Interaction of AR and iNOS in lens epithelial cell: a new pathogenesis and potential therapeutic targets of diabetic cataract

Xue Li^{1†}, Wenping Liu^{2†}, Xinduo Huang¹, Jianping Xiong³, Xiaoyong Wei^{1, 4*}

Abstract: Although there is significant interest in revealing the role of aldose reductase (AR) and inducible nitric oxide synthase (iNOS) in diabetic cataract (DC), the interaction of AR and iNOS remains unknown. The aim of this study is to investigate the pathogenesis mechanisms and explore as a new potential therapeutic targets for DC. This study investigated the interaction of AR-iNOS through the methods of enzyme kinetics, molecular docking and molecular dynamics simulation, co-immunoprecipitation and fluorescence resonance energy transfer (FRET). The IC₅₀ of AR for inhibition of iNOS activity is 0.04 μ M, and the IC₅₀ of iNOS for inhibition of AR activity is 0.042 μ M through enzyme kinetics; the interface showed that ARG99 on AR and GLU317 on iNOS played the key roles in the interaction of AR-iNOS predicted by molecular docking and molecular dynamics simulation. Co-immunoprecipitation of protein complexes in human lens epithelial cell (HLEC) demonstrated that AR could association with iNOS in cell; and the interaction distance of AR-iNOS was 6.50 ± 0.22 nm detected by FRET. This study exhibited a direct inhibition interaction between AR and iNOS in HLECs. It is the first report of inhibition interaction between AR and iNOS, suggesting a new pathophysiological mechanism and providing a new insight into the therapeutic mechanism of DC.

Keywords: diabetic cataract; aldose reductase; inducible nitric oxide synthase; interaction

1. Introduction

Diabetes cataracts (DC) has become the primary cause of visual loss and blindness with diabetes reaching global epidemic proportions [1-2]. It is well known that the primary damage of environmental or genetic factors of lens epithelial cell (LEC) may cause the abnormal differentiation and damage of cells in lens expressed as the opacity of lens tissues eventually and which resulted in cataracts [3-4].

It is well known that the major recognized pathogenesis of DC is polyol pathway, oxidative stress pathway and glycosylation pathway [5-7]. In polyol pathway, the intracellular increase of fluid in response to AR-mediated accumulation in LEC makes lens with complex biochemical changes ultimately leading to cataract formation; in oxidative stress pathway, diabetic lenses show an impaired antioxidant capacity, a susceptibility to oxidative stress, and inactivation of lens antioxidant enzymes like superoxide dismutases or activation of iNOS leads to the oxidative stress of lens; in glycosylation pathway, the increased glucose levels in lens induce glycation of lens proteins and result in generation of superoxide radicals and advanced glycation end products (AGE) in this process [8-10].

In DC, AR and iNOS played critical roles in the pathogenesis of DC in polyol pathway and oxidative stress pathway [11-12]. Aldose reductase (AR) is a key enzyme in polyol pathway, leads to accumulation of sorbitol and changes of intracellular osmotic pressure, which damages the LEC eventually [5, 11, 13]. And inducible nitric oxide synthase (iNOS), an isoform of nitric oxide synthase, catalyzes the oxidation of L-arginine, and the expression of iNOS in LEC can bring a lot of NO, further activates the oxidative stress response to damage LEC [6, 12, 14-15]. In LEC, AR and iNOS are both up regulated with hyperglycemia in DC [16-18]. It is reported that inhibition of AR can attenuate the stimulation and release of nuclear factor-kappaB

(NF- κ B) in human lens epithelial cell (HLEC), and regulate tumornecrosisfactor (TNF) induced expression of iNOS in human mesangial cell through AR's activation of NF- κ B [19-20]. Researches revealed that transcription of iNOS-gene depends on the NF- κ B signaling pathway and NF- κ B could enhance the transcription of iNOS-gene [21-22]. And NO is also reported to up-regulate the expression of AR which can relieve the cytotoxic action of NO [23]. According to that, there was indirect inhibition between AR and iNOS regulated by NF- κ B. But little is known about that whether there is a direct interaction between AR and iNOS.

Now, it is imperative for the comprehensive analysis of the complex pathogenic mechanisms in DC to find prompt and cost-effective interventions of DC [24-25]. And proteins become the important therapeutic targets and protein-protein interactions are considered the drug targets of great research value [26]. The pathogenesis mechanisms underlying AR and iNOS are mainly based on osmotic pressure theory in the polyol pathway and oxidative stress respectively in DC in previous reports [11-12]. AR inhibitors and iNOS inhibitors were considered as a treatment of up regulation of AR and iNOS in DC [14, 20], and they was deemed as drug targets respectively, but here, this study illustrated that there is direct interaction between AR and iNOS, and AR-iNOS were deemed as a whole for a new research target through the interaction between them, which provide a new insight into the pathogenic mechanism for DC and a new drug target of DC. In this paper, pEGFP-N1-AR plasmid was constructed and transfected into HLECs to form a DC model of up regulation of AR expression; then used co-immunoprecipitation(Co-IP) and fluorescence resonance energy transfer(FRET) to investigate the interaction between AR and iNOS, Co-IP enables their interactions in living cells [27-28].

2. Materials and Methods

2.1 Materials

The immortalized human lens epithelial cells (HLEC-B3) were obtained from Prof. ShangFu (Zhongshan Ophthalmic Center, Guangzhou, P.R. China).

2.1.1 Reagents

AR was purchased from ProSpec-Tany Techno Gene Ltd (Ness Ziona, Israel); iNOS was purchased from Cayman Chemical Company (Ann Arbor, USA); Reduced NADPH was purchased from Roth (Co, Italy); DL-glyceraldehyde was purchased from Sigma-Aldrich (ST. Louis, USA); iNOS kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, P.R. China); Fetal bovine serum (FBS), penicillin/streptomycin, and trypsin were purchased from GibcoBrl (NY, USA); disodium hydrogen phosphate and potassium phosphate dibasic were purchased from Amresco (OH, USA); iNOS Antibody co-immunoprecipitation kit, Turbofect Transfection Reagent were purchased from Thermo Fisher Scientific (Waltham, USA); AR antibody was purchased from Bestbio (Shanghai, P.R. China); and PVDF membrane was obtained from Life Scientific Research (USA).

