Beneficial Effects of Combined AT_1 Receptor/Neprilysin Inhibition (ARNI) Versus AT_1 Receptor Blockade Alone in the Diabetic Eye

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Purpose. Dysfunction of the renin-angiotensin system (RAS) contributes to pathogenesis of diabetic retinopathy (DR). Yet RAS blockers have only limited beneficial effects on progression of DR in clinical trials. The natriuretic peptide system offsets RAS, so that enhancing the activity of this system on top of RAS blockade might be beneficial. Neprilysin has an important role in the degradation of natriuretic peptides. Therefore, we hypothesize that dual angiotensin receptor-neprilysin inhibition (ARNI) may outperform angiotensin receptor blocker (ARB) in protection against DR. We tested this hypothesis in streptozotocin-induced diabetic transgenic (mRen2)27 rats.

Methods. Adult male diabetic (mRen2)27 rats were followed for 5 or 12 weeks. Treatment with vehicle, irbesartan (ARB), or ARB combined with the neprilysin inhibitor thiorphan (irbesartan+thiorphan [ARNI]) occurred during the final 3 weeks. Retinal cell death, gliosis, and capillary loss were evaluated. Real-time polymerase chain reaction (RT-PCR) analyses were performed to quantify the retinal level of inflammatory cell markers.

Results. Both ARB- and ARNI-treated groups showed similarly reduced retinal apoptotic cell death, gliosis, and capillary loss compared to the vehicle-treated group in the 5-week study. Treatment with ARNI reduced the expression of inflammatory markers more than ARB treatment in the 5-week study. In the 12-week study, ARNI treatment showed significantly more reduction in apoptotic cell death (51% vs. 25% reduction), and capillary loss (68% vs. 43% reduction) than ARB treatment.

Conclusions. Treatment with ARNI provides better protection against DR in diabetic (mRen2)27 transgenic rats, compared to ARB alone. This approach may be a promising treatment option for patients with DR.

Keywords: diabetes, diabetic retinopathy, renin-angiotensin system, AT1 receptor, angiotensin receptor blocker, irbesartan, neprilysin, thiorphan

Diabetes affects approximately 387 million people worldwide. Diabetic retinopathy (DR) is the most common complication of diabetes and the major cause of vision loss in middle-aged subjects.[1] The development of DR is strongly associated with the development of diabetic kidney disease, suggesting that the same pathogenic pathway underlies these complications.[2] The renal renin-angiotensin system (RAS) is upregulated in diabetes, evidenced by increased tissue levels of angiotensin II, the main effector peptide of the RAS. Elevated levels of angiotensin II contribute to pathogenic processes, such as inflammation, vascular remodeling, and oxidative stress,[3] that are associated with various renal and cardiovascular disorders, like heart failure, hypertension, diabetes, and other metabolic disorders.[4,5] Diabetic retinopathy is associated with activation of the local RAS in the eye, evidenced by an increase in retinal angiotensin II levels.[6,7] Blockers of the RAS, like angiotensin-converting enzyme inhibitors (ACEi) and angiotensin II type 1 receptor blockers (ARBs) provide protection against the progression of cardiovascular disorders, diabetic kidney disease, and diabetic retinopathy.[8] Still, these drugs are unable to completely reverse or halt the progression of these complications. The natriuretic peptide system regulates diuresis, natriuresis, and vasodilatory functions of the cardiovascular system, and it offsets the RAS, so that enhancing the activity of this system on top of RAS blockade might be beneficial.[9] Neprilysin (NEP) is a critical enzyme for the breakdown of a variety of substrates, including vasodilators, vasoconstrictors, and natriuretic peptides. The individual use of NEP inhibitors, however, did not prove to be very beneficial for the treatment of heart failure and other cardiovascular disorders, mainly due to its broad action on different vasodilators and vasoconstrictors. When a NEP inhibitor is combined with an angiotensin receptor blocker (the combination is referred to as "ARNI"), the balance shifts to the positive, blood pressure–lowering side. Indeed, ARNI has been shown to be more effective than RAS blockade alone in (1) reducing blood pressure,[10] (2) improving morbidity and mortality in heart failure,[11–13] and (3) improving surrogate cardiovascular outcomes in diabetic patients.[14,15] In diabetic, hypertensive rats, we have shown previously that ARNI lowers proteinuria and the development of focal segmental glomerulosclerosis, common features of diabetic kidney disease, more than ARB

TABLE 1. Real-Time PCR Primers

Gene	Accession Number	Sequences
ICAM-1	NM_012967	5′ CCCCACCTACATACATTCCTAC 3′ 5′ ACATTTTCTCCCAGGCATTC 3′
TNF-α	NM_012675	CCTTATCTACTCCCAGGTTCTC TTTCTCCTGGTATGAAATGGC
VEGF	NM_031836	TGC ACC CAC GAC AGA AGG GGA TCA CCG CCT TGG CTT GTC ACA T
MCP-1	NM_031550	GCAGCAGGTGTCCCAAAGAAGCT AGAAGTGCTTGAGGTGGTTGTGGAA

alone. This effect may even be independent of blood pressure.[16] Therefore, we hypothesize that dual inhibition also may provide better protection against DR, compared to ARB alone. We tested this hypothesis in transgenic (mRen2)27 (Ren2) rats made diabetic with streptozotocin (STZ). This is a well-known model of diabetic retinopathy.[17–20] We made use of the ARB irbesartan and the NEP inhibitor thiorphan.[16,21]

