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Title: AT1 receptor antagonism is proangiogenic in the brain: BDNF a novel mediator

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Abbreviations: AngII, angiotensin II; ARBs, angiotensin II receptor blockers; BDNF, brain derived neurotrophic factor; VEGF, vascular endothelial growth factor; cand, candesartan; GSK-3 β , Glycogen Synthase Kinase-3 β ; SHR, spontaneously hypertensive rats; hCMECs, human cerebromicrovascular endothelial cells. PD, PD-123319 (S-(+)-1-[(4-(Dimethylamino)-3-methylphenyl)methyl]-5-(diphenylacetyl)-4,5,6,7-tetrahydro-1H-imidazo[4,5-c]pyridine-6-carboxylic acid di(trifluoroacetate) salt hydrate); K252a, (9S,10R,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3',2',1'-*k*]pyrrolo[3,4-*l*][1,6]benzodiazocine-10-carboxylic acid methyl ester; CGP-42112A, N α -Nicotinoyl-Tyr-(N α -Cbz-Arg)-Lys-His-Pro-Ile; GSK-3 β , Glycogen Synthase Kinase-3 β ; SHR, spontaneously hypertensive rats; hCMECs, human cerebromicrovascular endothelial cells.

Abstract

Candesartan is an angiotensin II type 1 receptor blocker (ARB) that has been shown to limit ischemic stroke and improve stroke outcome. In experimental stroke, candesartan induces a proangiogenic effect that is partly due to vascular endothelial growth factor (VEGF). Brain derived neurotrophic factor (BDNF) is a member of the neurotrophin family that has been reported to have angiogenic effects and play an important role in recovery after stroke. The purpose of this investigation was to determine the role of BDNF in the proangiogenic effect of candesartan in the brain under hypertensive conditions. Accordingly, spontaneously hypertensive rats were treated with candesartan and brain tissues were collected for quantification of BDNF expression. In addition, human cerebrovascular endothelial cells were treated with either low dose (1fM) or high dose (1μM) angiotensin II alone or in combination with candesartan (0.16μM) to assess the effect of candesartan treatment and BDNF involvement in the behavior of endothelial cells. Candesartan significantly increased the expression of BDNF in the SHR ($p < 0.05$). In addition, candesartan reversed the antiangiogenic effect of the 1μM dose of AngII ($p = 0.0001$). The observed effects of candesartan were ablated by neutralizing the effects of BDNF. Treatment with the AT2 antagonist PD-123319 significantly reduced tube-like formation in endothelial cells. AT2 stimulation induced the BDNF expression and migration ($p < 0.05$). In conclusion candesartan exerts a proangiogenic effect on brain microvascular endothelial cells treated with angiotensin II. This response is due to increased BDNF expression and is mediated through stimulation of the AT2 receptor.

Introduction:

Angiotensin II type 1 receptor blockers (ARBs) have been shown to be vascular protective and seem to have a particularly robust effect in reducing the incidence of cerebrovascular events (Björn Dahlöf et al., 2002; Engelhorn et al., 2004; Kozak et al., 2009). Acutely, ARBs have been shown to improve outcome in experimental stroke (Fagan et al., 2006) and the long term functional benefit was accompanied by an augmented proangiogenic state (Kozak et al., 2009). This angiogenic effect was only partially attributed to an increase in VEGF expression (Kozak et al., 2009). Interestingly, the angiogenic response of candesartan was maintained even in non-stroked rats (Kozak et al., 2009). Subsequently, it was demonstrated that candesartan increased the expression of a number of genes for proangiogenic growth factors, including brain derived neurotrophic factor (BDNF), following experimental cerebral ischemia (Guan et al., 2011). BDNF is a member of the neurotrophin family that is expressed in a number of cell types and has been shown to have potent neurogenic, neuroprotective and angiogenic effects (Caporali and Emanuelli, 2009; Greenberg et al., 2009). The proangiogenic effects of ARBs are hotly debated, however, (Schieffer et al., 1994; Herr et al., 2008; Kozak et al., 2009) and may be tissue and situation dependent (Willis et al., 2011). In the brain, it is unclear whether the effects of ARBs are due to blood pressure lowering or a direct effect of candesartan on endothelial cells.

Glycogen synthase kinase- 3β (GSK- 3β) is a serine threonine kinase that plays a key role in gene expression regulation (Wada, 2009; Hur and Zhou, 2010). Recently, Li *et al.* demonstrated the involvement of GSK- 3β in the recovery after CNS ischemic insults (Li et al., 2011). They demonstrated the involvement of GSK inhibition in regulating

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neural stem cells-endothelial cells cross talk (Li et al., 2011). This cross talk was found to be mediated through soluble growth factors like BDNF (Madri, 2009).

The purpose of this investigation was to determine whether candesartan-mediated blood pressure lowering increased BDNF protein expression in the brain in vivo and whether BDNF is involved in the proangiogenic effect of candesartan in vitro. In addition the involvement of GSK-3 β in candesartan mediated effects was assessed.

Materials and methods:

Animals:

The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Charlie Norwood Veterans Affairs Medical Center (09-04-008). Male spontaneously hypertensive rats (SHRs) (280-300g, n=4-6 per group) were subjected to middle cerebral artery occlusion (MCAO) sham surgery and randomized to receive a single IV dose of candesartan 0.3mg/kg, hydralazine 1mg/kg, or saline. In addition, male wistar rats (280-300g, n=4 per group) were subjected to the same surgery and randomized to receive either a single IV dose of candesartan 1mg/kg or saline. Twenty four hours later the rats were euthanized and the brains were harvested and flash frozen in liquid nitrogen.

Western blotting:

To assess BDNF expression, the right and left hemispheres were separated and processed as described previously (Guan et al., 2011) and the blots probed with

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antiBDNF (1:250, abcam) and β -Actin (1:10000, sigma). For the in vitro experiments; human cerebrovascular endothelial cells (hCMECs) were cultured to confluence and serum starved for 10 hours followed by incubations with either 1fM or 1 μ M angiotensin II (AngII). After 6 hours, candesartan 0.16 μ M was added to the cells and incubated for 16 hours and then homogenized and processed for immunoblotting. To assess the involvement of AT2 receptor in BDNF expression HCMECs were serum starved for 16 hours and pretreated with PD-123319(0.1 μ M) 30 minutes before being incubated with AngII (1fM or 1 μ M) for 6 hours. Candesartan or vehicle were introduced in the media for 10 hours. To further confirm AT2 involvement, cells were serum starved for 16 hours followed by treatment with either the AT2 agonist CGP-121141A (0.1 μ M) or vehicle for 16 hours. The expression of AT1, AT2 and the phosphorylation status of GSK-3 β were assessed using the same above treatment paradigm. Blots were probed with mouse monoclonal AT1 antibody (Abcam; 1:1000); rabbit monoclonal AT2 antibody (Abcam; 1:1000); p-S9GSK-3 β (cell signaling; 1:1000) and total GSK-3 β (cell signaling; 1:1000) Protein expression was quantified as the relative optic density of the protein band normalized to actin using NIH-image J software.

Cell culture:

hCMECs were provided as a generous gift from Dr. J. Zastre (UGA College of Pharmacy). hCMECs were cultured in minimum essential media (ATCC) supplemented with EGM-2 SingleQuot Kit Suppl. & Growth Factors (Lonza) and 10% FBS (Atlanta Biologicals) and p30-34 were used in the experiments.

Treatments:

Candesartan was provided as a generous gift from Astra-Zeneca. Hydralazine was purchased from Sigma-Aldrich (St. Louis, MO) and was reconstituted with 0.9% normal saline. AngII was purchased from Sigma-Aldrich (St. Louis, MO) and was reconstituted and diluted to the desired concentration using serum free media. BDNF neutralization was achieved using 100 nM K252a (Trk receptor inhibitor) dissolved in 25% DMSO both purchased from Sigma-Aldrich (St. Louis, MO), 10ng/ml anti-BDNF neutralizing antibody (Abcam; Cambridge, MA) and 0.4ug/ml TrkB-Fc (soluble BDNF receptor chimera. R&D systems; Minneapolis, MN). The involvement of AT2 receptor was assessed using the AT2 antagonist PD-123319 (0.1 μ M) and the AT2 receptor agonist CGP-42112A (0.1 μ M) both purchased from Sigma-Aldrich (Sigma-Aldrich; St. Louis, MO). All the inhibitors were added to the media 30 minutes before AngII treatment.

Dose and time study:

hCMECs were cultured until confluence and serum starved for 10 hours . Cells were incubated with six different concentrations of AngII (0-1 μ M) for 2, 6and8 hours and the cells were homogenized and processed for immunoblotting. BDNF expression was quantified as the relative density of the BDNF band to the corresponding β -actin or GAPDH bands. The calculated relative density was normalized to the relative density of the control band, and reported as fold change.

Proliferation assay:

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Proliferation was assessed using BrdU incorporation (Cell Proliferation ELISA, BrdU (colorimetric); Roche Applied Science) according to the manufacturer recommendations. Briefly, 5000 hCMECs were seeded into each well of a 96 well plate and left to attach for 24 hours. Cells were serum starved for 10 hours and then treated with AngII (1fM or 1μM) for 6 hours. Following 6 hours, cells were treated with different combinations of candesartan, antiBDNF, TrkB-Fc, K252a, DMSO or IgG and incubated for 18 hours. The cells were then labeled with BrdU for 4 hours and then processed to quantify BrdU incorporation.

