



Renin-angiotensin-system increases phosphorylated tau and Reactive Oxygen Species in human cortical neuron cell line

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ARTICLE INFO

Keywords:

Renin-angiotensin-system
Angiotensin II
Losartan
Alzheimer's disease
Cortical neurons
Tau phosphorylation
Reactive oxygen species

ABSTRACT

Alzheimer's Disease (AD) is the most common cause of dementia. AD patients had increased extracellular amyloid β plaques and intracellular hyperphosphorylated tau (p-tau) in neurons. Recent studies have shown an association between the Renin-Angiotensin System (RAS) and AD. The involvement of RAS has been mediated through Angiotensin II (AngII), which is overexpressed in aging brains. However, the exact mechanism of how AngII contributes to AD is unknown. Thus, we hypothesize that AngII increases p-tau by activating its kinases, CDK5 and MAPK. In the human cortical neuron cell line, HCN2, treatment with AngII upregulated the gene expression of CDK5 (2.9 folds, $p < 0.0001$) and MAPK (1.9 folds, $p < 0.001$). The AT1R antagonist, Losartan, blocked the changes in tau kinases. Also, AngII-induced the MAPK activation, increasing its phosphorylation by 400% ($p < 0.0001$), an increase that was also blocked by Losartan. An increase in p-tau by AngII was observed using fluorescent microscopy. We then quantified Reactive Oxygen Species (ROS) production, and it was significantly increased by AngII ($p < 0.01$), and treatment with Losartan blunted their production ($p < 0.05$). The data obtained demonstrated that AngII might contribute to the pathogenesis of AD.

1. Introduction

Alzheimer's disease (AD) is the most common type of dementia. It has defined as a neurodegenerative disease characterized by the deterioration in cognition, function, and behavior [1]. In 2020, approximately 5.8 million Americans with ages 65 and older were diagnosed with AD, and studies show that the number of affected patients may triple by 2050 [1,2]. This makes prevention approaches one of the best options to reduce economic burden and mortality rates. AD has a progressive onset, with early signs beginning with short-term memory loss that impairs a patient's daily activities and quality of life. Autopsies of AD patients have shown increased extracellular amyloid- β plaques and intracellular hyperphosphorylated tau (p-tau) neurofibrillary tangles [1].

AD has been associated with chronic diseases, such as hypertension, diabetes mellitus, and obesity [3]. Recent studies have described the association of AD with the Renin-Angiotensin System (RAS). RAS works as part of the endocrine system to regulate the osmolarity of the circulatory system and the systemic vascular resistance [4]. This system is activated by releasing Renin from the juxtaglomerular cells in the renal glomeruli due to decreased renal blood flow and sodium concentration

in the filtrate [5]. Then Renin cleaves Angiotensinogen to Angiotensin I, which is the rate-limiting step of the cascade [4]. Angiotensin I is converted to Angiotensin II (AngII) by the intervention of the Angiotensin-Converting Enzyme (ACE), which is localized most commonly in the lung tissue [4]. AngII acts on the Angiotensin type 1 receptor (AT1R), inducing vascular constriction, increasing thirst, and stimulating salt and water retention [6]. In the brain, AT1R is a G-protein coupled receptor located in neurons of the cortex, hippocampus, and basal ganglia [7].

The RAS system in the brain, or b-RAS, is shown to have a different role than classical or circulatory RAS. B-RAS impacts Reactive Oxygen Species (ROS) production, endothelial dysfunction, neuroinflammation, cognition, and aging [4,8]. Studies had shown that b-RAS activates the Nicotinamide adenine dinucleotide phosphate [NADPH] oxidase (NOX), an important enzyme to produce ROS [9]. B-RAS have presented an association with AngII and p-tau [10].

However, how AngII induces p-tau formation is still unknown. Thus, we hypothesize that AngII induces p-tau by increasing the expression and activation of tau kinases, Cyclin-dependent kinase 5 (CDK5), and Mitogen-activated protein kinase (MAPK). This investigation aimed to evaluate CDK5 and MAPK expression and activation and measure p-tau

