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**Keywords:** Angiotensin II, Angiotensin receptor antagonists, Blood-brain barrier, Brain microvessel endothelial cells, losartan, PD 123,319

**Abstract:** The endothelial lining of the blood-brain barrier tightly controls the distribution of peptide hormones between the central nervous system and the circulation. Using primary cultures of brain microvessel endothelial cells, an *in vitro* model of the blood-brain barrier, we report here the uptake and transport of the octapeptide angiotensin II by a specific receptor population. Using the angiotensin II antagonists losartan ($AT_1$ specific) and PD 123,319 ($AT_2$ specific) we have shown that both the uptake and transport of angiotensin II were mediated by the $AT_1$ receptor. Western blot analysis confirmed the existence of the $AT_1$ receptor in our cell culture model. Rhodamine 123 studies also suggested that both angiotensin II antagonists, but not angiotensin II, were substrates for the P-glycoprotein efflux system thus restricting the transport of these compounds. These results suggest an $AT_1$ receptor mediates uptake and transport of angiotensin II at the blood-brain barrier and may contribute to the regulation of cerebrovascular levels of the peptide.

**Text of paper:**

# $AT_1$ receptors mediate angiotensin II uptake and transport by bovine brain microvessel endothelial cells in primary culture

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**running title:** $AT_1$ mediation of Ang II processing by BMECs

## Introduction

The octapeptide angiotensin II produces a number of physiological actions, the most common of these being its peripheral role as a potent vasoconstrictor (1). The renin angiotensin system is also known to exist in the brain (1-3). Brain angiotensin II appears to function in the central

regulation of blood pressure, the regulation of body fluid volume, and the control of hormone release (4). Angiotensin II does not readily penetrate the blood-brain barrier (BBB) and the effects of angiotensin II are generally considered to be mediated by receptors in the circumventricular regions of the brain which lack the BBB.

Speth and Harik (5) and Grammas et al. (6) observed that there were angiotensin II receptor binding sites on the brain microvessel endothelial cells (BMECs) lining the BBB and that these binding sites have biological activity (7,8). Subsequently, studies by Guillot and Audus reported that there were specific angiotensin II binding sites on BMECs in primary culture and that the cells internalized the peptide (9). Angiotensin II receptors are also found on peripheral vascular endothelial cells and mediate transendothelial transport of the peptide (10). The subtyping of these binding sites, however, has not been reported.

There are two major subtypes of angiotensin II receptors known to exist, designated $AT_1$ and $AT_2$. Both receptor subtypes have been found in brain tissue of rats, monkeys, and humans. Most of the known actions of angiotensin II are mediated through the $AT_1$ receptor (11). The development of nonpeptide antagonists has made it possible to distinguish between the two receptor subtypes. Losartan is a highly specific nonpeptide $AT_1$ antagonist and PD 123,319 is a highly specific nonpeptide $AT_2$ antagonist. These antagonists lack the partial agonist activities of peptide angiotensin II analogs, allowing study of angiotensin II processing without interfering side effects (11).

The objective of this study was to use primary cultures of BMECs to reveal the receptor subtype populations that regulate angiotensin II uptake and distribution across the BBB. The

nonpeptide antagonists losartan and PD 123,319 were used in this study to distinguish the $AT_1$ and $AT_2$ receptor subtypes on BMECs. The definition of receptor subtypes providing for the clearance of peptides at the BBB contributes to our knowledge of fundamental physiological mechanisms regulating cerebrovascular levels of vasoeffectors. In addition, characterization of BBB carrier mechanisms potentially can provide physiological strategies to beneficially circumvent this barrier by covalently linking pharmacophores to receptor ligands known to be transported across the barrier (12).

## Materials and methods

### Materials

[$^3$H]-angiotensin II (specific activity 45 Ci/mmol) was purchased from Amersham (Arlington Heights, IL). Losartan was donated by Merck & Co. (Rahway, NJ) and PD 123,319 ditrifluoroacetate was purchased from Research Biochemicals International (Natick, MA). Cyclosporin A was purchased from Sigma Chemical Company (St. Louis, MO). The $AT_1$ (N-10) rabbit polyclonal antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All other compounds used were of the highest commercially available quality.

