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Involvement of Calpain Isoforms in Retinal Degeneration in WBN/Kob Rats

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Results of our recent studies in rats suggested that calpains play an important role in retinal cell death induced by ischemia-reperfusion in vivo and by hypoxia in vitro. Study of spontaneous animal models could help determine the involvement of calpains in human retinopathy. The WBN/Kob rat is such a model for spontaneous retinal degeneration. The purpose of the study reported here was to determine the involvement of calpain isoforms during retinal degeneration in WBN/Kob rats. Histologic and functional retinal degeneration in WBN/Kob rats was observed by use of light microscopy and electroretinography, respectively. Proteolysis of α-spectrin in the retina was detected by use of immunoblot analysis in aging WBN/Kob rats. This proteolysis was associated with the increases of retinal calcium content and caseinolytic activity for calpains 1 and 2. Expression of calpain 1, calpain 2, and calpastatin mRNAs in the retina, as measured by use of reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, were only slightly up-regulated at 24 weeks of age. In contrast, expression of retina-specific calpains, such as Rt88, Rt88', and Rt90 mRNA, was markedly down-regulated at 12 weeks of age. Expression of calpain 10 mRNA in the retina was only slightly down-regulated at 12 weeks of age. In contrast to mRNA expression, various expression patterns of calpain 10 proteins were observed. Increased retinal calcium content, leading to activation of calpains 1 and 2, may be an important event in the sequential changes leading to degeneration of the retina in WBN/Kob rats. Activated calpain causing proteolysis of α-spectrin and changes in Rt88, Rt88', Rt90 and calpain 10 may also contribute to retinal degeneration.

The Wistar Bonn/Kobori (WBN/Kob) rat originated from a Wistar rat colony in the Institute of Experimental Gerontology in Basel and was maintained at the Institute of Pathology at the University of Bonn (9). In these rats, hyperglycemia is maintained for a long period and is associated with various secondary diseases (cataracts, nephropathy, neuropathy [12, 13, 15, 16, 27, 33]). Thus, the WBN/Kob strain is a model for spontaneous retinal degeneration and is considered useful as a model for human diabetes mellitus (23, 35, 36) and exo-endocrine pancreatic impairment. Retinal degeneration develops in male and female rats, but diabetes mellitus spontaneously develops only in the aging male rat. The rod and cone layers in the retina of the WBN/Kob rats degenerate, and gradually the thickness of outer and inner nuclear layers decreases with age (7). These retinal changes begin in males before hyperglycemia develops, and the retina from non-diabetic females has changes similar to those of age-matched males. Thus, retinal degeneration in WBN/Kob rats is considered to have no relation to diabetes mellitus and to be caused by hereditary factors.

The retinal changes in the WBN/Kob rat have been examined histologically and electrophysiologically (8), but only a few biological studies have been reported (19). The RCS rat and the rd and the rds mice are other models of retinal degeneration (4, 32, 37). Development of retinal degeneration in WBN/Kob rats is similar to that in the rds mouse (7). Except for the rod and cone layers, development of the retinal layers in the rds mice is normal; early changes are observed in the photoreceptor layer, and degeneration is more severe in the external retinal region.

Calpains (EC 3.4.22.17) are Ca^{2+}-activated cysteine proteases. Enzyme activity is regulated by an endogenous inhibitory protein, calpastatin, and by phospholipids (24). Two major classes of calpains are known: ubiquitous calpains (calpain 1/μ-calpain, calpain 2/m-calpain, and calpain 10), and tissue-specific calpains such as calpain 3/p94 in the muscle and its splice variants (3, 5, 14, 21, 25, 30). Proteolysis by calpains has been suggested to play an important role in visual functions (1, 2). α-Spectrin is one of the substrates for activated calpain, and is particularly vulnerable to calpain (26). Results of our previous in vivo study, using an experimental model, suggested that ubiquitous calpains 1 and 2 played an important role in ganglion cell death induced by ischemia-reperfusion in rat retina (29). Calpain inhibitor SJA6017 partially protected against loss of ganglion cells (29).

Atypical calpain 10, along with calpains 1 and 2, is the third ubiquitous calpain in tissues. Levels of calpain 10 in the lens changed with aging in male WBN/Kob rats; this change was associated with formation of cortical cataract (28). Calpain 10 was expressed in all tissues, including the retina. However, the biochemical function of calpain 10 in the retina is unknown.

The tissue-specific calpains may have unique properties or structures distinct from those of the ubiquitous calpains. Retina-specific calpain Rt88 is a splice variant of muscle-specific calpain 3 (3). Recombinant Rt88 was proteolytically active after activation by calcium, and intact Rt88 was rapidly broken due to the presence of the IS1 region in domain II. However, to the authors'

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knowledge, data for the role of Rt88 in the retina are not available. Calpain Rt88 is predicted to perform specific proteolytic functions during development, normal turnover, or pathologic degeneration of retinal proteins. We recently reported that ubiquitous calpains were involved in the biochemical mechanism of cataractogenesis in spontaneously diabetic WBN/Kob rats (28). Thus, the purpose of the study reported here was to examine the involvement of calpain isoforms such as calpain 1, calpain 2, Rt88, and calpain 10 in retinal degeneration in the WBN/Kob rat during aging.

Materials and Methods

Experimental animals. Male and female WBN/Kob rats and control Wistar rats were obtained from Shizuoka Laboratory Animal Center (Shizuoka, Japan). Rats were monitored and were maintained free of the following pathogens: *Pseudomonas aeruginosa*, *Salmonella* spp., *Pasteurella pneumotropica*, *Bordetella bronchiseptica*, *Streptococcus pneumoniae*, *Corynebacterium kutscheri*, *Clostridium piliforme*, sialodacryoadenitis virus, Sendai virus, hantavirus, pneumonia virus of mice, murine adenovirus, *Giardia* spp., *Spironucleus* spp., *Syphacia* spp., and *Aspiculuris tetraptera*. Rats were maintained at room temperature on a 12/12-h light/dark schedule, with ad libitum access to food and water. All experimental animals were handled in accordance with the Declaration of Helsinki and The Guiding Principles in the Care and Use of Animals (DHEW Publication, NIH 80-23) and according to the tenets of the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Hematoxylin and eosin staining. To assess morphologic changes in the retina, rats were sacrificed at 5, 12, 24, and 48 weeks of age. The enucleated eyes were immersed overnight in fixative solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1*M* phosphate buffer (pH 7.4), followed by dehydration and paraffin embedding. Two-micron-thick sections of the retina through the optic disk were stained with hematoxylin and eosin.

