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Angiotensin II down-regulates transferrin receptor 1 and ferroportin 1 expression in Neuro-2a cells via activation of type-1 receptor

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ABSTRACT

Angiotensin II (ANGII) modulates expression of iron intake and export proteins in cultured neurons. However, the relevant mechanisms have not been fully elucidated. Here, we investigated the effects of ANGII and/or candesartan, a ANGII-Type-1 Receptor (AT1R) antagonist, and PD123319, a ANGII-Type-2 Receptor (AT2R) antagonist on expression of transferrin receptor 1 (TfR1), ferroportin 1 (Fpn1)and ferritin as well as iron regulatory proteins (IRPs), hepcidin and nuclear factor E2-related factor 2 (Nrf2) in Neuro-2a cells. We demonstrated that ANGII induces a significant reduction in expression of TfR1, Fpn1, IRP2 proteins and Nrf2 mRNA and an increase in ferritin protein and hepcidin mRNA, while candesartan, but not PD123319, significantly attenuated or reversed all these ANGII-induced changes in Neuro-2a cells. These findings imply that ANGII down-regulates TfR1 expression likely via the AT1R/IRP2 pathway, and Fpn1 expression via ATR1/hepcidin and AT1R/ Nrf2 pathways.

1. Introduction

The Renin-Angiotensin System (RAS) is a vital modulator of the cardiovascular system, and Angiotensin II (ANGII) is an important hormone that regulates vasoconstriction, salt-water homeostasis and blood pressure [1]. Previous studies in the peripheral system have reported clinical and experimental evidence for the link between ANGII and iron metabolism. Significantly elevated serum ferritin levels and increased iron stores were observed in hypertension patients [2]. Studies have also observed that ANGII elevates tissue iron levels in the liver [3], cardiovascular system [4], and affects the process of cellular iron transport in the kidney and glomerular endothelial cells [5,6]. Mice treated with ANGII exhibit enhanced duodenal iron intake and increased iron content in macrophages and the kidney, accompanied by elevated hepcidin expression in the liver and serum and changes in expression of iron-regulating proteins (IRPs).

It has been demonstrated that all the required components of the RAS, including ANGII and its type 1 and 2 receptors [7–9], renin, angiotensinogen and angiotensin-converting enzyme (ACE) [10–12] and also all key proteins involved in iron metabolism such as transferrin receptor 1 (TfR1), ferroportin 1 (Fpn1) and IRPs and hepcidin are present in the mammalian brain [13–17]. Brain ANGII has been reported to be able to regulate iron homeostasis in primary mesencephalic cultures, the dopaminergic cell line MES23.5 and young adult rats [18]. The increased iron deposition and ferritin levels in substantia nigra observed in aged rats have been thought to involve activation of the ANGII/AT1R axis [19].

Our recent studies demonstrated that ANGII could modulate expression of iron intake and export proteins and influx and efflux of iron in cultured neurons [20,21]. However, the relevant mechanisms have not been fully elucidated. We therefore investigated the effects of ANGII and/or candesartan (AT1R antagonist) or PD123319 (AT2R antagonist) on the expression of iron intake proteinTfR1, iron export protein Fpn1, iron storage protein ferritin(Ft-H, ferritin light chain and Ft-L, ferritin

Abbreviations: ACE, Angiotensin-converting enzyme; ANGII, Angiotensin II; AT1R, ANGII-Type-1 Receptor; AT2R, ANGII-Type-2 Receptor; Cand, Candesartan; Fpn1, Ferroportin 1; Ft-H, Ferritin light chain; Ft-L, Ferritin heavy chain; IRPs, Iron regulatory proteins; Nrf2, Nuclear factor E2-related factor 2; RAS, Renin-Angiotensin System; TfR1, Transferrin receptor 1