### Cell Culture

Bovine brain microvessel endothelial cells (BMECs) were isolated from gray matter of cerebral cortices by enzymatic digestion and subsequent centrifugation, and seeded into primary culture as detailed by Audus and Borchardt (13,14). This preparation has been extensively

characterized as reported in the literature (13,15-18). Isolated BMECs were seeded at a density of 50,000 cells/$cm^2$ into 12-well plates and 100 mm tissue culture dishes (Corning Costar Corporation, Cambridge, MA), pretreated with rat-tail collagen and bovine fibronectin (Sigma Chemical Co.) in a culture medium consisting of 45 % minimum essential medium, 45 % F-12 Ham nutrient mixture (Gibco Life Technologies, Grand Island, NY), 10 mM HEPES, pH 7.4, 13 mM sodium bicarbonate, 10 % plasma-derived equine serum, 100 μg/ml heparin, 100 μg/ml streptomycin, 100 μg/ml penicillin G, 50 μg/ml polymyxin B, and 2.5 μg/ml amphotericin B (Sigma Chemical Co.). The cells were cultured at 37°C with 95 % humidity and 5 % $CO_2$. Cells were fed on the third day after seeding and then every two days until confluent monolayers were formed (10-14 days).

**Binding and internalization studies**

The effect of the nonpeptide antagonists on [$^3$H]-angiotensin II binding and internalization was studied in 12-well plates at 37°C. Aliquots of specified concentrations of losartan and PD 123,319 dissolved in culture medium, containing equine serum, were added to each well with a pulse of [$^3$H]-angiotensin II. The plates were incubated for 30 minutes at 37°C. At the conclusion of the experiment, the medium was aspirated from each well and the wells were rinsed three times with ice cold culture medium. The cell monolayers were then solubilized by the addition of 1.0 ml of 0.2 N NaOH / 0.5 % Triton X-100. An aliquot of 100 μl was removed to analyze protein content using the BCA protein assay kit (Pierce, Rockford, IL). The remaining lysate from each well was collected, placed in a scintillation vial with 10 ml of scintillation cocktail, and assayed with a Beckman LS 6800 scintillation counter.

Rhodamine 123 uptake studies were also performed on confluent monolayers in 12-well plates. Angiotensin II, losartan, PD 123,319, or cyclosporin A dissolved in culture medium was added to each well and the plate was preincubated for one hour at 37°C. After the one hour preincubation, rhodamine 123 was added to each well for a final concentration of 5 μM, and the plate was incubated at 37°C for an additional hour. After the second incubation, the plate was rinsed and the monolayers were solubilized as described above. The contents of each well were collected into cuvettes and assayed by fluorescence using an SLM-Aminco 4800 fluorometer (ex = 500 nm, em = 550 nm). After measuring relative fluorescence, 100 μl was removed from each cuvette and assayed for protein content with the BCA protein assay kit (Pierce).

**Transendothelial permeability studies**

Polycarbonate membranes (pore size 0.4 μm) were placed into 100 mm tissue culture dishes and coated with rat-tail collagen and bovine fibronectin. BMECs were grown to confluent monolayers as determined by inspection of the areas around the membranes using an inverted microscope. The basolateral side of the cells was defined as the side facing the collagen matrix. Once confluent monolayers were formed, the membranes were carefully placed into a horizontal Side-bi-Side™ diffusion apparatus (Crown Glass, Inc., Somerville, NJ) for transendothelial permeability studies. The area of the diffusion membrane was 0.636 $cm^2$. The donor and receiver chambers were filled with 3.0 ml culture medium, containing equine serum, and the temperature was maintained at 37°C with an external circulating water bath. The contents of each chamber were stirred with Teflon coated magnetic stir bars at a speed of 600

r/min driven by an external drive console (Crown Glass, Inc.). The apical to basolateral transport of a pulse of [$^3$H]-angiotensin II was studied for 30 minutes. An aliquot of 25 μl was removed from the donor chamber to be assayed for initial donor concentration. Sample aliquots of 100 μl were removed from the receiver chamber at various intervals. Following the 30 minute receiver sampling time, different concentrations of losartan or PD 123,319 were added to the donor chamber. Receiver samples were then taken for an additional 60 minutes. After each receiver sample, the volume was replaced with fresh medium. The samples were placed in a scintillation vial with 10 ml of scintillation cocktail and assayed by scintillation spectrometry. The flux was determined by plotting pmoles vs. minutes for each sampling interval (0-30 minutes and 30-90 minutes). The apparent permeability coefficient was calculated using the equation:

$$P = Flux / (A*C_{Do})$$

where flux is the slope of each line, A is the area of the membrane, and $C_{Do}$ is the initial donor concentration. The donor concentration did not change by more than 10 % during the time period of these experiments.