METHODS

Animal Studies

Heterozygous male Ren2 rats (age 10 weeks, weight 300–500 g) were obtained by crossing homozygous Ren2 and Sprague-Dawley (SD) rats. Only male Ren2 rats were used for this particular study as the females of this transgenic model develop a much milder phenotype.[22] Studies were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and under the regulation and permission of the Erasmus MC Animal Care Committee. Diabetes mellitus was induced by administering streptozotocin (55 mg/kg intraperitoneal [IP]; Merck Millipore, Amsterdam, The Netherlands), and diabetic animals were studied for 5 or 12 weeks. In the 5-week group, heart rate and BP were measured by radiotelemetry transmitters, implanted 2 weeks before induction of diabetes.[23] Rats were checked daily for nonfasting blood glucose and β-ketone levels until day 3 after STZ injection, and thereafter once weekly or every other week (5- and 12-week groups, respectively; Precision Xceed; Abbott, Zwolle, The Netherlands). Only rats with glucose >15 mmol/L were considered diabetic; they received 2 to 4 U insulin per day (Levemir; Novo Nordisk, Bagsvaerd, Denmark). Rats in both groups were treated during the final 3 weeks of the study (i.e., during weeks 2–5 or 9–12) with vehicle (saline containing 0.2% dimethylsulfoxide [DMSO]), the ARB irbesartan (15 mg/kg.day; Sanofi-Aventis, Chilly-Mazarin, France), or the ARNI irbesartan+thiorphan (0.1 mg/kg.day diluted in DMSO; Sigma-Aldrich Corp., St. Louis, MO, USA) making use of osmotic minipumps (2ML4; ALZET, Cupertino, CA, USA). After 3 weeks of treatment, animals were killed by IP pentobarbital injection (200–240 mg/kg). Renal, cardiac, and vascular data on these animals have been reported previously.[16] Each experimental group had 7 to 12 rats. In addition, eyes also were obtained from 8 age-matched untreated nondiabetic Sprague-Dawley rats that were used as wild-type controls. Eyes were harvested at the end of the 5-week or 12-week study, one eye from each animal was fixed in 4% paraformaldehyde (PFA) for histologic analysis and the other eye was snap frozen in liquid nitrogen for protein and gene expression analysis.

NEP Activity Assay

Frozen eyes from each experimental group were dissected to isolate retinal tissue that then were homogenized by sonication in NEP assay buffer (100 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 μM ZnCl2). Neprilysin activity assay was performed using 5 μg of retinal protein in black 96-well opaque plates with the fluorogenic peptide substrate Mca-RPPGFSAFK(Dnp)-OH (Enzo Life Science, Farmingdale, NY, USA) added at a final concentration of 20 μM. The enzymatic reaction was done at 37°C in the presence and absence of the NEP inhibitor thiorphan at 2 μM concentration. The enzymatic reaction was recorded in a SpectraMax M3 fluorescence microplate reader (Molecular Devices, Sunnyvale, CA, USA) for 1 hour with excitation at 320 nm and emission at 405 nm as described previously.[24,25] All measurements were performed in duplicate and the data represent the means of three assay results.

Immunofluorescence

Eyes fixed in 4% PFA were processed for paraffin embedding. Paraffin sections (4 μm thick) were cut and mounted on superfrost slides. Sections were deparaffinized in xylene and decreasing concentration of alcohol. This was followed by an antigen retrieval step where the slides were boiled for 20 minutes in sodium citrate buffer, pH 6.0. The sections then were incubated in blocking solution (5% BSA+ 0.3 % Triton X-100 in PBS) for 1 hour. This was followed by incubation with different primary antibodies: rabbit anti-glial fibrillary acidic protein (GFAP 1:1000, Sigma-Aldrich Corp., St. Louis, MO, USA), rabbit anti-Iba-1 (1:200; DAKO, Carpinteria, CA, USA), mouse anti-Brn3a (1:200; Millipore, Billerica, MA, USA) diluted in the same blocking solution (overnight at 4°C). The sections then were incubated with the appropriate secondary antibodies conjugated to Alexa 488 or 594 (Molecular Probes/Invitrogen, Eugene, OR, USA) for 1 hour at RT. Sections were washed in PBS containing the nuclear counterstain DAPI (4′,6 diamidino-2-phenylindole), and mounted in Dako mounting media (DAKO). For TUNEL staining the In Situ Cell Death Detection Kit, TMR red (Roche Applied Science, Indianapolis, IN, USA) was used on paraffin sections in accordance with the manufacturer's instructions. The images were captured on a Keyence confocal microscope (KEYENCE Corporation, Itasca,

TABLE 2. Main Characteristics of DM Ren2 Rats That Were Treated for 3 Weeks With Vehicle, ARB or ARNI

Parameter	5 Wks. DM			12 Wks. DM		
	Vehicle	ARB	ARNI	Vehicle	ARB	ARNI
Body weight, g	448 ±13	452 ± 16	450 ± 11	448 ± 9	482 ± 20	430 ± 23
Blood glucose, mmol/L	27 ± 0.6	27 ± 0.7	27 ± 0.5	27 ± 0.3	25 ± 1.9	26 ± 0.7
Baseline mean arterial pressure, mm Hg	157 ± 10	160 ± 10	154 ± 10	–	–	–
Change in mean arterial pressure, mm Hg	−9.5 ± 4.3	−45.0 ± 7.8*†	−47.7 ± 7.9*†	–	–	–

Treatment was started either 2 weeks ("5 weeks DM") or 9 weeks ("12 weeks DM") after the induction of DM. Data are mean + SEM of 7 to 12 rats.
* $P < 0.05$ versus baseline.
† $P < 0.05$ versus vehicle.

