Angiotensin II Type 1 Receptor Signaling Contributes to Synaptophysin Degradation and Neuronal Dysfunction in the Diabetic Retina

Toshihide Kurihara,[1,2,3] Yoko Ozawa,[1,2,3] Norihiro Nagai,[1,2] Kei Shinoda,[4] Kousuke Noda,[1,2] Yutaka Imamura,[5] Kazuo Tsubota,[2] Hideyuki Okano,[3] Yuichi Oike,[6] and Susumu Ishida[1,2]

OBJECTIVE—Pathogenic mechanisms underlying diabetes-induced retinal dysfunction are not fully understood. The aim of the present study was to show the relationship of the renin-angiotensin system (RAS) with the synaptic vesicle protein synaptophysin and neuronal activity in the diabetic retina.

RESEARCH DESIGN AND METHODS—C57BL/6 mice with streptozotocin-induced diabetes were treated with the angiotensin II type 1 receptor (AT1R) blocker telimsartan or valsartan, and retinal function was analyzed by electroretinography. Retinal production of the RAS components and phosphorylation of ERK (extracellular-signal regulated kinase) were examined by immunoblotting. Retinal mRNA and protein levels of synaptophysin were measured by quantitative RT-PCR and immunoblot analyses, respectively. In vitro, synaptophysin levels were also evaluated using angiotensin II–stimulated PC12D neuronal cells cultured with or without the inhibition of ERK signaling or the ubiquitin-proteasome system (UPS).

RESULTS—Induction of diabetes led to a significant increase in retinal production of angiotensin II and AT1R together with ERK activation in the downstream of AT1R. AT1R blockade significantly reversed diabetes-induced electroretinography changes and reduction of synaptophysin protein, but not mRNA, levels in the diabetic retina. In agreement with the AT1R-mediated post-transcriptional downregulation of synaptophysin in vivo, in vitro application of angiotensin II to PC12D neuronal cells caused the UPS–mediated degradation of synaptophysin protein via AT1R, which proved to be induced by ERK activation.

CONCLUSIONS—These data indicate the first molecular evidence of the RAS-induced synaptophysin degradation and neuronal dysfunction in the diabetic retina, suggesting the possibility of the AT1R blockade as a novel neuroprotective treatment for diabetic retinopathy. ***Diabetes*** **57:2191–2198, 2008**

From the [1]Laboratory of Retinal Cell Biology, Keio University School of Medicine, Tokyo, Japan; the [2]Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan; the [3]Department of Physiology, Keio University School of Medicine, Tokyo, Japan; the [4]Department of Ophthalmology, Oita University Faculty of Medicine, Hasama-machi, Yufu-shi, Oita, Japan; the [5]Inaida Laboratory for Anti-Aging Medicine, Keio University School of Medicine, Tokyo, Japan; the [6]Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan.

Corresponding author: Susumu Ishida, ishidasu@sc.itc.keio.ac.jp.

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T.K. and Y.O. contributed equally to this work.

Diabetic retinopathy is a vision-threatening disease with neurodegenerative change due to chronically progressive microangiopathy. The earliest functional disruption clinically detectable is changes in oscillatory potentials (OPs) measured by electroretinography (ERG) (1,2). The cellular source of OPs is regarded as retinal neurons with synapse formation in the inner retina, including bipolar and amacrine cells (3). At present, there is no established neuroprotective treatment for diabetic retinopathy, since molecular mechanisms underlying diabetes-induced retinal neuronal damage remain unclear.

We have recently demonstrated that angiotensin II type 1 receptor (AT1R) signaling contributes to diabetes-induced retinal inflammation such as leukocyte adhesion to the retinal vasculature (4). Angiotensin II functions as a proinflammatory factor to induce the activation of nuclear factor–κB pathway in microvascular endothelial cells (4). Angiotensin II is a final product of the renin-angiotensin system (RAS) produced from angiotensinogen through enzymatic cascade reactions, and the RAS components required for the generation of angiotensin II are reported to exist in the eye (5–7). Indeed, human surgical samples from eyes with diabetic retinopathy showed a significant increase in angiotensin II levels (8–10). Increasing evidence has suggested the contribution of the RAS to diabetes-induced retinal vascular complications including leukocyte adhesion (4), hyperpermeability (11), and impaired blood flow (12); however, little is known about the pathogenesis of angiotensin II–mediated neuronal dysfunction in the diabetic retina. Although AT1R blockade led to amelioration of hypertension-induced retinal dysfunction that was exacerbated with diabetes (13), no data have been reported that show the direct effect of AT1R signaling on diabetes-induced retinal dysfunction together with underlying molecular mechanisms.

Recently, we revealed the coexpression of AT1R and the synaptic protein synaptophysin in the inner retinal neurons (14), consistent with several previous reports showing synaptic expression of AT1R in the brain (15–18). Synaptophysin, the major synaptic vesicle protein, is a marker of synapses reported to be reduced in the postmortem brains affected by several neurodegenerative diseases (19). Considering that OPs in ERG are originated from inner retinal neurons bearing AT1R, we hypothesize that angiotensin II directly induces synaptophysin dysregulation and visual functional damage represented by ERG changes. In the present article, we report the first evidence showing that AT1R signaling contributes to diabetes-induced retinal dysfunction and

TABLE 1
Systemic data

	Nondiabetes	Diabetes		
		Vehicle	Telmisartan	Valsartan
n	17	17	18	18
Body weight (g)	26.0 ± 1.3	24.2 ± 1.3†	24.8 ± 1.1*	24.3 ± 1.2†
Blood glucose (mg/dl)	156 ± 22	544 ± 64†	539 ± 81†	516 ± 96†

*$P < 0.05$, †$P < 0.01$ vs. nondiabetes.

synaptophysin downregulation together with underlying molecular mechanisms.