**Western blot**

Total cellular extract was prepared using confluent monolayers of BMECs. The cells were lysed with a 45 min incubation in ice-cold phosphate buffer saline containing 3 % sodium dodecyl sulfate (SDS) and protease inhibitors (0.1mM leupeptin, 73 μM pepstatin A, and 100 μg/ml PMSF). The cell lysate was then centrifuged at 12,000 g for 15 min and the supernatant containing solubilized membrane proteins was stored at -20°C and used for further analysis.

Total cell protein was measured by the Micro BCA protein assay (Pierce) using bovine serum albumin as a standard. Cell plasma membranes were prepared using confluent monolayers of BMECs. The cells were removed from the tissue culture plates with a trypsin / EDTA solution. The cell suspension was then diluted in 5 volumes of ice-cold 10 mM Tris - HCl, 0.1 mM EDTA, pH 7.5 (TE buffer) and homogenized. The homogenate was passed through a fine-gauge syringe needle and then centrifuged at 500 g for 15 minutes to pellet unbroken cells and nuclei, leaving a membrane containing supernatant. The plasma membranes were collected by centrifugation at 40,000 g for 60 minutes. The pellets were washed in 10 volumes of TE buffer and re-centrifuged at 40,000 g for 60 minutes. The pellets were resuspended in TE buffer and stored at -70°C (19). The protein concentration was measured by the Micro BCA protein assay (Pierce) using bovine serum albumin as a standard. Cell proteins were electrophoresed on a 10 % Tris-Glycine gel (NOVEX, San Diego, CA) and transferred to a PVDF membrane. Immunoreactive protein was detected with the $AT_1$ polyclonal antibody using the enhanced chemiluminescence method (ECL) as per the manufacturer's protocol (Amersham, Downers Grove, IL). A positive control peptide (Santa Cruz Biotechnology, Inc.) and a negative control (serum albumin) were used to establish and confirm blot conditions.

**Statistical analysis**

The comparison between mean values within each experimental series was performed by one-way analysis of variance using Dunnett's test to compare several treatments against a control.

**Results**

The temperature and concentration dependence of the binding and internalization kinetics for angiotensin II were described in an earlier report (9). In this study, the uptake of [$^3$H]-angiotensin II alone and with different concentrations of losartan is shown in Fig. 1. When coincubated for 30 minutes at 37°C, micromolar concentrations of losartan were observed to significantly inhibit the binding and uptake of [$^3$H]-angiotensin II. In Fig. 2 the effect of PD 123,319 ditrifluoroacetate on angiotensin II uptake is shown. When different concentrations of PD 123,319 were coincubated with [$^3$H]-angiotensin II for 30 minutes, there was no significant change in the amount of angiotensin II taken up by the cells.

Since the concentrations of losartan required to inhibit angiotensin II binding and internalization were higher than expected and that earlier studies suggested a role for P-glycoprotein in mediating losartan distribution across the BBB (20), we performed rhodamine 123 uptake studies. The uptake of rhodamine 123 alone and with angiotensin II, losartan, PD 123,319, and cyclosporin A is shown in Fig. 3. The cells were exposed for one hour with 100 μM angiotensin II, 750 μM losartan, 500 μM PD 123,319, or 1 μg/ml cyclosporin A. After the one hour incubation, 5 μM rhodamine 123 was added to each well and the cells were incubated another hour. As shown in Fig. 3, losartan, PD 123,319, and cyclosporin A significantly increased the uptake of rhodamine 123 in BMECs, indicating that only the nonpeptide antagonists interact with P-glycoprotein.

The permeation of angiotensin II across BMECs was found to increase linearly over time as shown in Fig. 4 at 37°C. The transport of a pulse of [$^3$H]-angiotensin II was monitored alone for 30 minutes before addition of the antagonists. The effect of losartan on angiotensin II

transport is shown in Fig. 5 where again, micromolar concentrations of losartan were found to significantly decrease the transport of [$^3$H]-angiotensin II. There was no decrease in angiotensin II transport seen with PD 123,319 as shown in Fig. 6.

