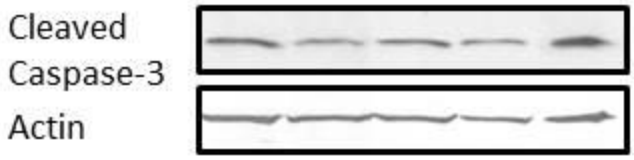
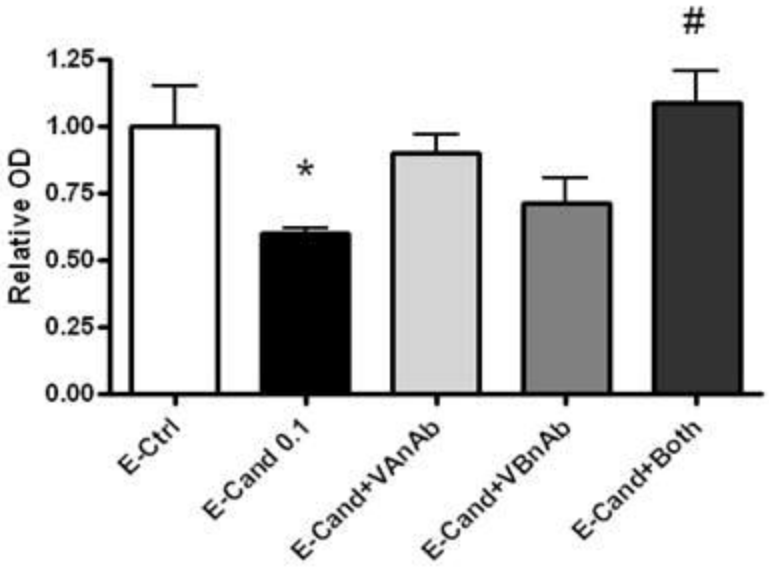

G
18 hours
24 hours
Relative Percent Migration
0
1
2
3
Ctrl
Cand 0.1
Cand+IgG
Cand+VAnAB
Cand+VBnAB
Cand+both

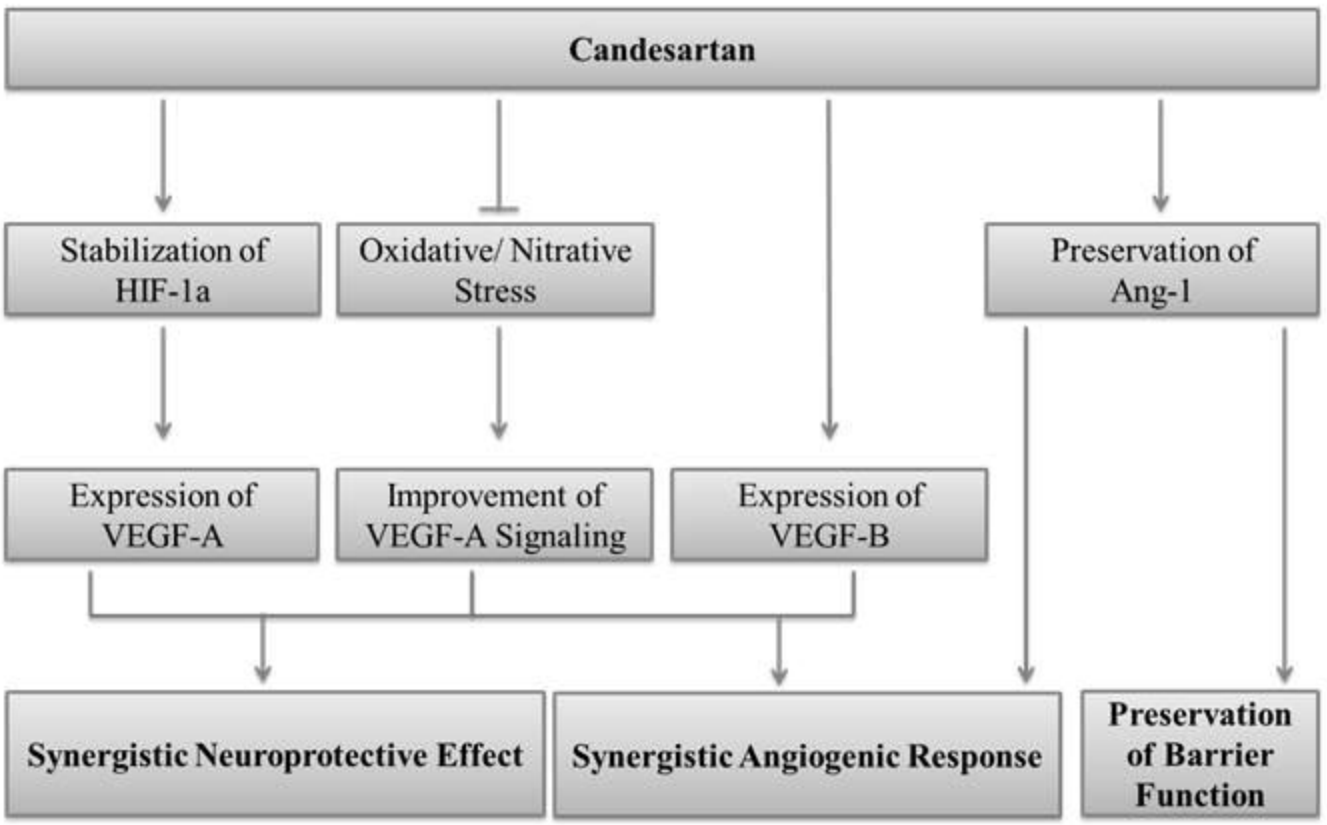
Figure 8

A


Cleaved
Caspase-3
Actin


Relative OD
1.25
1.00
0.75
0.50
0.25
0.00
*
#
E-Ctrl
E-Cand 0.1
E-Cand+VAnAb
E-Cand+VBnAb
E-Cand+Both

B


Candesartan
Stabilization of HIF-1a
Oxidative/ Nitrative Stress
Preservation of Ang-1
Expression of VEGF-A
Improvement of VEGF-A Signaling
Expression of VEGF-B
Synergistic Neuroprotective Effect
Synergistic Angiogenic Response
Preservation of Barrier Function