Electroretinography. Electroretinograms (ERG) were recorded at 3, 5, 12, 24, and 48 weeks of age. Before recording ERGs, rats were dark adapted for at least 2 h. All further procedures were performed under dim red illumination. Rats were anesthetized by intramuscular administration of a 1:1 mixture of Ketalar50 (ketamine hydrochloride, Sankyo, Tokyo, Japan) and Celactal (xylazine hydrochloride, Bayer, Leverkusen, Germany). The pupils had been dilated in advance by use of a mydriatic (Midorin-P, Santen, Osaka, Japan). The ERG was recorded using a platinum coil wire electrode positioned on the cornea. A similar wire electrode placed in the mouth served as the reference electrode, and a needle electrode inserted into the tail was used as the ground. The ERG response was amplified using a low-cut filter setting of 2 Hz and a high-cut filter setting of 1 kHz, and was recorded by use of a commercial system (Neuropack-II plus, Nihon Kohden, Tokyo, Japan). White (xenon) stroboscopic flashes (20 milliseconds) were presented by a Ganzfeld stimulator (VPA-10; Cadwell, Kennewick, Wash.) against a white adapting field. One flash was delivered every second, and responses to 16 successive flashes were averaged as one measurement. The amplitude of the a-wave was measured from the isoelectric line to the trough of the a-wave, and the amplitude of the b-wave was measured from the trough of the a-wave to the peak of the b-wave.

Measurement of total calcium content. Dry weight of the retinas was measured following heating at 100°C for 16 h. The retinas were then digested overnight in 0.2 ml of concentrated HCl at room temperature, with gentle agitation, 0.8 ml of water was added, and calcium content was measured by use of atomic absorption spectrophotometry (Polarized Zeeman Atomic Absorption Spectrophotometer Z-8100, Hitachi, Tokyo, Japan). Total calcium content in the retina was expressed as milliequivalents per kilogram of retinal dry weight.

Protein preparation. Retinas were homogenized in buffer containing 20 m*M* Tris (pH 7.5), 5 m*M* EGTA, 5 m*M* EDTA, and 2 m*M* dithioerythritol (DTE). The soluble and insoluble proteins were separated by centrifugation at 13,000 ×*g* for 20 min at 4°C. The insoluble fraction was further washed twice in buffer. Protein concentrations were measured using the BCA assay (Pierce Chemical Co., Rockford, Ill.), with bovine serum albumin as the standard.

Immunoblot analysis for calpain 2, calpain 10, and α-spectrin. Sodium dodecyl sulfide-polyacrylamide gel electrophoresis (SDS-PAGE) of soluble proteins (40 µg/lane for calpain 2 and calpain 10) or insoluble proteins (30 µg/lane for calpain 10) was performed on discontinuous, 12% (for calpain 2) or 8% (for calpain 10) gels using the glycine buffer system (10). Immunoblot analysis was performed by electrotransferring proteins from SDS-PAGE gels to polyvinylidene fluoride (PVDF) membranes at 100 V (constant) for 70 min at ice-cold temperature, using blotting buffer containing 25 m*M* Tris, 192 m*M* glycine, 0.1% SDS, and 20% methanol (34). Rabbit anti-calpain 2 or anti-calpain 10 (11) polyclonal antibodies were used at a 1:250 or 1:200 dilution. The SDS-PAGE of soluble proteins (30 µg/lane) was performed on discontinuous 8% gels using the tricine buffer system, and immunoblot analysis for α-spectrin was performed by electrotransferring proteins from SDS-PAGE gels to a PVDF membrane at 100 V (constant) for 90 min at ice-cold temperature using blotting buffer containing 25 m*M* Tris, 192 m*M* glycine, 0.2% SDS, and 20% methanol. A mouse anti-α-spectrin (nonerythroid) monoclonal antibody (Affiniti Research Product Ltd., Exeter, UK) was used at a 1:1,000 dilution. Immunoreactivity was visualized by addition of alkaline phosphatase conjugated to anti-rabbit IgG or anti-mouse IgG secondary antibody and BCIP/NBT (Bio-Rad, Hercules, Calif.). The staining intensity of the immunoblots of 150 kDa of spectrin breakdown products and calpain 2 were determined by use of densitometric image analysis with NIH Image software version 1.62.

Casein zymography. Casein zymography was performed using the method of Raser and co-workers (22). Eight percent acrylamide gels, copolymerized with 0.1% casein (TEFCO, Tokyo, Japan), were pre-run with buffer containing 25 m*M* Tris (pH 8.3), 192 m*M* glycine, 1 m*M* EGTA, and 1 m*M* dithiothreitol (DTT) for 15 min at 4°C. Fifty micrograms of retinal soluble proteins was then loaded and was electrophoresed. After electrophoresis, the gels were incubated overnight at room temperature in buffer containing 20 m*M* Tris (pH 7.4), 2 m*M* $CaCl_2$, and 10 m*M* DTT, with slow shaking. Gels were stained with Coomassie brilliant blue, and bands of caseinolysis appeared white. The intensity of the zymogram gels of calpain 2 were determined by use of densitometric image analysis.

Isolation of RNA and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Using a dissecting microscope, the eyes were bisected at the equator, the retinas were ob-

Table 1. Primer sequences and cycles for reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Name	GeneBank accession no.	Sequence (5'-3')	Cycle	Product size (bp)
Calpain 1	U53858	up: tgg aga tca gtg tca agg agt tac down: gca ctc atg ctg cca gac ttg tcc agg tca	28	235
Calpain 2	L09120	up: ggg cag acc aac atc cac ctc agc aaa aac down: atc tcc gca tcc tct cca gcc agc	26	320
Calpastatin	X56729	up: atc tga ttt cac ctg tag ctc tcc aac tg down: tgt gtc ctc atc ttc acc aca ttt ctc	26	292
Total Rt88	AF061726	up: ggt gac aga gtt ttt tga gat caa gg down: gca ccg gaa caattg tcc gtg acg gtc tgt	27	260
Rt88'	AF173834	up: ggg gct gtt ctg gag gct gcc gga act down: gaa gct ttt cgg gga gcc aca gac aga ctt	30	300
Rt90	AF184950	up: ggc ccc agc tgt ctg aaa gca gct cct cac down: tgg tga tga tgt cat aga gtt ggc tgt tga	30	300
Calpain 10	NM-031673	up: gag ctc ttc cgg gac gcc gca ttc down: cca aac tgc caa atc cga cag gtg	24	317

tained, and other visible contaminating tissues were removed. Total RNA was isolated by use of TRIzol reagent (Invitrogen Corp., Carlsbad, Calif.), then the RNA was treated with DNase I (Invitrogen Corp.) to remove genomic DNA. One microgram of total RNA was reverse transcribed using random primers (Invitrogen Corp.) for 50 min at 42°C in 20 µl of 50 m*M* Tris-HCl (pH 8.3), 75 m*M* KCl, 3 m*M* $MgCl_2$, 10 m*M* DTT, 0.5 m*M* dNTP mixture, and 200 U of Moloney murine leukemia virus reverse transcriptase (Invitrogen Corp.); then the mixture was heated to 70°C for 15 min.