Western blot studies, shown in Fig. 7A and 7B, were generated using an $AT_1$ receptor polyclonal antibody and show that the $AT_1$ receptor is present in BMECs. Fig. 7A shows the western analysis of total cell lysate and Fig. 7B shows the blot of BMEC membranes compared to total cell lysate. The blots indicated that there was a greater concentration of $AT_1$ receptors in the membrane portion of the cells.

**Discussion**

Speth and Harik (5) and Grammas et al. (6) originally described the presence of specific angiotensin II binding sites on brain microvessels. A hypothesis developed that angiotensin binding sites in the cerebrovasculature regulated water and ion transport (7,8). Guillot and Audus subsequently described angiotensin II binding sites on primary cultures of bovine BMECs, a well-characterized *in vitro* system representing BBB endothelium. Scatchard analysis of the concentration-dependent binding of angiotensin II to BMECs revealed a single population of binding sites with an apparent $K_d$ of 3.1 nM (9). In addition, angiotensin II was observed to be internalized intact by BMECs and apical application of the peptide regulated permeability properties of the BMEC monolayers at concentrations similar to the binding site affinity (9,21). The latter findings have been recently confirmed by other researchers in human brain microvessel endothelial cells (22).

In this study, we have shown that the binding and uptake of angiotensin II by BMECs was inhibited by losartan, the $AT_1$ receptor antagonist and not the $AT_2$ antagonist, PD 123,319. This observation was consistent with the presence of a single population of angiotensin II binding sites that were described by Guillot and Audus (9). Therefore, it appears that an $AT_1$ receptor mediates clearance of angiotensin II by BMECs. Although our data includes both binding and internalization, the concentrations at which an antagonist was inhibitory were higher than expected based on other receptor studies (11) and our observation that specific binding of angiotensin II to bovine BMECs could be inhibited by nM concentrations of peptide analogs, saralasin and sarathrin (9).

Our earlier studies on the uptake and transport of losartan itself indicated that the compound was an apparent substrate for P-glycoprotein at submicromolar concentrations (20). Therefore, P-glycoprotein may be a competing mechanism for losartan interactions with the BMEC membrane. To determine if the antagonists and angiotensin II were potential substrates for P-glycoprotein, the uptake studies were performed with rhodamine 123, a specific substrate for the P-glycoprotein efflux pump (23-26). The uptake of rhodamine 123 alone and with pretreatment of the monolayers using angiotensin II, losartan, PD 123,319, or a positive control, cyclosporin A (26), suggested that both antagonists were inhibitors of P-glycoprotein. Angiotensin II does not affect rhodamine 123 uptake, therefore it appears that the nonpeptide antagonists may not be as effective in blocking BMEC uptake and binding due to their added affinity for P-glycoprotein. On the other hand, the micromolar concentrations used here remain within the concentration range of losartan antagonist activity across a variety of different species, tissue types, and angiotensin II mediated biological functions (27).

Angiotensin II was previously shown to be transported across arterial endothelial cell monolayers by a specific, saturable mechanism that could be inhibited by unlabeled peptide. Mineo et al. required micromolar concentrations of angiotensin to inhibit arterial endothelial cell transport of the radiolabeled angiotensin II (10). We have observed that angiotensin II crosses monolayers of BMECs intact (28,29) at a rate slightly higher than expected based on its molecular weight. The transport was also a saturable process, $K_m$ 1.7 nM, and angiotensin II transport from the apical to the basolateral side was greater than the reverse direction (28,30). In the present study, the differential effects of the two angiotensin II receptor antagonists on the passage of the peptide through monolayers of BMECs affirmed the role of an $AT_1$ mediated mechanism with losartan inhibiting the transport of angiotensin II. As observed in the uptake studies, the $AT_2$ antagonist effect on angiotensin II transport was also absent. Consistent with the uptake studies, micromolar concentrations of losartan were required to inhibit angiotensin transport.

