



## Internalization of Angiotensin-(1–12) in Adult Retinal Pigment Epithelial-19 Cells

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### Abstract

**Purpose:** Angiotensin-(1–12) [Ang-(1–12)] serves as a primary substrate to generate angiotensin II (Ang II) by angiotensin-converting enzyme and/or chymase suggests it may be an unrecognized source of Ang II-mediated microvascular complication in hypertension-mediated retinopathy. We investigated Ang-(1–12) expression and internalization in adult retinal pigment epithelial-19 (ARPE-19) cultured cells. We performed the internalization of Ang-(1–12) in ARPE-19 cells in the presence of a highly specific monoclonal antibody (mAb) developed against the C-terminal end of the Ang-(1–12) sequence.

**Methods:** All experiments were performed in confluent ARPE-19 cells (passage 28–35). We employed high-performance liquid chromatography to purify radiolabeled, <sup>125</sup>I-Ang-(1–12) and immuno-neutralization with Ang-(1–12) mAb to demonstrate Ang-(1–12)'s internalization in ARPE-19 cells. Internalization was also demonstrated by immunofluorescence (IF) method.

**Results:** These procedures revealed internalization of an intact <sup>125</sup>I-Ang-(1–12) in ARPE-19 cells. A significant reduction (~53%,  $P < 0.0001$ ) in <sup>125</sup>I-Ang-(1–12) internalization was detected in ARPE-19 cells in the presence of the mAb. IF staining experiments further confirms internalization of Ang-(1–12) into the cells from the extracellular culture medium. No endogenous expression was detected in the ARPE-19 cells. An increased intensity of IF staining was detected in cells exposed to 1.0 μM Ang-(1–12) compared with 0.1 μM. Furthermore, we found hydrolysis of Ang-(1–12) into Ang II by ARPE-19 cells' plasma membranes.

**Conclusions:** Intact Ang-(1–12) peptide is internalized from the extracellular spaces in ARPE-19 cells and metabolized into Ang II. The finding that a selective mAb blocks cellular internalization of Ang-(1–12) suggests alternate therapeutic approaches to prevent/reduce the RPE cells Ang II burden.

**Keywords:** retinal pigment epithelial cells, angiotensin-(1–12), angiotensin II, internalization, peptide metabolism, monoclonal antibody therapy

### Introduction

THE DISCOVERY OF angiotensin-(1–12) [Ang-(1–12)] as a new member of the renin–angiotensin system (RAS) led us to explore the pathological role of this upstream angiotensin (Ang) II precursor in retinal vascular disorder associated with primary hypertension. Ang-(1–12) was first

detected in a strain of Japanese Wistar rats' blood and various tissues.<sup>1</sup> Since then, our laboratory is actively engaged in characterizing the role of this novel member of the RAS family in hypertension and vascular disease.

In circulation, primarily angiotensin-converting enzyme (ACE) sequentially cleaves the 2 amino acids from C-terminal end of the Ang-(1–12) peptide to release first

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Ang I and then Ang II; in cardiac tissue chymase mainly cleaves Ang-(1-12) directly into Ang II.<sup>2-8</sup> Recently, we showed Ang-(1-12) as a circulating RAS component in normal and hypertensive subjects.<sup>9</sup> In another study we found a significant increase of Ang-(1-12) levels in plasma of hypertensive patients with systolic blood pressure (SBP)  $\geq 140$  mm Hg compared with the group with SBP  $< 140$  mm Hg. Furthermore, our analysis shows that compared with Ang I levels reported in normal subjects, Ang-(1-12) levels in plasma and urine were severalfold higher in hypertensive patients.<sup>10</sup>

A robust literature shows that RAS is a key player in the regulation of various ocular disorders, including retinal vascular damage. However, the influence of systemic RAS at cellular and molecular levels in retinal pathology is not yet fully understood. The classical RAS including the biologically active Ang peptides, Ang II-forming enzymes (chymase and ACE), and receptors are expressed locally in the human eyes (retina, vitreous body, and aqueous humor).<sup>11-15</sup> Ang II is considered a main instigator of an inflammatory cascade causing retinal neurodegeneration through AT<sub>1</sub>R.<sup>16</sup> Significantly high levels of Ang II have been detected in the vitreous fluids of patients with proliferative diabetic retinopathy (DR) and diabetic macular edema.<sup>17,18</sup> Circulating and cellular Ang II plays an important role in the development of vascular diseases in the eye.<sup>11,19-21</sup>

The human retinal pigment epithelium (RPE) is responsible for the development and maintenance of the photoreceptors in retinas. Adult retinal pigment epithelial-19 (ARPE-19) is a spontaneously immortalized cell line of the human RPE derived from the normal eyes of a 19-year-old male donor who died in a vehicle accident.<sup>22</sup> Because of structural and functional similarities with *in vivo* RPE cells, the ARPE-19 cell line is widely used for *in vitro* eye research. This cell line holds epithelial characteristics after *in vitro* culturing for an extended period, making it beneficial for RPE physiological studies.<sup>22-24</sup> For the first time, we have utilized the ARPE-19 cells to investigate the Ang-(1-12) endogenous expression, cellular internalization, and hydrolysis. Furthermore, we investigated Ang-(1-12) uptake in the presence of a highly specific monoclonal antibody (mAb) developed by us toward the C-terminal end of the Ang-(1-12).