Angiogenesis Assays

Cell migration:

Wound recovery assay was used to assess cell migration where hCMECs were cultured in a 12 well plate to confluence and then serum starved for 10 hours followed by 6 hours of AngII (1fM or 1μM) treatment. A wound was introduced in the monolayer of endothelial cells and the cells treated with AngII (1fM or 1μM) with different combinations of candesartan, antiBDNF, TrkB-Fc, K252a, DMSO or IgG. In some experiments, cells were pretreated with PD-123319 (0.1μM) 30 minutes before AngII treatment. In another set of studies cells were treated with CGP-42112A (0.1μM), candesartan (0.16μM) or their combination. Scratch recovery was assessed by taking images of the scratch at baseline and at 16 hours after scratch introduction and the width of the scratch was measured at both time points using NIH image J software. The percentage wound recovery was calculated as the percent decrease in scratch width at

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16 hours and the data was presented as percentage scratch recovery as compared to the control.

Tube formation:

2×10^4 hCMECs were suspended in serum free media and mixed with matrigel (BD Biosciences; San Jose, CA) in a 60:30 ratio and plated in a 96 well plate and then treated with different combinations of AngII (1fM or 1 μ M, sigma), candesartan, antiBDNF, TrkB-Fc, K252a, DMSO, IgG ,or PD-123319. Tube like structure formation was assessed using a digital camera attached to an Olympus microscope. Three images from each well were photographed at 24 hours and the number of tube like structures was quantified.

Statistical analysis:

All experiments were repeated three times in triplicate and data was quantified in a blinded manner. Statistical significance was detected by one-way ANOVA for in vitro data followed by post-hoc Tukey test. Unpaired t-test was used to determine the significance of BDNF expression in the right and left brain hemispheres of candesartan treated SHRs as compared to the corresponding hemisphere in saline treated animals. Statistical analyses were performed using GraphPad prism software (5.1). $P < 0.05$ was considered significant.

Results:

Candesartan increases the expression of BDNF in SHR brain:

BDNF has been shown to exert a beneficial effect in a variety of CNS pathologies (Greenberg et al., 2009); the direct use of BDNF in therapy is limited by its pharmacokinetic profile (Greenberg et al., 2009). A plausible alternative approach is to use either synthetic BDNF mimetics or agents that can induce BDNF expression in the brain. Treatment with a single dose of candesartan (0.3mg/kg) dramatically reduced the blood pressure from 150 mmHg at baseline to 120 mmHg after treatment (Figure 1A). Candesartan treatment significantly increased the expression of BDNF in both right and left hemisphere of SHR animals 24 hours after sham surgery (Figure 1B). The effect of candesartan on BDNF expression was maintained in wistar rats (supplemental data S3). In contrast, hydralazine treatment did not affect BDNF expression in SHRs (supplemental data S4).

Angiotensin II modulates the expression of BDNF in hCMECs.

Angiotensin II has been reported to affect the expression of BDNF in the adrenals (Szekeres et al., 2010) and brain (Chan et al., 2010); but its effect on BDNF expression in endothelial cells has never been reported. In hCMECs treatment with AngII was found to induce a dose and time-dependent modulation of BDNF expression that was maximal at 6 hours after incubation with either AngII 1fM or 1μM (Figure 2A). These two concentrations were used in the following experiments.

Candesartan increases BDNF expression in hCMECs:

After establishing the effect of ARBs on the expression of BDNF in brain tissue in vivo, the effect of candesartan on the expression of BDNF in AngII treated cells in vitro was investigated. Candesartan significantly increased the expression of BDNF in hCMECs

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treated with AngII 1fM in comparison to AngII 1fM alone and in hCMECs treated with both concentrations of AngII as compared to the control as assessed after 16 hours of incubation with candesartan (Figure 2B).

Candesartan has a dose dependent modulatory effect on the angiogenic potential of hCMECs.

Data from clinical studies in stroke presented conflicting results on whether the hypotensive effect of ARBs is an essential requirement for its reported benefit (Schrader et al., 2003; Schrader et al., 2005). Consequently, we were interested in assessing whether a therapeutically relevant concentration of candesartan would have an in vitro effect in hCMECs in the absence of AngII. We calculated the amount of candesartan that can give a concentration similar to that achieved in patients receiving the drug (0.16 μ M). The angiogenic effect of a range of candesartan concentrations, including the concentration under consideration, was assessed using an in vitro matrigel tube formation assay (Figure 2C). The observed response was further confirmed using the wound recovery migration assay (Figure 2B). Candesartan induced a dose dependent, bell-shaped modulatory effect on the tube formation rate in hCMECs. The 0.16 μ M candesartan concentration (therapeutically relevant) significantly increased the tube formation rate, whereas the other concentrations did not affect the rate of tube formation (Figure 2C). A similar effect was observed in the wound recovery migration assay, except for an increased migration rate in hCMECs treated with candesartan 1.6 μ M concentration (Figure 2D). This differential effect of the 1.6 μ M candesartan concentration might be attributed to dose dependent effects of candesartan on the different processes involved in migration and tube formation.

Candesartan modulates the proliferation of hCMECs:

Angiotensin II has been reported to induce the proliferation of cells in vitro (Kou et al., 2007; Herr et al., 2008). This proliferative effect has been shown to be blocked with ARBs (Kou et al., 2007; Herr et al., 2008); but the reported concentration of ARBs in these studies was supratherapeutic (Herr et al., 2008). Consequently, we attempted to assess the effect of the therapeutically relevant concentration on the proliferative effect of AngII in hCMECs. Both low and high concentrations of AngII significantly increased the proliferation of hCMECs (Figures 3A and D). Treatment with candesartan maintained and further enhanced this proliferative effect (Figures 3A and D).

Candesartan modulates the migration rate of hCMECs:

Similar to its proliferative effect, AngII has been reported to affect the migration of cells in vitro (Buharalioglu et al., 2011). Since we have found a proliferative effect of candesartan on AngII treated hCMECs, we were interested in assessing the effect of this dose of candesartan on two critical steps of angiogenesis: migration and tube formation in AngII treated hCMECs. We observed a significant increase in the migration of hCMECs in response to treatment with AngII 1fM (Figure 4A). Interestingly, the higher AngII concentration did not have an effect on the migration of hCMECs (Figure 4D) in our model. While candesartan maintained the increased hCMECs migration in the low dose AngII group (Figure 4A), candesartan increased hCMECs migration in the high dose AngII dose treated cells (Figure 4D). Cells migration was not affected by K252a, DMSO or IgG (Figures 4C and F).

Candesartan has a proangiogenic effect in hCMECs:

The pro-angiogenic effect of AngII has been reported previously (Hu et al., 2007; Buharalioglu et al., 2011). In hCMECs there was a significantly increased rate of tube formation in response to AngII 1fM treatment (Figure 5A) and a reduction in tube formation in AngII 1μM treated cells (Figure 5D). Interestingly, candesartan maintained AngII 1fM induced tube formation and reversed the antiangiogenic effect of AngII 1μM (Figure 5A and D).

BDNF mediates the effects of candesartan on hCMECs:

Our in vivo results demonstrated the ability of candesartan to increase the expression of BDNF in SHR's brain. Previously, BDNF has been demonstrated to have an angiogenic effect which shifted our interest to assess whether BDNF is involved in the observed effects of candesartan. Upon neutralizing BDNF using three different methods of BDNF neutralization, the observed effects of candesartan were consistently ablated (Figures 3B and E; 4B and E, 5B and E); a finding that identifies BDNF as an essential mediator of candesartan effects in hCMECs. Interestingly, the angiogenic effect of AngII 1fM was significantly inhibited upon BDNF neutralization (Figure 5C), which suggests the involvement of BDNF in AngII induced angiogenesis, which is confirmed by the robust angiogenic response to BDNF treatment (Figures 5C and F).

AT2 receptor mediates the angiogenic response to angiotensin II in hCMECs.

Candesartan mediates its effects through AT1 blockade (Engelhorn et al., 2004; Willis et al., 2011), which induces an unopposed stimulation of AT2 receptor (Hashikawa-Hobara et al., 2012). Findings in this investigation suggest a possible involvement of AT2 receptor in mediating candesartan effects.

AT2 blockade using PD-123319 significantly inhibited the angiogenic response induced by either angiotensin II or the combination of angiotensin II and candesartan (Figure 6 A and B). In addition the AT2 agonist, CGP-24112A, significantly increased the migration of hCMECs. This migratory response was not affected by the concomitant treatment with candesartan (Figure 6C). These findings demonstrate an essential role of AT2 in the angiogenic process of hCMECs.

Candesartan induced BDNF expression is mediated through the AT2 receptor.