2.1.2 Instruments Inverted fluorescence microscope (LX71, Olympus, Japan); multimode plate reader (BioTek, Vermont, USA); laser confocal microscope (LSM710, ZMISS, Germany); CO₂ cell incubator (Scientific, Thermo, Waltham, USA); automatic chemiluminescence image analyzer (Tanon5200, Shanghai, P.R. China).

2.2 METHODS

2.2.1 Enzyme kinetics

First, we investigated the inhibitory activity of AR against iNOS with gigantol. The

concentrations of AR (2, 4, 8, 16, 32 μ M), gigantol (6 μ M), and iNOS (3 μ M) was selected in this experiment. The iNOS activity was evaluated with a nitric oxide synthase assay kit. The iNOS activity was assessed by NO production, which was based on the method of Fröhlich et al [29], calculated as inhibition rate using the equation: $\% = [(B_1 - B_2)/(B_1 - B_0)] \times 100\%$, where B_0 and B_1 represent the absorbance values of the blank group and standard group, respectively, and B_2 represents the absorbance value of the samples. This experiment has investigated the AR's IC₅₀ with iNOS and gigantol. All the assays were performed in sextuplet.

Then the inhibitory activity of iNOS against AR was measured as previously described by Sahoo [30]. The reaction mixture of comprised AR (0.5 μ M, 20 μ L), gigantol solution (6 μ M, 10 μ L), NADPH (0.104mM, 50 μ L), DL-glyceraldehyde (10mM, 50 μ L), PBS (0.1M, pH6.2, 50 μ L), and selected concentrations of iNOS (0.0625, 0.125, 0.25, 0.5, 1 μ M, 10 μ L) to the total volume of 200 μ L. The reaction was initiated by the addition of DL-glyceraldehyde as the substrate, and double-distilled H₂O was used as the blank control. AR activity was shown by decrease of NADPH absorption values at 25°C for 10min at 40s intervals at 340 nm using multimode plate reader. AR inhibition rate was calculated as inhibition rate (%) = $[1 - (A_2 - A_0)/(A_1 - A_0)] \times 100\%$ [31], where A_0 represents the decrease in NADPH absorbance without enzyme, substrate and sample; A_1 represents the decrease in NADPH absorbance prior to sample addition; A_2 represents the decrease in NADPH absorbance with sample addition. This experiment has investigated the iNOS's IC₅₀ with AR and gigantol. All the assays were performed in sextuplet.

In the enzyme kinetics, Gigantol, a multi-target therapeutic drug for AR and iNOS, which can inhibit the activity of AR and iNOS in LEC simultaneously, was chosen as

the substrate to investigate the inhibitory effect between AR and iNOS [32-33].

2.2.2 Molecular docking and Molecular Dynamics simulation

The free AR (PDB code 1USO) was downloaded from PDB database and regarded as the ligand with receptor iNOS (PDB code 2NOS). Docking of AR to iNOS was performed with Swarm Dock server (<http://bmm.crick.ac.uk/~SwarmDock/>) [34]. Docking progress consisted of three steps. First, our input structures were progressed and minimized. Then docking was conducted using a hybrid particle swarm optimization/local search. Last, 458 complex structures (Numbered 0a to 117d) were generated and they were minimized, ranked and clustered. Top ten structures with lowest energies were sorted out, named complex 1 to 10, and treated as initial conformations for following MD simulations.

MD simulations were conducted to obtain the critical residues in the interface because ten complexes were not equilibrated structures if they are surrounded with water molecules under physiological thermal environment and MD simulations were the only tool to describe protein dynamics by tracking the precise position of each atom at any instant in time [35]. Two software packages, Visual Molecular Dynamics (VMD) for visualization and modeling and NAnoscale Molecular Dynamics (NAMD) 2.9 program for MD simulations [36-37], were used here. Ten AR/iNOS complexes generated by Swarm Dock were solvated with TIP3P water molecules in a rectangular box, respectively. The walls of the ten water boxes were at least 15 Å away from any protein atoms. Then Na⁺ and Cl⁻ ions were added to neutralize the systems at a 150mM salt concentration. The CHARMM22 all-atom force field, along with cMAP correction for backbone, particle mesh Ewald algorithm for electrostatic interaction and a 12Å cutoff for electrostatic and van der Waals interaction, was used to perform

MD simulations with periodical boundary condition and time step of 2fs [38].

First, each system was energy-minimized for 15,000 steps with all protein atoms fixed and for another 15,000 steps with all atoms free. Then ten systems were equilibrated for 2ns with pressure and temperature control, respectively. The temperature was held at 310K using Langevin dynamics, and the pressure was held at 1 atmosphere by the Langevin piston method. Root mean square deviation (RMSD) of heavy atoms was used to observe whether the system had been equilibrated.

The salt bridges and/or hydrogen bonds (H-bonds) in simulations were detected by VMD. A hydrogen bond was defined if the donor-acceptor distance was $<3.5\text{\AA}$ and the donor-hydrogen-acceptor angle was $>150^\circ$. But only the bond-length cut off of $3.5\text{\AA}$ was applied to examine the salt bridges in binding site. Survival ratio of an H-bond (salt bridge) was defined as the ratio of bond survival time in the period of simulation, which could be calculated through the Formula (1).

$$(1)$$

2.1.3. Cell culture and transfection

HLECs were maintained in minimum essential medium (MEM) supplemented with 10% FBS. HLECs were transfected by Turbofect transfection Reagent. Results of transfection have used inverted fluorescence microscope to observe the expression of green fluorescent protein (pEGFP). Briefly, 200,000cells/well were seeded in 6-well plates daily in Co-IP and 60,000cells/well seeded on glass coverslips in 12-well plates daily for transfection in FRET. In Co-IP, the design of experiment was grouped as control group and transfection group. The control group was treated with complete medium, and transfection group was transfected with $2\mu\text{g}$ of pEGFP-N1-AR. After rinsing twice with cold PBS, cells were lysed in RIPA buffer on ice for 30min with

occasional vortexing. After centrifugation at 12,000 g, 4°C for 20 min, proteins were quantified by the BCA Protein Assay Reagent, using bovine serum albumin as the standard. In FRET, the design of experiment was grouped as control group, transfection group, and the control group was transfected with 2µg of pEGFP-N1, and transfection group was transfected with 2µg of pEGFP-N1-AR for 24h. Cells were cultured in serum-free media for 12h prior to measurement.