## Methods

ARPE-19 cells (passage 27) were kindly provided by Dr. Goldis Malek lab (Duke University Medical Center, Durham, NC) and all experiments reported in this article were performed from passages 28 to 35. Ang peptides (purity  $\geq 98.5\%$ ) were custom synthesized by GenScript (Piscataway, NJ). Radiolabeled iodine-125 (<sup>125</sup>I) was obtained from PerkinElmer (Waltham, MA). The remaining analytical grade chemicals were purchased from Sigma (St. Louis, MO) and Fisher Scientific (Atlanta, GA). The hybridoma clones producing the mAb directed toward the C-terminus of the human Ang-(1-12) were developed by us in collaboration with GenScript USA, Inc. The hybridoma clones were maintained, purified, and characterized by us, as described previously.<sup>25</sup> Protein L column purified Ang-(1-12) mAb IgG from hybridoma clone# 14B3 is highly specific to detect the C-terminus of the human Ang-(1-12) amino acids and does not cross-react with the angiotensinogen protein and any other closely related Ang peptides.<sup>25</sup>

## Radiolabeling of Ang peptides and high-performance liquid chromatography purification

Ang peptides [human Ang-(1-12), Ang I, and Ang II] radiolabeling with <sup>125</sup>I and high-performance liquid chromatography (HPLC) purifications were done by us as described elsewhere.<sup>10</sup> The purified <sup>125</sup>I-Ang-(1-12) trace (purity  $\geq 99\%$ ) was free of unlabeled Ang-(1-12) and <sup>125</sup>I.<sup>10</sup> The purity of <sup>125</sup>I-Ang-(1-12) peptide was routinely monitored on the HPLC before each experiment at the time it was used for internalization, neutralization, and metabolism studies.

## Radiolabeled Ang-(1-12) internalization and hydrolysis by ARPE-19 cells

In this study, we used ARPE-19 cells (a primary cell line, passage 28) grown in 10% fetal bovine serum (FBS) supplemented Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F12) medium at 37°C in a humidified incubator with an atmosphere of 5% CO<sub>2</sub>. We replaced the culture media with fresh 10% FBS supplemented DMEM/F12 medium every 2-3 days. All experiments were performed between passages 28-35.

For internalization experiments, we grew the ARPE-19 cells (seeding density  $1 \times 10^5$  cells/3.5 cm<sup>2</sup>) in 12-well plates in 10% FBS supplemented DMEM/F12. Internalization of the radiolabeled <sup>125</sup>I-Ang-(1-12) peptide was performed when cells reached confluence as described by us.<sup>26</sup> On the day of internalization study, the 12-well plates were washed 3 times with serum-free media and 1.0 mL of FBS-free media containing RAS and peptidase inhibitors cocktail was added as described in Table 1. After 10 min preincubation

TABLE 1. THE RENIN-ANGIOTENSIN SYSTEM AND PEPTIDASE INHIBITOR COCKTAIL USED TO STOP THE ANGIOTENSIN-(1-12) HYDROLYSIS

<i>Enzyme inhibitor cocktail (final concentration)</i>	<i>Inhibits, enzyme</i>
<b>RAS inhibitors</b>	
Lisinopril (50 $\mu$ M)	ACE
Chymostatin (50 $\mu$ M)	Chymase
SCH39370 (50 $\mu$ M)	Nepirylisin
MLN-4760 (50 $\mu$ M)	ACE2
<b>Peptidase inhibitors</b>	
Amastatin hydrochloride (10 $\mu$ M)	Leucine aminopeptidase
Bestatin hydrochloride (50 $\mu$ M)	Aminopeptidase enzyme
Benzylsuccinic acid (50 $\mu$ M)	Carboxypeptidase A
4-Chloromercuribenzoic acid (250 $\mu$ M)	Cysteine proteases

The inhibitor cocktail contains all these RAS and peptidase inhibitors. This inhibitor cocktail completely stops the Ang-(1-12) peptide hydrolysis and is reported by us previously.<sup>3,26</sup> To demonstrate the ACE and chymase-mediated hydrolytic products formation from the substrate Ang-(1-12), the ACE and chymase inhibitors (lisinopril and chymostatin) were removed from the reaction mixture.

ACE, angiotensin-converting enzyme; Ang, angiotensin; RAS, renin-angiotensin system.

in the inhibitors cocktail media, the radiolabeled  $^{125}\text{I}$ -Ang-(1–12) peptide (1 nM) alone or with human Ang-(1–12) mAb (3.0 mg/mL) were added in each well and placed at 37°C for 4 h in a humidified incubator with an atmosphere of 5%  $\text{CO}_2$ .

The cultured media were collected after 4 h from each well and stored at  $-80^\circ\text{C}$  for HPLC analysis. Finally, each well was washed with 3 mL of ice-cold phosphate-buffered saline (PBS) 3 times and 1 time with 3 mL of ice-cold glycine-HCl (0.05 M, pH 4.0) to remove the free and outer membrane-bound radiolabeled tracers. The thoroughly washed cells were lysed with 1 N NaOH (1.0 mL) overnight on a plate shaker and counted (gamma counter) to detect the internalized radiolabeled  $^{125}\text{I}$ -Ang-(1–12) contents. Internalization of  $^{125}\text{I}$ -Ang-(1–12) by ARPE-19 cells were expressed as counts per min per mg protein.

HPLC analysis was also performed to demonstrate that the intact radiolabeled  $^{125}\text{I}$ -Ang-(1–12) peptide was internalized in ARPE-19 cells. For this, the cells (seeding density  $1 \times 10^6$  cells/60  $\text{cm}^2$ ) were grown in 100 mm culture dishes. The ARPE-19 cells were incubated with radiolabeled  $^{125}\text{I}$ -Ang-(1–12) with or without mAb for 4 h at 37°C in a humidified incubator with an atmosphere of 5%  $\text{CO}_2$ . After aspirating the medium, dishes were washed with 5 mL of ice-cold PBS 3 times and 1 time with 5 mL of 0.05 M ice-cold glycine-HCl (pH 4.0) to remove the free and membrane-bound radiolabeled tracers.