Polymerase chain reaction analysis was performed in 20 m*M* Tris-HCl (pH 8.4), 50 m*M* KCl, 1 m*M* $MgCl_2$, 0.2 m*M* dNTP mixture (Invitrogen Corp.), and 0.2 µ*M* each primer (Table 1) with 1 µl of cDNA solution. Platinum *Taq* DNA polymerase (1.875 U; Invitrogen Corp.) was added in a final volume of 25 µl. To allow direct comparison between multiple samples, QuantumRNA 18S internal standards (Ambion Inc., Austin, Tex.) were used as the internal controls. The PCR conditions for calpain 1, calpain 2, calpain 10, total-Rt88 (Rt88 + Rt88' + Rt90), Rt88', Rt90, and calpastatin were 94, 60, and 72°C, respectively, each for 1 min, followed by 72°C for 7 min. The amplification for calpains and calpastatin was performed using adequate cycles (Table 1), and the PCR products were evaluated during the exponential phase of the amplification process. Each PCR reaction mixture (10 µl) was size separated by use of electrophoresis on 1.5 % agarose gels. The gels were stained with ethidium bromide and were scanned by use of Gel Doc 2000 (Bio-Rad). The NIH Image software version 1.61 was used to invert the image and to measure the uncalibrated optical density of the bands corresponding to PCR products. To compare samples, the ratio of densities for target/18S rRNA was calculated.

In situ hybridization. For calpain 2 mRNA detection, rat cDNA fragments for calpain 2 (Genbank Accession No. NM_017116) were isolated by use of RT-PCR analysis, and were subcloned into pGEM-T easy vector (Promega, Madison, Wis.). This template was linearized, and digoxigenin-labeled riboprobes for sense and antisense were synthesized using the T7 and SP6 RNA polymerases (Roche, Indianapolis, Ind.). Two-micrometer-thick sections were deparaffinized, treated with 10 µg of proteinase K/ml for 15 min at 37°C, washed in phosphate-buffered saline (PBS), further fixed in 4% paraformaldehyde for 5 min at room temperature, washed in PBS again, and rinsed in 50% formaldehyde (FA)/2× SSC (0.3 M sodium chloride and 30 mM sodium citrate). Hybridization was performed in 50 % FA, 10 m*M* Tris-HCl (pH 8.0), 0.2 µg of yeast tRNA/µl, 1× Denhardt's solution, 10 % dextran sulfate, 600 m*M* NaCl, 1 m*M* EDTA, 0.25% SDS, and digoxigenin-labeled antisense or sense probes at 45°C overnight. The slides were washed in 50% FA/2× SSC at 45°C for 1 h and 50% FA/0.1× SSC at 45°C for 1 h.

After equilibration in 10 m*M* Tris-HCl (pH 8.0) with 500 m*M* NaCl, blocking was performed by addition of 1.5% blocking reagent (Roche) for 1 h at room temperature, then the solution was incubated with anti-digoxigenin antibody conjugated to alkaline phosphatase (Roche) at a 1:1,000 dilution for 30 min at 37°C. For visualization, the tissue sections were washed in PBS, then were incubated overnight with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) (Roche) at room temperature to develop color. The reaction was stopped by addition of 10 m*M* Tris-HCl (pH8.0)/1 m*M* EDTA. As a control, hybridization was also performed using the sense probe.

Statistical analysis. Statistical analysis of data was performed by use of Student's *t* test. Data from WBN/Kob rats were compared with data from age- and sex-matched Wistar rats. Significance was set at $P < 0.05$.

Results

Light microscopy revealed development of retinal degeneration in WBN/Kob rats with age (Fig. 1). There were no substantial differences in the retinal structures between 5-week-old WBN/Kob rats (Fig. 1B) and control Wistar rats (Fig. 1A), although the thickness of inner nuclear layer cells in 5-week-old WBN/Kob rats was slightly thinner than that in Wistar rats. Obvious changes in retinal thickness were observed in 12-week-old WBN/Kob rats (Fig. 1D). The photoreceptor layer appeared thin, and the number of outer and inner nuclear layer cells was reduced compared with that in Wistar rats (Fig. 1C). These changes in WBN/Kob rats became severe with age (Fig. 1F). At 48 weeks of age, only a single outer nuclear cell layer remained, and photoreceptor cells almost disappeared in WBN/Kob rats (Fig. 1H). This was in contrast to the retinas in age-matched Wistar rats, where the retinal layers did not disappear, although retinal thickness gradually decreased with age (Fig. 1G). There was no difference in progression of retinal degeneration in age-matched male and female WBN/Kob rats. In contrast to retinal degeneration, only male WBN/Kob rats developed cataracts at 48 weeks of age, because only male WBN/Kob rats develop diabetes mellitus (data not shown).

To assess functional changes, an electroretinographic study was performed. Representative ERG waves are shown in Fig. 2A, and the amplitudes of a- and b-waves are shown in Fig. 2B and Fig. 2C. The amplitudes of the a-wave from 12-week-old