RESEARCH DESIGN AND METHODS

Induction of diabetes. C57BL/6 mice (Clea, Tokyo, Japan) at the age of 6 weeks were used in diabetes induction. All animal experiments were conducted in accordance with the ARVO (Association for Research in Vision and Ophthalmology) Statement for the Use of Animals in Ophthalmic and Vision Research. Animals received intraperitoneal injections of streptozotocin (Sigma, St. Louis, MO) at the dose of 60 mg/kg body weight for 3 days. Blood glucose concentrations were measured from the tail vein using Medisafe mini GR-102 (Terumo, Tokyo, Japan). Development of diabetes was defined by blood glucose >250 mg/dl 7 days after the first injection of streptozotocin.

AT1R blockade in vivo. Mice were intraperitoneally injected with the AT1R blocker (ARB) telmisartan or valsartan (U.S. Pharmacopeia, Rockville, MD) at the dose of 5 or 10 mg/kg body weight, respectively, or vehicle (0.25% DMSO in PBS). The ARB treatment started 22 days after the first injection of streptozotocin for 6 consecutive days and continued until the end of the study (4-week diabetes at evaluation). The doses used in each ARB group were determined according to our previous study on diabetes-induced retinal inflammation (4). Telmisartan was a kind gift of Boehringer Ingelheim (Ingelheim, Germany).

ERG analyses. Animals were dark-adapted for 12 h and prepared under dim red illumination. Mice were anesthetized with pentobarbital sodium at the dose of 70 mg/kg body weight and placed on a heating pad that maintained their body temperature at 35–36°C throughout the experiments. The pupils were dilated with a mixed solution of 0.5% tropicamide and 0.5% phenylephrine (Mydrin-P; Santen, Osaka, Japan). The ground electrode was a subcutaneous needle in the tail, and the reference electrode was placed subcutaneously between the eyes. The active contact lens electrodes (Mayo, Inazawa, Japan) were placed on the cornea. Recordings were performed with PowerLab system 2/25 (AD Instruments, New South Wales, Australia). Responses were differentially amplified at the gain of 1,000 times using an AC (alternate current)-coupled bioamplifier ML132 (AD Instruments) and filtered through a bandpass filter ranging from 0.3 to 500 Hz to yield a- and b-waves. OPs were simultaneously recorded using a high-pass filter set to 100 Hz, so that an overall bandpass ranging from 100 to 500 Hz was achieved. Light pulses of 800 cd-s/m^2 were delivered via Ganzfeld System SG-2002 (LKC Technologies; Gaithersburg, MD). The amplitude and implicit time of the a- and b-waves and OPs were measured and compared among age-matched nondiabetic controls and 4-week diabetic animals treated with vehicle, telmisartan, or valsartan.

In vitro assays. The neuronal cell line PC12D cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 10% horse serum (Medical & Biological Laboratories, Nagoya, Japan). Cells were incubated with 50 μg/μl of nerve growth factor (Millipore, Billerica, MA) for differentiation to neurons for 2 days. The neuronal cells were applied with the ARB telmisartan (10 μmol/l) or valsartan (25 μmol/l), the proteasome inhibitor MG132 or lactacystin (20 μmol/l for both; Calbiochem, San Diego, CA), the lysosome inhibitor E64 (50 μmol/l; Sigma), the extracellular signal–regulated kinase (ERK) kinase inhibitor U0126 (10 μmol/l; Cell Signaling Technology, Beverly, MA) or PD98059 (10 μmol/l; Calbiochem), the phosphatidylinositol 3-kinase inhibitor LY294002 (20 μmol/l; Calbiochem) or wortmannin (100 nmol/l; Sigma), the Janus kinase inhibitor AG490 (1, 10, or 100 μmol/l; Calbiochem), or vehicle. After 5 min, the pretreated cells were subsequently stimulated with 100 nmol/l angiotensin II for 30 min. Immediately after the exposure to angiotensin II, the neuronal cells were subjected to quantitative RT-PCR and immunoprecipitation for synaptophysin and to immunoblot analyses for synaptophysin and phosphorylated forms of ERK1/2 (p44/p42), Akt (protein kinase B), and signal transducer and activator of transcription (STAT)-3.

AT1R and ERK1/2 knockdown by RNA interference. Transfections were performed using Lipofectamine 2000 (Invitrogen) and the SureSilencing short hairpin RNA (shRNA) kit (SuperArray Bioscience, Frederick, MD) according to the manufacturers' instructions. Briefly, 4 μg Rat Agtr1a (AT1R), Rat Mapk3 (ERK1), and Rat Mapk1 (ERK2) or control shRNA was incubated with 10 μl of the transfection reagent in 500 μl serum-free medium for 20 min to facilitate complex formation. The resulting mixture was added to PC12D cells cultured in a six-well plate with 2 ml medium for 48 h before differentiation.

Quantitative RT-PCR analyses for synaptophysin. Animals were killed with an overdose of anesthesia. The eyes were immediately enucleated and the retina was carefully isolated. Total RNA was extracted from the retinas or PC12D cells using an extraction reagent (TRIzol; Invitrogen), and cDNA was synthesized with SuperScript III reverse transcriptase (Invitrogen). Real-time PCR was performed using MX3000 (Stratagene, La Jolla, CA) with PCR primers for synaptophysin designed by TaqMan Gene Expression Assays (Applied Biosystems, Foster, CA). The mRNA levels were normalized to β-actin as an internal control.