The $AT_1$ receptor belongs to the class of G-protein-coupled seven-transmembrane receptors and is made up of 359 amino acids with an unmodified molecular weight of 41,000 (11). Using western blot analysis we confirmed the expression of the $AT_1$ receptor in BMECs. Further, we demonstrated that the $AT_1$ receptor resides in the membrane portion of BMECs. The $AT_1$ receptor is responsible for most of the known biological effects of angiotensin II, including those of the central nervous system (11,31). Therefore, expression in the BMECs was consistent with most other angiotensin II mediated functions. At the time of this study, antibodies for the $AT_2$ receptor were not available to potentially rule out expression of this class of receptors in BMECs.

In addition to peripheral endothelia (10), other cell types have been shown to internalize angiotensin II including adrenocortical cells (32) and vascular smooth muscle cells (33,34). Our results suggest that the presence of the $AT_1$ receptor populations mediating BMEC uptake and transport of angiotensin II may serve as a local physiological mechanism for controlling angiotensin II distribution in the cerebrovasculature. This observation appears consistent with the local control of the renin-angiotensin systems associated with specific tissues (1-3). Alternatively, the $AT_1$ receptors might provide a mechanism that could be exploited for facilitating delivery of some substances via chimeric constructs into the central nervous system like other endogenous peptides, insulin and transferrin (35,36). However, like insulin, it is also apparent that angiotensin II has significant biological activity at the BBB, regulating the BMEC secretion of other vasoeffectors (9,21,22,37) and the permeability properties of the cells (7-9) that would preclude its use in chimeric constructs. The nonpeptide antagonists such as losartan could provide alternative targeting ligands in a chimeric construct; unfortunately, losartan appears to be a substrate for BMEC efflux mechanisms (20). Therefore, future works in our laboratory will focus on the transport of alternative ligands for the carrier, including metabolically stable angiotensin II peptide analogs.

In summary, we have shown that the uptake and transport of angiotensin II by BMECs appears to be mediated by the $AT_1$ receptor. These results form a better understanding of the regulation of cerebrovascular levels of angiotensin II by the BBB and may lead to possible delivery strategies for therapeutic chimeric peptides.