Findings in this study demonstrated the ability of candesartan to increase the expression of BDNF both in vivo and in vitro; in addition the in vitro effects of candesartan were shown to be mediated through the AT2 receptor. Accordingly, the involvement of AT2 receptor in BDNF expression was evaluated. As has been demonstrated earlier candesartan significantly increased the expression of BDNF in AngII treated cells (Figure 2B, 7A). Pretreatment with the AT2 antagonist PD-123319 significantly inhibited candesartan induced BDNF expression, which suggests the involvement of AT2 receptor. To confirm this findings hCMECs were treated with the AT2 agonist CGP-42112A (0.1 μ M) and the expression of BDNF was assessed. CGP-42112A significantly increased t expression of BDNF in hCMECs by about one fold as compared to vehicle treated cells (Figure 7B).

AT1 antagonism regulates the expression of AT1 in an AT2 dependent manner.

Candesartan treatment significantly increased the expression of AT1 receptor in cells treated with high dose of AngII as compared to both control and AngII alone. This

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response was ablated when the AT2 receptor was blocked using PD-123319 (Figure 7B). Additionally, the AT2 agonist CGP-24112A induced a significant fourfold increase in AT1 receptor expression in hCMECs (supplementary data). In cells treated with low dose AngII, candesartan significantly increased the expression of AT1 compared to control alone (Figure 7A). The expression of AT2 was not changed under the different treatments used (Figure 7 C and D).

AT1 antagonism modulates the activity of GSK-3 β in an AT2 dependent manner.

GSK-3 β was found to modulate BDNF expression (Hur and Zhou, 2010; Li et al., 2011). Our results demonstrated the ability of candesartan to increase the expression of BDNF in both in vivo and in vitro settings. Accordingly we were interested in assessing the activity of GSK-3 β under the different treatment conditions we were using. Candesartan treatment significantly increased the phosphorylation of GSK-3 β at the inhibitory serine 9 residue in cells treated with high dose AngII (Figure 8A). The higher phosphorylation was reversed by the concomitant treatment with AT2 blocker PD-123319 (Figure 8A).

Discussion:

Our results demonstrate, for the first time, the ability of candesartan to promote a proangiogenic state in hCMECs in an AngII concentration independent manner, where candesartan was able to maintain the angiogenic effect of AngII at the low dose, and reverse the antiangiogenic effect of the high dose. The proangiogenic effect of candesartan was found to be mainly dependent on BDNF and is mediated through AngII stimulation of the AT2 receptor. In addition, we have demonstrated the ability of

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candesartan to induce an angiogenic response in endothelial cells; even in the absence of exogenously added AngII.

Following an ischemic insult in the brain, induction of angiogenesis has been shown to ameliorate the damage and is coupled to neurogenesis leading to enhanced recovery and better outcome (Navaratna et al., 2009; Xiong et al., 2010). ARBs administration was found to increase vascular density in heart and brain following MI (Sladek et al., 1996) and stroke (Kozak et al., 2009; Guan et al., 2011) which was attributed to an increase in VEGF expression in stroke (Guan et al., 2011). In contrast to in vivo data, in vitro angiogenesis studies demonstrated an antiangiogenic effect of ARBs in endothelial cells treated with AngII (Hu et al., 2007; Herr et al., 2008). This antiangiogenic effect was mediated mainly through inhibiting AngII induced increase in VEGF signaling (Fujiyama et al., 2001; Herr et al., 2008). The majority of these studies were conducted in either HUVECs (Kou et al., 2007; Buharalioglu et al., 2011) or coronary artery endothelial cells (Fujiyama et al., 2001; Hu et al., 2007) and none of them evaluated the effect of AngII or AngII and ARBs in hCMECs which are phenotypically different from other endothelial cells (Feletou, 2011). In addition, the majority of the studies focused on the involvement of VEGF (Buharalioglu et al., 2011), angiopoietins (Herr et al., 2008) and EGFR transactivation (Fujiyama et al., 2001) in the angiogenic response to AngII. This ignores the possible role of other angiogenic mediators, like BDNF, which has been shown to induce VEGF expression (Caporali and Emanuelli, 2009) and produce an angiogenic response comparable to that of VEGF in endothelial cells (Li et al., 2006). Results from this study demonstrate a dose dependent effect of AngII on brain angiogenesis, where low concentrations of AngII induce angiogenesis and higher doses

inhibit it. This is consistent with that shown in other vascular beds (Kou et al., 2007; Buharalioglu et al., 2011).

The design of this study is unique in many aspects. In a typical in vitro study, cells are initially treated with candesartan followed by AngII treatment. This precludes detection of a possible AngII independent interaction between candesartan and endothelial cells. This design has limited clinical relevance since candesartan will be introduced to the system in response to the effects of AngII or other circulating vasoactive mediators. In our design, we attempted to model the temporal relationship of treatment introduction. Data about the effect of AngII on BDNF expression in endothelial cells were lacking and the only reports addressing the effect of AngII on BDNF expression were in the adrenal cortex (Szekeres et al., 2010) and the brain (Chan et al., 2010). Accordingly, we did a time and dose response study in hCMECs to determine the incubation time and AngII dose to be used in the in vitro studies. This dose and time response study was followed by assessing the effect of those AngII on the viability of hCMECs (supplementary data S1). Also, the concentration of candesartan used in the in vitro studies was calculated to produce the midpoint therapeutic steady state concentration in humans (Schulz and Schmoldt, 2003). The calculated concentration was tested for its angiogenic and migratory effect and compared to other candesartan doses in a dose response curve (Figures 2C and D). The in vivo dose was determined based on previously reported data demonstrating neurovascular protection in SHR (Kozak et al., 2008).

Our findings support and expand our previously reported data on the ability of cerebral spinal fluid (CSF) from candesartan treated non stroked animals to induce angiogenesis in endothelial cells (Kozak et al., 2009). This proangiogenic effect of candesartan was

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only partially attributed to VEGF (Fagan et al., 2006), suggesting the involvement of other angiogenic factors. In this study we have identified BDNF as another important mediator of the angiogenic effect of candesartan. BDNF neutralization using receptor inhibitor K252a, antiBDNF antibody or TrkB-Fc almost ablated the effects of candesartan in all reported assays.

The proliferative and angiogenic effects of AngII have been largely attributed to AT1 mediated signaling (Otani et al., 1998; Fujiyama et al., 2001; Hu et al., 2007; Herr et al., 2008; Buharalioglu et al., 2011) while AT2 mediated signaling was thought to either counteract AT1 induced angiogenesis (Fujiyama et al., 2001; Javier Carbajo-Lozoya and Hans-Peter Hammes, 2012) or not have an effect on AngII induced angiogenesis (Otani et al., 1998; Hu et al., 2007). In contrast to this notion, AT2 mediated signaling has been demonstrated to promote angiogenesis in ischemic myocardium (Munk et al., 2007) and retinal endothelial cells (Sarlos et al., 2003). In this study, AT2 mediated signaling was found to be largely responsible for the angiogenic response in hCMECs. AT2 involvement in angiogenesis was initially suggested by the lack of effect on AngII – mediated angiogenesis when AT1 was blocked using candesartan and was further confirmed when the angiogenic response was totally prevented upon AT2 blockade using PD-123319. This finding suggests the importance of unopposed AT2 stimulation following stroke (McCarthy et al., 2009; Oprisiu-Fournier R, 2009) and may explain the lack of protective effect of ARBs in the absence of AT2 signaling following cerebral ischemia (Iwai et al., 2004; Li et al., 2005; Lu et al., 2005; Faure et al., 2008) . Our data demonstrates the indispensable role of AT2 for angiogenesis in hCMECs which has

been linked to neurogenesis and improving recovery after CNS ischemic insults (Madri, 2009; Navaratna et al., 2009; Xiong et al., 2010).

The activity of glycogen synthase kinase 3 beta (GSK-3 β) has been shown to be involved in the expression of neurotrophins in the brain (Wada, 2009) and in the cross talk between endothelial and neural stem cells through regulating BDNF and VEGF expression (Li et al., 2011). In addition GSK-3 β has been recently demonstrated to regulate the angiogenic response in endothelial cells (Flugel et al., 2012). Data from this study highlights a possible involvement of GSK-3 β in mediating the effects of AT1 antagonism in hCMECs in an Ang II dose-dependent manner. Candesartan increased phosphorylation of GSK-3 β at the inhibitory serine 9 residue which will inhibit the activity of GSK-3 β when used alone or in combination with 1 μ M AngII the concentration that induced antiangiogenic effects in hCMECs. Concomitantly this increased inhibition of GSK-3 β was associated with increased BDNF expression and angiogenic response in hCMECs. These findings are consistent with previously published data about the interaction between GSK-3 β and BDNF expression (Wada, 2009; Li et al., 2011).

In this study, candesartan demonstrated the ability to modulate the angiogenic response of hCMECs in the absence of exogenously added AngII. This effect was found to be dose dependent. The therapeutically relevant candesartan concentration induces a proangiogenic effect; whereas the other two concentrations didn't affect the angiogenic potential of the cells. This finding may help explain the controversial angiogenic effect of ARBs between the in vivo and the in vitro data (Willis et al., 2011). Previously published reports on the in vitro antiangiogenic effect of ARBs used supra-therapeutic concentrations (Hu et al., 2007; Herr et al., 2008). As demonstrated by the findings of

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this study, the supra-therapeutic doses of ARBs might have direct antiangiogenic effects on endothelial cells.