The thoroughly washed cells were harvested in hypotonic buffer (10 mM NaCl, 25 mM HEPES, and 5 mM EDTA, pH 7.4). The harvested cells were lysed using a tight-fitting glass mortar and pestle Dounce homogenizer with 7–10 strokes. The lysed cells were centrifuged (28,000  $g$ ) for 20 min to remove the cell membranes and debris. The pellet [native plasma membranes (nPMs)] and cytosolic cell fractions were counted on a gamma counter. The clear supernatants (pooled cytosolic fractions) were analyzed by HPLC described as follows.

#### *HPLC of ARPE-19 cells culture media and cytosolic fraction*

As described earlier, the collected cytosolic fractions and cultured medium were processed on C18 HPLC column to detect the  $^{125}\text{I}$ -Ang-(1–12) cellular internalization and hydrolytic products generation in culture medium by ARPE-19 cells. In brief, we added an equal volume of 1% phosphoric acid (PA) to the pooled cytosolic fractions and cultured medium, filtered the mixture through a 0.2- $\mu\text{m}$  syringeless filter device and injected ( $\sim 50,000$  cpm) on C18 HPLC column to separate the  $^{125}\text{I}$ -Ang-(1–12) substrate and its metabolic products as previously described by us.<sup>3,5,6,26</sup>

#### *Immunofluorescence imaging of Ang-(1–12) internalization*

Immunofluorescence (IF) technique was also utilized to demonstrate the internalization of Ang-(1–12) by ARPE-19 cells using Ang-(1–12) mAb and anti-mouse Alexa Fluor 488 secondary antibody.<sup>27</sup> For IF study, cells ( $2.5 \times 10^4$  cells/1.7  $\text{cm}^2$  in 1.0 mL DMEM/F12 medium) were seeded in 4-well glass chamber slides (Lab-Tek, Rochester, NY). The cells were washed as described earlier and 1.0 mL of serum-free medium containing a chemical preparation of

RAS and peptidase inhibitors (Table 1) was added to stop the hydrolysis of Ang-(1–12). After 10 min of preincubation, the Ang-(1–12) was added only in third well (0.1  $\mu\text{M}$ ) and fourth well (1  $\mu\text{M}$ ) and incubated for 4 h at 37°C with an atmosphere of 5%  $\text{CO}_2$ . Ang-(1–12) added was not added in first and second wells and served as untreated control.

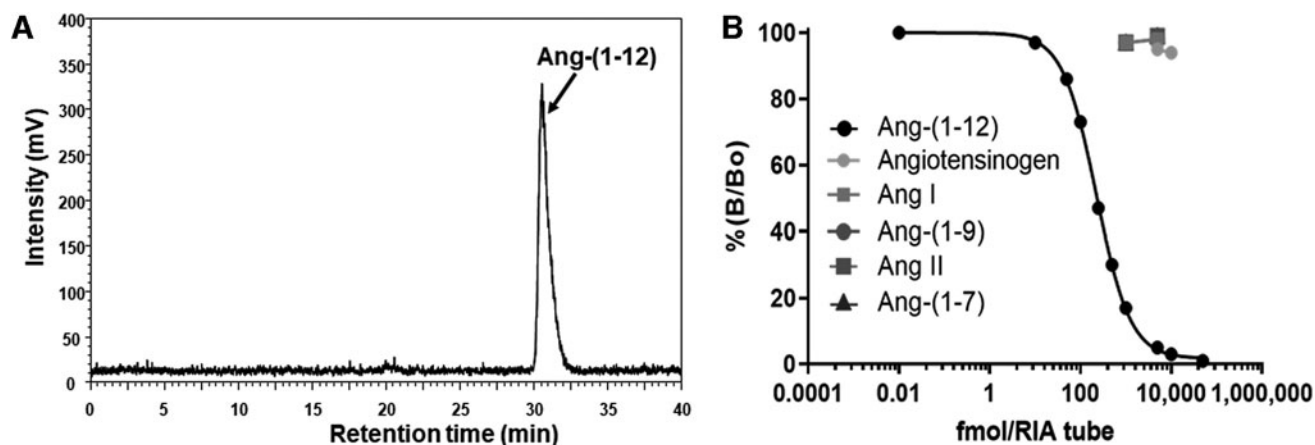
After 4 h of exposure, the culture media were aspirated and each well was washed 4 times with 1 mL of Dulbecco's phosphate-buffered saline (DPBS) and 1 time with 1.0 mL of 0.05 M glycine-HCl (pH 4.0) to remove free and outer membrane-bound Ang-(1–12) peptide. The thoroughly washed cells were fixed with 1.0 mL of 4% paraformaldehyde for 10 min, permeabilized with 0.3% Triton X-100 in DPBS for 15 min, and blocked with 1.0 mL of 5% fat-free bovine serum albumin with 0.1% Tween-20 in DPBS for 1 h at room temperature (RT). After 1 h, the blocking buffer was removed from the wells (second, third, and fourth) and 1.0 mL of Ang-(1–12) mAb (primary antibody 1:5,000 dilution in blocking buffer) was added in the wells (second, panel B; third, panel C and fourth, panel D) and incubated overnight at 4°C.