## Acknowledgments

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**Fig. 1**

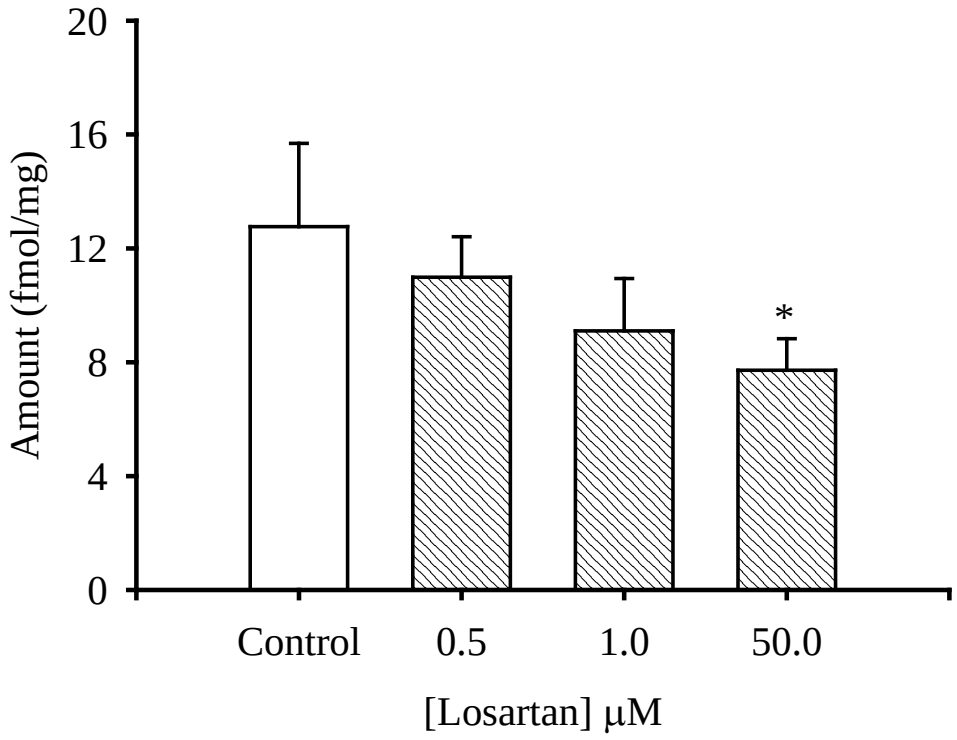

**Fig. 2**

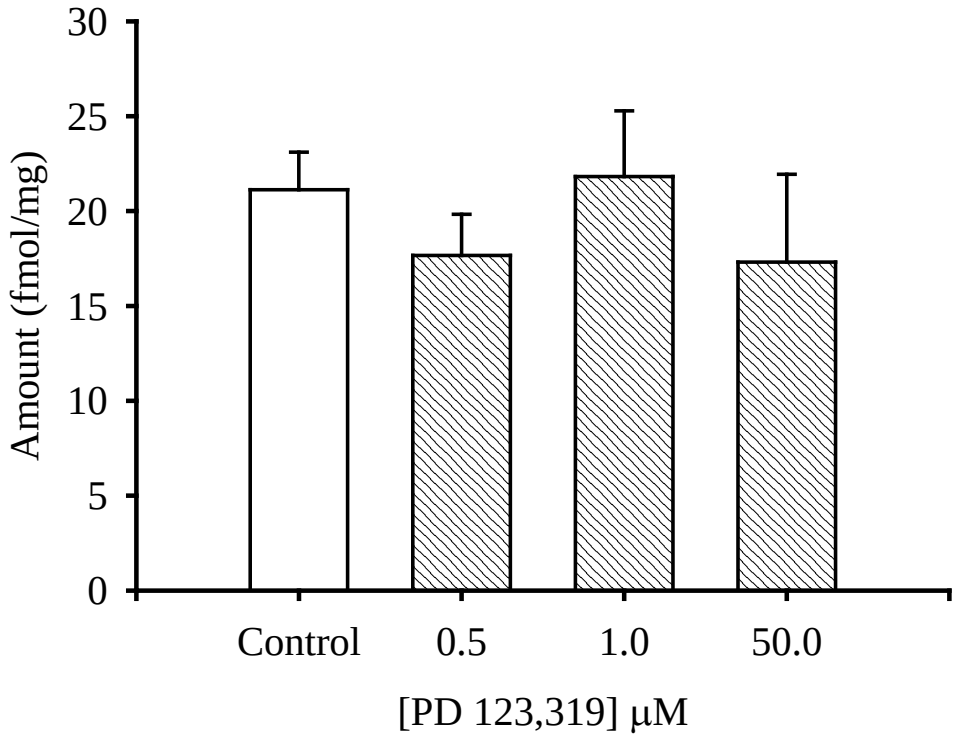


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**Fig. 3**

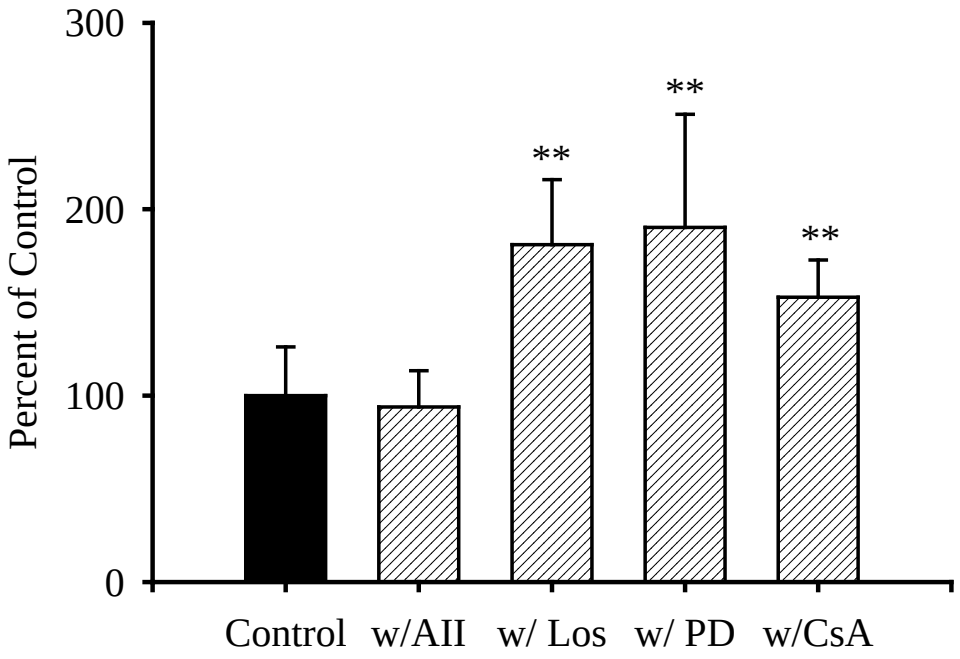