Another unique aspect of this work is the demonstration of candesartan's ability to increase the expression of BDNF in both hCMECs and the brain tissue of hypertensive animals. Hashikawa-Hobara et al. recently demonstrated the ability of candesartan to stimulate neurite growth in an AT2 dependent manner through Akt signaling (Hashikawa-Hobara et al., 2012). This is consistent with our findings, highlighting the involvement of the AT2 receptor in BDNF expression which is known to stimulate neurite growth (Parrish et al., 2007) and Akt signaling (Caporali and Emanuelli, 2009). Interestingly, the effect of candesartan on BDNF expression appears to be independent of blood pressure lowering. Candesartan increased BDNF expression in wistar rats (supplemental data S3); whereas, hydralazine had no effect on BDNF expression in SHRs (supplemental data S4).

An interesting finding in this study is the involvement of AT2 in AT1 expression. Cross-talk between AngII receptors have been previously reported at both expression and functional levels (De Paolis et al., 1999; Saavedra, 1999; Miura et al., 2010). These reports consistently demonstrated the involvement of AT1 in AT2 expression (De Paolis et al., 1999). In addition, it has been shown that AT2 mediated signaling antagonizes some aspects of AT1 mediated effects (Miura et al., 2010). Our results demonstrate for the first time that AT1 expression in hCMECs is positively regulated by AT2 mediated signaling.

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In this study all efforts were made to confirm each result using different methods but the following limitations can be identified; the in vitro studies were conducted in an endothelial cell line rather than primary endothelial cells. In addition, BDNF neutralization studies were performed using mainly pharmacologic methods although a genetic approach using RNA interference would provide more power to the study. In addition, in our study we have used human derived cell line in our in vitro study while using a murine model as an in vivo model. In our research the main goal and focus is to model and understand the changes that accompany ischemic stroke in humans. Because of the inability to directly probe human brain samples, we are using rats to study the in vivo changes in response to candesartan or any pharmacologic agent of interest. In addition whenever we had the chance to use human derived tissues or cells we do use them to give a better understanding of what changes are taking place. In this investigation we employed human cerebromicrovascular endothelial cells as an in vitro system and we used SHRs as an in vivo system.

In conclusion, our findings demonstrate the ability of candesartan to modulate the behavior of endothelial cells to promote a proangiogenic state. In hCMECs, this modulatory effect of candesartan can be largely attributed to BDNF and is mediated through the AT2 receptor. In addition, the dose dependent proangiogenic effect of candesartan and even the mechanism involved may help explain the disparate findings on the angiogenic potential of ARBs that prevails in the biomedical literature.

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Authorship contributions:

Participated in research design: Alhusban, Ergul, and Fagan.

Conducted experiments: Alhusban, and Kozak.

Performed data analysis: Alhusban, Ergul, and Fagan.

Wrote or contributed to the writing of the manuscript: Alhusban, Kozak, Ergul, and Fagan.

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Footnotes:

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Figure legends:

Figure 1: Hypertension and AT1 blockade affects the expression of BDNF. Blood pressure transmitters were implanted intraperitoneally in SHR. Animals had sham surgery and received a single dose of candesartan (0.3mg/kg) and the mean arterial blood pressure was monitored (A). Arrow indicates time of candesartan administration; n=6. SHR underwent sham surgery and randomized to receive either candesartan (0.3mg/kg) or saline intravenously (n=6 per group). 24 hours later the animals were sacrificed and the brains were extracted and. Right and left hemispheres were separated and processed for immunoblotting (B).

Figure 2: Angiotensin II and AT1 blockade affects the expression of BDNF and the angiogenic potential in hCMECs. hCMECs were cultured to confluence followed by serum starvation for 16 hours. Cells were treated with a concentration range of Angiotensin II (AngII) for different time periods. The expression of BDNF was assessed using immunoblotting (A) n=3-5. hCMECs were treated with AngII (1fM or 1μM) for 6 hours followed by treatment with candesartan (0.16μM) for 10 hours and the expression of BDNF was assessed (B) n=3. The ability of candesartan to modulate the angiogenic response of hCMECs was evaluated using in vitro matrigel tube formation assay (C) and wound recovery assay (D) in response to treatment with a concentration range of candesartan (0-1.6μM) n=3. For panels A and B, data presented as mean±SEM, * p<0.05. For panels C and D * significantly different from control; \$ significantly different from cand 0.16μM. Overall p=0.0014, F=8.19 (C) and p<0.0001; F=22.44 (D).

Figure 3: Angiotensin II modulates the proliferation of hCMECs. Proliferative response of HCMECs was evaluated using BrdU incorporation assay. hCMECs were treated with AngII (1fM or 1μM) for 6 hours followed by candesartan (0.16μM) alone or in combination with other treatments. The plates were then processed according to the manufacturer recommendations. Data are presented as mean±SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, \$ significantly different from AngII+cand in the same group; overall p=0.005, F=8.85 for AngII 10⁻⁹ and p<0.0001, F=34.97 for AngII 1μM

Figure 4: Angiotensin II modulates the migration of hCMECs. Insert; representative image of control, AngII 1μM and AngII+candesartan showing candesartan induced migration of hCMECs. hCMECs were cultured to confluence followed by 10 hours serum starvation. Cells were treated with either AngII 1fM or 1μM for 6 hours and then a scratch was introduced in the monolayer. Cells were then incubated with AngII 1fM or 1μM alone or with candesartan (A and D). The involvement of BDNF was assessed using a number of inhibitors for BDNF functions (B and E) which were added to the media 30 minutes before AngII treatment. Data are presented as mean±SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, \$ significantly different from AngII+cand in the same group; overall p<0.0001, F=16.08 for AngII 1fM and p<0.0001, F=22.08 for AngII 1μM.

Figure 5: Angiotensin II modulates the angiogenic potential of hCMECs. Insert; representative images of in vitro tube formation (arrows) showing reduced tube

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formation rate in AngII 1 μ M treated hCMECs and the reversal of AngII 1 μ M antiangiogenic effect by candesartan.

hCMECS (2 \times 10⁴ cells/well) were suspended in a 30:60 solution of matrigel and serum free media. Angiogenic response of hCMECs to AngII 1 fM (A) and 1 μ M (D) in the presence and absence of candesartan was evaluated 24 hours after treatment. The involvement of BDNF was assessed through using K252a (Trk inhibitor) or TrkB-Fc (soluble chimeric receptor) (B and E). Data are presented as mean \pm SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, \$ significantly different from AngII+cand in the same group; overall p<0.0001, F=12.74 for AngII 1 fM and p<0.0001, F=9.54 for AngII 1 μ M.

Figure 6: AT2 receptor mediates the angiogenic response in hCMECs. The involvement of AT2 receptor in the angiogenic response to AngII 1 fM alone or in combination with candesartan (A) and to the combination of AngII 1 μ M and candesartan (B) was assessed using the AT2 antagonist PD-123319 (0.1 μ M). The angiogenic response was evaluated using matrigel tube formation assay as described in the methods section. Data are presented as mean \pm SEM of 3 different experiments each in triplicate. * Significantly different from control, # significantly different from AngII in the same group, \$ significantly different from AngII+cand in the same group; overall p<0.0001, F=8.779 for both AngII 1 fM and AngII 1 μ M.

Figure 7: Candesartan induced BDNF expression is mediated through AT2 receptor. To evaluate the involvement of AT2 receptor in candesartan induced BDNF

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expression hCMECs were pretreated with PD-123319 or vehicle followed by AngII (1fM or 1μM) in the presence or absence of candesartan (0.16μM) (Figure 7A). To further confirm the role of AT2 hCMECs were treated with CGP-42112A (0.1μM) or vehicle for 16 hours (Figure 7B). * Significantly different from control, # significantly different from AngII in the same group, \$ significantly different from AngII+cand in the same group; overall $p=0.02$, $F=3.086$. $n=4-6$.

Figure 8: AT1 antagonism affects the expression of AT1 receptor in an AT2 receptor mediated manner. To assess the expression of AT1 (A and B) and AT2 (Cand D) receptors in response to the different treatments used. Cells were incubated with PD-123319 (0.1uM) or vehicle for 30 minutes followed by 6 hours of AngII 1fM (A and C) or 1μM (B and D). After 6 hours of AngII treatment cells were co-incubated with candesartan or vehicle for 10 hours. Receptor expression was assessed using immunoblotting. Data presented as mean \pm SEM; $n=3-5$. * Significantly different from control, # significantly different from AngII in the same group, \$ significantly different from AngII+cand in the same group; overall $p<0.0042$, $F=8.742$ for AngII 1μM.