IL, USA) or on a spinning disc confocal microscope (Ultra VIEW Vox; PerkinElmer, Waltham, MA, USA) using a ×20, ×40, and/or ×60 objective lens and were prepared for presentation using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA, USA). For GFAP quantification, digital images captured in Adobe Photoshop were adjusted similarly for brightness and contrast. Images were thresholded in ImageJ (National Institutes of Health [NIH], Bethesda, MD, USA) and the fluorescence intensity and area were quantified using its Measure module. For cell counts and GFAP expression, each quantification was performed on at least 5 sections from at least 6 animals (i.e., at least 30 sections each experimental group).

Western Blot Analysis

To quantify the level of GFAP expression in the different experimental groups, retinal tissues were collected in cold RIPA buffer (Sigma-Aldrich Corp.) supplemented with protease inhibitors and were homogenized by sonication. Homogenized tissues were centrifuged at 12,000*g* for 15 minutes at 4°C and the supernatant was collected. The protein concentration was detected by Bio-Rad Protein Assay kit (Bio-Rad Life Sciences, Hercules, CA, USA). Protein samples (20 μg) were loaded and separated on 10% gels for 1 hour at 120 V in Tris-glycine buffer and electrophoretically transferred onto polyvinylidene difluoride membrane. Immunodetection was performed on blots blocked in fluorescence-blocking buffer for 1 hour (Rockland Immunochemicals, Inc., Gilbertsville, PA, USA) and then incubated with primary (rabbit anti- GFAP; 1:2000; Sigma-Aldrich Corp.), and secondary (goat anti-rabbit IR Dye 800; 1:5000; Rockland Immunochemicals, Inc.) antibodies. β-Actin antibody (mouse anti-β-actin; 1:5000; Sigma-Aldrich Corp.) immunoblotting was used as a loading control. Immunoblots were visualized and quantified by using a Li-Cor odyssey infrared imager after normalizing it to β-Actin (Odyssey; Li-Cor, Lincoln, NE, USA).

Trypsin Digest Preparations of Retinal Vasculature

Trypsin digest preparations of retinal vasculature were made as described previously.[26] Briefly, the retinas were dissected from the 4% PFA fixed eye cups, washed in water for an hour, and digested in 3% trypsin for 2 to 3 hours at RT. The tissue then was transferred into water and the network of vessels was freed from adherent retinal tissue by gentle shaking and manipulation under a dissection microscope. The vessels then were mounted on clean slides and allowed to dry and stained with periodic acid Schiff-hematoxylin and eosin (PAS-H&E; Gill No.3; Sigma-Aldrich Corp.). After the tissue was stained and washed in water, it was dehydrated and mounted (Permount mounting media; Fisher Scientific, Pittsburgh, PA). The prepared retinal vessels were photographed by a Zeiss microscope equipped with a high-resolution digital camera (AxioCam, MRC5, Zeiss Axionvert 200; Carl Zeiss Meditec, Jena, Germany) using ×20 and ×40 objective lenses, 6 to 8 representative nonoverlapping fields from each quadrant of the retina were imaged. Acellular capillaries were counted from images for each retina and expressed as the number of acellular vessels per square millimeter.

Real-Time PCR Analysis

Total RNA was isolated from frozen rat retinal samples using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instruction. Reverse transcription was performed using Enhanced Avian HS RT-PCR kit (Sigma-Aldrich Corp.) following manufacturer's instructions. Real-time PCR

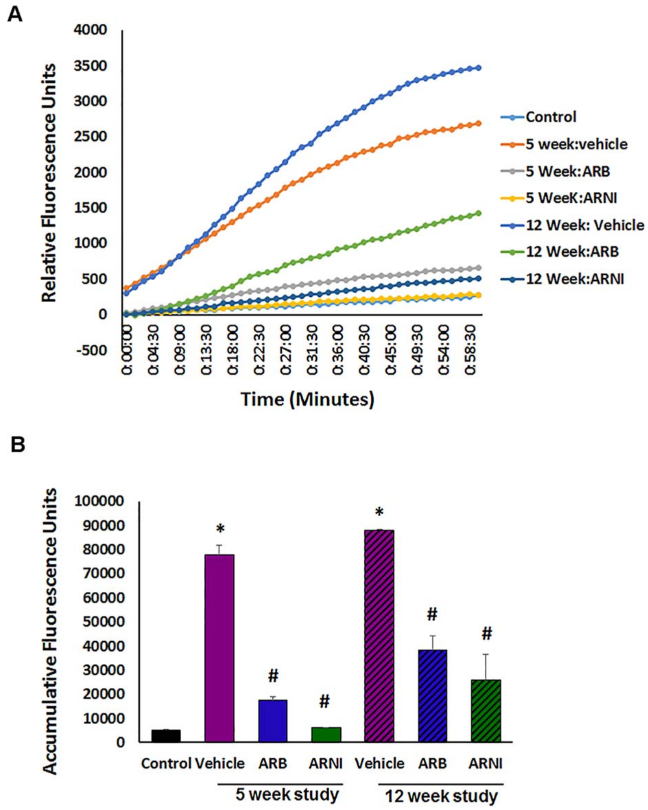

Figure 1. Neprilysin activity assay in STZ-induced diabetic Ren2 rat retinas and control retinas obtained from non-diabetic SD rats. (**A**) Relative fluorescence intensity and (**B**) Accumulative fluorescence intensity as a measure of NEP activity level using protein samples extracted from Ren-2 rat retinas show an increase in NEP activity in STZ-induced diabetic Ren2 rat retinal samples when compared to control nondiabetic SD rat retinal samples. Angiotensin receptor blocker and ARNI treatment resulted in reduction in the level of NEP activity. All measurements were performed in duplicate and the experiment was repeated at least twice with similar results. *$P < 0.05$ compared to control. #$P < 0.05$ compared to vehicle; $N = 3$ per group.