Immunoblot analyses for angiotensin II, AT1R, synaptophysin, and phosphorylated forms of ERK, Akt, and STAT3. The isolated retinas or PC12D cells were placed into lysis buffer (10 mmol/l Tris-HCl [pH 7.6], 100 mmol/l NaCl, 1 mmol/l EDTA, 1% Triton X-100, protease inhibitors). Each sample was separated with SDS-PAGE and electroblotted to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). After nonspecific binding was blocked with 4% skim milk, the membranes were incubated at 4°C overnight with a rabbit polyclonal antibody against angiotensin II, AT1R (1:200, 1:100, respectively; Santa Cruz Biotechnology, Santa Cruz, CA), or phosphorylated forms of ERK, Akt, or STAT3 (1:1,000, 1:500, or 1:1,000, respectively; Cell Signaling Technology) or a mouse monoclonal antibody against synaptophysin (1:500; Sigma) or α-tubulin (1:2,000; Sigma). The membranes were then incubated with a horseradish peroxidase–conjugated goat antibody against rabbit or mouse immunoglobulins or with a biotinylated secondary antibody followed by avidin-biotin horseradish peroxidase complexes (Vectastain Elite ABC Kit; Vector, Burlingame, CA). The signals were visualized with chemiluminescence (ECL Blotting Analysis System; Amersham, Arlington Heights, IL), measured by ImageJ software (National Institutes of Health, Bethesda, MD) and normalized to α-tubulin.

Ubiquitination assays. PC12D cells were transfected with a hemagglutinin-ubiquitin plasmid (provided by Keiji Tanaka, the Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) as described above at the time of differentiation. Forty-five hours after transfection, the cells were incubated for 3 h with 20 μmol/l MG132. The transfected cells were then pretreated with telmisartan, valsartan, or vehicle and followed by stimulation with angiotensin II as described above. The stimulated cells were placed into the lysis buffer, and the cell lysate was applied with a rabbit polyclonal antibody against synaptophysin (1:25; Sigma). After rocking at 4°C for 1 h, the lysate treated with anti-synaptophysin antibodies were then added with protein G-sepharose beads (Sigma) and incubated overnight at 4°C. After washes of nonspecific binding to beads, the bound protein was solubilized in sample buffer, loaded on an SDS-PAGE, and processed for immunoblotting with a rat monoclonal antibody against hemagglutinin (1:500; Roche Applied Science, Mannheim, Germany) to detect multi-ubiquitinated synaptophysin and with a mouse monoclonal antibody against synaptophysin (1:500; Sigma) to confirm the equal amount of synaptophysin protein applied in each lane.

Statistical analysis. All results were expressed as means ± SD. The values were processed for statistical analyses (one-way ANOVA with Tukey's post hoc test), and differences were considered statistically significant at $P < 0.05$.

RESULTS

Diabetes-induced retinal production of angiotensin II and AT1R led to ERK activation. Mice with streptozotocin-induced diabetes showed a significant ($P < 0.05$) decrease in body weight and a significant ($P < 0.01$) increase in blood glucose, compared with age-matched nondiabetic controls (Table 1). Treatment with telmisar-

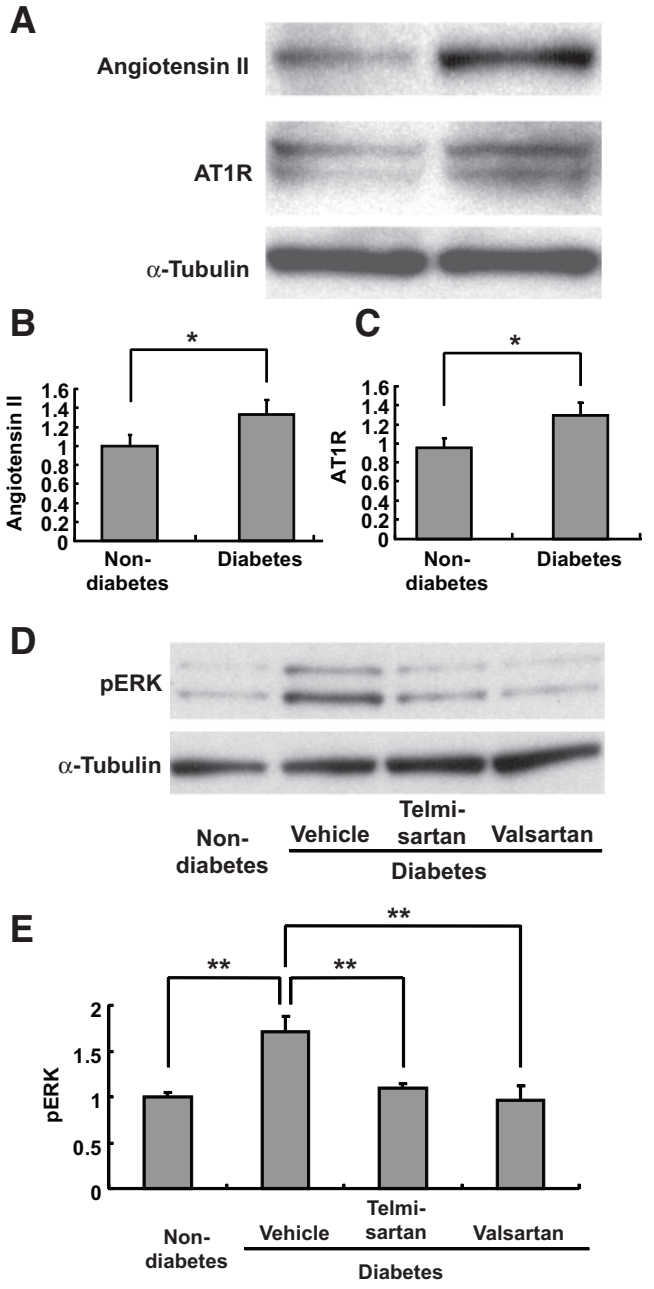

FIG. 1. Diabetes-induced retinal production of angiotensin II and AT1R led to ERK activation. Immunoblot analyses showing the production of the RAS components (*A–C*) and ERK activation in the diabetic retina (*D* and *E*). Retinal levels of angiotensin II and AT1R were significantly higher in 4-week diabetic than in nondiabetic mice (*A–C*). ERK phosphorylation, elevated in 4-week diabetic mice, was significantly attenuated by AT1R blockade with telmisartan or valsartan (*D* and *E*). *n* = 4 for all. **P* < 0.05.