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heavy chain) as well as some key iron-regulatory-molecules including IRP1, IRP2, hepcidin and Nuclear factor E2-related factor 2(Nrf2) in Neuro-2a cells. Our findings imply that ANGII down-regulates TfR1 expression likely via the AT1R/IRP2 pathway, and Fpn1 expression via ATR1/hepcidin and AT1R/ Nrf2 pathways.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemicals were obtained from the Sigma Chemical Company, St. Louis, MO, USA. Mouse monoclonal anti-rat TfR1 was purchased from Invitrogen, Carlsbad, CA, USA; rabbit polyclonal anti-mouse Fpn1 from Novus Biologicals, Littleton, CO, USA; anti-Ft-Land anti-Ft-H from Proteintech, Chicago, IL, USA; goat anti-rabbit or anti-mouse IRDye 800 CW secondary antibodies from LI-COR Bio Sciences, Lincoln, Nebraska, USA; ANGII from APExBIO, Houston, TX, USA; candesartan and PD123319 from Selleck, Houston, TX, USA; TRIzol reagent from Life Technologies, Carlsbad, CA, USA; RevertAid First Strand cDNA Synthesis Kits from Thermo Scientific, Waltham, MA, USA; FastStart Universal SYBR Green Master and LightCycler® 96 System from Roche, Nutley, NJ, USA; and BCA protein Assay kits and RIPA lysis buffer from Beyotime Institute of Biotechnology, Haimen, JS, China.

2.2. Neuro-2aneuroblastoma cells

Neuro-2a cells (mouse neuroblastoma N2a cells) were grown in DMEM supplemented with 10 % Fetal Bovine Serum (FBS) and antibiotics (penicillin 100 U/ml, streptomycin 100 mg/ml), maintained in a 5 % CO2 incubator with 37 °C, and culture medium changed every 2 days. All experimental protocols were performed according to the Animal Management Rules of the Ministry of Health of China, and approved by the Animal Ethics Committees of Fudan University.

2.3. Determination of cell viability

Cell viability was determined using an MTT assay as described previously [22,23]. Neuro-2a cells were incubated for 24-h in a 37 °C incubator, and then treated for another 24-h with designed concentrations of ANGII, candesartan and PD123319. Optical density (OD) was measured at 570 nm by the use of the ELX-800 microplate assay reader (Bio-tek, USA).

2.4. Western blot

Cells were washed with ice-cold PBS, lysed, sonicated and collected into Eppendorf tubes, and centrifuged at 14,000 rpm under 4 °C for 15-min. Supernatant was transferred into another tube for protein concentration tests using BCA protein assay kit. The primary antibodies used were: anti-rat TfR1 (1:500), anti-mouse Fpn1 (1:1000), anti-Ft-L (1:2000) and anti-Ft-H (1:2000), anti-IRP1 and anti-IRP2 (1:1000), anti-Nrf2 antibody (1:1000), and β-actin (1:10,000) [24,25]. Blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (1:5000) for 2-h under room temperature. Band intensities were detected using the Odyssey infrared image system (Li-Cor, Lincoln, NE, USA).

2.5. Real-time PCR

Total RNA was extracted and cDNA was generated with the RevertAid First Strand cDNA Synthesis Kit. Real-time PCR analysis of hepcidin, IRP1 and β-actin mRNA expression was conducted using FastStart Universal SYBR Green Master and LightCycler® 96 System (Roche).The primer sequences used are: hepcidin forward, 5′-gaaggcaagatggcactaagca-3′; reverse, 5′-tctcgtctgttgccggagatag-3′; IRP1 forward, 5′-acaggccgcgaggaaga-3′; reverse, 5′-gaaacatgcctacagcctgaagat-3′; IRP2 forward, 5′-gccatagcaggcacagtgaata-3′; reverse, 5′-tttccttgcccgtagagtcagt-3′;Nrf2 forward, 5′-cgagatatacgcaggagaggtaaga-3′; reverse, 5′-gctcgacaatgttctccagctt-3′; β-actin forward, 5′-aaatcgtgcgtgacatcaaaga-3′; reverse, 5′-gccatctcctgctcgaagtc-3′ [26]. The Ct values of each target gene were normalized to that of the β-actin mRNA. Relative gene expression was calculated by the $2-\Delta\Delta Ct$ method.