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**Fig. 4**

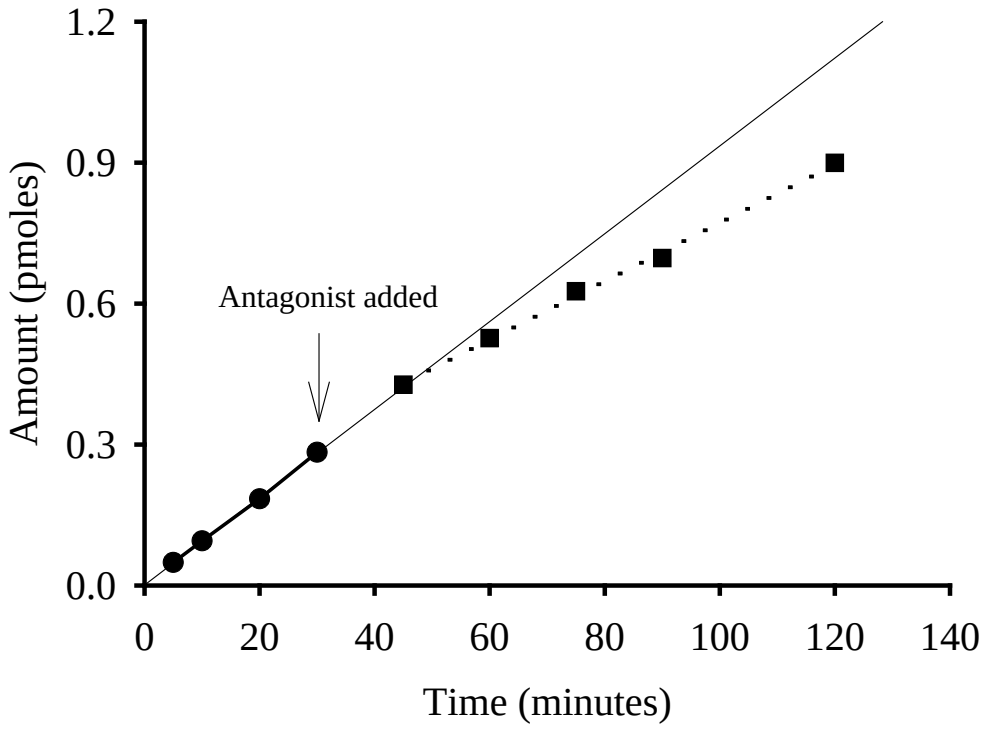


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**Fig. 5**

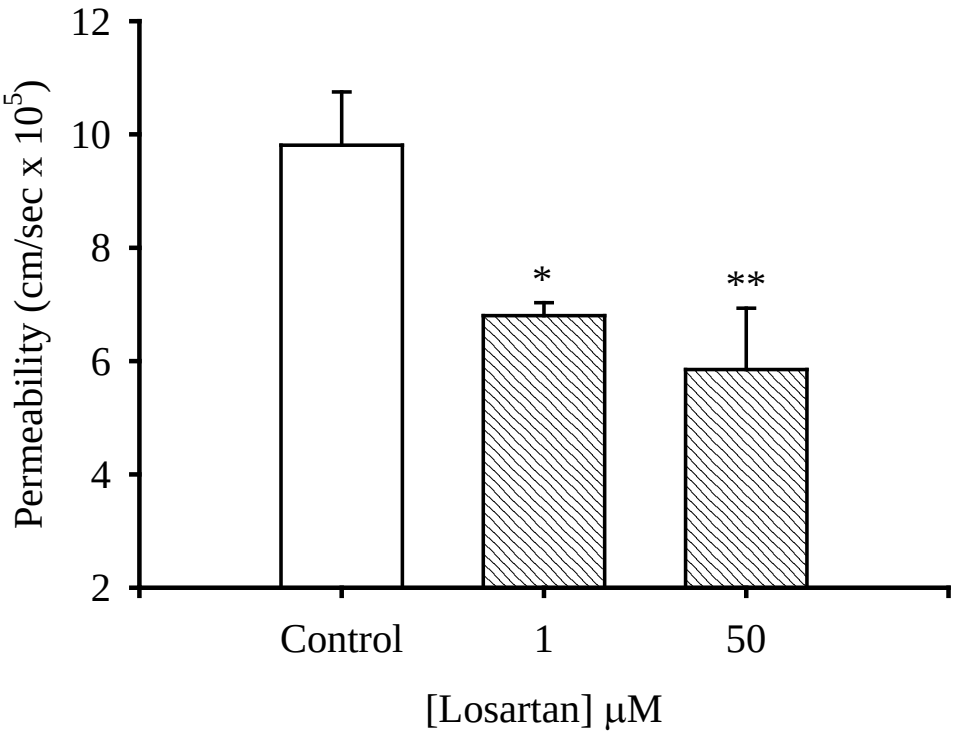


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**Fig. 6**