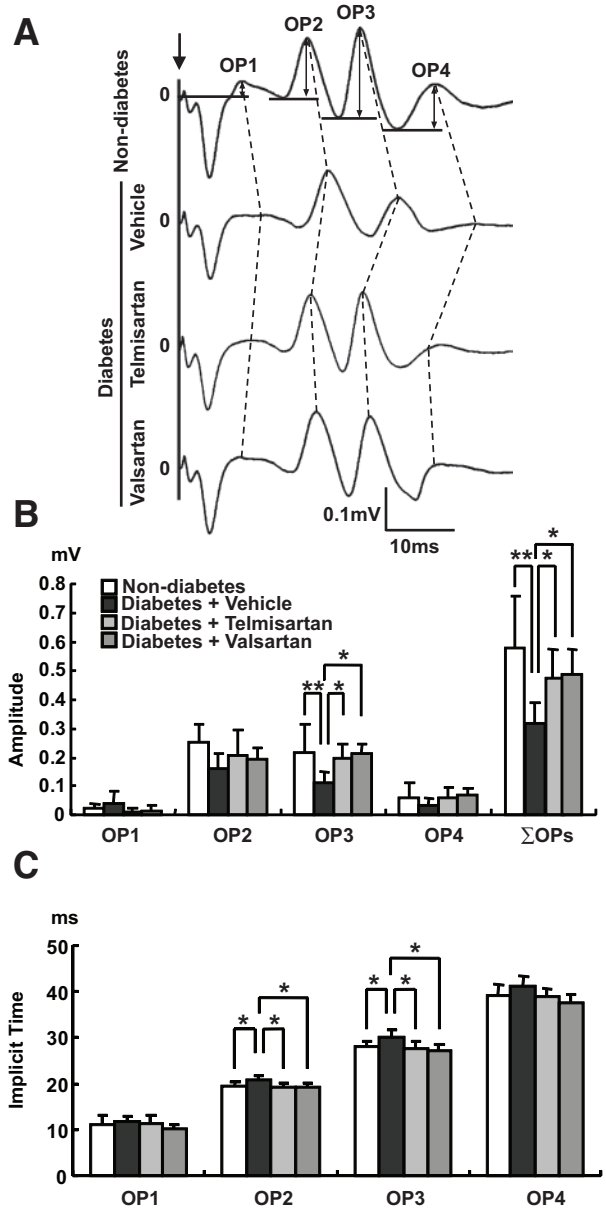

FIG. 2. Diabetes-induced visual dysfunction was suppressed by AT1R blockade. *A*: Representative wave responses from an individual mouse in each group to one flash. *A* and *B*: ERG analyses showing that AT1R blockade with telmisartan or valsartan significantly reversed the reduction of OP3 and total OPs (ΣOPs) amplitude in 4-week diabetic mice. Similarly, the implicit time of OP2–3, prolonged in 4-week diabetic mice, was significantly recovered by AT1R blockade (*A* and *C*). *n* = 7–9. **P* < 0.05, ***P* < 0.01.

tan or valsartan of 4-week diabetic mice did not significantly ($P > 0.05$) change these metabolic parameters (Table 1). The retinas from 4-week diabetic mice were subjected to immunoblot analyses to detect the production of the RAS components and its downstream ERK activation (Fig. 1). Retinal levels of angiotensin II and its receptor AT1R were significantly ($P < 0.01$) higher in 4-week diabetic than in nondiabetic animals (Fig. 1*A–C*). Consistently, ERK phosphorylation, enhanced in 4-week diabetic mice ($P < 0.01$), was significantly ($P < 0.01$) attenuated by AT1R blockade with telmisartan or valsartan to the level equivalent with that in nondiabetic mice (Fig. 1*D* and *E*). In contrast, AT1R blockade did not attenuate the baseline level of retinal pERK in nondiabetic mice (data not shown).

Diabetes-induced visual dysfunction was suppressed by AT1R blockade. To determine the effect of the RAS activation in the diabetic retina (Fig. 1) on visual function, we performed ERG analyses (Fig. 2). OP changes including the reduced amplitude and prolonged implicit time are known to occur in the early stage of murine and human diabetic retinopathy (1,2,20,21). There was no remarkable difference in a- or b-wave among nondiabetes and 4-week diabetes treated with vehicle, telimsartan, and valsartan (data not shown). Importantly, AT1R blockade with telmisartan or valsartan significantly reversed the reduction of