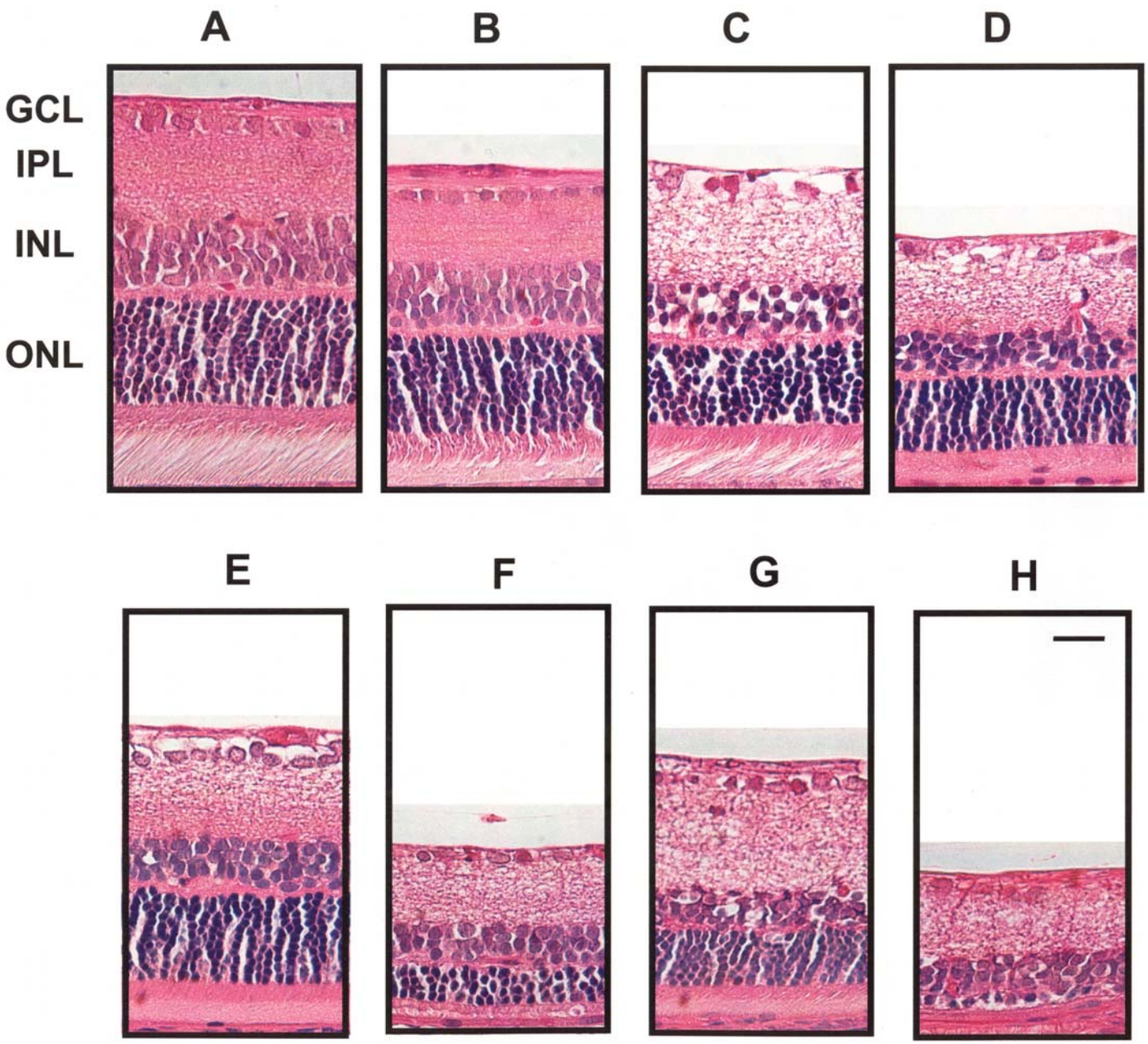

Figure 1. Photomicrographs of sections of the retina from control male Wistar rats (A, C, E, G) and male WBN/Kob rats (B, D, F, H) aged 5 weeks (A, B), 12 weeks (C, D), 24 weeks (E, F), and 48 weeks (G, H). Obvious changes in retinal thickness were observed in 12-week-old WBN/Kob rats, and these changes became severe with age. Progression of retinal degeneration in female WBN/Kob rats was almost the same as that in age-matched male WBN/Kob rats (data not shown). GCL = ganglion cell layer, IPL = inner plexiform layer, INL = inner nuclear layer, ONL = outer nuclear layer. H&E stain; bar = 20 μm.

male WBN/Kob rats were significantly less than those in 12-week-old male Wistar rats, and in contrast to that in 12-week-old female Wistar rats, the amplitudes of the a-wave from 12-week-old female WBN/Kob rats had a tendency to decrease. The amplitudes of a-waves from 24- and 48-week-old WBN/Kob rats also were significantly less than those in age- and sex-matched Wistar rats. The a-wave originates from the photoreceptor cells, and the decrease of a-waves in WBN/Kob rats with age was strongly associated with the histologic changes, as mentioned previously. The amplitude of the b-waves from 5-week-old or older WBN/Kob rats was significantly less than that in age- and sex-matched Wistar rats. The b-wave originates from the bipolar cells in the inner nuclear layer, and the decrease of b-waves with age in WBN/Kob rats also was associated with histologic changes. The ERG became non-recordable at 48 weeks in male and non-diabetic female WBN/Kob rats. The wave patterns were similar between age-matched male and female WBN/Kob rats.

Proteolysis of α-spectrin, a well-known and sensitive substrate for calpain, was examined by use of immunoblot analysis (Fig. 3). New 150-kDa bands of proteolyzed α-spectrin were observed in 12-week-old WBN/Kob rats. Only weak 150-kDa bands were visible in age-matched control Wistar rats. At 12 weeks of