2.6. Statistical analysis

All data were presented as mean ± SEM. Graphpad Prism (GraphPad Software Inc., La Jolla, USA) was used for statistical analysis. The differences between groups were analyzed using one-way or two-way ANOVA, followed by the Tukey test. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effect of ANGII and AT1R and ATR2 antagonists on Neuro-2a cell viability

Firstly, we evaluated the effects of different concentrations of ANGII, candesartan and PD123319 on Neuro-2a cell viability by incubating the cells with 0, 0.1, 1, 10, 100 or 1000 nM of ANGII;0, 0.1, 1, 10 or 100 μM of candesartan;or0, 0.1, 1, 10 or 100 μM of PD123319.Significant reduction in cell viability was observed only when Neuro-2a cells were treated with the highest concentration of ANGII (1000 nM) (Fig. 1A), candesartan (100 μM) (Fig. 1B) or PD123319 (100 μM) (Fig. 1C). There were no differences in cell viability between the cells treated with 0 and 0.1 − 100 nM of ANGII, 0 and 0.1 − 10 μM of candesartan, or 0 and 0.1 − 10 μM of PD123319

3.2. ANGII inhibited TfR1 and Fpn1 and promoted ferritin expression in Neuro-2a cells

Neuro-2a cells were cultured with 0 (Control), 10, 100 and 1000 nM of ANGII for 24-h. Western blot assay was performed to detect the expression of TfR1, Fpn1, Ft-L and Ft-H proteins. Results indicated that the expression of TfR1 (Fig. 1D) andFpn1 (Fig. 1E) was significantly suppressed, while the expression of Ft-L (Fig. 1F) and Ft-H (Fig. 1G) were increased by all three concentrations of ANGII. The expression of TfR1 and Fpn1 in the cells treated with ANGII were about 75 % and 50 % of the control values in average (Fig. 1D & E), inducing a more significant inhibition by ANGII on Fpn1 expression as compared with TfR1.As 100 nM of ANGII showed a significant impact on expression of these two iron transport proteins without affecting Neuro-2a cell viability, 100 nM was therefore selected as the concentration of ANGII treatment for subsequent experiments.

3.3. Candesartan reversed ANGII-induced down-regulation of TfR1 and Fpn1 and up-regulation of Ft-L and Ft-H expression in Neuro-2a cells

To find out how ANGII affects iron homeostasis, we first examined the effects of candesartan on expression of TfR1, Fpn1, Ft-L and Ft-H in the cells treated with ANGII. The cells were pretreated with 0 (Control) or 10 μM of candesartan for 1-h, followed with 100 nM ANGII for another 24-h. Western blot analysis demonstrated that there were no differences in the expression of TfR1 (Fig. 2A), Fpn1 (Fig. 2B), Ft-L (Fig. 2C) and Ft-H (Fig. 2D) between the cells treated with 0 (Control) or 10 μM of candesartan, indicating that candesartan had no effects on the expression of these four proteins in the cells treated without ANGII. However, the expression of TfR1 (Fig. 2A) and Fpn1 (Fig. 2B) was found to be significantly higher, while ferritin (Fig. 2C & D) lower, in the cells treated with candesartan and ANGII (ANGII + Cand) than that in the cells treated by ANGII only, demonstrating that candesartan could