2.2.4 Co-IP experiment

Co-IP procedures were performed at 4°C unless otherwise indicated, using a centrifuge tube which was capped with a bottom for incubation and remove the supernatant by centrifugation at 10000g, for 10min. Then the protein was pre-incubated by untreated beads for 1h at 4°C. The binding of iNOS (IP) antibody and proteinG-Agarose was accomplished with Pierce crosslink immunoprecipitation kits. The antibody/G agarose complex slurry was washed with PBS, and incubated with bovine serum albumin (BSA, 0.1%) prepared in PBS at 4°C for 1h and the supernatant was discarded three times. After the proteins' pre-incubation, the supernatant of each group was distributed into two centrifuge tubes by centrifugation at 2000g, for 5min. Then the one was added 10µl binding iNOS antibody and proteinG-Agarose beads, the other one was added 10µl IgG as the negative group. The mixed protein was incubated for 12h at 4°C. Then the protein added 5µl untreated beads to each centrifuge tube for 5h on a shaker at 4°C, followed by centrifugation at 2,000g for 5min. After removing the supernatant and washing three times with washing buffer, the immunoprecipitates were added to 2× loading buffer at 100 °C for 10min. The complex was subjected to SDS-PAGE followed by Western blotting analysis [39]. Samples were electrophoresed in a 9% Bis-Tris gels at 120v using SDS running

buffer. The AR was transferred to a PVDF membrane for 60 min at 70v, and iNOS was for 120min at 70v. The PVDF membrane was washed three times with TBS containing 0.1% tween 20 (TBS-T), and blocked with 5% BSA in TBS-T at room temperature for 1h. Then the membrane was washed three times with TBS-T and incubated with primary antibody of AR (at 1/1000 ratio) at 4°C overnight, washed three times with TBS-T, and incubated with secondary antibody for 1h on a shaker at room temperature. After washing three times with TBS-T, the membrane was incubated with BeyoECL Plus detection reagent for 5min. Then the membrane was used automatic chemiluminescence image analyzer to detect. Western blot analysis was performed by Tanon Gel image system ID4.2 analysis software.

2.2.5 Cell imaging with photo-bleaching reveals interaction between AR and iNOS in HLEC

After the cells transfect were washed three times with PBS and fixed with 4% paraformaldehyde for 30min. Then the cells washed three times with PBS and treatment with 0.1% Triton-100 5min, added 2% BSA 1h after washed three times with PBS. Then iNOS antibody was added on the cover slips at 4°C overnight, washed three times with PBS 5min/time, and added second-antibody-cy3 for 2h in wet state. The cover slips were washed with PBS three times and mounted onto microscope glass. Cell imaging was performed using a Zeiss LSM710 Laser Scanning Confocal Microscope and Cell Chamber System at 37 °C temperature control and CO₂ control 3700. The pEGFP-N1-AR was cloned into HLEC and iNOS was labeled with Cy3. The FRET experiments were performed using the pEGFP and Cy3 as fluorophore pairs. The fluorescence recovery after photo-bleaching with Cy3 was performed by bleaching a region of interest (ROI) in the cytoplasm and nucleus at

100% power of an argon laser (488nm for pEGFP, 558nm for Cy3) for duration of 30s. The photoactivation experiments were performed under the following settings: 558 nm laser for Cy3 excitation and bleaching, and 488nm laser for pEGFP. The Cy3-iNOS was bleached and the resultant energy transfer to GFP-AR was quantified. The FRET efficiencies were calculated by curve fitting analysis of Cy3 fluorescence changes. The interaction distance between the donor and the acceptor (R) was calculated using the equation [40]: $E^{-1} = 1 + (R/R_0)^6$, wherein R_0 is the Foster distance when the energy transfer efficiency between donor and acceptor is 50%, and R represents the actual distance between donor and acceptor. The FRET efficiency formula is as follows [41]:

$$(2)$$

Where I^{DD} is the fluorescence intensity of the donor channel under the excitation wavelength of the donor before bleaching, I^D is the fluorescence intensity of the donor channel after donor excitation, and the P_B denotes the degree of bleaching.

Data analysis

Data are presented as means $\pm$ SD. All statistical analysis was performed using the two-tailed student's t-test. P values <0.05 were considered significant.

3. RESULTS AND DISCUSSION

3.1 Analysis of the activity and the synergistic mutual inhibitory effects of AR and iNOS

We found that the IC_{50} of AR for inhibition of iNOS activity was $0.04\mu M$, and the IC_{50} of iNOS for inhibition of AR activity was $0.042\mu M$ through enzyme kinetics. The IC_{50} of AR was based on the calculation method of iNOS, and the IC_{50} of iNOS was based on the calculation method of AR respectively, results as shown in Table1,

2. From this result, it showed that the mutual inhibitory effects were existed between AR and iNOS. AR-iNOS could interplay directly extracellular, and exhibited an inhibition effect based on the enzyme kinetics.

3.2 The potential interface between AR and iNOS provided by Docking and Molecular Dynamics simulations

In the docking of AR and iNOS, altogether 458 poses were generated and we sorted the top ten complexes with the lowest free energy, named complex 1 to 10. They were shown in Figure1 A with AR (blue) aligned. From the time-curves of the NANOScale Molecular Dynamics (NAMD) of heavy atoms (Figure2), we could see that complex 1 to 5 and 7 to 9 were equilibrated after 0.5ns, while complex 6 and 10 had great conformation changes after 1ns, which indicated that their interface were not stable.