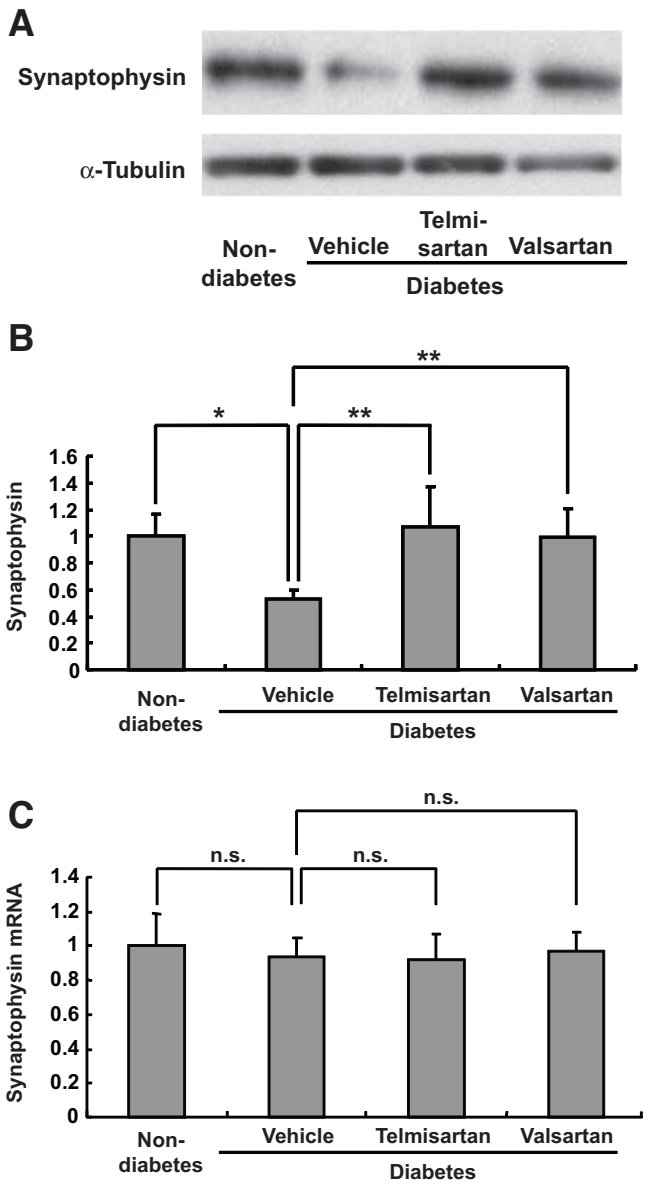

FIG. 3. Posttranscriptional reduction of synaptophysin in the diabetic retina was reversed by AT1R blockade. The retinal levels of synaptophysin protein, reduced by inducing diabetes, were significantly reversed in 4-week diabetic mice by AT1R blockade with telmisartan or valsartan (*A* and *B*). In contrast, mRNA levels of synaptophysin were unaltered in 4-week diabetic mice treated with vehicle, telmisartan, or valsartan compared with age-matched nondiabetic control animals (*C*). $n = 4–8$. *$P < 0.05$, **$P < 0.01$.

OP3 and total OPs (ΣOPs) amplitude ($P < 0.05$ for both) in 4-week diabetic mice (Fig. 2*A* and *B*). Similarly, the implicit time of OP2 and OP3, prolonged in 4-week diabetic mice, was significantly ($P < 0.05$ for both) recovered by AT1R blockade to the normal level (Fig. 2*A* and *C*). In contrast, AT1R blockade did not alter normal ERG waves in nondiabetic mice (data not shown).

Posttranscriptional reduction of synaptophysin in the diabetic retina was reversed by AT1R blockade. To investigate the molecular mechanisms underlying the AT1R-mediated impairment of ERG responses (Fig. 2), we analyzed the regulation of the synaptic vesicle protein synaptophysin (Fig. 3), which is abundantly expressed in the inner retinal neurons (14), the cellular source of OPs (1,3,22,23). Synaptophysin is indispensable for several presynaptic functions, including the release of neurotransmitters (24). The retinal levels of synaptophysin protein, reduced by inducing diabetes, were significantly ($P < 0.01$) reversed in 4-week diabetic mice by AT1R blockade with telmisartan or valsartan (Fig. 3*A* and *B*). In contrast, mRNA levels of synaptophysin were unaltered in 4-week diabetic mice treated with vehicle, telimsartan, or valsartan, compared with those in age-matched nondiabetic controls (Fig. 3*C*). The discrepancy between the protein and mRNA levels indicated that the AT1R-mediated decline of synaptophysin in the diabetic retina was posttranscriptionally regulated. In contrast, AT1R blockade did not affect the physiologic level of synatophysin mRNA or protein in the nondiabetic retina (data not shown).

AT1R-mediated posttranscriptional reduction of synaptophysin was regulated by the ubiquitin-proteasome system in neuronal cells. To elucidate the molecular mechanisms involving the AT1R-mediated posttranscriptional decrease in synaptophysin in the diabetic retina (Fig. 3), we used the in vitro culture system with PC12D neuronal cells stimulated with angiotensin II (Fig. 4). In agreement with the in vivo data (Fig. 3), in vitro application with angiotensin II significantly ($P < 0.01$) reduced synaptophysin protein (Fig. 4*A* and *B*), but not mRNA (Fig. 4*C*), levels in a posttranscriptional manner, which were significantly ($P < 0.05$ for all) reversed by AT1R blockade with telmisartan, valsartan, or shRNA for AT1R knockdown (Fig. 4*A*, *B*, and *D–F*). Because synaptic vesicle proteins including synaptophysin have recently proven to be physiologically degraded by the UPS for the maintenance of synaptic plasticity (25–27), we examined the involvement of the ubiquitin-proteasome system (UPS) (Fig. 4*G–I*) with AT1R-mediated posttranscriptional decrease in synaptophysin protein (Fig. 4*A–F*). Multi-ubiquitinated synaptophysin as a high–molecular weight smear, enhanced by stimulation with angiotensin II, was substantially attenuated by AT1R blockade with telmisartan or valsartan (Fig. 4*G*). Application with the proteasome inhibitor MG132 or lactacystin, but not the lysosome inhibitor E64, led to significant ($P < 0.05$) suppression of angiotensin II–induced degradation of synaptophysin (Fig. 4*H* and *I*). In contrast, the baseline levels of synaptophysin in PC12D cells not stimulated with angiotensin II were unaffected with these inhibitors (data not shown).