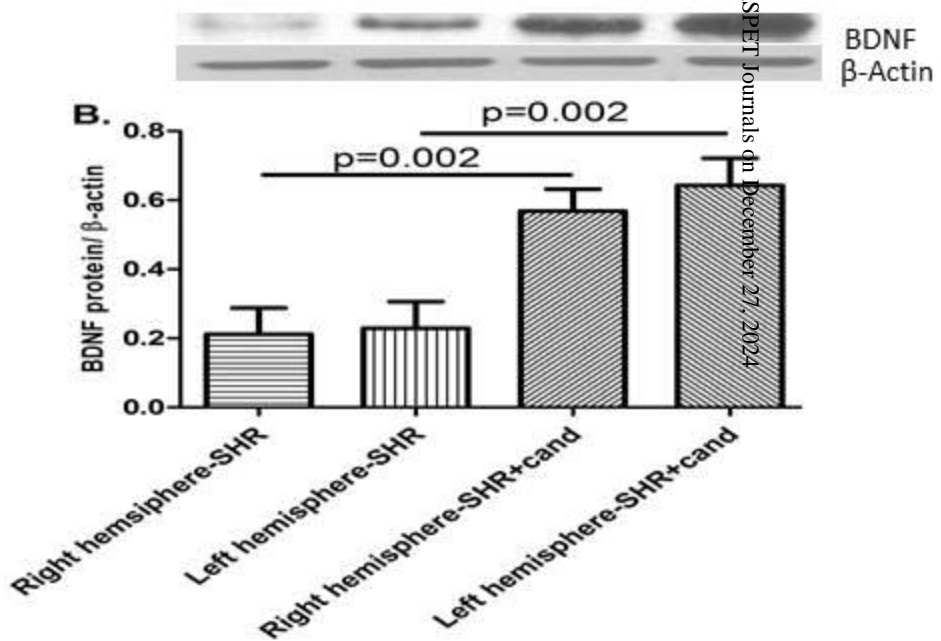
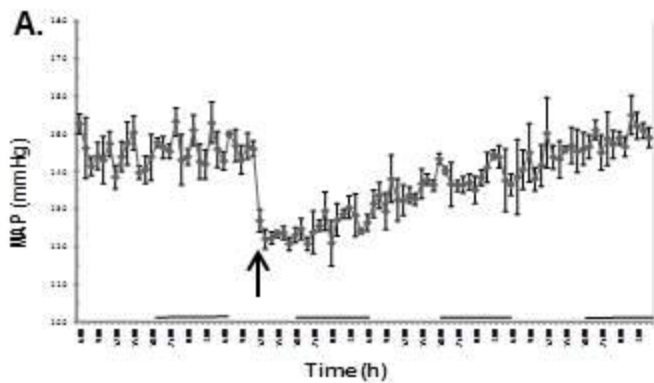
Figure 9: AT1 antagonism modulates the phosphorylation of GSK-3β in an AT2 receptor mediated manner. To assess the phosphorylation of GSK-3β at the inhibitory serine 9 residue, hCMECs were treated as described for AngII receptors expression evaluation. Panel (A) represents the response to AngII 1fM while (B) represents AngII 1μM response. Data presented as mean \pm SEM; $n=3-5$. * Significantly different from

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control, # significantly different from AngII in the same group, \$ significantly different from AngII+cand in the same group; overall $p=0.0038$, $F=6.94$ for AngII 1 μ M.

Figure 10: A schematic representation of the results. By blocking AT1 receptors ARBs induce an unopposed stimulation of AT2 receptors. AT2 stimulation induces the expression of BDNF which will bind to its TrkB receptor to promote a proangiogenic state in hCMECs.

Figure 1



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Figure 2

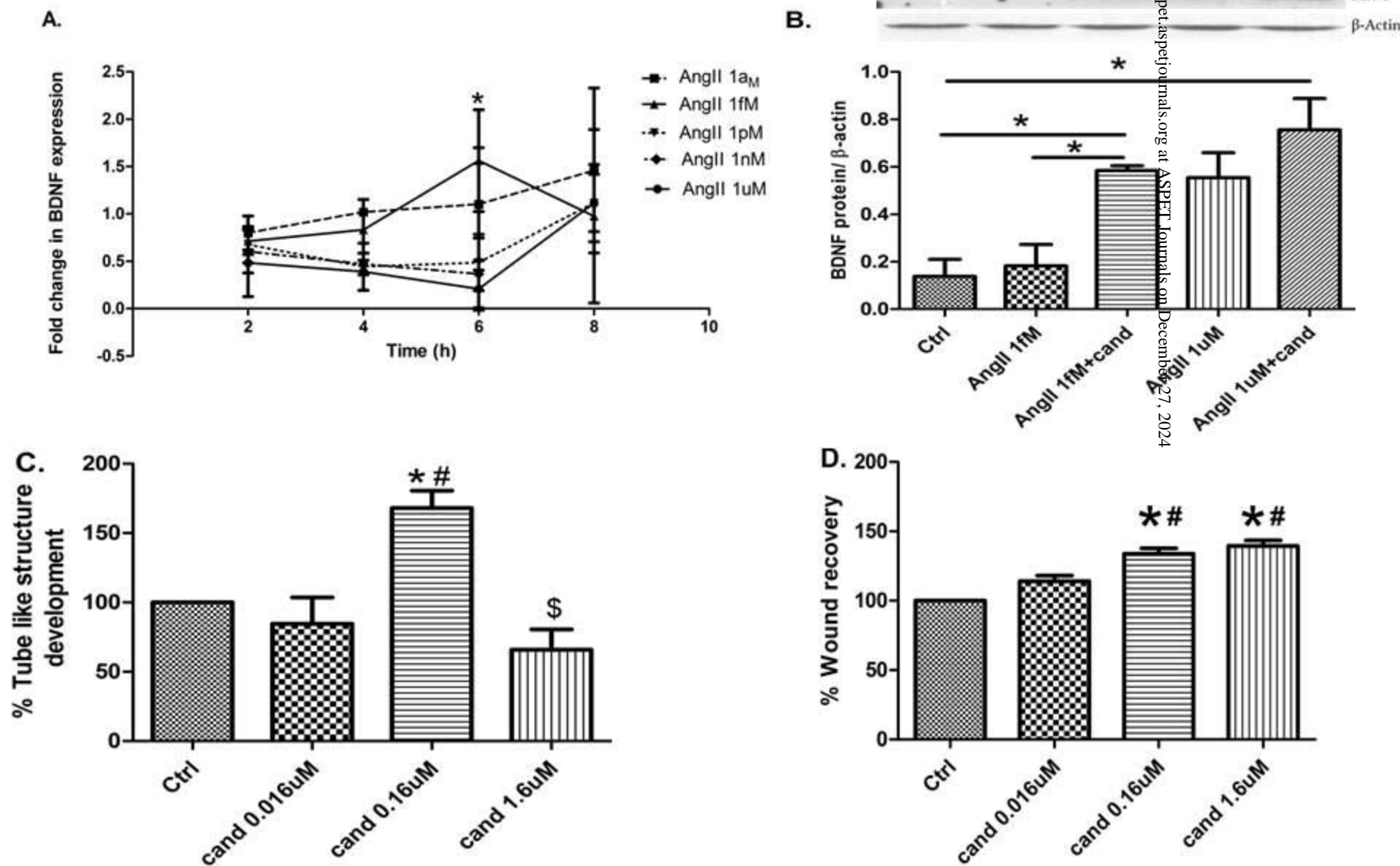
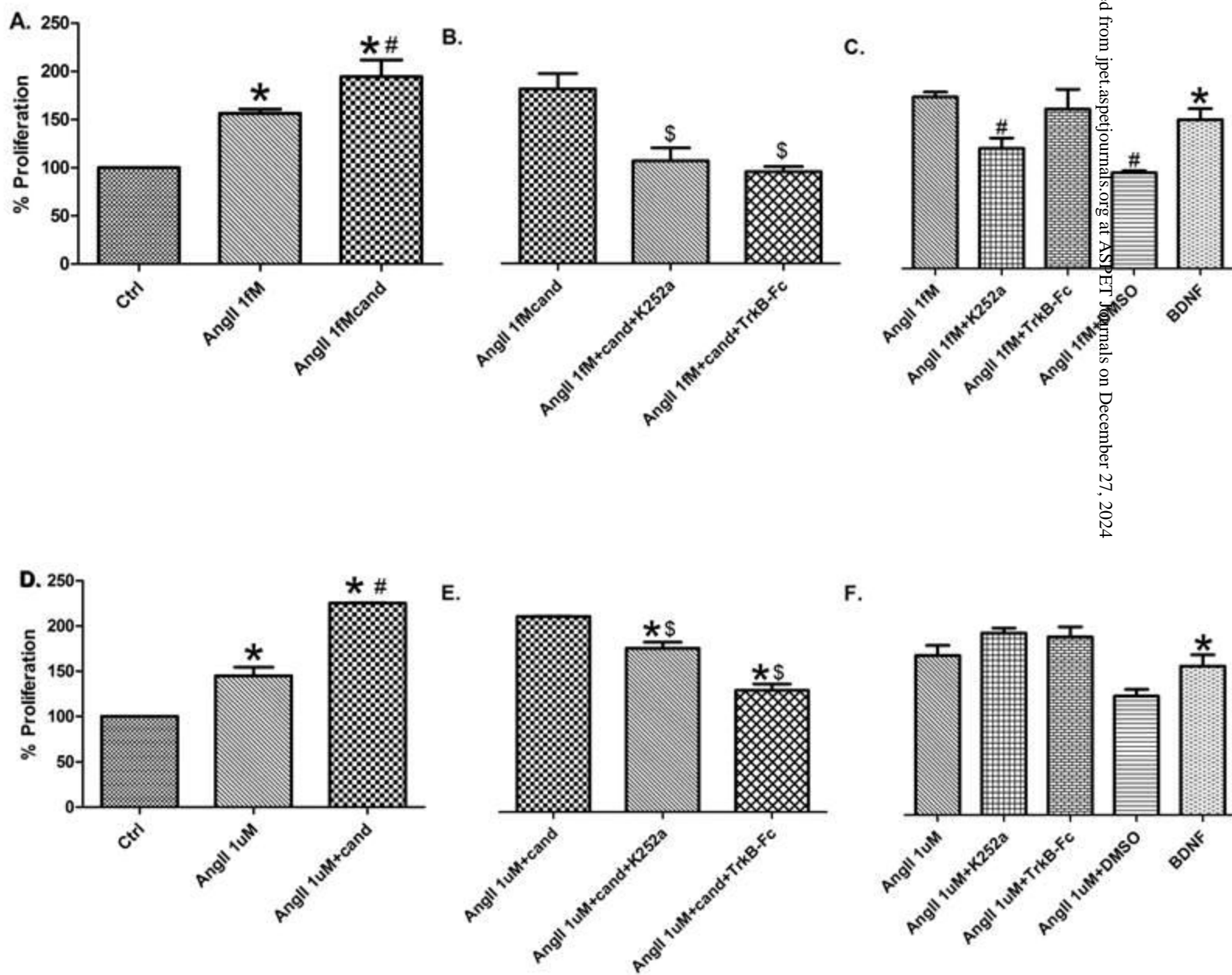
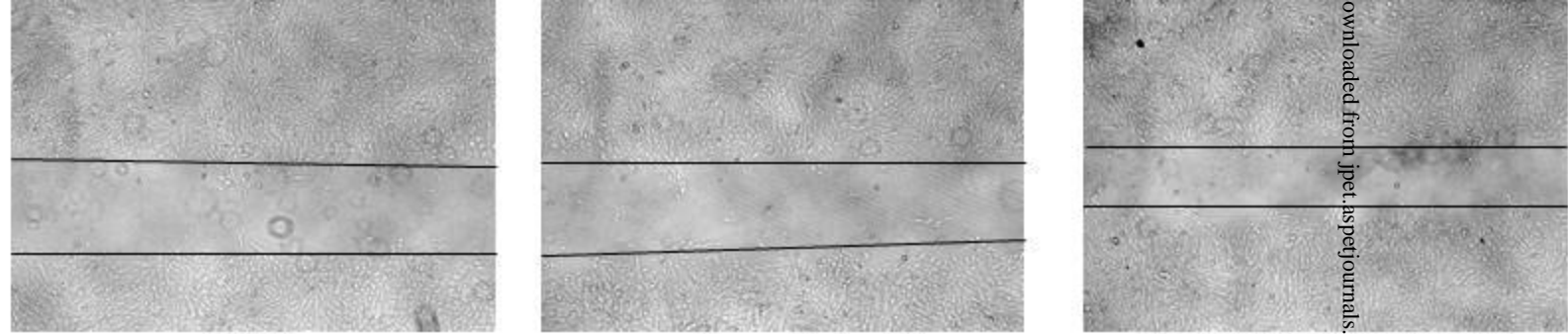