Survival ratios of hydrogen bonds and salt bridges in the interface of eight complexes during equilibriums were calculated except for complex 6 and 10. H-bonds and salt bridges with survival ratio above 0.5 in any simulation were sorted out and listed in supplementary data. There were altogether forty-four hydrogen bonds and salt bridges, involving twenty-six residues on AR and twenty-nine residues on iNOS. To detect the main binding sites in the interface, we calculated the number of hydrogen bonds (salt bridges) for each residue and plotted them in Figure3. Results showed that residues with number from 92 to 100 formed the main interface on AR, especially ARG99, which formed six hydrogen bonds with GLU317 and ALA162 on iNOS (Figure1 B and Figure3.). Residues on iNOS with number from 314 to 320 mainly contribute to the binding, especially GLU317, which could form eight hydrogen bonds (salt bridges) with ASN97, ARG99 and ARG107 on AR (Figure1 B

and Figure 3). Therefore, docking and Molecular Dynamics (MD) simulation results again suggested that AR could form complex with iNOS, and ARG99 of AR and GLU317 of iNOS were believed to play important roles in the binding (Figure 1 B and Figure 3).

3.3 Analysis of the interaction between AR and iNOS in HLEC

To biochemically confirm the results of docking and MD simulation that AR could form complex with iNOS, we using the Co-IP experiments for AR and iNOS. As shown in Figure 4, the experimental results of Co-IP showed that the control group and transfection group exhibited positive hybridizing bands at 37KD. As expected, preadsorbed iNOS has immunoprecipitated with AR in cells both in control group and transfection group. Probing the blots for total AR verified that AR reduced the level of total iNOS in transfection group, as evidenced by the AR and iNOS signal in the Co-IP lanes. The expression of AR was up regulation after transfection with pEGFP-N1-AR compared with control group, the differences were statistically significant ($p < 0.01$), but expression of iNOS was decreased after the up regulation of AR expression compared with the control group, so it showed that there might be inhibition interaction between AR and iNOS.

3.4 Analyses of the interaction distance between AR and iNOS in HLEC by FRET

In FRET, we selected the pEGFP-N1-AR as a donor, and Cy3-iNOS as a receptor and FRET receptor photo-bleaching technique to further validate the interaction between AR and iNOS. Using laser confocal fluorescence microscopy, AR and iNOS labeled with pEGFP and Cy3, respectively, significant fluorescence of pEGFP and Cy3 was observed in cell. AR and iNOS were assayed for interaction via co-

localization using FRET (Figure 5). ROI in the cytoplasm was treated with FRET for regional co-localization. The interaction distance between AR and iNOS was evaluated based on altered fluorescence intensity in each channel. Receptor fluorescence intensity of Cy3 decreased rapidly in the control group, transfection group, after photo-bleaching. Donor fluorescence intensity rapidly increased while the receptor Cy3 intensity plummeted in transfection group. The results demonstrated FRET phenomenon between the pEGFP-AR (donor) and Cy3-iNOS (receptor) (Figure6). FRET rate E was calculated using the photo-bleaching formula. In Figure 6, the energy transfer rate of transfection group was $38.37 \pm 4.76 \%$, compared with the control group at $4.23 \pm 1.04 \%$, with significant differences ($p < 0.01$). We computed the energy transfer rate using the formula $E^{-1} = 1 + (R / R_0)^6$ to calculate the interaction distance between AR and iNOS, as shown in Figure 7. The transfection group distance R was $6.50 \pm 0.22 \text{ nm}$, and compared with the control group distance R was $10.14 \pm 0.44 \text{ nm}$; the differences were statistically significant ($p < 0.01$).

3.5 DISCUSSION

This investigation is the first to provide the evidence that there is a direct inhibition interaction between AR and iNOS in HLEC. The major findings in this paper that are:

- 1). AR and iNOS exhibited significant inhibitory interaction in enzyme kinetics test;
- 2). AR could form complex with iNOS, and ARG99 of AR and GLU317 of iNOS were believed to play important roles in the binding in docking and MD simulation.
- 3). AR could be co-immunoprecipitated with iNOS in cell through Co-IP; 4). The interaction distance of AR and iNOS in HLEC was 6.5nm ($< 10\text{nm}$) using FRET.

Now there are a number of hypotheses to explain why the hyperglycemia in diabetic could lead to diabetic complication of DC. Among them three received strong

experimental supports. They are Polyol pathway, oxidation mechanism and glycation pathway [42-45]. The three mechanisms contributed to the pathogenesis of DC in varying degree. As reported that there was interrelation between Polyol pathway and oxidation mechanism [46]. And AR and iNOS play important roles in the Polyol pathway and oxidation mechanism respectively in pathogenic mechanisms of DC [47-49]. The increase of glucose through AR in the Polyol pathway lead to consequent expenditure to redox state change, but the production of NO through iNOS in oxidation mechanism suppress the depletion of NADPH to release this state [50-51]. So the Polyol pathway and oxidation mechanism in DC could combine through AR and iNOS, but rarely research reports of a shared mechanism through AR and iNOS in DC, so this paper starts from the connection between AR and iNOS to investigate a new pathogenic mechanism for DC, and studying of DC targeting on the pathogenic mechanisms to find new treatment for DC apparently is necessary.

Now AR was reported that its inhibitors could cause an increase in expression of inflammatory markers such as iNOS and cyclooxygenase-2 [52], and AR was reported suppress the transcription of iNOS through other mediating factors such as NF- κ B [19]. In this paper, we found the inhibition effect between AR and iNOS not through other mediating factors by enzyme kinetics, and there was a significant inhibition effect between AR and iNOS according to the results of enzyme kinetics. In enzyme kinetics, we choose gigantol as the substrate to investigate the inhibitory effect between AR and iNOS. We found gigantol is a multi-target therapeutic drug for AR and iNOS, which can improved the lens opacification significantly through inhibit the activity and gene expression of AR and iNOS in LEC simultaneously [32-33, 53]. And the result of enzyme kinetics found that there was shown an inhibition effect

between AR and iNOS, according to this, an inhibition effect was existed between AR and iNOS in extracellular.