No primary antibody was added in well-1 (panel A) serves as the negative control. After overnight incubation at 4°C, the wells were washed 4 times with 1.0 mL of PBS and anti-mouse Alexa flour 488 fluorescent secondary antibody (1:1,000 dilution in blocking buffer) was added in each well and incubated for 1 h (RT) in the dark. At the end of treatment, wells were washed with 1 mL of PBS 4 times. Finally, the chamber wall and gasket assembly were removed and 10–15  $\mu\text{L}$  of Mounting Medium containing DAPI (Vector Laboratories, Burlington, CA) was added to each well and sealed by a glass coverslip. The immunoreactive fluorescent staining of the internalized Ang-(1–12) in ARPE-19 cells was visualized and imaged using a confocal microscope (Nikon Instruments, Melville, NY).

#### *Metabolism of $^{125}\text{I}$ -Ang-(1–12) by ARPE-19 cells membrane*

The metabolism of radiolabeled Ang-(1–12) substrate by ACE and chymase using ARPE-19 cells membrane was performed in the presence or absence of RAS inhibitors cocktail as previously described by us.<sup>3,5,6,26</sup> In this study, the ARPE-19 cells were cultured and the postconfluence cells were collected in PBS buffer. The cells were lysed by sonication and centrifuged (28,000  $g$ ) for 20 min at 4°C to collect the nPMs. The nPMs were solubilized in triton X-100 (0.5%) containing PBS for overnight (4°C) and insoluble nPMs were removed by centrifuging the tubes at 16,000  $g$  for 10 min at 4°C.

The solubilized proteins were used for  $^{125}\text{I}$ -Ang-(1–12) hydrolysis. In brief, the soluble proteins ( $\sim 50$   $\mu\text{g}/200$   $\mu\text{L}$ ) were preincubated (10 min at 37°C) with all RAS inhibitor cocktail or inhibitors cocktail without lisinopril and chymostatin (Table 1). After preincubation for 10 min, the reactions were started by adding  $^{125}\text{I}$ -Ang-(1–12) substrate [1 nM] and stopped after 60 min by adding 1% PA (200  $\mu\text{L}$ ). Finally, the reaction mixtures were centrifuged at 16,000  $g$  for 10 min, filtered, and injected ( $\sim 100,000$  cpm) to separate the  $^{125}\text{I}$ -Ang-(1–12) substrate and its metabolic products on C18 column by HPLC, and the radiolabeled peaks were analyzed as described by us.<sup>3,5,6,26</sup>



**FIG. 1.** HPLC chromatogram of purified  $^{125}\text{I}$ -Ang-(1-12) and mAb specificity and cross-reactivity. The human Ang-(1-12) was radiolabeled with Iodine-125 and was purified on C18 column by HPLC. As shown in HPLC chromatogram, (A) the purified radiolabeled  $^{125}\text{I}$ -Ang-(1-12) peptide fraction was highly pure ( $\geq 99\%$  purity) and was used in internalization and metabolism studies. (B) The Protein L column purified mAb shows high specificity for Ang-(1-12) (filled black circle) and does not cross-react with closely related Ang peptides (1,000 and 5,000 fmol) and human angiotensinogen (5,000 and 10,000 fmol) as demonstrated by RIA. Ang, angiotensin; HPLC, high-performance liquid chromatography; mAb, monoclonal antibody.

### Statistical analysis

All experiments were performed at least 3 or more times. Values are presented as mean  $\pm$  SD (standard deviation). The statistical analysis between 2 groups were performed by Student's *t*-test where  $P < 0.05$  was set as statistical significance (GraphPad Prism 9 Software, San Diego, CA).

## Results

### HPLC purification of $^{125}\text{I}$ -Ang-(1-12) and mAb cross-reactivity

As shown in Fig. 1A, our radiolabeled human  $^{125}\text{I}$ -Ang-(1-12) substrate was highly purified (purity  $\geq 99\%$ ) and utilized in cellular internalization and metabolism studies. Also the purified Ang-(1-12) mAb used in these experiments were highly specific in recognizing the C-terminal end of the Ang-(1-12) sequence, possessed no cross-reactivity with angiotensinogen and other Ang peptides, including Ang I and Ang II (Fig. 1B).

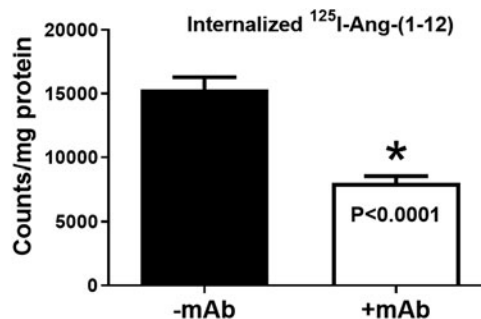
### Cellular internalization of Ang-(1-12)

Figure 2 shows that radiolabeled Ang-(1-12) was internalized in ARPE-19 cells from extracellular culture medium. Importantly, our finding further shows that the internalization of radiolabeled Ang-(1-12) in ARPE-19 cells significantly decreased ( $P < 0.0001$ ) when co-incubated with Ang-(1-12) mAb. The cell associated radiolabeled counts (mean  $\pm$  SD) after 4 h exposure was  $15,307 \pm 3,711$  cpm/mg protein for Ang-(1-12) alone and  $8,058 \pm 1,926$  cpm/mg protein in the presence of the Ang-(1-12) mAb. We found a 53% decrease in ARPE-19 cellular internalization of  $^{125}\text{I}$ -Ang-(1-12) during co-incubation with the mAb.