was done on a real-time thermal cycler (iCycler; Bio-Rad Life Sciences) using iQTM Syber Green Supermix (Bio-Rad Life Sciences). The threshold cycle number (Ct) for real-time PCR was set by the cycler software. Optimal primer concentration for PCR was determined separately for each primer pair. Each reaction was run in duplicate or in triplicate, and reaction tubes with target primers and those with actin primers always were included in the same PCR run. The expression levels of the different genes were established based on the Ct compared to control housekeeping gene β-actin in each sample (amount of target = $2^{-\Delta\Delta Ct}$) and presented as fold change. The rat specific primers used in this experiment are listed in Table 1.

Results

Induction of Diabetes Mellitus and General Physiologic Characteristics

Ren2 rats developed hypertension but had a normal blood glucose level, identical to that in SD rats as reported previously.[16,17] Streptozotocin-induced diabetes mellitus increased blood glucose levels (Table 2). Treatment with ARB or ARNI did not affect the glucose level nor the body weight, but significantly reduced mean arterial blood pressure (Table 2). Streptozotocin-induced diabetic retinopathy in Ren2 rats is

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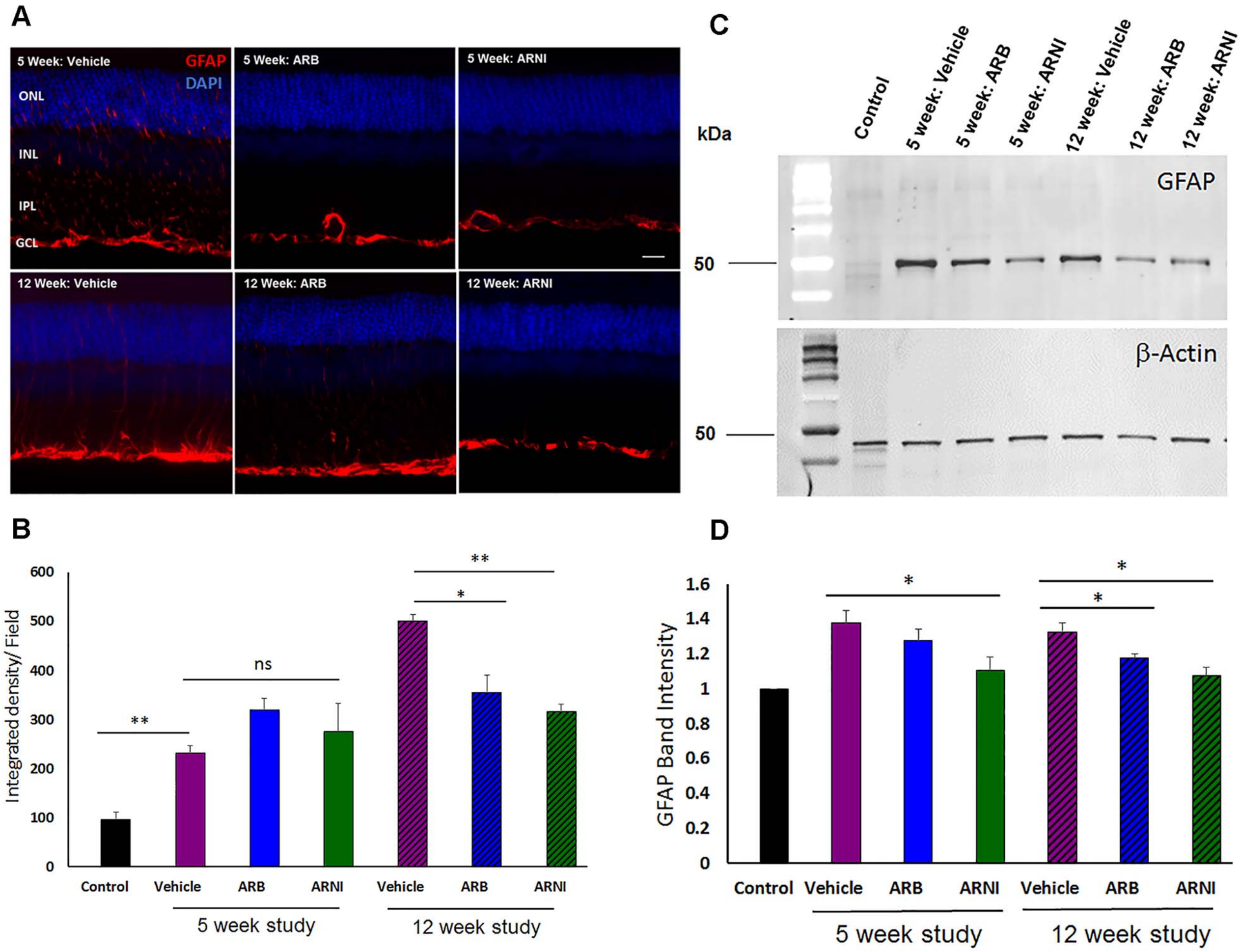