To preliminarily validate the interface of AR and iNOS, we performed docking and the MD simulation to reveal it. The docking and the MD simulation were used to reveal more atom-level details of the molecular mechanism underlying the interaction of AR and iNOS. Thus, docking and the MD simulation were effective used to show protein-ligant interaction and obtain accurate indication of the dynamic evolution of the binding site interactions [54]. And there was reported that docking and the MD simulation was used to investigate the connection between biological activity and conformational changes of AR or iNOS with their inhibitors[55-56], but the conformational changes and binding modes between AR and iNOS have not been reported. In this paper, ten complex structures were obtained through docking and eight stable ones were sorted out to conduct MD simulations to find out key residues in the interface by detecting hydrogen bonds and salt bridges. The interface of the complex was supposed to consist of twenty-six residues on AR and twenty-nine residues. Residues with number from 92 to 100 on AR and residues with number from 314 to 320 on iNOS were believed to be the critical areas for binding. Among them, ARG99 on AR and GLU317 on iNOS were the most important residues with the former forming six hydrogen bonds with GLU317 and ALA162 on iNOS and the latter forming eight hydrogen bonds (salt bridges) with ASN97, ARG99 and ARG107 on AR.

To further confirm the result of enzyme kinetics, docking and the MD simulation experiment, we used the assays of Co-IP and FRET experiment with AR and iNOS. In the two experiments, the up regulation of AR expression was transfected with pEGFP-

N1-AR plasmid into HLECs to form the DC model, and pEGFP-Cy3 was used as fluorescent matching markers in FRET. In Co-IP, the band of AR showed AR and iNOS had been co-immunoprecipitated simultaneously, and level of iNOS was diminished with up regulation of AR. Indeed, this study was demonstrated that AR could suppress the expression of iNOS as the previous study that over-expression of AR could inhibit the TNF α -induced iNOS expression [19]. And the FRET with photo-bleaching revealed the interaction between AR and iNOS through the distance between the two proteins in cells [57], and showed that the co-localization of AR-iNOS, and the distance of AR and iNOS is 6.5 nm to explain the interaction between AR and iNOS. Previous study demonstrated that AR or iNOS in HLECs could lead lens opacity to cataract through polyol pathway and oxidation mechanism [32-33], and the connection of two pathogenic mechanisms had some reports that lead to the same products such as reactive oxygen species, and AR-iNOS was regulated by the same medium such as NF-kB showing an indirect inhibition effect [58-59]. This study also showed the inhibition effect between AR and iNOS, and provided the new evidence of the direct interaction between AR and iNOS for the first time. From this results, taking AR and iNOS as a whole is a new idea for research about the new pathogenic mechanism and therapeutic targets of DC. AR inhibitors and iNOS inhibitors are not the only treatment for the up regulation of AR and iNOS expression in DC, and a new drug targets of AR-iNOS become the new drug treatment for DC. It also showed that osmotic pressure mechanism, oxidative stress mechanism in DC were not only influence each other indirectly, and leading a new common mechanism by taking AR-iNOS as a whole that this two mechanism could interact through the direct interaction between AR and iNOS.

4. Conclusions

In this work, AR and iNOS were found a significant inhibition interaction extracellular. It was found that AR may repress the activity of iNOS, and there was a direct inhibition interaction between AR and iNOS in HLECs. This finding demonstrate a new pathogenic mechanism of the interaction of AR and iNOS and a new research targets in DC, and may cast light on a new potential role for AR and iNOS in DC, leading to a better understanding of clinical drug in DC.

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References:

1. S. Rusnak, J. Vrzalova, M. Sobotova, L. Hecova, R. Ricarova, O. Topolcan, The Measurement of Intraocular Biomarkers in Various Stages of Proliferative Diabetic Retinopathy Using Multiplex xMAP Technology, Journal of Ophthalmology Volume 424783 (2015) 1-6.
2. P. Alexiou, K. Pegklidou, M. Chatzopoulou, I. Nicolaou, V.J. Demopoulos, Aldose reductase enzyme and its implication to major health problems of the

21(st) century, *Current Medicinal Chemistry*. 16 (2009) 734–752.

3. H. Ochiai, N. Kanemaki, R. Sato, K. Onda, Distribution, Molecular Structure and Functional Analysis of Carnitine Transporter (SLC22A5) in Canine Lens, Epithelial Cells *Exp Anim*. 63 (2014) 467–73.
4. V.A. Padgaonkar, V.R. Leverenz, A.V. Bhat, S.E. Pelliccia, F.J. Giblin, Thioredoxin reductase activity may be more important than GSH level in protecting human lens epithelial cells against UVA light, *Photochem Photobiol*. 91(2015) 387-96.
5. M. Lorenzi, The polyol pathway as a mechanism for diabetic retinopathy: attractive elusive, and resilient. *Exp Diabetes Res*. 2007 (2007) 61038.
6. S. Shin, S. Na, O.S. Kim, Y.S. Choi, S.H. Kim, Y.J. Oh, Effect of pneumoperitoneum on oxidative stress and inflammation via the arginine pathway in rats, *Yonsei Med. J*. 57 (2016) 238-46.
7. S.A. Kandarakis, C. Piperi, F. Topouzis, A.G. Papavassiliou, Emerging role of advanced glycation-end products (AGEs) in the pathobiology of eye diseases, *Prog. Retin. Eye Res*. 42 (2014) 85-102.
8. J.H. Kinoshita, S. Fukushi, P. Kador, L.O. Merola, “Aldose reductase in diabetic complications of the eye,” *Metabolism*. 28 (1979) 462–469.
9. A.W. Stitt, “The Maillard reaction in eye diseases”, *Annals of the New York Academy of Sciences*. 1043 (2005) 582–597.
10. T. Ookawara, N. Kawamura, Y. Kitagawa, N. Taniguchi, “Site-specific and random fragmentation of Cu, Zn-superoxide dismutase by glycation reaction. Implication of reactive oxygen species”, *Journal of Biological Chemistry*. 267 (1992) 18505–18510.