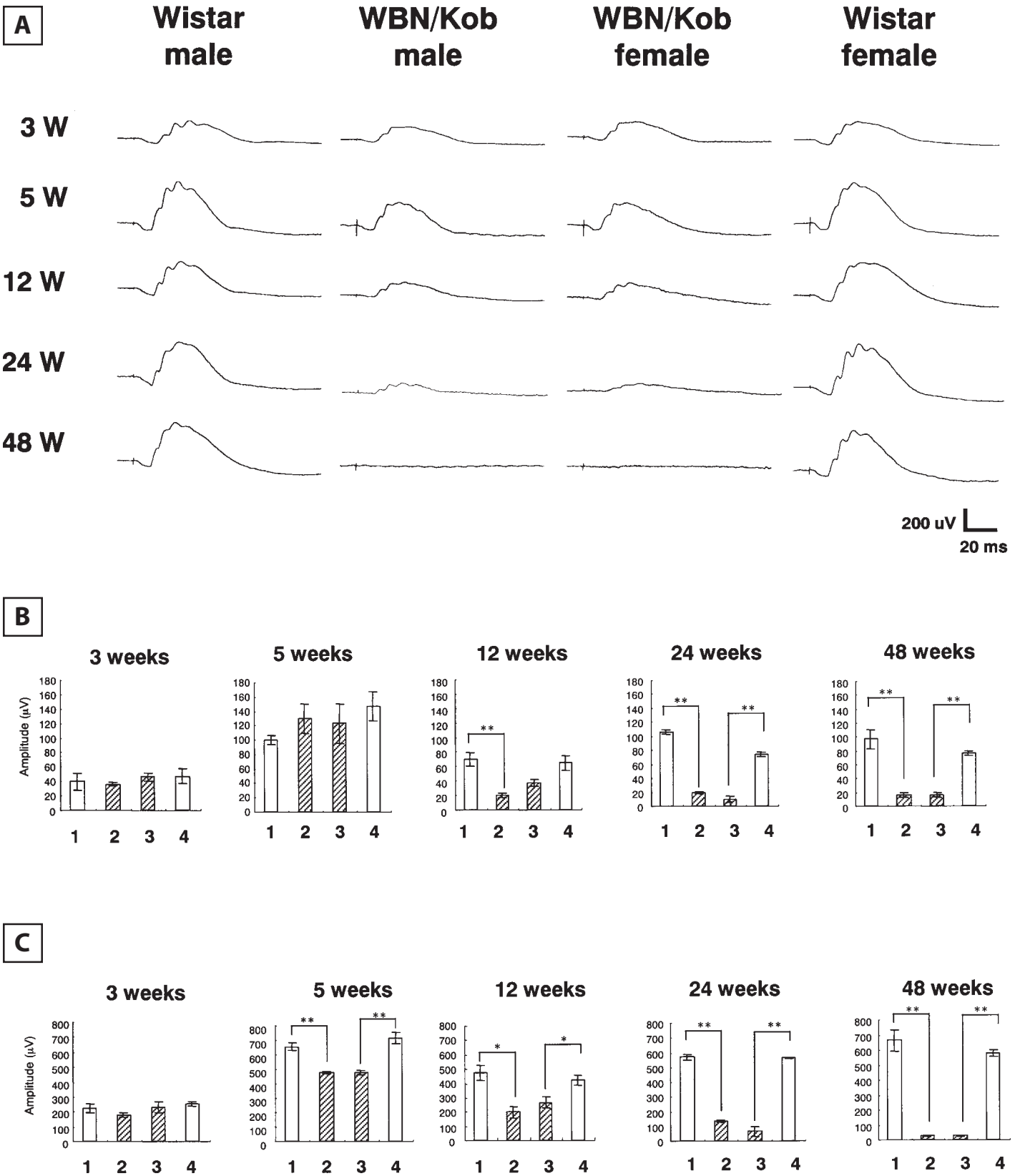

Figure 2. Electroretinograms from rats of the study. (A) Comparison of scotopic electroretinogram (ERG) wave form changes in the left eyes of each indicated group after dark adaptation for 2 h. The ERG responses were recorded at 3, 5, 12, 24, and 48 weeks old of age. Obvious changes in a- and b-waves were observed in 12-week-old WBN/Kob rats, and these changes became severe with age. Amplitude of a-waves (B) and b-waves (C) in the retinas from male Wistar rats (lane 1), male WBN/Kob rats (lane 2), female WBN/Kob rats (lane 3), and female Wistar rats (lane 4). Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$ and $^{**}P < 0.01$ relative to value for age- and sex-matched Wistar rats.

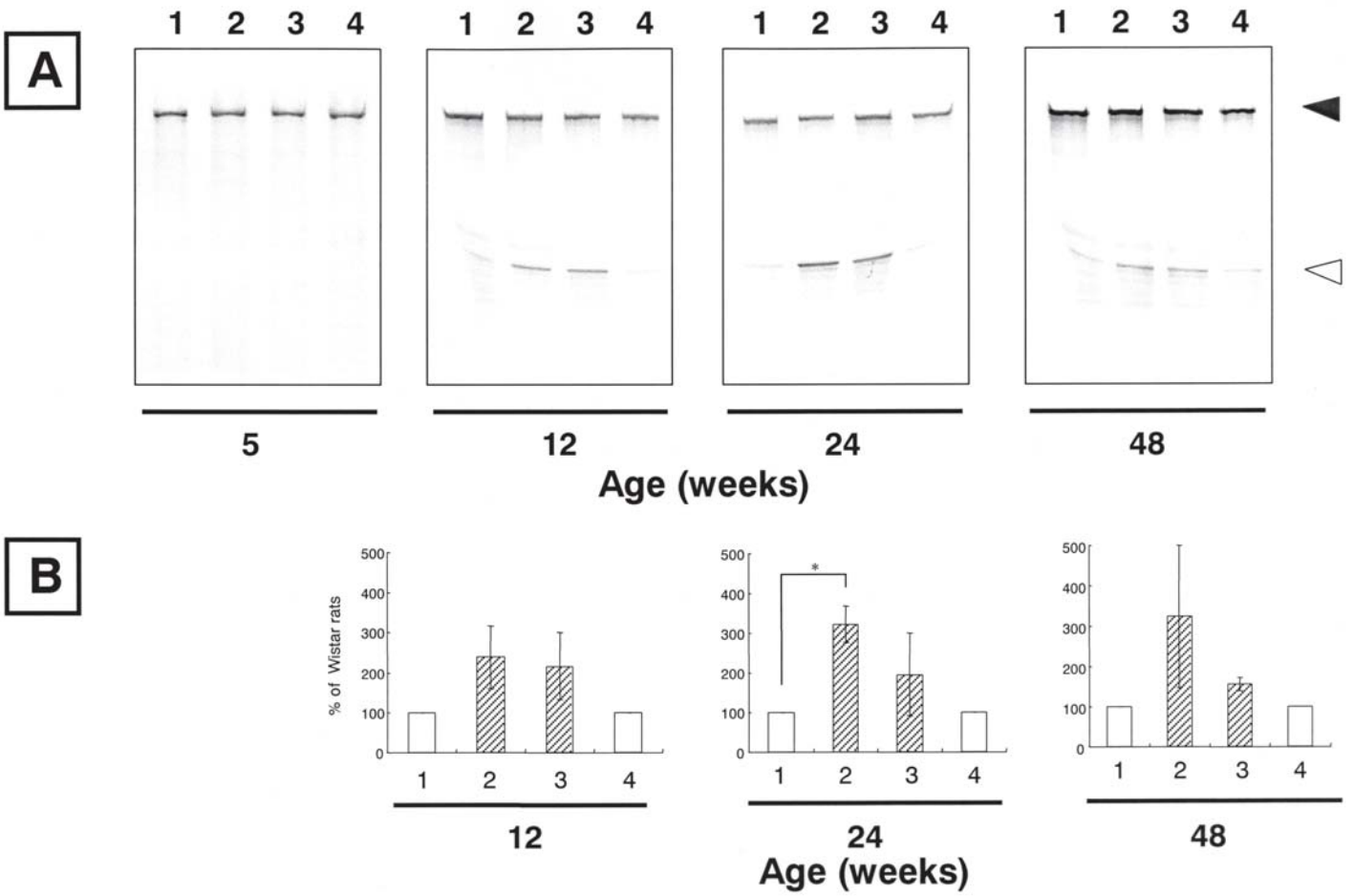

Figure 3. (A) Representative immunoblots for α-spectrin in retinal soluble proteins from male Wistar rats (lane 1), male WBN/Kob rats (lane 2), female WBN/Kob rats (lane 3), and female Wistar rats (lane 4). The solid and open arrowheads indicate intact 230-kDa α-spectrin, and the 150 kDa α-spectrin breakdown products, respectively. New bands of proteolyzed α-spectrin appeared at 150 kDa in 12-week-old WBN/Kob rats. (B): Densitometric analysis of the band for 150 kDa α-spectrin breakdown products indicated by the open arrowhead in (A). Data are expressed as mean ± SEM (n = 2 to 3). $^{*}P < 0.05$, relative to value for age- and sex-matched Wistar rats.

age, a weak 145-kDa band appeared only in WBN/Kob rats. A similar pattern of proteolysis was observed at 24 and 48 weeks. Densitometric analysis also revealed the increase of 150-kDa breakdown products in 12-, 24-, and 48-week-old WBN/Kob rats (Fig. 3B).

To test whether increased calcium content was associated with the calpain-induced proteolysis, total calcium content was measured (Fig. 4). At 5 weeks of age, the calcium content in whole retinas of WBN/Kob rats was approximately twice that of control Wistar rats. After that, the calcium content of retinas from WBN/Kob rats gradually increased, and it was significantly higher than that in control Wistar rats.