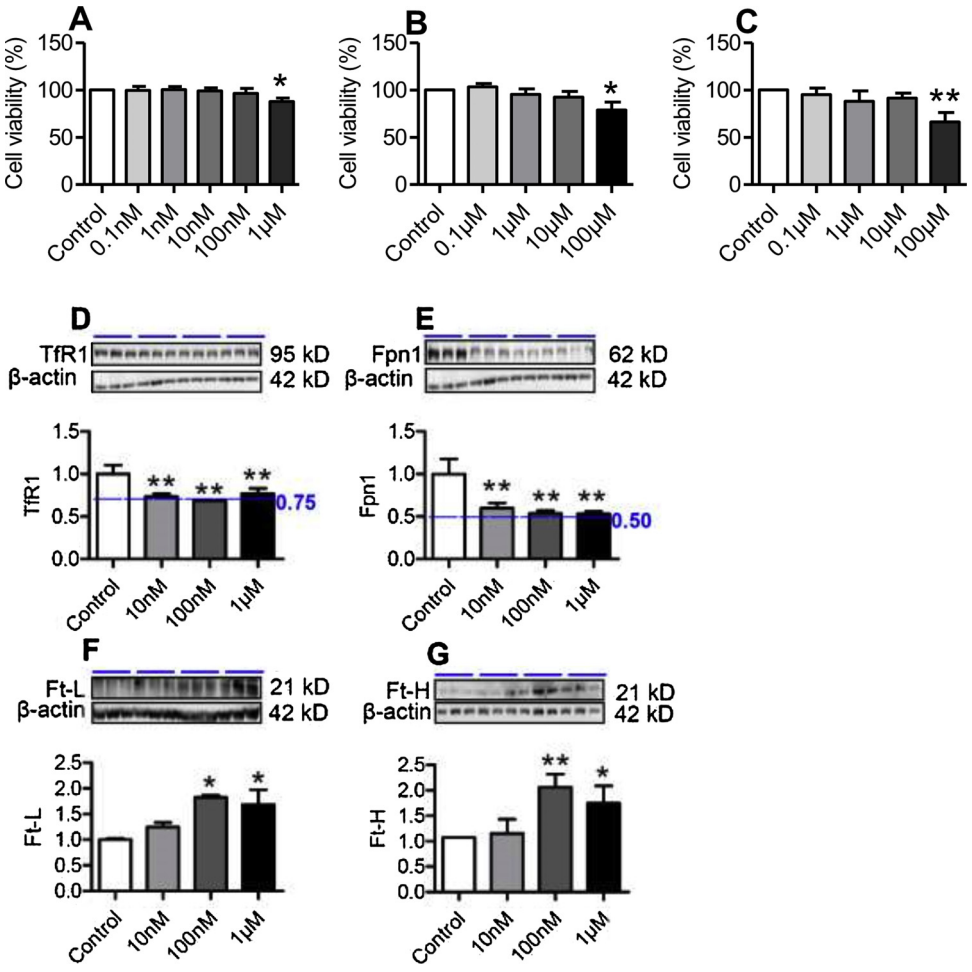

Fig. 1. A-C: Effect of ANGII and AT1R and ATR2 antagonists on cell viability. Neuro-2a cells were incubated with 0, 0.1, 1, 10, 100 or 1000 nM of ANGII (A); 0, 0.1, 1, 10 or 100 μM of candesartan (B); and 0, 0.1, 1, 10 or 100 μM of PD123319 (C) for 24-h, cell viability was then determined. D–G: ANGII inhibited TfR1 and Fpn1 and promoted ferritin expression. Neuro-2a cells were cultured with 0 (Control), 10, 100and 1000 nM of ANGII for 24-h, and the expression of TfR1 (A), Fpn1 (B), Ft-L (C) and Ft-H (D) proteins was then detected by Western blot analysis. The values were mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. Control.

significantly reverse ANGII-induced down-regulation of TfR1 and Fpn1 and up-regulation of Ft-L and Ft-H expression in Neuro-2a cells.

3.4. Candesartan reversed ANGII-induced down-regulation in expression of IRP2 protein and mRNA in Neuro-2a cells

We then investigated the effects of ANGII and/or candesartan on expression of IRP1 and IRP2 proteins and mRNAs. Western blot and RT-PCR analysis showed that there were no differences in expression of IRP1 protein (Fig. 2E) and mRNA (Fig. 2G) between the control cells and cells treated with candesartan (Card), ANGII, or ANGII + Card. However, expression of IRP2 protein (Fig. 2F) and mRNA (Fig. 2H) was significantly higher in the cells treated with ANGII + Cand than that in the cells treated with ANGII only. The findings demonstrated that ANGII could down-regulate expression of IRP2 protein and mRNA, which could be reversed by candesartan in Neuro-2a cells.