11. Y. Hashimoto, S. Yamagishi, H. Mizukami, C. Yabe-Nishimura, S.W. Lim, H.M. Kwon, S. Yagihashi, Polyol pathway and diabetic nephropathy revisited: Early tubular cell changes and glomerulopathy in diabetic mice overexpressing human aldose reductase, *J. Diabetes Investiq.* 2 (2011) 111-22.
12. T.L. Poulos, H. Li, Structural basis for isoform-selective inhibition of nitric oxide synthase, *Acc. Chem. Res.* 46 (2013) 390-398.
13. R. Ananthakrishnan, Q.T. Li, A.M. Gomes, R. Schmidt, Ramasamy, Aldose reductase pathway contributes to vulnerability of aging myocardium to ischemic injury, *Exp. Gerontol.* 46 (2011) 762-7.
14. R.B. Silverman, H. Huang, M.A. Marletta, P. Martasek, Selective inhibition of neuronal nitric oxide synthase by N omega-nitroarginine and phenylalanine-containing dipeptides and dipeptide esters, *J. Med. Chem.* 40 (1997) 2813–2817.
15. M. Barua, Y. Liu, M.R. Quinn, Taurine chloramine inhibits inducible nitric oxide synthase and TNF- α gene expression in activated alveolar macrophages: decreased NF- κ B activation and I κ B kinase activity, *J. Immunol.* 167 (2001) 2275-81.
16. A.Y. Lee, S.K. Chung, S.S. Chung, Demonstration that polyol accumulation is responsible for diabetic cataract by the use of transgenic mice expressing the aldose reductase gene in the lens, *Proc. Natl. Acad. Sci.* 92 (1995) 2780-2784.
17. H.J. Shin, Y.H. Park, S.U. Kim, H.B. Moon, D.S. Park, Y.H. Han, C.H. Lee, D.S. Lee, I.S. Song, D.H. Lee, et al. Hepatitis B virus X protein regulates hepatic glucose homeostasis via activation of inducible nitric oxide synthase, *J. Biol. Chem.* 286 (2011) 29872-29881.
18. S. Shinozaki, C.S. Choi, N. Shimizu, M. Yamada, M. Kim, T. Zhang, G.

- Shiota, H.H. Dong, Y.B. Kim, M. Kaneki, Liver-specific inducible nitric-oxide synthase expression is sufficient to cause hepatic insulin resistance and mild hyperglycemia in mice, *J. Biol. Chem.* 286 (2011) 34959-34975.
19. J. Zhao, T. Jiang, H. Li, Y. Zhang, N. Zhang, Aldose reductase regulates TNF- α -induced inducible nitric oxide synthase expression in human mesangial cells, *Mol. Biol. Rep.* 39 (2012) 1815-1822.
 20. R. Maccari, R.M. Vitale, R. Ottanà, M. Rocchiccioli, A. Marrazzo, V. Cardile, A.C. Graziano, P. Amodeo, U. Mura, A. Del, Corso, Structure-activity relationships and molecular modelling of new 5-arylidene-4-thiazolidinone derivatives as aldose reductase Inhibitors and potential anti-inflammatory agents, *Eur. J. Med. Chem.* 81 (2014) 1-14.
 21. M.K. Varsha, R. Thiagarajan, R. Manikandan, G. Dhanasekaran, Vitamin K1 alleviates streptozotocin-induced type 1 diabetes by mitigating free radical stress, as well as inhibiting NF- κ B activation and iNOS expression in rat pancreas, *Nutrition.* 31 (2015) 214-222.
 22. K.V. Ramana, B. Friedrich, A. Bhatnagar, S.K. Srivastava, Aldose reductase mediates cytotoxic signals of hyperglycemia and TNF- α in human lens epithelial cells, *FASEB. J.* 17 (2003) 315-317.
 23. H.G. Seo, T. Nishinaka, C. Yabe-Nishimura, Nitric oxide up-regulates Aldose Reductase expression in rat vascular smooth muscle cells: a potential role for aldose reductase in vascular remodeling, *Mol. Pharmacol.* 57 (2000) 709-717.
 24. M. Porta, F. Bandello, Diabetic retinopathy A clinical update, *Diabetologia.* 45 (2002) 1617-1634.
 25. J. Ruberte, E. Ayuso, M. Navarro, A. Carretero, V. Nacher, V. Haurigot, M.

- George, C. Llombart, A. Casellas, C. Costa, A. Bosch, F. Bosch, Increased ocular levels of IGF-1 in transgenic mice lead to diabetes-like eye disease, *J. Clin. Invest.* 113 (2004) 1149-1157.
26. R. Cao, J. Cheng, Integrated protein function prediction by mining function associations, sequences, and protein-protein and gene-gene interaction networks, *Methods.* 93 (2016) 84-91.
 27. J. Mehla, J.H. Caufield, P. Uetz, The yeast two-hybrid system: a tool for mapping protein-protein interactions, *Cold Spring Harb Protoc.* 2015 (2015) 425-430.
 28. P. Chen, C. Wang, X. Ma, Y. Zhang, Q. Liu, S. Qiu, Q. Liu, J. Tian, J. Ni, Direct Interaction of Selenoprotein R with Clusterin and Its Possible Role in Alzheimer's Disease, *PLoS One.* 8 (2013) e66384.
 29. L.G. Fröhlich, P. Kotsonis, H. Traub, S. Taghavi-Moghadam, N. Al-Masoudi, H. Hofmann, H. Strobel, H. Matter, W. Pfeleiderer, H.H. Schmidt, Inhibition of neuronal neuronal nitric oxide synthase by 4-amino pteridine derivatives: structure-activity relationship of antagonists of (6R)-5,6,7,8-tetrahydrobiopterin cofactor, *J. Med. Chem.* 42 (1999) 4108-4121.
 30. T. Onoda, C. Ishikawa, T. Fukazawa, W. Li, M. Obayashi, K. Koike, Inhibitory activities of selected Kampo formulations on human aldose reductase. *BMC Complement Altern Med*, 14 (2014) 435.
 31. N. Halder, S. Joshi, SK. Gupta, Lens aldose reductase inhibiting potential of some indigenous plants. *J Ethnopharmacol*, 86 (2003) 113-116.
 32. H. Fang, X.H. Hu, M.L. Wang, W.C. Wan, Q.H. Yang, Anti-osmotic and antioxidant activities of gigantol from *Dendrobium aurantiacum* var. *denneanum* against cataractogenesis in galactosemic rats, *Journal of Ethnopharmacology.* 172