Figure 3



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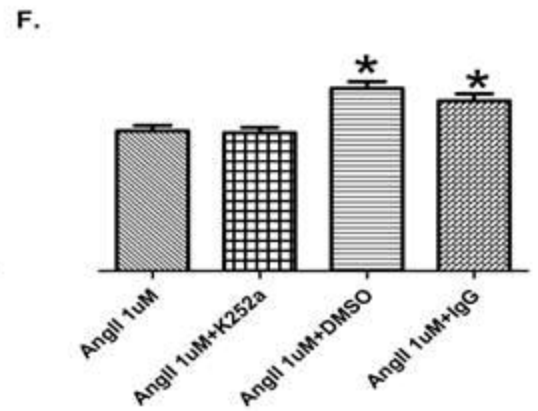
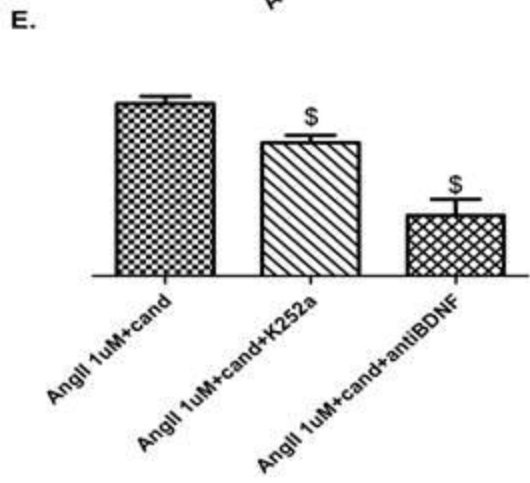
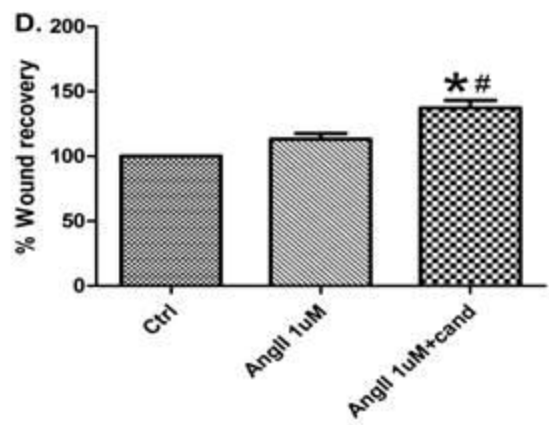
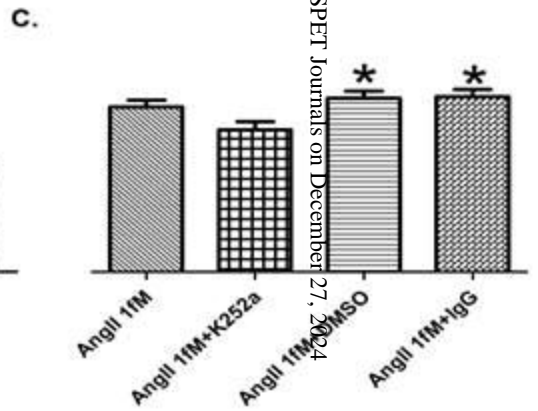
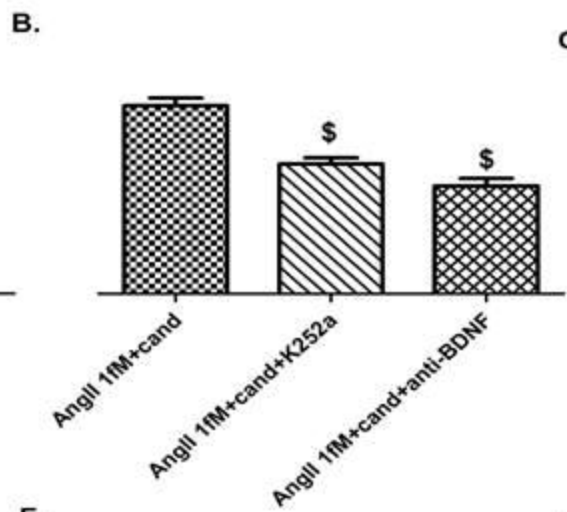
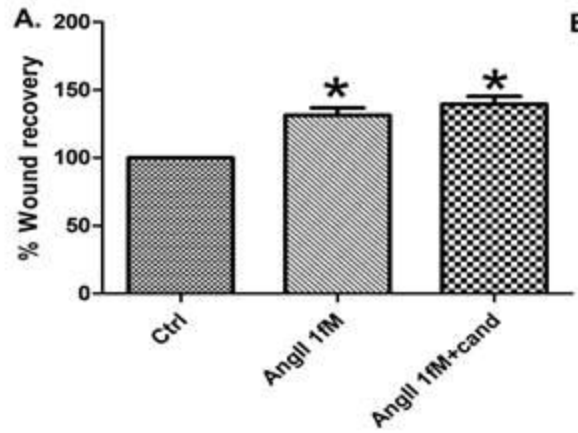
Figure 4