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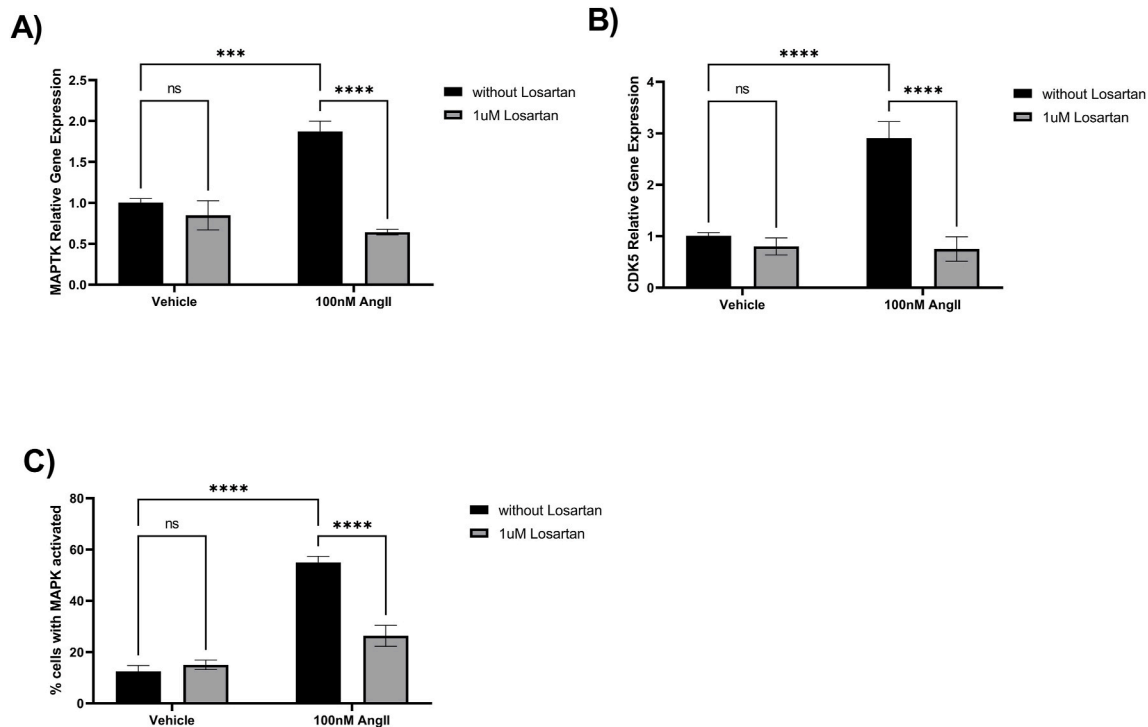


Fig. 1. Angiotensin II increases expression and activation of the tau kinases CDK5 and MAPK. HCN2 cells were incubated with 100 nM Ang II in absence or presence of 1 μ M Losartan for 24 h at 37 °C and compared to vehicle (PBS) treatment. Gene expression of MAPK (A) and CDK5 (B) was quantified by qRT-PCR using Taqman probes. Values were normalized using GAPDH as house-keeping gene. (C) Graphical representation of MAPK activation (phospho-ERK1/2 (Thr202/Thr204, Thr185/Thr187)). Data represents mean \pm SD (n = 4). *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

and ROS production in human cortical neuron cells line, HCN2, treated with AngII.

2. Materials and methods

2.1. Cell culture

Human cortical neuron cell line, HCN2 (ATCC, CRL-10742) were maintained in DMEM with 10% FBS (Sigma Aldrich) and 100 U/ml penicillin/streptomycin (Life Technologies) at 37 °C in a 5% CO₂-humidified atmosphere. Briefly, 12 h before treatment, cells were serum-starved in DMEM with 0.2% FBS. At the treatment time, cells were washed with PBS and incubated with vehicle, AngII, or Losartan in DMEM with 0.2% FBS. The cells were harvested for analyses after 24-h incubation.

2.2. RNA extraction and quantitative real-time PCR

Total RNA was prepared with 1 mL of TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used to make 20 μ L of cDNA from 2 μ g of RNA. Gene expression was analyzed using real-time PCR using TaqMan gene expression assay for CDK5, MAPK, NOX1, and GAPDH (Applied Biosystems) in a StepOne Plus from ABI. The $\Delta\Delta$ cycle threshold method was used to determine mRNA levels. Gene expression was normalized to GAPDH levels.

2.3. Determination of ROS generation

ROS generation of HCN2 cells was measured by the Muse Oxidative stress kit using the Muse cell analyzer (Millipore, Billerica, MA, USA) fluorescent-based analysis. The manufacturer-specific protocol was followed for the assay. In brief, HCN2 cells were treated with 100 nM AngII with or without Losartan treatment and incubated for 24 h 1×10^7 cells/

mL samples were prepared in 1X assay buffer and treated with an Oxidative stress reagent based on dihydroethidium (DHE) used to detect ROS oxidized with superoxide anion procedure, the DNA-binding fluorophore ethidium bromide, which intercalates with DNA resulting in red fluorescence. This method was validated using N-Acetyl-L-Cysteine (Sigma-Aldrich) as ROS inhibitor [9] and Antimycin A (Sigma-Aldrich) as ROS inducer [11].