(2015) 238-246.

33. J. Wu, C. Lu, X. Li, H. Fang, W. Wan, Synthesis and biological evaluation of novel gigantol derivatives as potential agents in prevention of diabetic cataract. *Plos One*, 10 (2015) e0141092.
34. M. Torchala, P.A. Bates, Predicting the structure of protein-protein complexes using the SwarmDock Web Server, *Methods Mol. Biol.* 1137 (2014) 181-197.
35. H.M. Wang, G.X. Li, H.S. Zheng, X.Z. Wu, Protective effect of resveratrol on lens epithelial Cell apoptosis in diabetic cataract rat, *Asian. Pac. J. Trop. Med.* 8 (2015) 153-156.
36. W. Humphrey, A. Dalke, K. Schulten, VMD: visual molecular dynamics, *J. Mol. Graph.* 1 (1996) 33-38.
37. J.C. Phillips, R. Braun, W. Wang, J. Gumbart, E. Tajkhorshid, E. Villa, C. Chipot, RD. Skeel, L. Kale, K. Schulten, Scalable molecular dynamics with NAMD, *J. Comput. Chem.* 26 (2005) 1781-1802.
38. P.C. Whitford, J.K. Noel, S. Gosavi, A. Schug, K.Y. Sanbonmatsu, J.N. Onuchic, An all-atom structure-based potential for proteins: bridging minimal models with all-atom empirical forcefields, *Proteins.* 75 (2009) 430-441.
39. S.Y. Hwang, J.S. Hwang, S.Y. Kim, I.O. Han, O-GlcNAcylation and p50/p105 binding of c-Rel are dynamically regulated by LPS and glucosamine in BV2 microglia cells, *British Journal of Pharmacology.* 169 (2013) 1551–1560.
40. S. Rashidipour, S. Naeeminejad, J. Chamani, Study of the interaction between DNP and DIDS with human hemoglobin as binary and ternary systems: spectroscopic and molecular modeling investigation, *J. Biomol. Struct. Dyn.* 34 (2016) 57-77.

41. A.D. Elder, A. Domin, G.S. Kaminski Schierle, C. Lindon, J. Pines, A quantitative protocol for dynamic measurements of protein interactions by Förster resonance energy transfer-sensitized fluorescence emission, *J. R. Soc.* 24 (2008) 59-81.
42. Yabe-Nishimura. Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications. *Pharmacological reviews*, 50 (1998) 21-33.
43. T. Nishikawa, D. Edelstein, X.L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M.A. Yorek, D. Beebe, P.J. Oates, H.P. Hammes, I. Giardino, M. Brownlee, Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage, *Nature*. 404 (2000) 787-790.
44. H. Vlassara, J. Uribarri, Advanced glycation end products (AGE) and diabetes: cause, effect, or both? *Curr. Diab. Rep.* 14 (2014) 453.
45. A.G. Miranda-Díaz, L. Pazarín-Villaseñor, F.G. Yanowsky-Escatell, J. Andrade-Sierra, Oxidative Stress in Diabetic Nephropathy with Early Chronic Kidney Disease, *J. Diabetes. Res.* 2016 (2016) 7047238.
46. C. Yabe-Nishimura, Aldose reductase in glucose toxicity: a potential target for the prevention of diabetic complications, *Pharmacol. Rev.* 50 (1998) 21-33.
47. A.S. Grewal, S. Bhardwaj, D. Pandita, V. Lather, B.S. Sekhon, Updates on aldose reductase inhibitors for management of diabetic complications and nondiabetic diseases, *Mini. Rev. Med. Chem.* 16 (2016) 120-162.
48. M. Kawakami, M. Kuroki, Aminoguanidine and other AGE inhibitors, *Nihon Rinsho*. 60 (2002) 601-605.
49. A. Pollreis, U. Schmidt-Erfurth, Diabetic Cataract—Pathogenesis, Epidemiology and Treatment, *Journal of Ophthalmology Volume*. 2010 (2010)

608751.

50. N.E. Cameron, M.A. Cotter, K.C. Dines, E.K. Maxfield, Pharmacological manipulation of vascular endothelium function in non-diabetic and streptozotocin-diabetic rats: effects on nerve conduction, hypoxic resistance and endoneurialcapillarization. *Diabetologia*, 36 (1993) 516–522.
51. M.J. Stevens, J. Dananberg, E.L. Feldman, S.A. Lattimer, M. Kamijo, T.P. Thomas, H. Shindo, A.A. Sima, D.A. Greene, The linked roles of nitric oxide, aldose reductase and, (Na⁺,K⁺)-ATPase in the slowing of nerve conduction in the streptozotocin diabetic rat, *J. Clin. Invest.* 94 (1994) 853–859.
52. U.C. Yadav, M. Shoeb, S.K. Srivastava, K.V. Ramana, Aldose reductase deficiency protects from autoimmune- and endotoxin-induced uveitis in mice, *Invest. Ophthalmol. Vis. Sci.* 52 (2011) 8076-8085.
53. Z. Yu, C. Gong, B. Lu, L. Yang, Y. Sheng, L. Ji, Z. Wang, *Dendrobium chrysotoxum* Lindl Alleviates Diabetic Retinopathy by Preventing Retinal Inflammation and Tight Junction Protein Decrease, *Journal of Diabetes Research*. 2015 (2015) 518317.
54. R.M. Almeida, S. Dell'Acqua, L. Krippahl, J.J. Moura, S.R. Pauleta, Predicting Protein-Protein Interactions Using BiGGER: Case Studies, *Molecules*. 21 (2016) DOI: 10.3390.
55. H. Zhang, J. Zan, G. Yu, M. Jiang, P. Liu, A Combination of 3D-QSAR, Molecular Docking and Molecular Dynamics Simulation Studies of Benzimidazole-Quinolinone Derivatives as iNOS Inhibitors, *Int. J. Mol. Sci.* 13 (2012) 11210-11227.
56. P. Antony, R. Vijayan, P. Antony, Identification of Novel Aldose Reductase