Figure 2. Immunofluorescence and Western blot detection of GFAP expression in retina. (**A**) GFAP immunostaining in 5-week (*top*) and 12-week (*bottom*) diabetic rats, treated for 3 weeks with vehicle, ARB, or ARNI. *Scale bar*: 20 μm. ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer. (**B**) Quantification of GFAP immunofluorescence in representative rat retinal sections from each experimental group, including nondiabetic (control) rats. $n = 8$ per group (5–6 sections/animal). (**C**) GFAP Western blot showing a reduction in GFAP expression in ARB- and ARNI-treated groups when compared to the vehicle group. *Upper*: GFAP and *bottom*: β-actin. (**D**) Quantification of GFAP band intensity in Western blot normalized to β-actin. $n = 4$. ns, not significant, $^*P < 0.05$, $^{**}P < 0.001$.

associated with a corresponding increase in the activity of NEP enzyme (Fig. 1). Treatment with ARB and ARNI reduced the level of NEP activity in the 5- and 12-week studies, with ARNI having a stronger effect than ARB in the 5-week study but appear to have almost a similar effect in the 12-week study (Fig. 1B).

ARNI Reduced Gliosis More Strongly Than ARB After 12 Weeks of Diabetes

Immunostaining for GFAP, an astrocyte-specific marker, was done in each group to evaluate the level of gliosis. In a normal healthy retina, GFAP expression is confined to the astrocytes at the inner limiting membrane. However, under pathologic conditions, like DR, GFAP expression is elevated and also observed in the Müller cell processes that extend across the retina from the inner limiting membrane to the outer limiting membrane. In a previous study in nondiabetic Ren2 rats, we observed that retinal GFAP expression is slightly elevated versus age-matched SD controls in astrocytes.[17] In diabetic Ren2 rats, retinal GFAP expression was greatly elevated, and seen along the Müller cell processes (Fig. 2). The longer the duration of diabetes, the higher the level of GFAP expression that was observed. Angiotensin receptor blocker– and ARNI-treated groups showed similarly reduced retinal gliosis compared to the vehicle-treated group in the 5-week study. In the 12-week study, the ARNI-treated group showed a marked reduction in the level of GFAP expression when compared to the vehicle and the ARB-treated group (Figs. 2A–D). Retinal sections from each group also were immunostained for Iba-1, which is a marker for activated microglial cells. In the 5-week study, ARB- and ARNI-treated groups displayed almost the same number of Iba-1–positive cells as the vehicle-treated group. In the 12-week study, the total number of Iba-1–positive cells was significantly ($P < 0.01$) reduced in the ARNI-treated group when compared to the vehicle and ARB-treated groups (Figs. 3A, 3B). Angiotensin receptor blocker treatment alone also reduced the total number of Iba-1–positive cells versus vehicle.

ARNI Is More Effective Than ARB in Reducing Apoptotic Cell Death After 12 Weeks of Diabetes

Paraffin sections from each group were processed for TUNEL labeling to evaluate retinal degeneration. Compared to the nondiabetic control group the diabetic vehicle-treated groups showed a marked increase in the number of cells undergoing apoptotic cell death in the 5-week (2.4-fold increase) and 12-week (2.8-fold increase) study. The ARB- and ARNI-treated groups showed similarly reduced apoptotic cell death com-

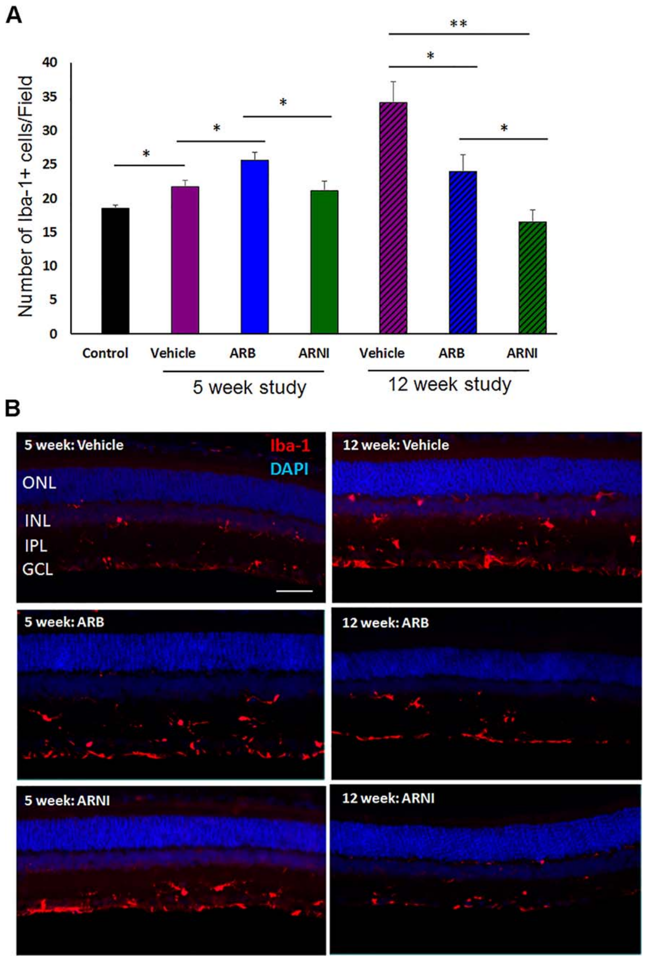

Figure 3. Immunofluorescence detection of activated microglial cells in retina. (**A**) Quantification of Iba-1 positive cells in the different experimental groups, including nondiabetic control rats. (**B**) Representative images of Iba-1 Immunostaining in rat retinal sections from different groups. *Scale bar*: 20 µm. $^{*}P < 0.05$. $n = 6$ per group (5–6 sections/animal).