2.4. Detection of MAPK activation

STAT-1 activation in HCN2 cells was measured by the Muse® MAPK Activation Dual Detection Kit using the Muse cell analyzer (Millipore, Billerica, MA, USA). The manufacturer-specific protocol was followed for the assay. In brief, HCN2 cells were treated with 100 nM AngII with or without Losartan treatment and incubated for 24 h. Cells were fixed for 5 min and then permeabilized for 5 min. The antibody cocktail was added to the samples and incubated at room temperature and dark for 30 min. Samples were analyzed in the Muse cell analyzer.

2.5. Immunofluorescence

HCN2 cells were cultured in two-well Nunc Lab-Tek II chamber slides. After 24 h of treatment, the cells were fixed in a 4% paraformaldehyde solution for 15 min and permeabilized with 0.5% Triton X-100 in DPBS for 10 min at room temperature. The cells were blocked with 1% bovine serum albumin for 30 min at room temperature, then probed with Phospho-Tau (Ser214) Polyclonal Antibody (Invitrogen, 44-742G) for 2 h at room temperature. The cells were washed with PBS and incubated with Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 (Invitrogen, A-11035) for 30 min at room temperature. Cells were fixed again in 4% paraformaldehyde, and ProLong® Diamond Antifade Mountant with DAPI (Invitrogen) was added.