3.5. Candesartan reversed ANGII-induced increase in hepcidin mRNA and -reduction in Nrf2 mRNA expression in Neuro-2a cells

Hepcidin is known to induce the internalization and degradation of the iron-export protein Fpn1 and reduce iron efflux [27]. Nrf2 has also been known to affect Fpn1 expression and cell iron release [28]. Therefore, we subsequently investigated the effects of ANGII or/and candesartan on the expression of hepcidin and Nrf2 mRNAs. Real-time PCR showed that candesartan could induce a reduction in hepcidin and an increase in Nrf2 mRNA expression (Fig. 3A).On the contrary, ANGII induced an increase in hepcidin and a reduction in Nrf2 mRNA expression (Fig. 3B). The expression of hepcidin mRNA was significantly lower and Nrf2 higher in the cells treated with ANGII + Cand than that in the cells treated with 100 nM of ANGII only (Fig. 3A & B), implying that candesartan has the ability to reverse ANGII-induced increase in hepcidin mRNA and reduction in Nrf2 mRNA expression in Neuro-2a cells.

3.6. PD123319 failed to reverse ANGII-induced changes in the expression of TfR1, Fpn1, Ft-L and Ft-H in Neuro-2a cells

We also examined the effects of PD123319, an AT2R selective blocker, on expression of TfR1, Fpn1, Ft-L and Ft-H by incubating the cells with 10 μM of PD123319 for 1-h and then 100 nM of ANGII for another 24-h. Western blot analysis showed that there were no significant differences in expression of these four proteins between the control and PD123319-treated cells or ANGII-treated and ANGII + PD123319-treated cells, demonstrating that PD123319 had no effects on expression of TfR1 (Fig. 3C), Fpn1 (Fig. 3D), Ft-L (Fig. 3E) and Ft-H (Fig. 3F) proteins in the cells treated with or without ANGII, and also implying that AT2R may not be involved in the effects of ANGII on expression of iron uptake, release and storage proteins in Neuro-2a cells.