AT1R-mediated ERK activation was required for synaptophysin degradation in neuronal cells. Because ERK was activated in the downstream of AT1R signaling in the diabetic retina (Fig. 1), we examined the involvement of ERK activation (Fig. 5) with AT1R-mediated degradation of synaptophysin in the neuronal cells (Fig. 4). Consistent with AT1R-mediated ERK activation in the diabetic retina (Fig. 1), angiotensin II administration induced the phosphorylation of ERK in the neuronal cells, which was significantly ($P < 0.05$) inhibited by AT1R blockade with telmisartan or valsartan (Fig. 5*A* and *B*). Inhibition of ERK activation with U0126, PD98059, or shRNA for ERK1/2 knockdown led to significant ($P < 0.05$ for all) suppression of angiotensin II–induced degradation of synaptophysin (Fig. 5*C–H*). In addition to ERK signaling, phosphatidylinositol 3-kinase/Akt and Janus kinase/STAT pathways are known to be in the downstream of AT1R. In the neuronal cells, angiotensin II treatment also increased the phosphorylated forms of Akt and STAT3 (data not shown). In contrast to ERK involvement (Fig. 5*C–H*), however, the phosphatidylinositol 3-kinase inhibitor wortmannin or LY294002 or the Janus kinase inhibitor AG490 did not

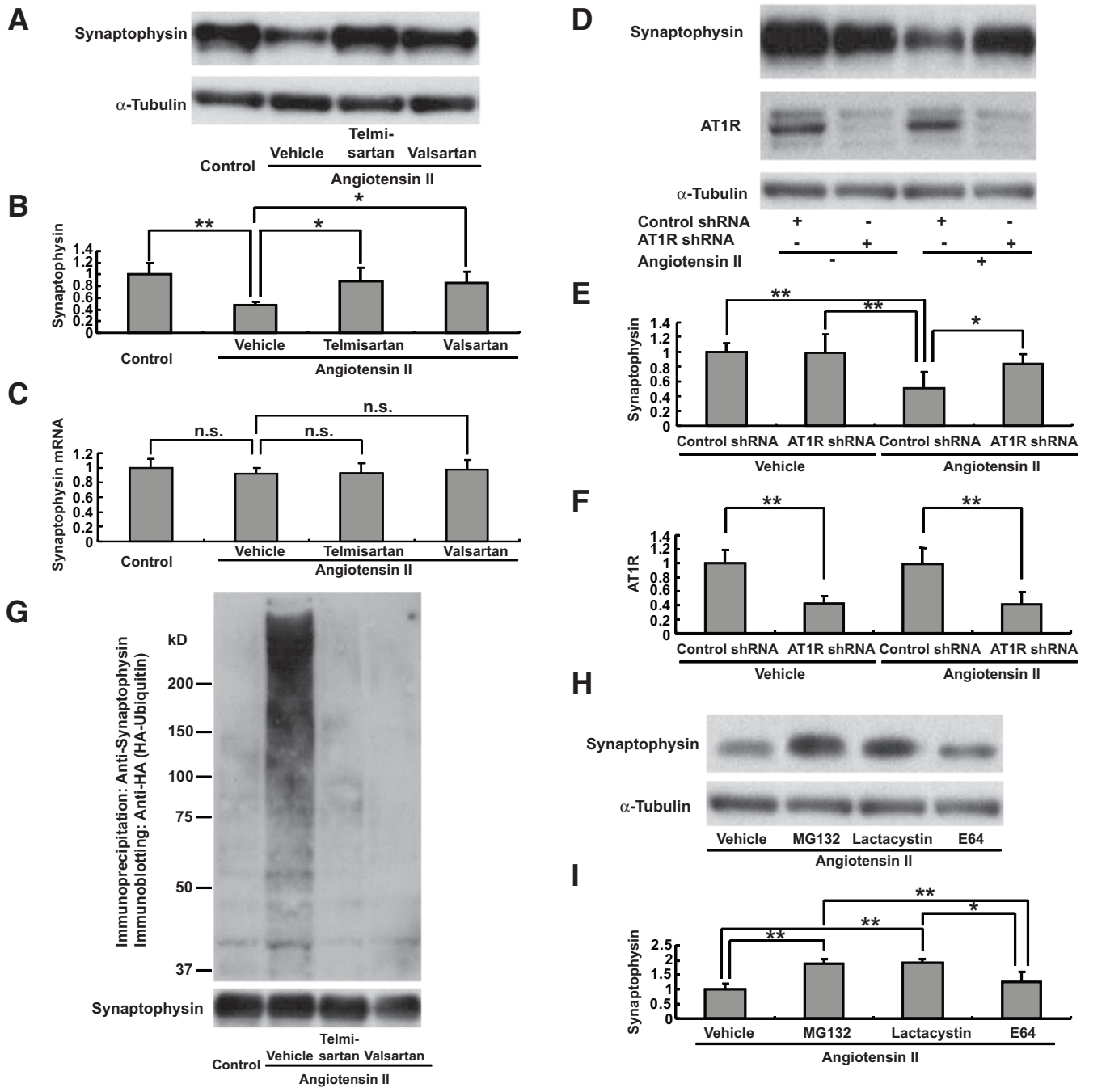

FIG. 4. AT1R-mediated posttranscriptional reduction of synaptophysin was regulated by the UPS in neuronal cells. In vitro application with angiotensin II significantly reduced synaptophysin protein (*A* and *B*), but not mRNA (*C*), levels in a posttranscriptional manner, which were significantly reversed by AT1R blockade with telmisartan or valsartan. The data with the pharmacologic AT1R blockade (*A–C*) were reproduced via shRNA for AT1R knockdown (*D–F*). Multi-ubiquitinated synaptophysin as a high–molecular weight smear, enhanced by stimulation with angiotensin II, was substantially attenuated by AT1R blockade (*G*). Application with the proteasome inhibitor MG132 or lactacystin, but not the lysosome inhibitor E64, led to significant suppression of angiotensin II–induced degradation of synaptophysin (*H* and *I*). $n = 4–6$. *$P < 0.05$, **$P < 0.01$.

reverse angiotensin II–induced degradation of synaptophysin in vitro (data not shown).