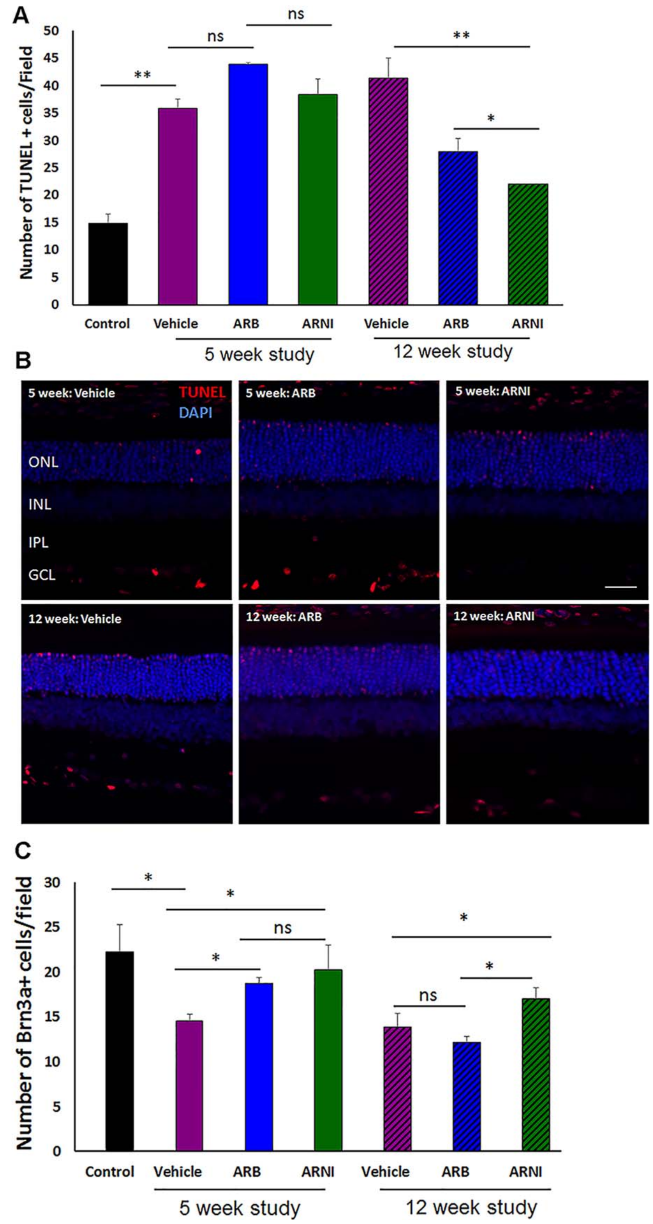

Figure 4. In situ cell death detection by TUNEL assay in retina. (**A**) Quantification of TUNEL-positive cells in the different experimental groups, including nondiabetic control rats. (**B**) Representative images of TUNEL immunostaining in rat retinal sections from different groups. (**C**) Quantification of Brn3a-positive cells in different experimental groups, including nondiabetic control rats. *Scale bar*: 20 µm. $^{*}P < 0.05$, $^{**}P < 0.001$, $n = 6$ per group (5–6 sections/animal).

pared to the vehicle-treated group in the 5-week study. In the 12-week study, the ARNI-treated group showed a marked reduction (51%) in the number of apoptotic cell deaths when compared to the vehicle-treated group, while the ARB-treated group showed only a 25% reduction versus vehicle (Figs. 4A, 4B). Immunostaining for Brn3a which is a specific retinal ganglion cell (RGC) marker was used to quantify the number of RGC in retinal sections from each experimental group as another measure for retinal neurodegeneration. Number of Brn3a+ cells in the control group was significantly higher when compared to the 5-week and 12-week vehicle-treated group. In the 5-week study, ARB and ARNI treatment resulted in a statistically significant increase in number of Brn3a+ RGC cells when compared to the vehicle-treated group. In the 12-week study ARNI-treated group showed an increase in number of Brn3a+ RGC cells while the ARB and vehicle-treated group showed a reduced Brn3a+ RGC cells (Fig. 4C).

ARNI Reduced Capillary Loss More Strongly Than ARB After 12 Weeks of Diabetes

We have reported previously that the Ren2 rat retina shows an increased loss of capillaries when compared to age-matched SD rats.[17] Streptozotocin-induced diabetes in Ren2 rats further exacerbated this loss. Angiotensin receptor blocker- and ARNI-treated groups showed a similar reduction in the number of acellular capillaries compared to vehicle-treated group in the 5-week study. In the 12-week study, ARNI treatment showed significantly more reduction in capillary loss (68% vs. 43% reduction) than ARB treatment (Fig. 5).