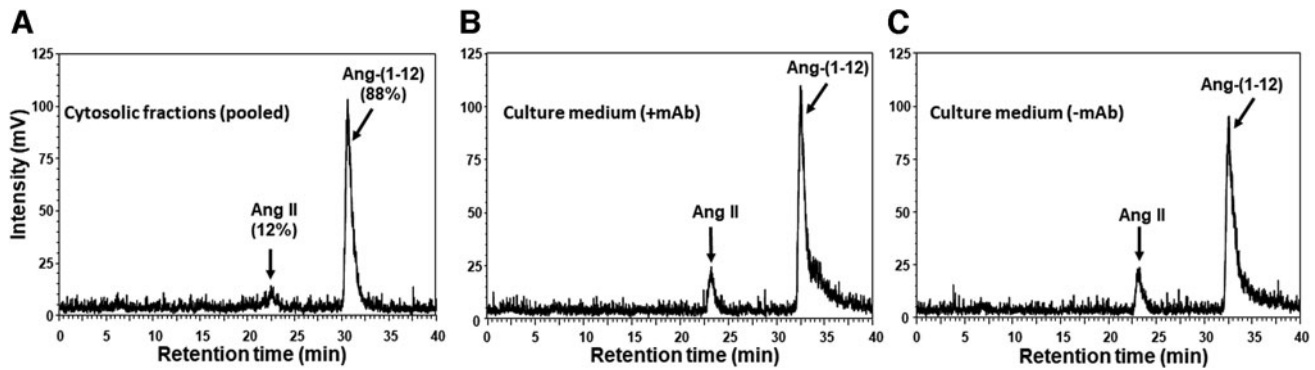
The internalization of  $^{125}\text{I}$ -Ang-(1-12) in ARPE-19 was calculated based on a total radiolabeled trace ( $1.8 \times 10^6$  cpm) added into the culture medium. We found that after 4 h of exposure, a total  $5.5 \times 10^4$  cpm (3.1%) and  $3.5 \times 10^3$  cpm (0.2%) were detected in the cells' cytosolic and membrane

fractions, respectively. Furthermore, the cytosolic cell fraction was injected on C18 column and performed HPLC analysis to confirm that an intact  $^{125}\text{I}$ -Ang-(1-12) is internalized in ARPE-19. As shown in Fig. 3A, the internalized radiolabeled content (88%) eluted at a retention time identical to standard intact  $^{125}\text{I}$ -Ang-(1-12) peptide. A small peak ( $\sim 12\%$ ) corresponds to the retention time of Ang II, which was also detected in the cytosolic fraction.

The cultured supernatants collected after 4 h exposure from ARPE-19 cells treated with radiolabeled Ang-(1-12) with or without mAb added to culture medium were injected on HPLC C18 column to identify the metabolic products. We found that the majority of radiolabeled Ang-(1-12) peptide remained intact and not hydrolyzed in the presence of inhibitors cocktail (Fig. 3B, C). As shown in the HPLC chromatograms, the Ang-(1-12) peptide remained intact (84% and 86% for -mAb and +mAb, respectively) and only a small peak ( $< 16\%$  for -mAb and 14% for +mAb, respectively)



**FIG. 2.** Radiolabeled  $^{125}\text{I}$ -Ang-(1-12) Internalization -/+mAb. Internalization of radiolabeled  $^{125}\text{I}$ -Ang-(1-12) in confluent ARPE-19 cells at 37°C for 4 h in the presence and absence of mAb (1 mg/mL). All experiments were performed at least 3 times or more on different passages. Values are expressed in terms of counts per milligram protein ( $*P < 0.0001$  vs. -mAb). Details are in Methods section. ARPE-19, adult retinal pigment epithelial-19.



**FIG. 3.** HPLC chromatograms of cytosolic fractions (from 100 mm dishes) and culture media (from 12-well plates). ARPE-19 cells were treated with  $^{125}\text{I}$ -Ang-(1-12) for 4 h at 37°C. The cells and culture media were collected after 4 h of exposure. The radiolabeled substrate and its metabolic products were analyzed by HPLC. Chromatograms; (A) pooled cytosolic fractions, (B) culture medium in the presence of mAb, and (C) culture medium in the absence of mAb. Details are in Methods section.

corresponding to the retention time of Ang II was detected in the culture supernatants.

The IF staining results (Fig. 4A–D) demonstrate that Ang-(1-12) is not expressed endogenously in ARPE-19 cells and is internalized from extracellular space. As shown in Fig. 4B, in the absence of exogenous Ang-(1-12) in culture media, no immunoreactive fluorescent green staining of Alexa flour 488 was detected in ARPE-19 cells. Whereas, in the presence of exogenous Ang-(1-12) in the culture media, the immunoreactive fluorescent green staining was detected in the ARPE-19 cells (Fig. 4C, D). An increased intensity in immunoreactive fluorescent green staining was detected with increasing concentration of Ang-(1-12) in the culture medium (panel C, 0.1  $\mu\text{M}$  vs. panel D, 1.0  $\mu\text{M}$ ). Figure 4A serves as the negative control (secondary antibody only).

#### Metabolism of $^{125}\text{I}$ -Ang-(1-12)

The HPLC chromatogram (Fig. 5A, B) shows the metabolism of radiolabeled Ang-(1-12) peptide by soluble membrane fractions of ARPE-19 cells. We found that in the presence of all inhibitors, including lisinopril and chymostatin (Table 1), >94% of the radiolabeled Ang-(1-12) substrate had remained intact and only a minute Ang II peak (<6%) was detected on the HPLC chromatogram (Fig. 5A). However, in the absence of both ACE and chymase enzyme inhibitors (lisinopril and chymostatin), only 70% of the  $^{125}\text{I}$ -Ang-(1-12) substrate remained intact (Fig. 5B) and the remaining 30% hydrolyzed into Ang I (17%) and Ang II (13%) products.