Ctrl

AngII 1μM

AngII 1μM +cand



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Figure 5

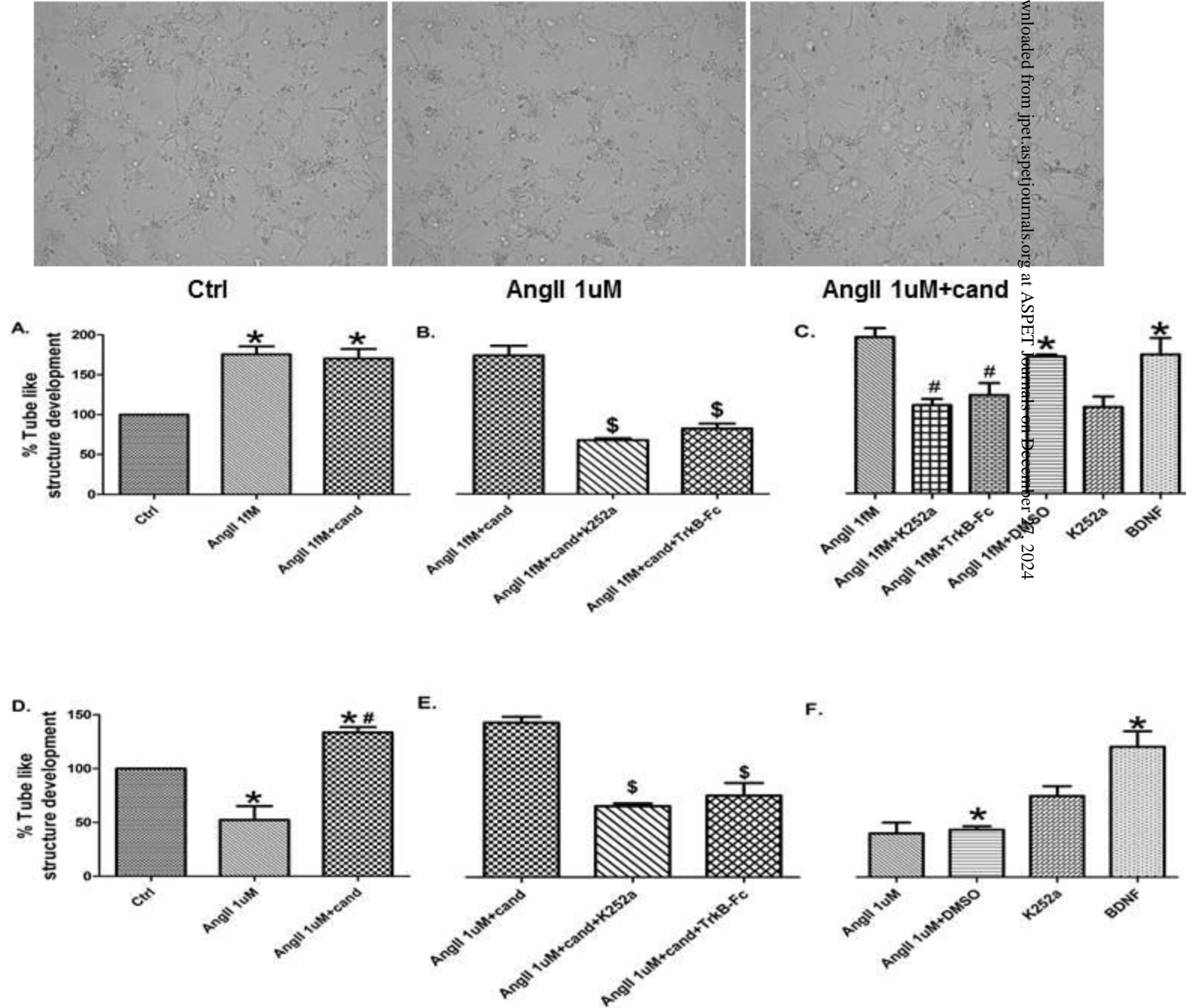


Figure 6

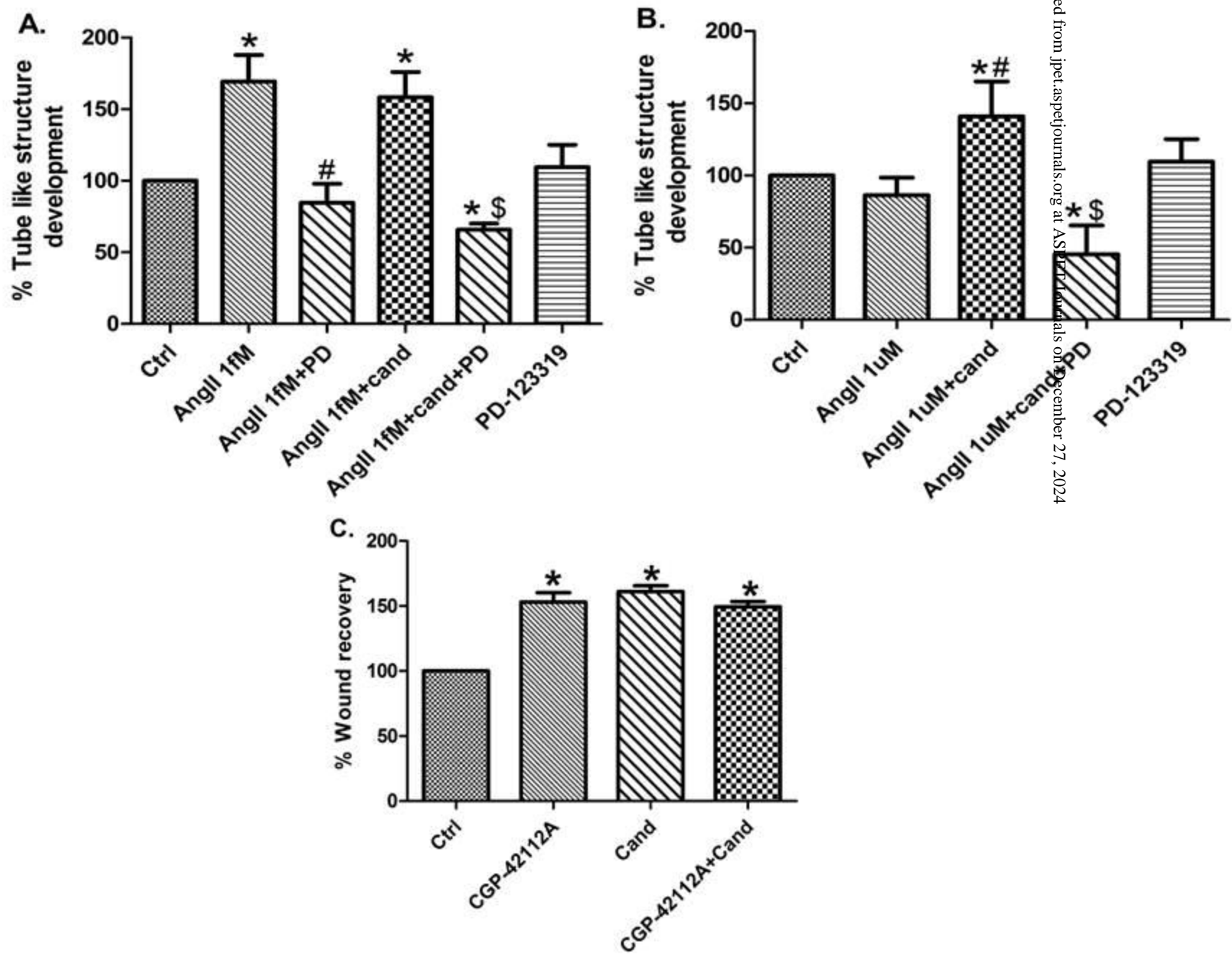


Figure 7

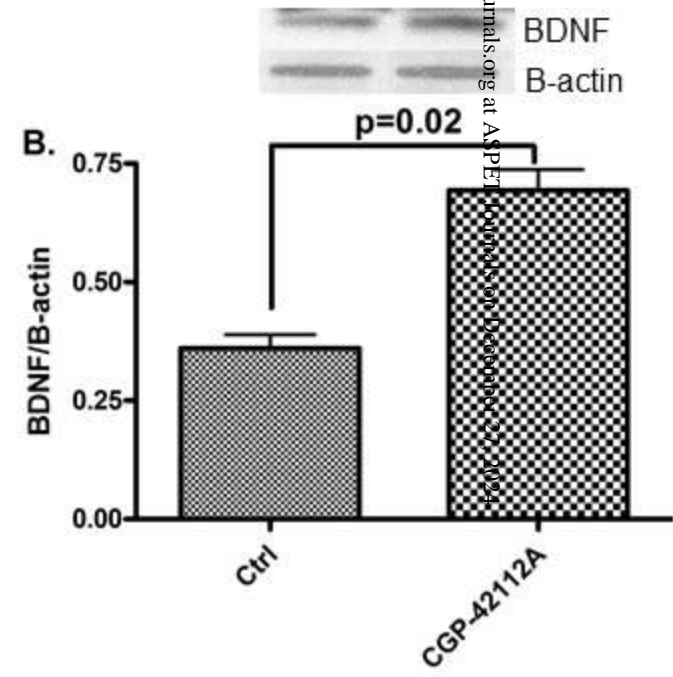
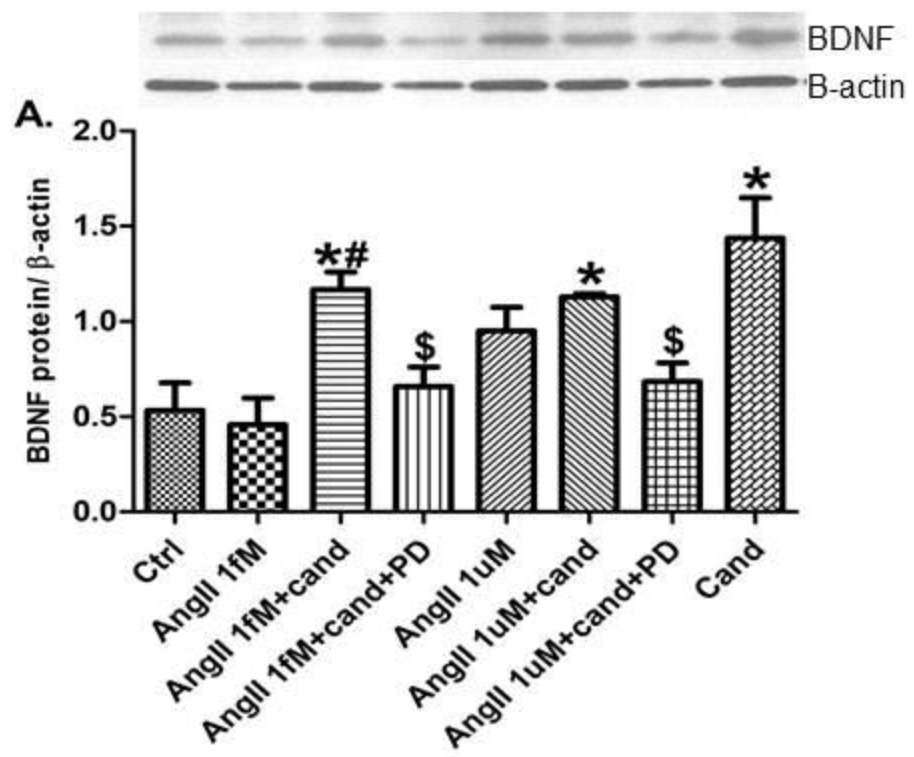