ARNI Decreased Expression Levels of Inflammatory Markers More Strongly Than ARB After Both 5 and 12 Weeks of Diabetes

Real-time RT-PCR was used to evaluate the expression level of inflammatory cytokines and angiogenic factors in the

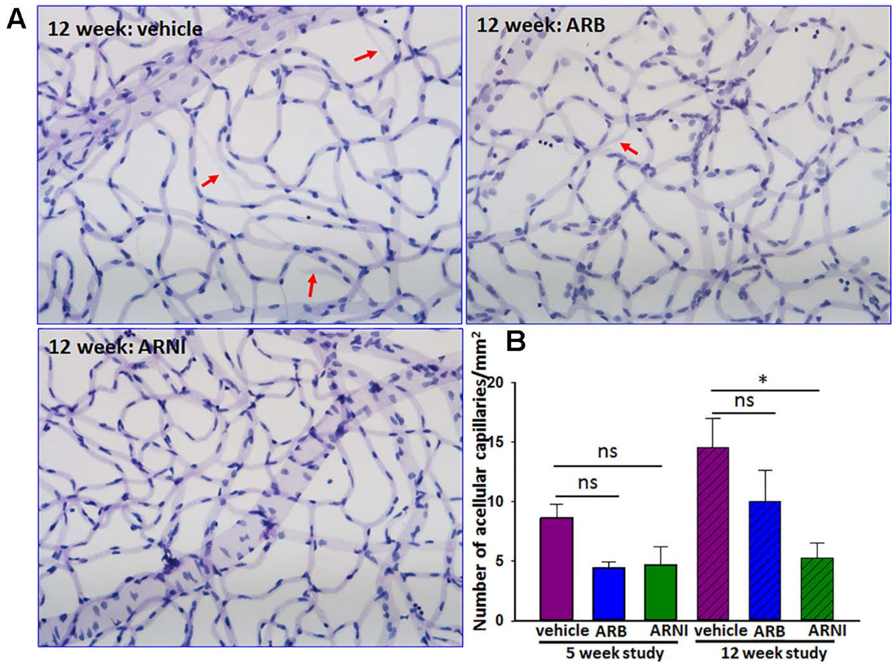

Figure 5. Histologic detection of acellular capillaries in retina. (**A**) Representative images of trypsin-digested retinal vascular preparations from different groups of the 12-week study. (**B**) Quantification of acellular capillaries in the different experimental groups. $^*P < 0.01$, $n = 4$ per group.

retina from each experimental group. In the 5-week study and the 12-week study ARB and ARNI treatment significantly reduced the mRNA levels of TNF-α, intercellular adhesion molecule-1 (ICAM-1), VEGF, and monocyte chemoattractant protein-1 (MCP-1) when compared to the vehicle treated groups (Fig. 6).

Discussion

The present study first showed that STZ-induced diabetes in Ren2 rats resulted in increased levels of NEP activity, capillary loss, inflammatory cytokine expression, gliosis, and neuronal apoptotic cell death in the retina, which all are indicators of

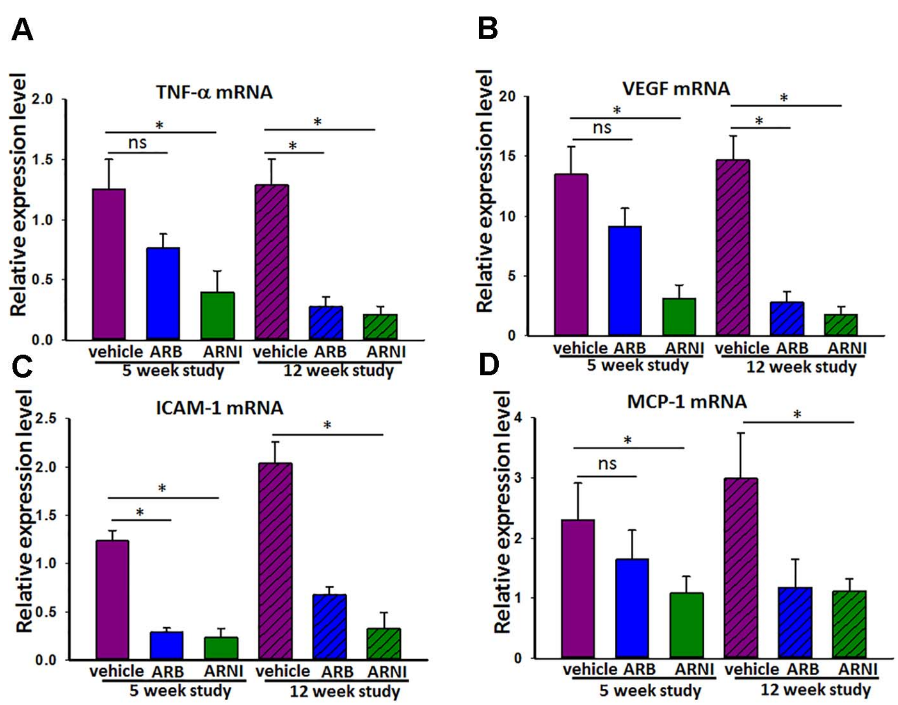

Figure 6. Real-time RT-PCR detection of inflammatory cytokines in retina. Real-time PCR analysis of mRNA extracted from rat retinas of different experimental groups for inflammatory marker genes: *TNF-α*, *VEGF*, *ICAM-1*, and *MCP-1*. *Y*-axis shows the fold change in the gene expression level normalized to β-actin. $^*P < 0.01$, $n = 4$ per group.