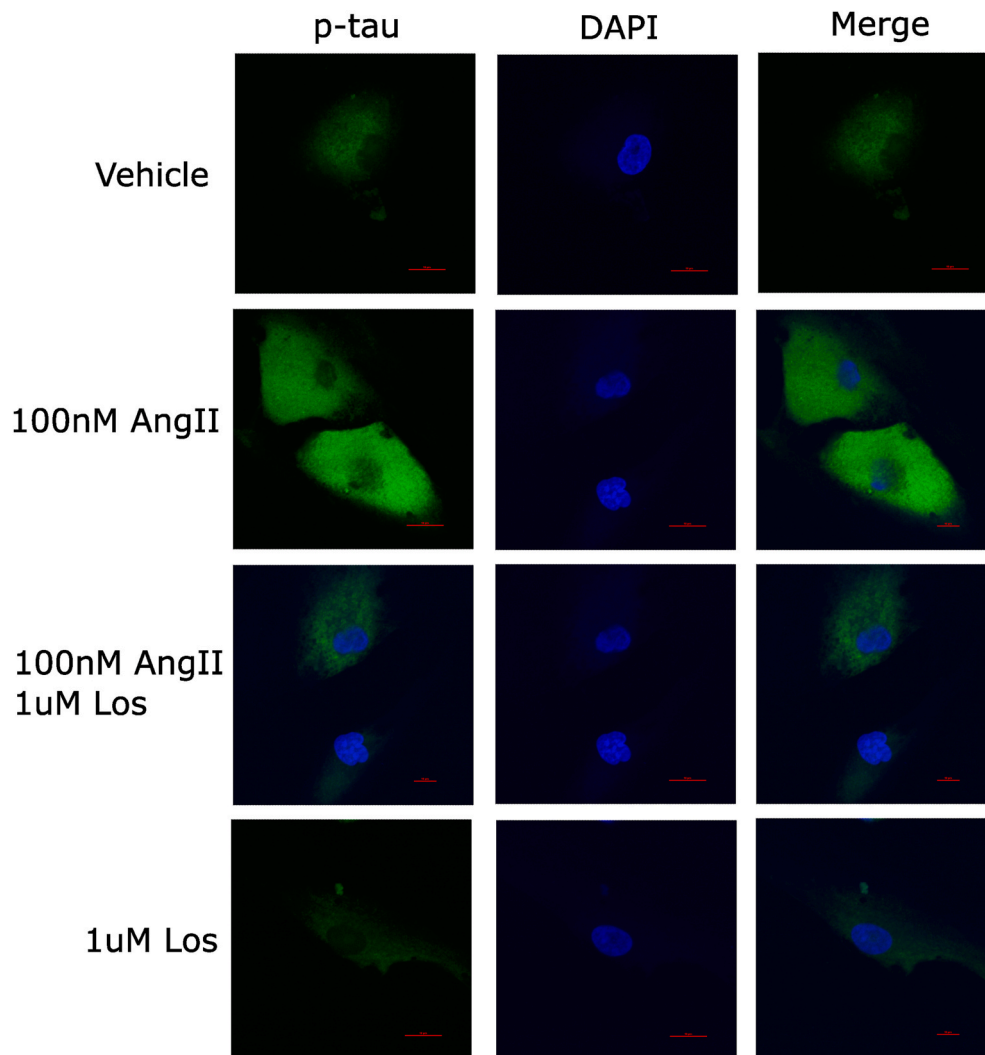


Fig. 2. Angiotensin II increases tau phosphorylation. Tau phosphorylation was observed with confocal microscopy using Phospho-Tau (Ser214) (green). Nuclei were stained with DAPI (blue), and the images were merged. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2.6. Statistical analysis

Data were analyzed by one-way or two-way ANOVA with Bonferroni post-test or Student's t-test when appropriate. The p-value was set to be < 0.05 . Data are expressed as mean \pm SD of (n) independent experiments unless otherwise stated.

3. Results

3.1. AngII increases CDK5 and MAPK gene expression in cortical neurons

To study the effect of AngII on CDK5 and MAPK gene expression, human cortical neuron cells, HCN2, were treated with 100 nM AngII with or without 1 μ M Losartan (Fig. 1). AngII upregulated the gene expression of MAPTK (1.9 folds, $p < 0.001$, $n = 4$) (Fig. 1A) and CDK5 (2.9 folds, $p < 0.0001$, $n = 4$) (Fig. 1B). The AT1R antagonist, Losartan, blocked the changes in tau kinases.

The Muse® Cell Analyzer was used to measure the amount of MAPK activated in HCN2 cells treated for 24 h with 100 nM AngII in the absence or presence of Losartan. The results in Fig. 1C demonstrated higher activated MAPK in the AngII treated HCN2 cells (54.92% \pm 4.815). With Losartan treatment, the levels significantly decreased to 26.35% \pm 8.185 ($p < 0.0001$). This suggests that AngII increases tau

kinases gene expression and activation by AT1R.

3.2. AngII increases tau phosphorylation in cortical neurons

AngII was associated with p-tau in rats' brains [12]. To study the direct effect of AngII in cortical neurons, HCN2 cells were treated with AngII for 24 h in the presence or absence of Losartan. Then, p-tau was studied using Phospho-Tau (Ser214) antibody. AngII induces an increase in p-tau, seen in the HCN2 cells treated with 100 nM of AngII (Fig. 2). HCN2 cells in the presence of Losartan showed a decrease in p-tau. These results show the protective role Losartan has on the neuron cells by decreasing the production of p-tau.

3.3. AngII increases ROS production in cortical neurons

We study ROS production in HCN2 cells using the Muse oxidative assay system based on flow cytometry. AngII significantly increased ROS production ($p < 0.01$, $n = 4$), 45.93% \pm 11.57 of cells had ROS compared to 17.79% \pm 2.161 in the vehicle. Treatment with Losartan decreased ROS production caused by AngII to 18.71% \pm 5.335 ($p < 0.05$, $n = 4$) (Fig. 3). In addition, AngII significantly increase NOX1 gene expression by 2.6 \pm 0.9 compared to vehicle ($p < 0.05$, $n = 4$) (Fig. 3C).