Inhibitors from Spices: A Molecular Docking and Simulation Study, PLoS One. 10 (2015) e0138186.

57. T. Ungvári, P. Gogolák, M. Bagdány, L. Damjanovich, L. Bene, Perrin and Förster unified: Dual-laser triple-polarization FRET (3polFRET) for interactions at the Förster-distance and beyond. *Biochim. Biophys. Acta*, 1863 (2016) 703-716.
58. Z.Y. Yu, B.C. Kone, The STAT3 DNA-binding domain mediates interaction with NF-KB p65 and inducible nitric oxide synthase transrepression in mesangial cells, *J. Am. Soc. Nephrol.* 15 (2004) 585–591.
59. U.C. Yadav, A.S. Naura, L. Aguilera-Aguirre, K.V. Ramana, I. Boldogh, S. Sur, H.A. Boulares, S.K. Srivastava, Aldose reductase inhibition suppresses the expression of Th2 cytokines and airway inflammation in ovalbumin-induced asthma in mice. *J. Immunol*, 183 (2009) 4723–4732.

Table1.The inhibition ratio of different concentrations of AR against iNOS in extracellular

enzyme AR (μM)	Inhibition ratio Gigantol($6\mu\text{M}$)/iNOS($3\mu\text{M}$)
2	61.01 ± 1.8756
4	69 ± 3.5689
8	76.8 ± 2.5696
16	89.68 ± 6.2582
32	92.79 ± 3.8691
IC50	0.04

Table2.The inhibition ratio of different concentrations of iNOS against AR in extracellular

enzyme iNOS (μM)	Inhibition ratio Gigantol(6μM)/AR(0.5 μM)
0.0625	52.15±2.5895
0.125	61.27±1.5892
0.25	70.77±6.2591
0.5	80.28±5.5691
1	85.56±4.3656
IC50	0.042

Figure 1. A. Top ten complex structures with the lowest energies: Ten structures were shown in New Cartoon with AR aligned in docking. AR was shown in blue, iNOS of complex 1 was shown in red and iNOS of complex 2 to 10 was shown in light red. B. Critical residues in the interface between AR and iNOS obtained through docking and MD simulation. AR and iNOS of complex 1 were shown in New Cartoon, with AR in silver and iNOS in green. THR92 to GLU100 on AR were shown in red and TYR314 to LYS320 were shown in blue. Those two regions were believed to mainly contribute to the binding. Two critical residues, ARG99 and GLU317, were shown in Visual Molecular Dynamics.

Figure 2. Variation of RMSD versus simulation time for ten complexes of AR and iNOS through docking.

Figure3.A. Number of hydrogen bonds and salt bridges for each residue on AR. Red dotted lined showed the main binding sites. B. Number of hydrogen bonds and salt bridges for each residue on iNOS. Red dotted lined showed the main binding sites.

Figure 4. Co-immunoprecipitation of AR with iNOS in HLECs

HLECs were transfected with pEGFP-N1-AR plasmid: A. Control group; B. Transfection group (transfection with pEGFP-N1-AR); (1) the up regulation of AR expression in HLECs treated with transfection pEGFP-N1-AR for 24h through western blot; (2) AR immune complex were immunoprecipitated with iNOS antibody and analysed for the presence of AR by western blot with AR antibody. AR-IP (immunoprecipitation): Western blot results of AR after treatment with anti-iNOS antibody in IP; INPUT: Western blot results of internal reference GAPDH. (3) Western blot analysis was performed as mentioned in Materials and Methods. Levels of AR protein was also up regulated on immunoblot of cell extracts 24h after transfection with pEGFP-N1-AR from duplicate samples. And Levels of iNOS was diminished on immunoblot after up regulation of AR expression in HLECs. Histogram represents the data from three independent experiments. ** $P < 0.01$, compared with the expression of protein in the same group; ## $P < 0.01$, compared with the control group.

Figure 5. Cell imaging with photo-bleaching reveals the interaction between AR and iNOS in cells analyzed by FRET (A) transfection with plasmid pEGFP-N1-AR (donor), resulted in green fluorescence of the whole cells, indicating adequate AR expression in HLEC cells; (B) iNOS labeled with Cy3 (receptor), with the whole cell showing red fluorescence, indicating iNOS expression in HLEC cells; (C) co-localization of the two proteins showing yellow fluorescence. The ROI in cell (red arrow box) was subjected to laser for photo-bleaching of pEGFP-Cy3.

Figure 6.Quantification of photo-bleaching the Cy3-iNOS acceptor and the concomitant changes of fluorescence in pEGFP-N1-AR through FRET: Control group (transfected with pEGFP-N1 plasmid); Transfection group (transfected with pEGFP-N1-AR plasmid).

Figure 7. FRET efficiency and distance was measured between Cy3-iNOS and pEGFP-AR: (1) Efficiency of Energy transfer rate for Cy3-iNOS and pEGFP-N1-AR; (2) the interaction distance between Cy3-iNOS and pEGFP-AR; ** $p < 0.01$, compared with the control group;