DISCUSSION

The present study reveals, for the first time to our knowledge, several important findings concerning the relationship of the RAS with diabetes-related neuronal damage in vivo and in vitro. In the diabetic retina, the RAS components angiotensin II and AT1R were upregulated together with AT1R's downstream ERK activation (Fig. 1). AT1R blockade with telmisartan or valsartan significantly reversed diabetes-induced OP changes measured by ERG (Fig. 2), known functional abnormalities in human diabetic retinopathy. Moreover, AT1R signaling caused a posttranscriptional decrease in the synaptic vesicle protein synaptophysin in the diabetic retina, which was rescued by the ARB application to diabetic mice (Fig. 3). The protein, but not mRNA, reduction of synaptophysin in neuronal cells was shown to depend on the UPS, which was enhanced via AT1R signaling (Fig. 4). Angiotensin II–induced neuronal ERK

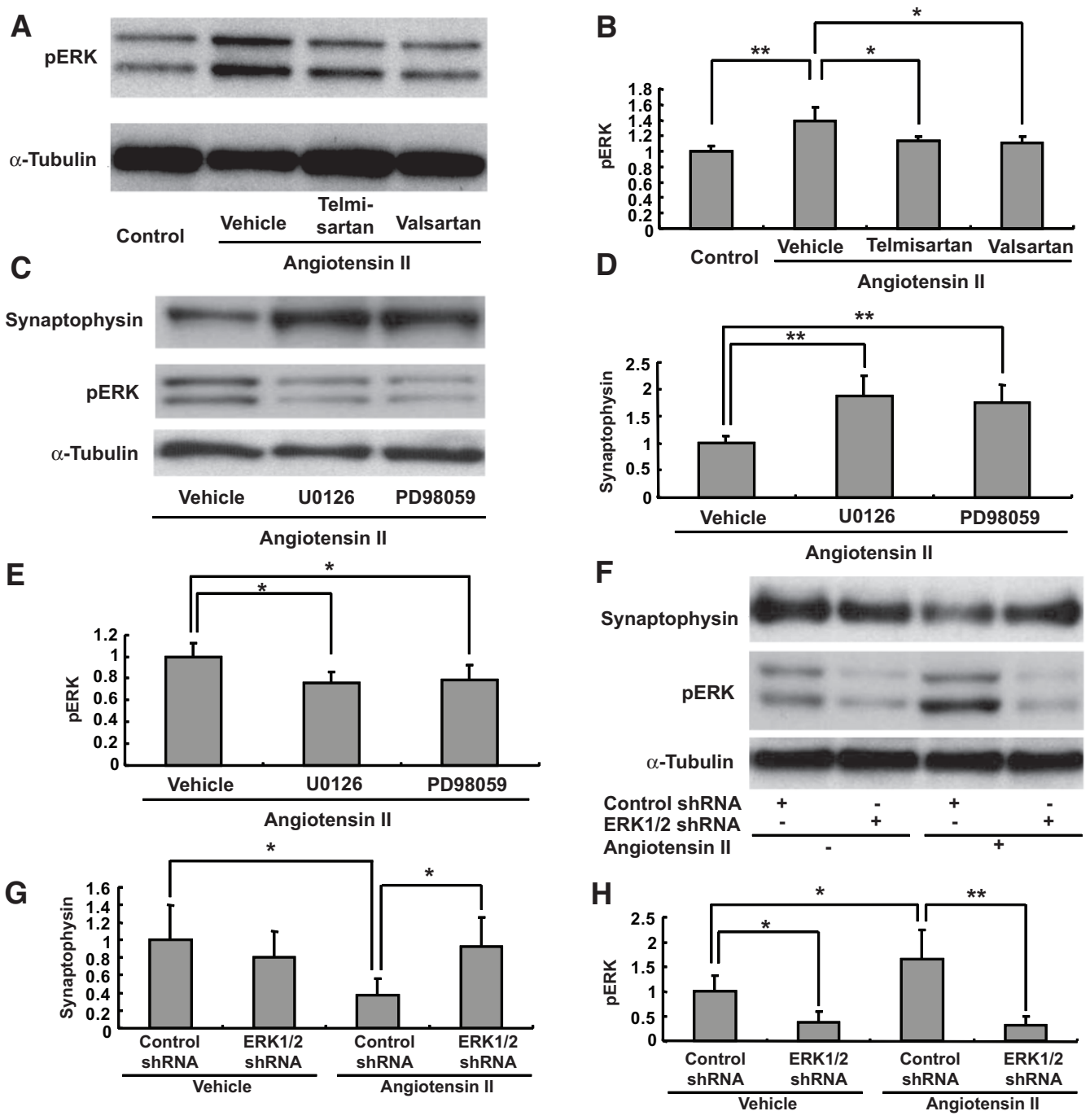

FIG. 5. AT1R-mediated ERK activation was required for synaptophysin degradation in neuronal cells. Angiotensin II administration induced the phosphorylation of ERK in the neuronal cells, which was significantly inhibited by AT1R blockade with telmisartan or valsartan (*A* and *B*). Inhibition of ERK activation with U0126 or PD98059 led to significant suppression of angiotensin II–induced decline of synaptophysin (*C–E*). The data with the pharmacologic ERK inhibition (*C–E*) were reproduced via shRNA for ERK1/2 knockdown (*F–H*). $n = 4–6$. *$P < 0.05$, **$P < 0.01$.

activation, also observed in the diabetic retina (Fig. 1), resulted in the degradation of synaptophysin protein in vitro (Fig. 5). The use of two different ARBs throughout the experiments confirmed that the suppression of diabetes-induced retinal dysfunction and synaptophysin degradation is a class effect for ARBs. Additionally, our in vitro results were further confirmed via gene-specific shRNA knockdown technique for AT1R and ERK.