Casein zymography was used to measure calpain activities in the retinas of WBN/Kob rats during aging. Compared with those of age- and sex-matched control Wistar rats, activities of intact calpains 1 and 2 increased in 5-week-old WBN/Kob rats and remained increased at 48 weeks (Fig. 5A). Densitometric analysis confirmed the increase of calpain 2 activity in 5-, 12-, and 24-week-old WBN/Kob rats (Fig. 5B). Immunoblot analysis for calpain 2 in WBN/Kob rats indicated a small increase in calpain 2 protein values in (data not shown).

To help explain changes in calpain activities, the expression patterns of mRNAs for calpains and the endogenous inhibitor,

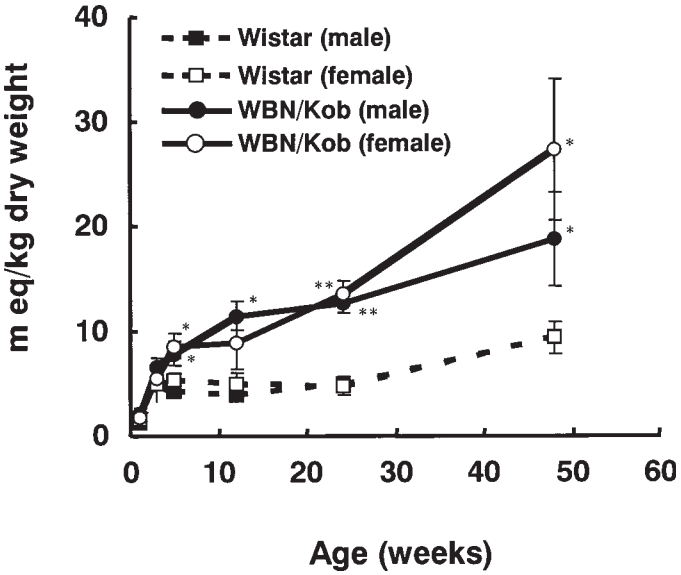

Figure 4. Total calcium content in whole retina. Notice minimal increase in 5-week-old WBN/Kob rats, and thereafter, gradual increase with age. Data are expressed as mean ± SEM (n = 2 to 4). $^{*}P < 0.05$, $^{**}P < 0.01$ relative to the value for age- and sex-matched Wistar rats.

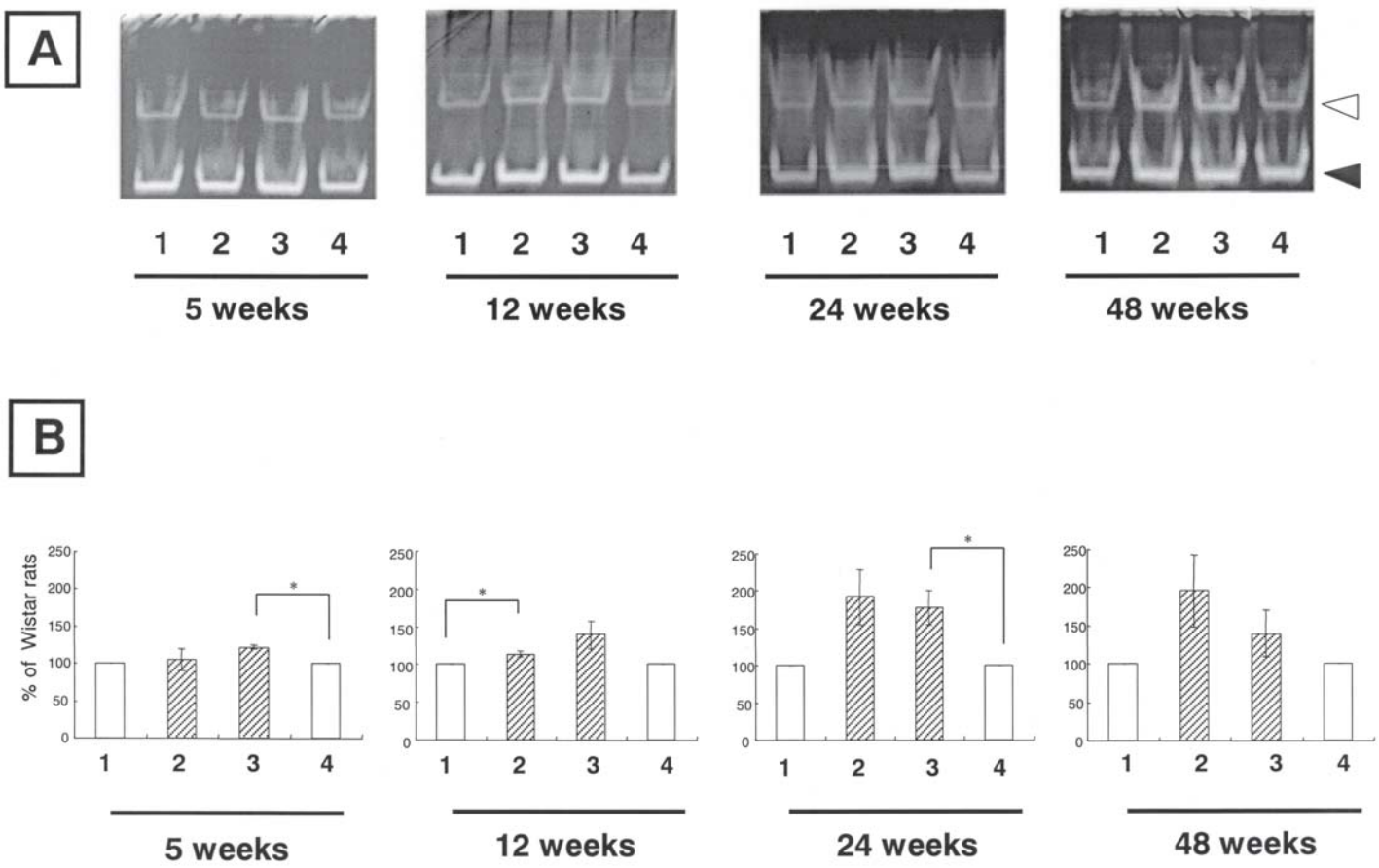

Figure 5. (A) Representative casein zymograms for calpain in retinal soluble proteins from male Wistar rats (lane 1), male WBN/Kob rats (lane 2), female WBN/Kob rats (lane 3), and female Wistar rats (lane 4). The solid and open arrowheads indicate calpain 2 and calpain 1, respectively. (B) Densitometric analysis of the band for calpain 2 indicated by the solid arrowhead in (A). Caseinolytic activity for calpains 1 and 2 tended to increase minimally in 5-week-old WBN/Kob rats and further increase in some WBN/Kob groups during aging. Data are expressed as mean ± SEM (n = 3). $^{*}P < 0.05$ relative to the value for age- and sex-matched Wistar rats.