#### Discussion

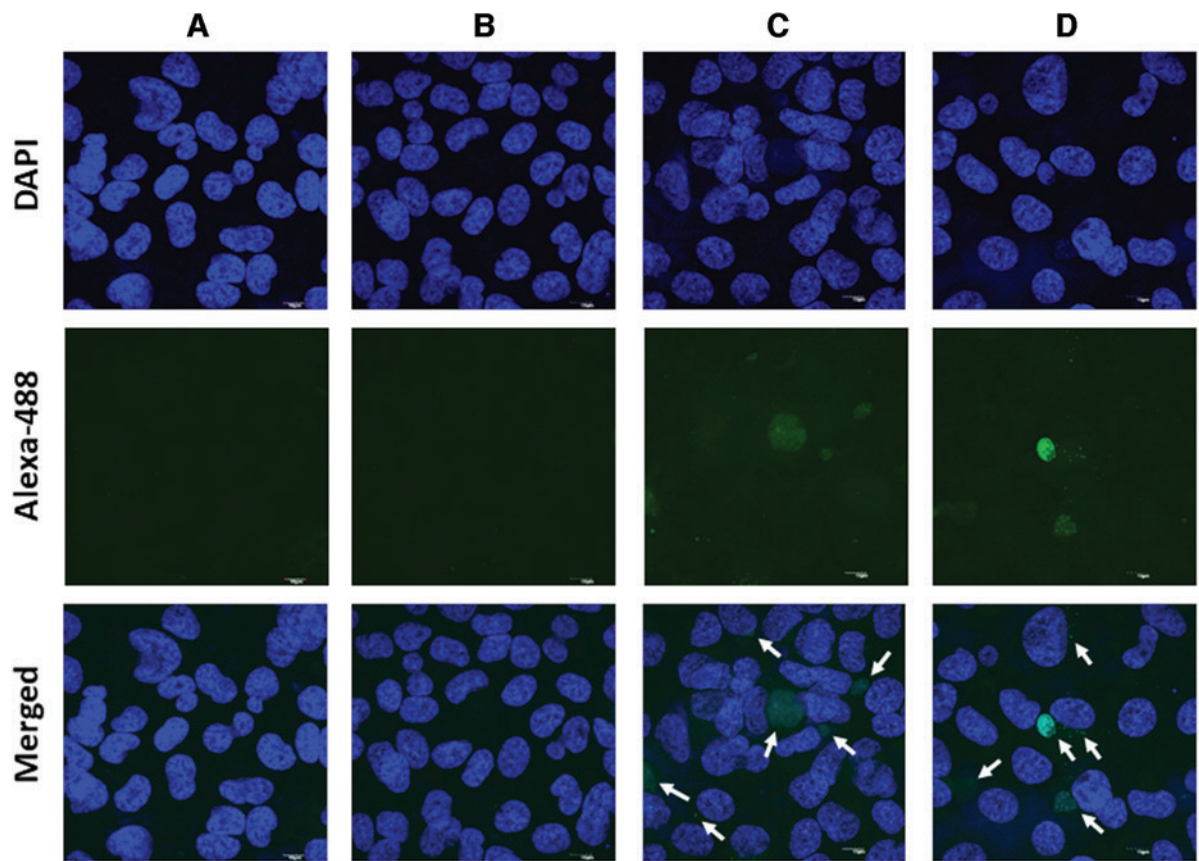
The characterization, by our laboratory, that an Ang I upstream precursor “Ang-(1-12)” serves as a primary substrate for Ang II generation in humans is expanded in this study, which revealed a significant intake of  $^{125}\text{I}$ -Ang-(1-12) in a cell line that is a critical tool in exploring the role of endocrine and cytokine mechanisms of retinopathy. Our current HPLC data show that an intact radiolabeled Ang-(1-12) peptide is uptaken by ARPE-19 cells. This internalization was blocked with co-incubation of Ang-(1-12) mAb present in the culture medium. The cellular internalization depends on the amount of Ang-(1-12) present in the

extracellular culture medium. We found that the IF intensity was increased with increasing the Ang-(1-12) concentrations in the culture medium.

Recently, we reported that Ang-(1-12) levels were severalfold elevated in the plasma of human hypertensive patients compared with the Ang I in healthy humans.<sup>10</sup> The high levels of circulating Ang-(1-12) may contribute as a source for extracellular and intracellular production of Ang II. Earlier, we demonstrated an uptake of intact Ang-(1-12) peptide in myocytes isolated from 1- to 3-day-old pups of spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY).<sup>26</sup> We showed that radiolabeled Ang-(1-12) was internalized in neonatal myocytes (SHR and WKY) from the culture medium. Furthermore, we found that at all time points Ang-(1-12) internalization rate was significantly higher in SHR myocytes when compared with WKY myocytes. In another study, a significantly higher level of endogenous Ang-(1-12) content was detected in adult SHR rats (heart and kidney) compared with WKY.<sup>28</sup> A recent study shows that radiolabeled angiotensinogen protein is internalized by ARPE-19 cells.<sup>29</sup>