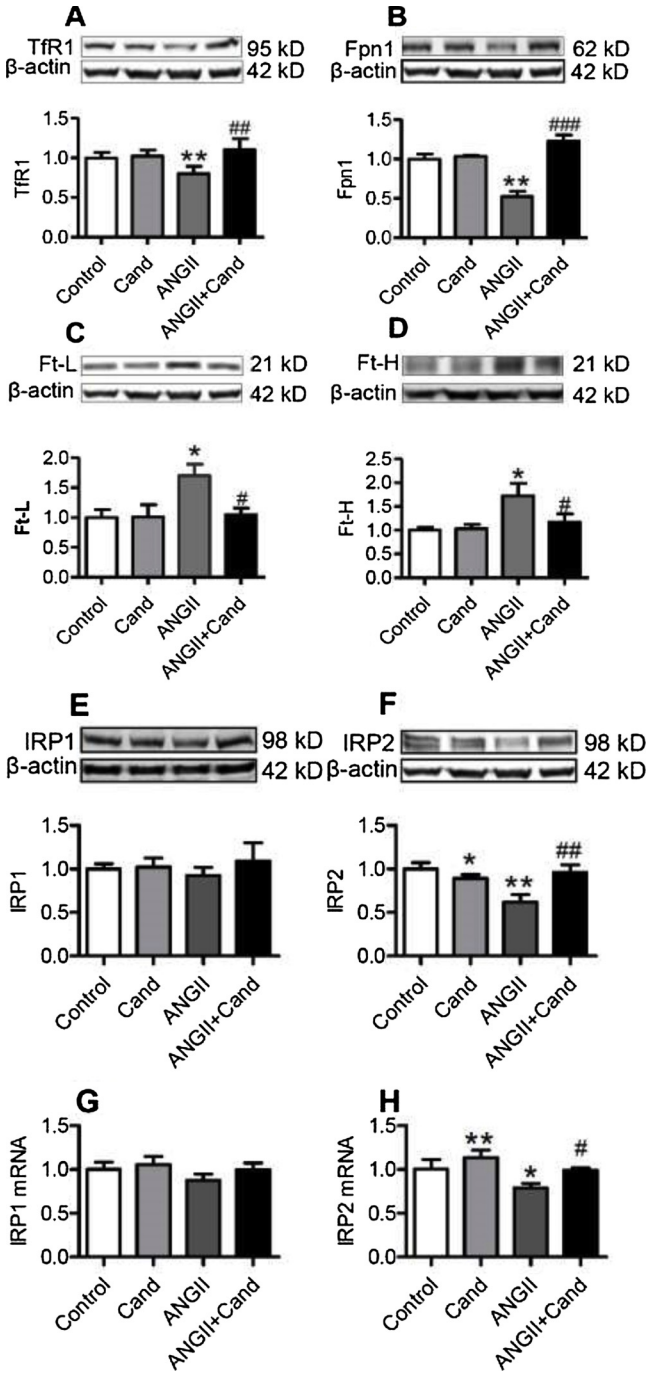

Fig. 2. Candesartan reversed ANGII-induced down-regulation of TfR1, Fpn1 and IRP2 and up-regulation of Ft-L and Ft-H expression. Neuro-2a cells were pretreated with 0 (Control) or 10 μM of Cand for 1-h and then with 100 nM of ANGII for another 24-h. The expression of TfR1 (A), Fpn1 (B), Ft-L (C), Ft-H (D), IRP1 and IRP2(E and F) proteins and mRNAs (G and H) was then detected by Western blot ordeal-time PCR analysis respectively. The values were mean ± SEM (N = 3). *P < 0.05, **P < 0.01 vs. Control; #P < 0.05, ##P < 0.01, ###P < 0.001vs. ANGII.