calpastatin, were analyzed in rat retinas by use of RT-PCR analysis. The expression of calpain 2 mRNAs was slightly up-regulated at 24 weeks in WBN/Kob rats compared with that in age- and sex-matched control Wistar rats; the values gradually increased at 48 weeks of age (Fig. 6A). To determine whether up-regulation of calpain 2 mRNA in retinas from WBN/Kob rats was due to the decreased retinal cell layer (Fig. 1) or to specific increase in mRNA expression, in situ hybridization analysis of calpain 2 was performed (Fig. 7). The mRNA for calpain 2 was distributed throughout the retina, especially in the outer nuclear layer, where the number of cells decreased with age. This suggested that up-regulation of calpain 2 mRNA was not due to the decreased thickness of the retinal cell layer. The expression of mRNAs for calpain 1 (Fig. 6B) and calpastatin (Fig. 6C) was only slightly up-regulated at 24 and 48 weeks in WBN/Kob rats. In contrast, mRNAs for retina-specific calpains such as total Rt88 (Rt88, Rt88' and Rt90, Fig. 6D), Rt88' (Fig. 6E), and Rt90 (Fig. 6F), were markedly decreased in WBN/Kob rats.

Since the expression of mRNA for calpain 10 was only slightly down-regulated in WBN/Kob rats (Fig. 6G), immunoblot analysis was performed to determine changes in expression of calpain 10 proteins in the retina. The human calpain 10 gene was reported to generate at least eight forms due to alternative splicing (6), and two calpain 10 proteins were found in the lens of young rats (11). In the study reported here, the water-soluble fraction from the retina had one dense band for calpain 10 in one-week-old rats, and this was similar in WBN/Kob and Wistar rats (data not shown). Several dense bands for calpain 10 were observed at 3 weeks, and the pattern of calpain 10 proteins differed between WBN/Kob rats and control Wistar rats. At 24 weeks, the number of calpain 10 bands increased, and the pattern of calpain 10 differed between WBN/Kob rats and control Wistar rats. The expression patterns of calpain 10 proteins were similar in age-matched, male and non-diabetic, female WBN/Kob rats. In the water-insoluble fraction of the retina, only weak bands for calpain 10 were detected, and the pattern of calpain 10 proteins differed between WBN/Kob rats and control Wistar rats (data not shown). The expression patterns for calpain 10 proteins were almost the same in age-matched male and non-diabetic female WBN/Kob rats.

Discussion

The major finding of this study was that activation of ubiquitous calpains 1 and 2 was temporally associated with retinal degeneration in WBN/Kob rats. This conclusion was supported by the following observations. Retinal degeneration with age was observed histologically and functionally in male and female WBN/Kob rats. The photoreceptor cell layer appeared thin, and the

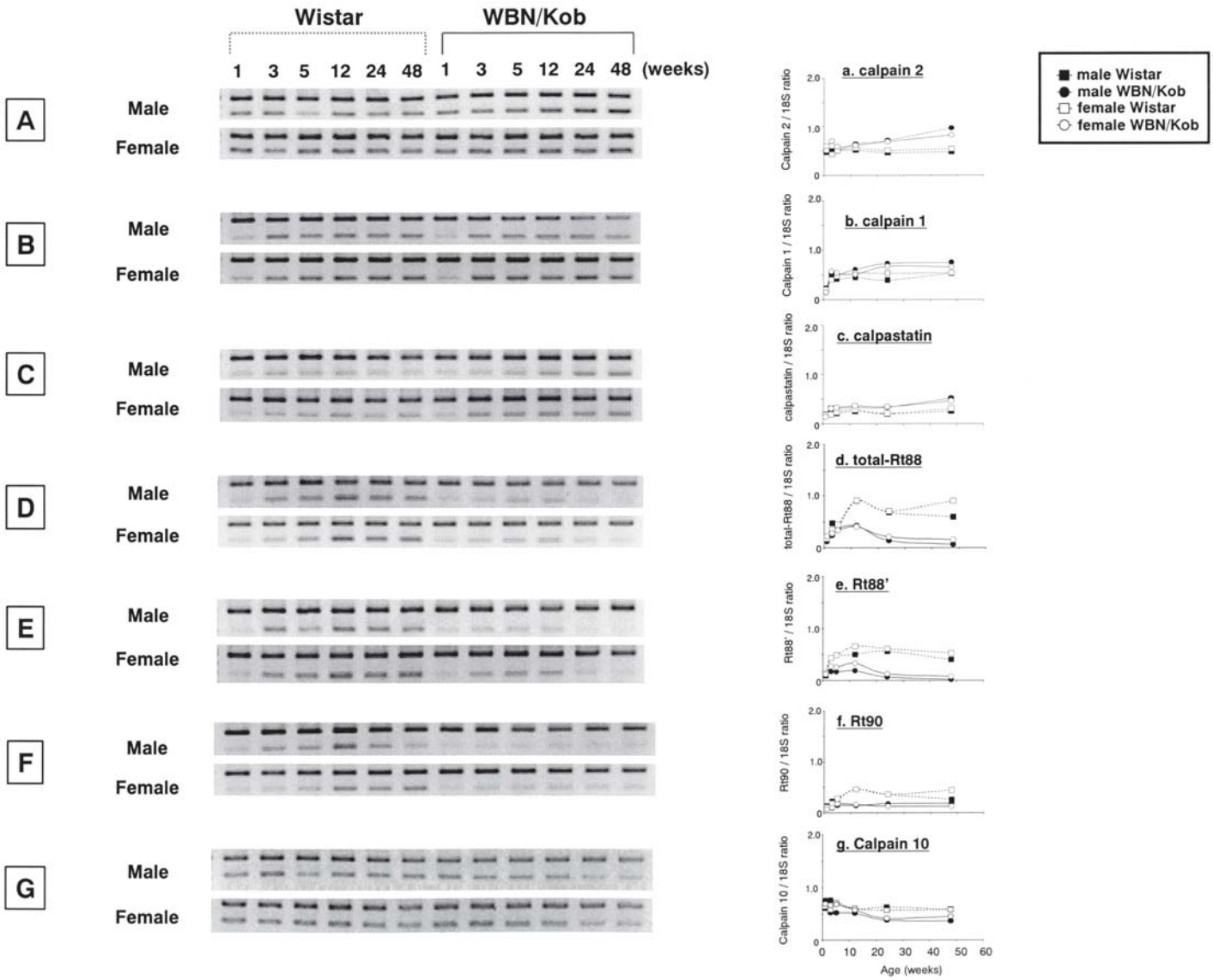

Figure 6. Representative ethidium bromide-stained gels from reverse transcriptase-polymerase chain reaction (RT-PCR; left) and densitometric image (right) analyses of gels for measurement of calpain 2 (A), calpain 1 (B), calpastatin (C), total-Rt88 (D), Rt88' (E), Rt90 (F), and calpain 10 (G) in retinas from WBN/Kob and control Wistar rats. The mRNA levels for calpain isoforms and calpastatin from densitometric image analysis were normalized to 18S ribosomal RNA as an internal standard. The expression of mRNAs for calpains 1 and 2 and calpastatin was slightly up-regulated in WBN/Kob rats. In contrast, mRNAs for retina-specific calpains were markedly decreased in WBN/Kob rats.

number of cells in the outer and inner nuclear layers was reduced in 12-week-old WBN/Kob rats. These changes became severe with age, only the single outer nuclear cell layer remained, and photoreceptor cells almost disappeared at 48 weeks of age. The decreases in a- and b-waves with age in WBN/Kob rats were strongly associated with histologic changes. These changes became severe with age, and the ERG became non-recordable at age of 48 weeks.