In the diabetic retina, the production of angiotensin II and AT1R was upregulated, leading to ERK activation in the downstream of AT1R signaling (Fig. 1). Since ERK is known to be one of AT1R's signaling pathways, the present data are compatible with clinical (5,7,28,29) and experimental (4) studies showing increased angiotensin II generation in the diabetic eye. The cellular source of angiotensin II was shown to be retinal glial cells by immunohistochemistry for human postmortem eyes (30). In the liver and kidney as well (31–33), diabetes-induced activation of the RAS causes elevated concentration of tissue angiotensin II. The tissue RAS activation in diabetes is explained in part by the findings that in vitro stimulation with high glucose enhanced the transcriptional activity of angiotensinogen gene encoding a precursor protein for angiotensin I and II (34,35).

Our ERG analyses demonstrated the reduced amplitude and prolonged implicit time of OPs in diabetic mice, in accordance with clinical and experimental data on attenuated OP changes in early diabetes (1,2,29). Importantly, AT1R blockade significantly improved these functional parameters in the diabetic retina to the normal levels observed in nondiabetic controls (Fig. 2). Reasonably, the present ERG findings, in concert with our recent data on AT1R expression in the inner retinal neurons (14), the cellular origins of OPs, suggest that the functional recovery of the diabetic inner retina was attributable in part to the direct effect of AT1R blockade on the inner retinal neurons.

Several previous studies on neuronal damage in the diabetic retina showed the cellular changes including the apoptosis of ganglion and amacrine cells and the activation of glial cells (36–38). In contrast, the present study revealed, with the molecular changes in the diabetic retina, a significant decrease in synaptophysin (Fig. 3), the synaptic vesicle protein capable of maintaining the function of retinal neurons. Furthermore, AT1R signaling was shown to play a crucial role in the reduction of synaptophysin protein (Fig. 3). Pharmacologic studies using tetrodotoxin and glycine suggested the cellular origins of OPs as neurons with synapse formation in the inner retina including bipolar and amacrine cells (3,22,23), both of which develop a retinal neuronal network contributing to visual function. Synatophysin, a well-known marker for the presynapse, was shown to decrease with the progression of Parkinson's disease and Alzheimer's disease (19). In the retina as well, pathogenic conditions such as retinal detachment and inflammation caused reduction of synaptophysin (14,39). Mice deficient in synaptophysin exhibited a significant decrease in the number of synaptic vesicles (24). The neuron maintains its viability and activity by interacting with other neurons via synapses; however, synaptophysin knockouts were for the most part functionally compensated possibly because of biological redundancy (24). Although it is reasonable to think about the possibility of cooperative involvement of several other synaptic proteins, the diabetes-induced reduction of synaptopysin (Fig. 3) was attributable, at least in part, to the functional disturbance of neuronal activity in the diabetic retina. The currently observed suppression of synaptophysin decrease by AT1R blockade (Fig. 3) is likely to rescue the activity of the AT1R-bearing inner retinal neurons, leading to the improvement of OP changes (Fig. 2).

The present in vitro data (Figs. 4 and 5) further elucidated the detailed molecular mechanisms underlying the AT1R-mediated posttranscriptional decrease in synaptophysin observed in vivo (Fig. 3). Angiotensin II directly exerted its bioactivity to the neuronal cells via AT1R, leading to a posttranscriptional decrease in synatophysin (Fig. 4*A–F*), which mimicked the in vivo data (Fig. 3). Interestingly, angiotensin II/AT1R signaling proved to cause the activation of the UPS, leading to synaptophysin degradation in neuronal cells (Fig. 4*G–I*). Although the UPS was recently shown to play a role in the degradation of several synaptic proteins (25–27), the present data are the first to reveal the RAS-mediated UPS activation for synaptic protein degradation in neurons. Out of several signaling molecules in the downstream of AT1R, the phosphorylation of ERK, but not Akt or STAT3, was required for synaptophysin degradation (Fig. 5), suggesting that the UPS-dependent synaptophysin degradation is mediated by ERK activation. This is supported by several recent studies showing that the activation of the ERK pathway promoted the UPS-mediated degradation of the cell cycle protein Cdc25 (40,41) and the cell survival factor Bim (42). In the *Drosophila* eye, ERK signaling was shown to be required for the physiologic activity of Sina (seven in absentia), a key molecule for normal photoreceptor development (43). Interestingly, the mammalian homologues of *Drosophila* Sina, Siahs (seven in absentia homologues), proved to be synaptophysin-binding proteins functioning as E3 ubiquitin ligases to regulate the UPS-mediated degradation of synaptophysin (26). Reasonably, baseline ERK phosphorylation in the normal retina (Fig. 1) is likely to play a physiologic role in the UPS-mediated degradation of synaptophysin for the maintenance of synaptic plasticity (25–27). In the diabetic retina, however, excessive ERK activation mediated by AT1R (Fig. 1) is thought to induce the pathologic decline of synaptophysin (Fig. 3), contributing to neuronal dysfunction demonstrated by ERG (Fig. 2).

In the current study, diabetes-induced retinal dysfunction and synaptophysin downregulation were successfully reversed by AT1R signaling blockade in vivo (Figs. 2 and 3). Although future studies are required to determine whether angiotensin II/AT1R signaling leads to diabetes-induced neuronal damage systemically or locally in specific target organs, AT1R blockade has proven to be neuroprotective at least in the diabetic eye. Collectively, our present data show the possibility of AT1R blockade as a novel therapeutic strategy for neuronal dysfunction in vision-threatening diabetic retinopathy.

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