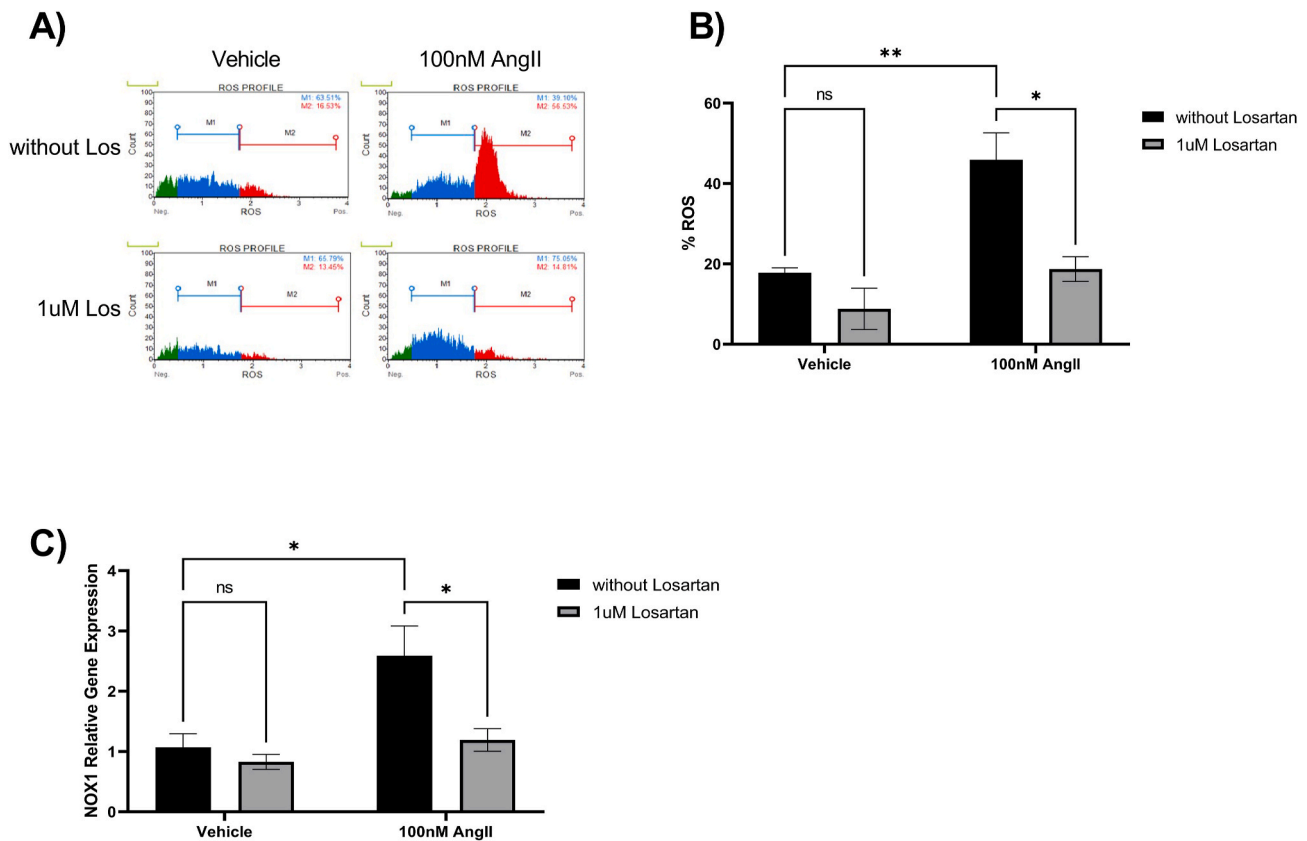


Fig. 3. Angiotensin II increases ROS production in HCN2 cells line. HCN2 cells were incubated for 24 h in the absence or presence of 100 nM Ang II with or without Losartan (1uM) and analyzed by flow cytometric assay. (A) The figure represents the population and ROS profile of the cell. (B) Graphical representation of ROS formation. (C) Gene expression of NOX1 was quantified by qRT-PCR using Taqman probes. Values were normalized using GAPDH as house-keeping gene. Results represent the mean \pm SD of $n = 4$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

4. Discussion

RAS has given a new perspective to AD pathology, given that some RAS molecules are present in the brain and lead to hyperphosphorylation of tau and ROS production [4,8]. One of the RAS molecules that have been related to AD is AngII [4]. The latter has different types of receptors in the brain, but specifically, its AT1R has been found in areas involved in cognition and behavior [7]. The activation of the AT1R in these areas has been shown to cause neuroinflammation, cognitive impairment, and neuronal death [4,13]. This study investigated the role of AngII on tau phosphorylation and ROS production in a human cortical neuron cell line.

AngII induces tau hyperphosphorylation in normal brain rats [12]. Similar results were obtained in human studies [8]. We found that AngII increases p-tau in HCN2 cells, and this effect was blocked by Losartan, indicating that AngII's effect is through AT1R. In addition, AngII increases the expression and activation of certain tau kinases, CDK5 and MAPK. Studies focused on these tau kinases have found that CDK5 is activated in post-mitotic neurons and hyperactivated in diseased brains [13]. Some MAPK enzymes are upregulated in AD [14]. More specifically, selective pharmaceuticals that target MAPK have neuroprotective against tau hyperphosphorylation and aggregation [15]. We show that AngII increases the expression of tau kinases CDK5 and MAPK. Specifically, we prove that AngII's effects on these tau kinases were through AngII's AT1R signaling mechanism, given that their up-regulation was attenuated by Losartan (Fig. 1 A and Fig. 1 B). With Losartan we were able to show the pharmacological effect of an AT1R blocker as tau kinases attenuator. The mechanism of how tau kinases increase activity and expression is currently unknown. Still, studies hypothesize that in terms of MAPK, AngII can increase its activity through RAS/Raf-1

signaling of the AT1R [16].