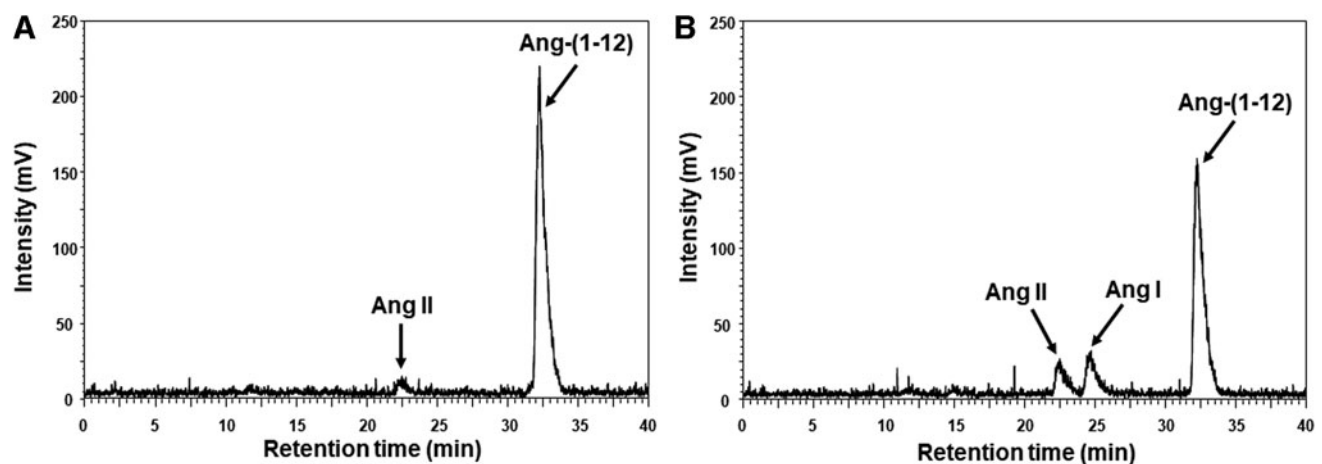
The blood-retinal barrier (BRB) is made of prominent types of cells, the endothelial cells present on the inner side and a monolayer RPE cells on the outer side of BRB.<sup>30</sup> This RPE is heavily involved in the membrane trafficking burden (transports the nutrients into the retinal neurons and removes the waste products from the retina into the blood through the transporters, receptors, and channels present on both apical-side and basolateral-side plasma membranes of the cells).<sup>31–34</sup> Receptors for proteins and peptides (such as AT<sub>1</sub>R, MasR, natriuretic peptides receptors, and glucagon-like peptide-1 receptor) are present in human RPE and cultured ARPE-19 cells at the apical and basal plasma membrane.<sup>35–38</sup> The multiligand megalin and cubilin receptors, heavily involved in the endocytic uptake of many ligands (peptides, hormones, proteins, enzymes, and drugs), are contained by ARPE-19 cells.<sup>39–43</sup>

Megalyn has been recognized to regulate homeostasis of many RAS molecules and significantly involved in pathophysiological functions and neurodegenerative disorders.<sup>44</sup> Several studies demonstrated that angiotensinogen, renin, Ang II, and Ang-(1-7) are endocytosed through megalin present on the cell surface, including the retinal cells.<sup>29,41,42,44–48</sup> Our



Treatments	Well-1 (Panel A)	Well-2 (Panel B)	Well-3 (Panel C)	Well-4 (Panel D)
Ang-(1-12)	Untreated	Untreated	Treated (0.1 $\mu\text{M}$ )	Treated (1.0 $\mu\text{M}$ )
1 <sup>o</sup> Ab [mAb]	No 1 <sup>o</sup> Ab/Control	[+] 1 <sup>o</sup> Ab	[+] 1 <sup>o</sup> Ab	[+] 1 <sup>o</sup> Ab
2 <sup>o</sup> Ab	[+] Alexa 488	[+] Alexa 488	[+] Alexa 488	[+] Alexa 488

**FIG. 4.** ARPE-19 cells immunofluorescence staining. The ARPE-19 cells grown on 4-well chambered slides and treated with or without Ang-(1–12) for 4 h at 37°C. Well-1 [A, untreated Ang-(1–12) and mAb] and well-2 [B, untreated Ang-(1–12)] serve as negative control and endogenous Ang-(1–12) expression, respectively. Well-3 (C) and well-4 were treated with Ang-(1–12) (0.1 and 1.0  $\mu\text{M}$ , respectively) serve as internalization of Ang-(1–12) from extracellular culture medium. Endogenous (B) and arrows indicate the Ang-(1–12) internalization (C, D) were detected by immunofluorescence staining using Ang-(1–12) mAb as primary and anti-mouse Alexa Fluor 488 for secondary antibody as described in Methods section. Color images are available online.



**FIG. 5.** HPLC chromatograms of radiolabeled  $^{125}\text{I}$ -Ang-(1–12) substrate and its hydrolytic products generation by ARPE-19 cells. HPLC analysis of the hydrolytic products generated from  $^{125}\text{I}$ -Ang-(1–12) substrate by solubilized plasma membranes in the absence of both ACE and chymase inhibitors. The HPLC chromatograms, represent the hydrolytic products are generated in presence of ACE and chymase inhibitors (panel A) and in the absence of both inhibitors (panel B). Details are in Methods section. ACE, angiotensin-converting enzyme.

current study on human ARPE-19 cells and previous data from the cultured neonatal hypertensive rat myocytes<sup>26</sup> suggest that circulating Ang-(1–12) may be a source of tissue and cellular accumulation through receptor-mediated internalization process. Plasma-derived angiotensinogen and renin in the interstitial fluid is a potential source of cardiac angiotensins.<sup>49</sup> Further studies are in progress to demonstrate the receptor-ligand internalization process of Ang-(1–12) from the basal-to-apical and apical-to-basal compartments using transwell inserts.