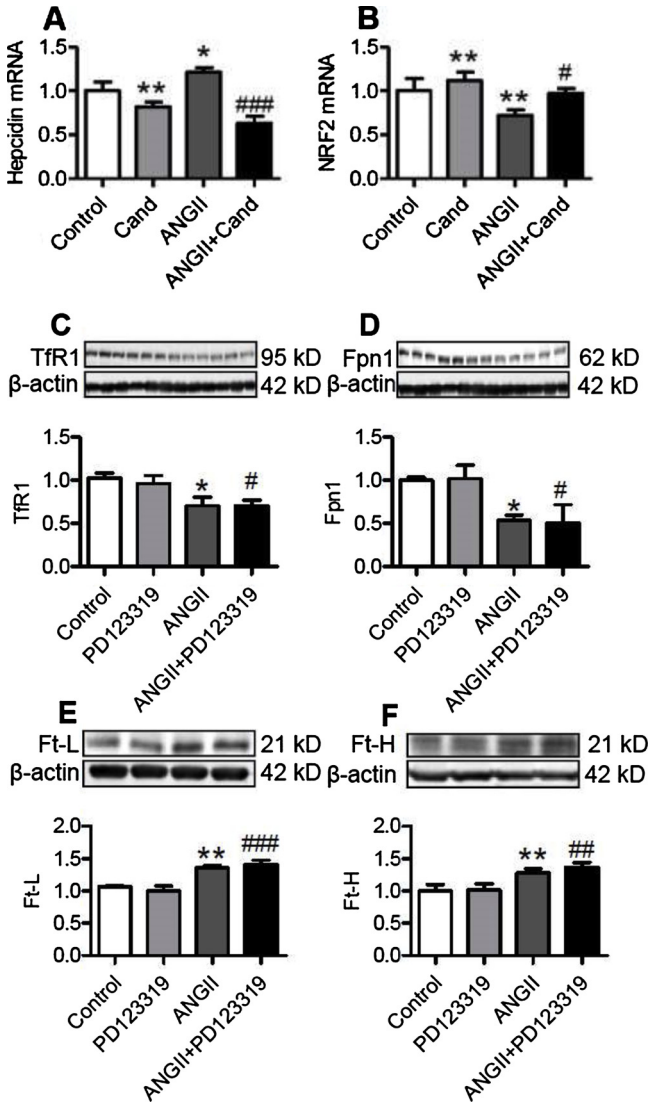

Fig. 3. A-B: Candesartan reversed ANGII-induced increase in hepcidin mRNA and reduction in Nrf2 mRNA expression. Neuro-2a cells were pretreated with 0 (Control) or 10 μM of Cand for 1-h and then with 100 nM of ANGII for another 24-h. The expression of hepcidin (A) and Nrf2 (B) mRNAs was determined by Real-time PCR analysis. C-F: PD123319 failed to reverse ANGII-induced changes in the expression of TfR1, Fpn1, Ft-L and Ft-H. Neuro-2a cells were pretreated with 0 (Control) or 10 μM of PD123319 for 1-h and then with 100 nM of ANGII for another 24-h, and the expression of TfR1 (C), Fpn1 (D), Ft-L (E) and Ft-H (F) proteins was then detected by Western blot analysis. The values were mean ± SEM (n = 3). *P < 0.05, **P < 0.01 vs. Control; #P < 0.05, ##P < 0.01, ###P < 0.001 vs. ANGII.

4. Discussion

In the present study, we demonstrated that ANGII could induce a significant reduction in expression of both TfR1 and Fpn1 and an increase in ferritin (Ft-H and Ft-L) in Neuro-2a cells, consistent with what we found in cultured neurons [21]. We also showed that AT1R antagonist candesartan could significantly reverse ANGII-induced down-regulation of TfR1, Fpn1 and IRP2 proteins and Nrf2 mRNA expression and up-regulation of ferritin (Ft-L and Ft-H) protein and hepcidin mRNA expression in Neuro-2a cells, while AT2R antagonist PD123319 had no such effects. These findings imply that ANGII down-regulates TfR1 expression probably via the AT1R/IRP2 pathway and Fpn1 expression via ATR1/hepcidin and AT1R/ Nrf2 pathways.

Iron is the most abundant trace metal in the brain. As in all cells, neurons and glia require iron for many aspects of their physiology, including electron transport, NADPH reductase activity, myelination of axons, and as a cofactor for several enzymes involved in neurotransmitter synthesis [29]. Hence, an imbalance in brain iron results in dysfunction in iron related metabolism [13]. Iron deficiency has been reported to have a role in brain development and the pathophysiology of restless legs syndrome [30,31]. Iron accumulation has been related to

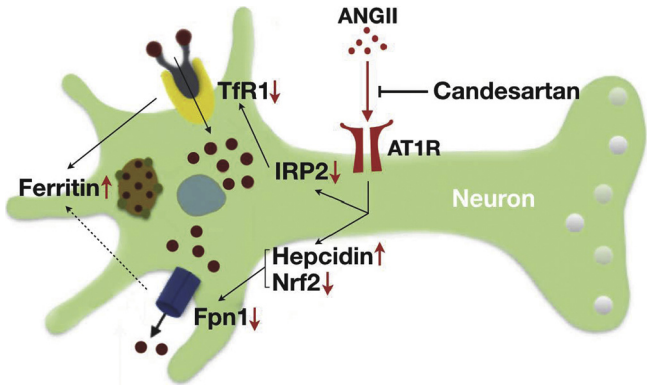

Fig. 4. A hypothetical scheme for ANGII to influence the expression of TfR1, Fpn1 and Ferritin in Neuro-2a cells.

some neurologic disorders, such as neurodegeneration with brain iron accumulation, Alzheimer's disease and Parkinson's disease [32].