These results can provide knowledge of one of the AD's known histopathologic presentations, which is the formation of neurofibrillary tangles because of tau hyperphosphorylation. Tau's physiological role is to stimulate and stabilize microtubule assembly [17]. However, when it suffers mutations or post-transcriptional phosphorylation in certain Ser and Thr residues can inhibit microtubule assembly and promote aggregation [18,19]. These tau oligomers are toxic to neurons, leading to neuronal death [16].

ROS could also be one mechanism that leads to AD pathology, given studies have demonstrated that ROS promotes tau protein modification into p-tau in neurons [9,20]. Previously was shown that there is an increase in ROS production in aging brains, causing neurotoxicity and neuronal death, which could also precipitate the pathogenesis of AD [8]. We found that AngII increases ROS production, and that Losartan attenuates this effect. Literature has discussed that AngII increases ROS by activating NOX, which to produce superoxide, a type of ROS [9]. We found that AngII increase NOX1 gene expression generating the increase in ROS production. However, more investigation is needed to elucidate other signaling pathways that increase ROS production through AngII.

This study shows how AngII could be a mechanism of AD pathology and presents Losartan as a possible treatment for AD. Currently, this drug is used as an antihypertensive treatment to prevent vasoconstriction and aldosterone secretion [21]. However, studies have discussed that Losartan can also lower the incidence of AD [22]. We show that Losartan decreases the activation of tau kinases and the production of p-tau and ROS in the HCN2 cell line. Losartan could have a protective role in reducing neurotoxicity and neuronal death, leading to dementia [23]. Losartan also has been shown to increase cognitive function [23] by improving the surrogate marker for cognitive performance, Cerebral

Blood Flow [24]. This provides a form of treatment that can help the patients delay the onset of AD and slow the progression of the disease. Current medication for AD lacks the aim of reducing the progression of AD, given that they only treat the patients' symptoms to improve their quality of life [25]. Losartan could be the treatment to reduce the incidence of AD and health care costs. However, most importantly, it helps tackle the pathophysiology of the disease to decrease the mortality rate of AD.

This study has limitations that need to be considered when interpreting our results. The method used to quantify ROS, DHE, can form two fluorescent products, ethidium, which is formed by nonspecific redox reactions, and 2-hydroxyethidium, a specific superoxide adduct. We are reporting the fluorescence of both products because we were unable to separate the signals and only measure superoxide. In addition, AngII concentration used were above physiological levels; it would be interesting to determine if AngII upregulates p-tau at concentrations of <100 nM.

5. Conclusions

In conclusion, AngII is demonstrated to contribute to AD pathogenesis by increasing the expression and activation of tau kinases, CDK5 and MAPK, and the increase in p-tau in HCN2 cells. In addition, AngII increases NOX1 gene expression and ROS production in cortical neurons. Finally, we were able to show that the AngII's effects under this study were mediated through the ATR1 receptor, given that Losartan could blunt such effects. This suggests that Losartan could be considered a possible therapeutic agent for AD due to its protective role against AngII's effects.

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Funding

This work was supported by the San Juan Bautista School of Medicine Pilot Projects Program to YIN. The confocal microscopy work was supported by the National Institute of General Medical Sciences (NIGMS) of the National Institutes of Health (NIH) under Award Number P20GM103642.

Author contributions

Conceptualization, YIN; methodology, YIN.; formal analysis, LDD, and YIN; investigation, CC; writing—original draft preparation, CC and LDD; writing—review and editing, LDD and YIN; supervision, YIN; funding acquisition, YIN. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors are very grateful to Mr. Bismark Madera, M.T., for his outstanding work and dedication to the confocal imaging experiments.

List of Abbreviations

AD	Alzheimer's Disease
RAS	Renin-Angiotensin System
AngII	Angiotensin II
p-tau	hyperphosphorylated tau
AT1R	Angiotensin type 1 receptor
ROS	Reactive Oxygen Species
CDK5	Cyclin-dependent kinase 5
MAPK	Mitogen-activated protein kinase
DHE	dihydroethidium

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