In this study, we have demonstrated that in the absence of both lisinopril and chymostatin, the solubilized plasma membranes from ARPE-19 cells metabolize Ang-(1–12) substrate into Ang I and Ang II. Our current findings are consistent with our previous studies that ACE sequentially cleaves the 2 residues at a time from the C-terminal end by cleaving the bond (first Leu<sup>10</sup>-Val<sup>11</sup> and then Phe<sup>8</sup>-His<sup>9</sup>) of the Ang-(1–12) substrate to release the products Ang I and Ang II, whereas chymase directly generates Ang II product by cleaving the Phe<sup>8</sup>-His<sup>9</sup> bond.<sup>2,5,8</sup> The RAS components (including ACE and chymase enzymes) necessary for the generation of biologically active Ang II peptide have been demonstrated in experimental animals and human retinal tissue, subretinal fluid or vitreous.<sup>13,18,50–53</sup>

Chymase is an enzyme highly expressed in various tissues, including blood vessels and ocular tissue. Study shows that in idiopathic macular hole disease an increased levels of chymase was detected in experimental animals.<sup>53</sup> Extracellularly and intracellularly produced Ang II are exerting trophic, pro-fibrotic, pro-inflammatory, and proliferative actions in target organs of diabetes (including eye).<sup>54–56</sup> Earlier, we have demonstrated that the generation of Ang II primarily depends on ACE in circulation and on chymase in tissues/cells.<sup>2,3,5–7</sup> Regardless of the generation Ang II by ACE and/or chymase, this biologically active peptide responsible for reactive oxygen species production, extracellular matrix remodeling, apoptosis, and gene expression can damage the tissues in various organs by activating the intracellular signaling pathways (including retinal microvasculature).<sup>11,16,57</sup>

Both laboratory and clinical studies indicate that Ang II plays a critical role in retinal tissue pathogenesis (retinopathy). However, involvement of the circulatory RAS [particularly Ang-(1–12)] at the cellular and molecular level in progression and development of retinopathy remains to be elucidated. Hypertension is linked in the development and progression of retinopathy and significantly contributes in several other retinal diseases in nondiabetic subjects.

However, the retinal disease risk factors are increased in hypertensive patients with diabetes. Studies indicate that the DR relative risk factor is increased by 70% with coexistence of both hypertension and diabetes.<sup>58–60</sup> Our recent data suggest that Ang-(1–12) may represent a source of intracellular Ang II production escaping the alternative hydrolysis by ACE or uptake by Ang II receptor blockers (ARBs).<sup>7</sup> Administration of anti-hypertension drugs [ACE inhibitor (ACEi) and ARBs or both] have the expected effects on plasma Ang II, but we showed that cellular (myocardial) Ang II content remains unchanged.<sup>61</sup>

To date, a precise medical treatment to prevent RAS-mediated retinopathy remains elusive. Although there is no cure for RAS-mediated retinopathy, the progression of the

disease can be prevented, delayed, or reduced by strict control of blood sugar, antihypertensive medications, laser surgery, vitrectomy, and anti-inflammatory medicines.<sup>62–66</sup> Lack of therapies targeting specific pathogenic mechanisms remain a serious limitation to prevent blindness in diabetic patients with hypertension. Experimental evidence suggests the involvement of circulating and ocular RAS components in neurodegenerative progression of DR.<sup>11,16,19,67–69</sup> ACEi and/or ARBs are currently favored to treat patients with hypertension and diabetes. These medications are only partially effective to control the retinopathy progression.<sup>70,71</sup>

Intracellular Ang II generation and effects are not improved by ACEi and/or receptor blockers as these molecules cannot penetrate the cell plasma membrane layers.<sup>72–76</sup> This discrepancy may result from renin-independent noncanonical pathways where Ang-(1–12) serves as a substrate to generate in circulation and in cellular/tissue levels. In retinal tissues, the Ang-(1–12)/Ang II/chymase/ACE axis may be a primary pathogenic factor for retinopathy progression complicated by hypertension and diabetes. In this study, we have found that the mAb against Ang-(1–12) neutralizes the Ang II-forming substrate and blocks the internalization of Ang-(1–12) into the ARPE-19 cells from the extracellular space. The data suggest that the mAb could be used as a therapeutic agent to prevent/reduce the hypertension-mediated retinopathy by neutralizing the effects of the circulatory Ang-(1–12).

For the past few decades, the FDA has approved a number of mAb to treat cancers, atherosclerosis, and immunologic diseases.<sup>77–80</sup> To date, the therapeutic use of mAb has not been investigated in RAS-associated neurovascular disease in hypertension and diabetes. We demonstrated in a humanized transgenic hypertensive rat model that circulating Ang-(1–12) serves as an Ang II-forming substrate endogenously and showed that this mAb directed toward Ang-(1–12) by itself decreases arterial pressure.<sup>25</sup> Neutralization of Ang-(1–12) by mAb will block the non-renin pathway of Ang II generation and also prevent the circulating Ang-(1–12) internalization into retinal cells. Our mAb has high specificity for Ang-(1–12) and does not cross-react with the angiotensinogen protein and angiotensinogen-derived angiotensin peptides. Also our mAb completely blocks the Ang-(1–12) hydrolytic sites and prevents Ang II formation through ACE and chymase.<sup>25</sup>

## Conclusions

This is the first line of evidence that ARPE-19 cells do not express Ang-(1–12) endogenously and as intact Ang-(1–12) peptide sequence is internalized from extracellular space and is metabolized into Ang II. Furthermore, the selective mAb blocks the cellular internalization of Ang-(1–12) suggests alternate therapeutic approaches to prevent/reduce the RPE cells Ang II burden.

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## Authors' Contributions

S.A., C.M.F., and R.M.S. designed the experiments and wrote original draft of the article. S.A., K.N.W., and J.R.G. conducted the experiments. S.A., K.N.W., and C.M.F. performed the data analysis. J.L.V., K.N.W., M.S.O., M.C., G.M., J.R.G., and R.M.S. were involved in proof reading and editing the article. All contributing authors approved the final version of this article and agreed for its submission.

## Author Disclosure Statement

No competing financial interests exist.

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