DR. This is consistent with our earlier work in this model.[17] Secondly, these symptoms exacerbated after a longer duration of diabetes; the increase in capillary loss, inflammatory cytokine expression, gliosis, and apoptotic cell death were all significantly worsened after 12 weeks of diabetes compared to 5 weeks of diabetes. This could have important clinical significance in terms of developing potential therapeutics, as DR, a progressive retinal neurovascular disorder, is strongly associated with prolonged duration of diabetes in patients.[1] Thirdly, this study provides strong evidence that, in the 12-week study, ARNI is much more effective than ARB alone in ameliorating the retinal neurovascular dysfunctions observed in diabetic Ren2 rats, while the two treatments were equally effective at 5 weeks. Thus, the use of a NEP inhibitor in addition to an ARB seems to provide additional benefits effects on the retinal neurovascular system, making ARNI a promising therapeutic approach in the treatment of DR. To what degree the beneficial effects of ARNI in the eye at 12 weeks occurred independently of blood pressure still must be investigated. Nevertheless, given the identical blood pressure lowering effects of ARNI and ARB at 5 weeks, at this stage there is no reason to believe that this would be entirely different at 12 weeks.

A large body of evidence suggests that a deficiency in the natriuretic peptide system in addition to a hyperactive RAS is associated with various renal and cardiovascular diseases like heart failure, hypertension, and metabolic syndromes.[27–29] Natriuretic peptides are a family of hormones that help maintain sodium and fluid balance. This system comprises three structurally similar peptides: atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP).[30] All three peptides have cardiorenal protective properties. Natriuretic peptides, in addition to having potent natriuretic, diuretic, and vasodilator properties, also lower sympathetic drive, and have antiproliferative and antihypertrophic effects.[31] These actions are mediated by the binding of ANP and BNP to the type A receptor, which is coupled to guanylyl cyclase. C-type natriuretic peptide is found mostly in the central nervous system, kidneys, and vascular endothelial cells; it has antithrombotic and antifibrotic effects and binds to the type B receptor. The activation of both these receptors increases the expression of the second messenger cGMP, which mediates most of the natriuretic peptide mechanisms of action. The natriuretic peptides are cleared from circulation via receptor-mediated internalization (involving the natriuretic peptide clearance receptor) and/or degradation by extracellular proteases, like NEP.[32,33] Natriuretic peptides and their receptors are expressed and functional in the rodent and human eyes in lens epithelial cells, vitreous, neural retina, and RPE.[34–36] Although their role in the retina is not yet clear, induction of diabetes in rats causes downregulation of retinal ANP expression. The activity level of NEP in the vitreous of patients with proliferative DR is increased compared to control patients.[36] Additionally, in our present study we showed that STZ-induced DR in Ren2 rats results in increased level of NEP activity, which can be significantly reduced by ARB and ARNI treatment. Moreover, it has been shown that ANP and CNP can suppress endothelial and RPE leakage, prevent choroidal neovascularization,[37,38] and reverse advanced glycation end-product-induced retinal blood-barrier dysfunction in RPE.[35] All these processes precede the development of DR. Thus, inhibiting NEP and increasing the level of natriuretic peptides in the retina could be beneficial in the treatment of DR.

Increasing the levels of natriuretic peptide by NEP inhibitors has been investigated as a therapeutic approach for treatment of hypertension and heart failure.[39,40] However, the use of NEP inhibitors individually was not very beneficial, due to its broad effects on a variety of substrates other than natriuretic peptides.[31] Indeed, NEP is critical for the processing and catabolism of vasoactive peptides and peptides involved in diuresis, natriuresis, and blood pressure regulation, like angiotensin II, bradykinin, substance P, adrenomedullin, glucagon, vasoactive intestinal peptide, and endothelin-1.[30] Therefore, in recent studies NEP inhibitors were used in combination with other cardiovascular agents, such as ACEi and ARBs, to at least counteract their potentiating effects on angiotensin II.[31,41,42] The combination of a NEP inhibitor and an ACEi was not useful because of a high incidence of angioedema, due to increased bradykinin levels[43,44] related to the fact that NEP and ACE are the main enzymatic pathways for the breakdown of bradykinin. An alternative combination is that of an ARB and a NEP inhibitor (ARNI). Angiotensin receptor blockers do not disrupt bradykinin metabolism, and patients on ARNI did not seem to develop angioedema.[41,42] This combination could be additionally beneficial because blockade of the angiotensin II type 1 receptor by ARB facilitates the binding of angiotensin II to the angiotensin II type 2 receptor that elicits several favorable actions. Clinical studies using ARNI for the treatment of heart failure and hypertension have shown much stronger favorable results when compared to ARB, without any extra associated negative side effects.[10,12,42,45]

Possible chronic adverse effects of NEP inhibitors on the eye have not specifically been assessed yet. Neprilysin has a possible neuroprotective effect as it brings about degradation of the amyloid β peptide.[36,46] Elevated levels of amyloid β peptide in the brain is associated with cognitive impairment, and extracellular deposition leads to the development of Alzheimer's disease. Elevated levels of amyloid β peptide in the eye have been found in different progressive retinal neurodegenerative disorders, such as age-related macular degeneration.[47–49] Hence, further studies must be done to better understand the long-term effects of NEP inhibitors on the eye before they can be applied for the treatment of DR. Nevertheless, the beneficial effect that ARNI has over ARB on retinal neurovascular symptoms in diabetic Ren2 rats suggest that this treatment holds promise as a novel potential therapeutic strategy for treatment of patients with DR.

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