Cellular iron content is mainly dependent on expression of iron uptake and release proteins. TfR1 is a key-uptake protein and TfR1-mediated endocytosis of transferrin-bound iron (Tf-Fe) is the main route for cellular iron accumulation [33], while Fpn1 is the currently only cellular iron exporter that has been identified [34]. In general, down-regulation of TfR1, induced by ANGII, will lead to reduced cellular iron uptake and cellular iron content. In the present study, however, we found the content of iron storage protein ferritin to be significantly increased, rather than reduced as we expected, by treatment with ANGII in Neuro-2a cells. This is likely because inhibition of ANGII on Fpn1 (75 %) is more significant than that on TfR1 (50 %) expression, leading to a relative increase in iron intake compared to iron release in the cells treated with ANGII. The ANGII-induced increase in ferritin content suggests that iron accumulation may be partly associated with RAS-mediated Parkinson's disease in the aging process [19].

Mammalian iron metabolism is regulated cellular by IRPs [35]. IRPs are known to regulate post-transcription ally the expression of TfR1 and ferritin, which contain iron responsive elements (IREs) in their 3′ untranslated regions (UTRs) and 5′ UTRs respectively. Binding of IRPs may stabilize genes with IREs on 3′ UTR (TfR1) and suppress the translation of genes with IREs on 5′ UTR (Ferritin) [36]. ANGII down-regulatesTfR1 and up-regulates Ft-L and Ft-H expression, while these effects of ANGII could be reversed by treatment with candesartan, but not PD123319, in Neuro-2a cells. This suggests that inhibition of ANGII on TfR1 expression is mediated by binding of ANGII with AT1R, rather than AT2R, and then inhibiting IRP2 expression (Fig. 7). Currently, the connection between the activated AT1R and reduced expression of IRP2 are unknown.

Hepcidin is a principal regulator of systemic iron homeostasis [35]. Hepcidin binds to its membrane receptor Fpn1, which induces the internalization and degradation of the Hepcidin/Fpn1 complex, suppressing the export of iron from the cells [27]. In addition, Nrf2 has been known to affect Fpn1 expression [28]. Impaired Nrf2 expression can reduce Fpn1 transcription and then diminish iron egress in mouse and human cells [28]. To find out whether the effect of ANGII on Fpn1 is associated with hepcidin and Nrf2, we therefore also investigated the effects of ANGII and/or AT1R and AT2R antagonists on the expression of hepcidin and Nrf2 in Neuro-2a cells. We showed that ANGII has a role to increase hepcidin mRNA and to reduce Nrf2 mRNA expression, suggesting that the reduced expression of Fpn1 may be partly due to the increased hepcidin and impaired Nrf2 expression. Candesartan, but not PD123319, reversed ANGII-induced increase in hepcidin mRNA and reduction in Nrf2 mRNA expression in Neuro-2a cells, indicating that ANGII regulates Fpn1 expression via AT1R/hepcidin and AT1R/Nrf2 pathways (Fig. 4). Currently, how the binding of ANGII withAT1R or activation of AT1R, up-regulates hepcidin and down-regulates Nrf2 expression remains unknown.

5. Conclusion

Our findings collectively imply that ANGII down-regulates TfR1 expression, likely via the AT1R/IRP2 pathway, and Fpn1 expression, via ATR1/hepcidin and AT1R/ Nrf2 pathways.

CRediT authorship contribution statement

Yun-Jin Chen: Methodology, Investigation. **Zhong-Ming Qian:** Conceptualization, Validation, Formal analysis, Resources, Data curation, Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Yuan Sheng:** Methodology, Formal analysis, Investigation. **Jie Zheng:** Formal analysis, Investigation. **Yong Liu:** Conceptualization, Validation, Data curation, Visualization, Funding acquisition.

Declaration of Competing Interest

The Authors declare that there is no conflict of interest.

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