Figure 8

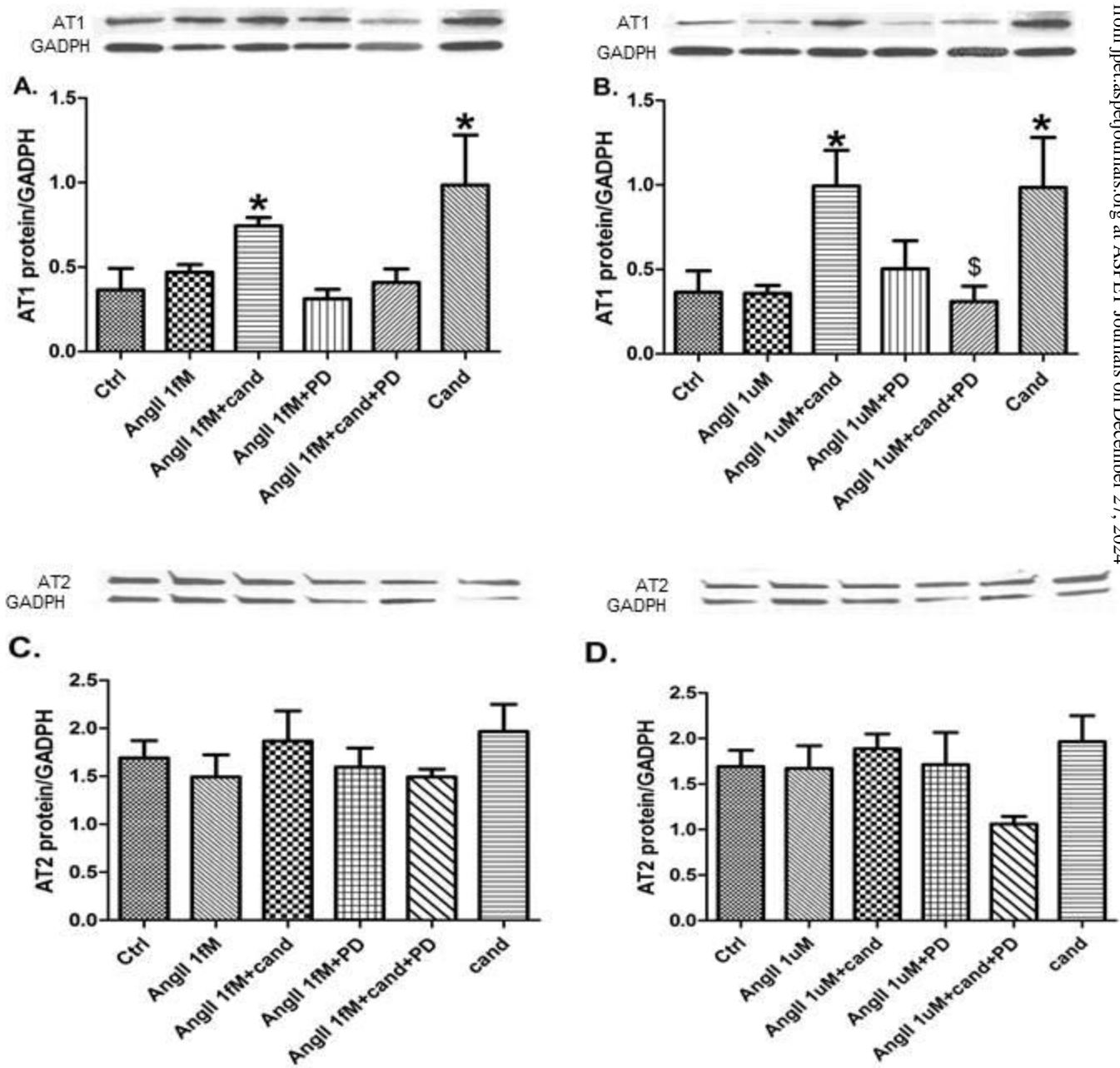


Figure 9

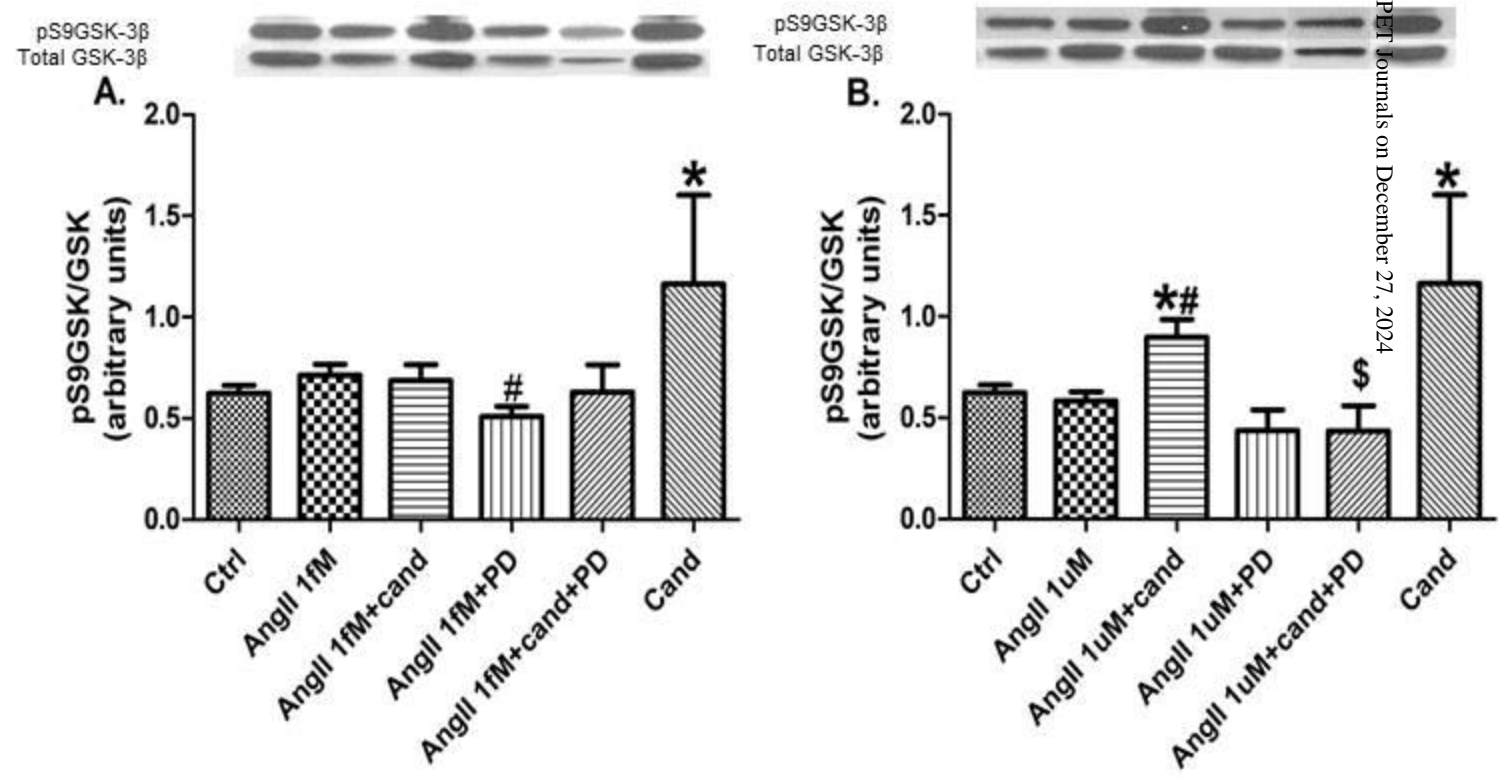


Figure 10