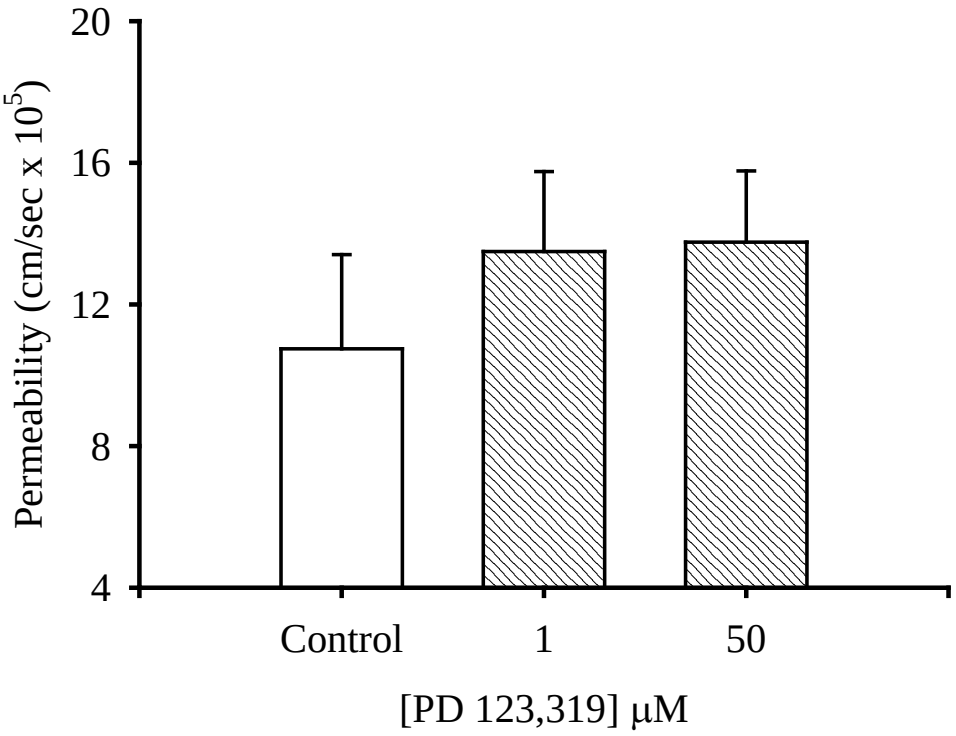


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**Fig. 7A**

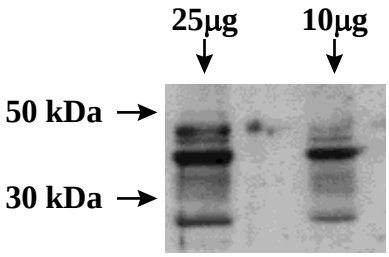


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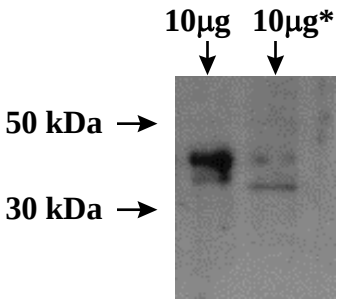
**Fig. 7B**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