α-Spectrin breakdown products of 145/150 kDa were observed in retinas from male and female WBN/Kob rats. Proteolysis of α-spectrin was strongly associated with retinal degeneration. The 150-kDa fragment of α-spectrin is produced not only by calpain but also by caspase-3, whereas the 145-kDa band is produced only by calpain (18, 38). The 150- and 145-kDa fragments are produced sequentially. Retinal ischemia followed by reperfusion and hypoxia treatment in vitro induced proteolysis of α-spectrin. Calpain inhibitor SJA6017 protected against proteolysis of α-spectrin (31). Compared with results of previous studies, proteolysis of α-spectrin in this study was less severe. One possible explanation for this difference was the massive loss of damaged cells in the retina. Our results suggested that calpain-induced proteolysis may induce retinal cell death in WBN/Kob rats.

Caseinolytic activity of calpains 1 and 2 increased in male and female WBN/Kob rats after the age of 12 weeks. Increased calpain was also confirmed by results of immunoblot analysis. Calpain activity was temporally associated with proteolysis of α-spectrin. However, these results were different from those of previous studies, which indicated a decrease in caseinolytic activity in retina after ischemia-reperfusion (29). Decreased calpain activity has been interpreted as indirect evidence of calpain activa-

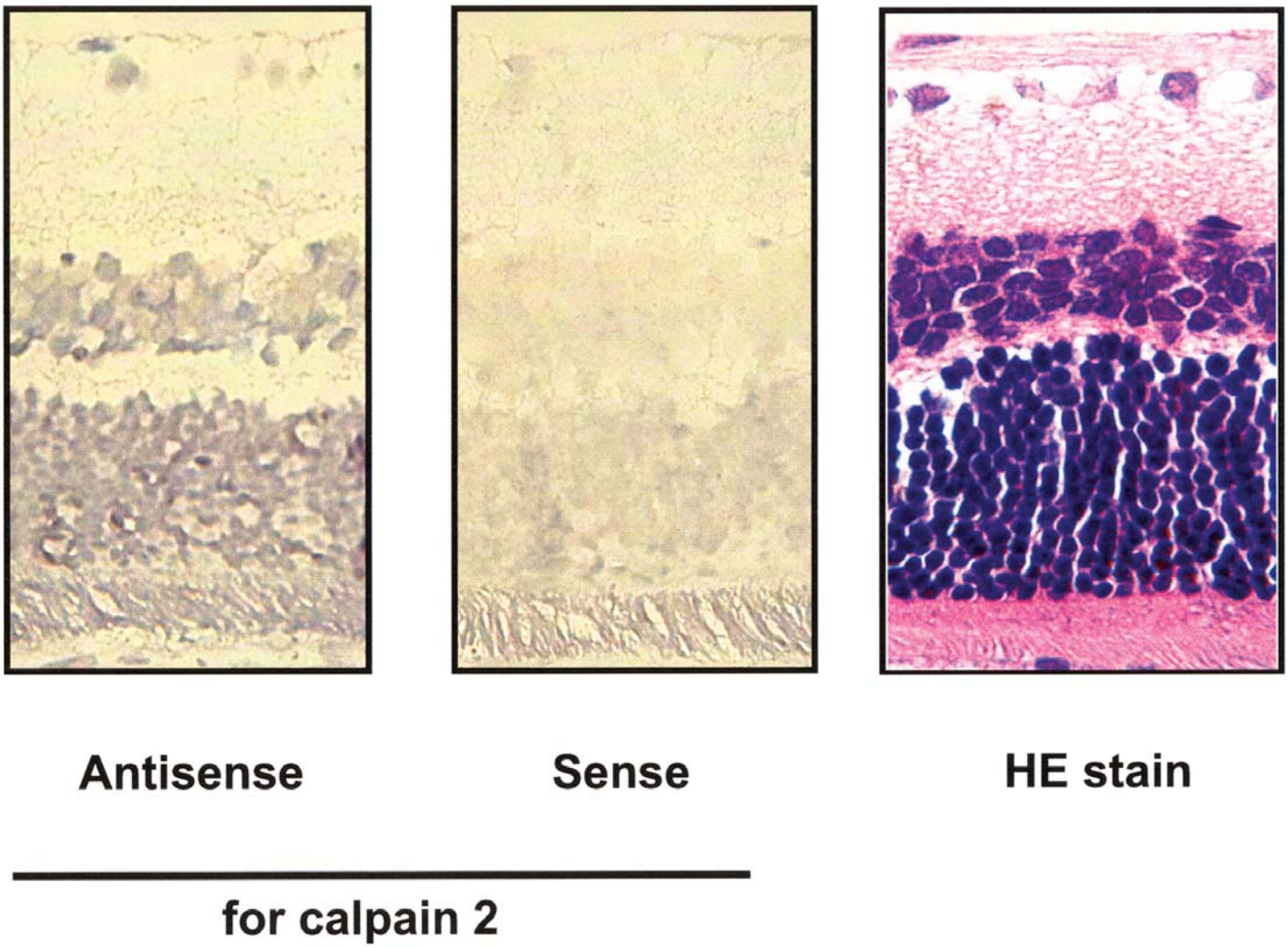

Figure 7. In situ hybridization analysis for calpain 2 in retinas from 3-week-old male WBN/Kob rats. Left: hybridization of the antisense probe to rat calpain 2 showing substantial staining distributed throughout the retina, middle: hybridization of the sense probe to rat calpain 2 as negative control, and right: H&E stain; original magnification = 400×.

tion due to autodegradation of calpain following its activation (17). The massive loss of damaged cells in the retina may have contributed to our results. Along with calpain activation, changes in expression levels of mRNAs for calpains were observed. Calpains 1 and 2 mRNA were mildly up-regulated. This was markedly associated with increased activity of calpains 1 and 2 proteins. Thus, it is quite likely that ubiquitous calpains 1 and 2 were activated and caused proteolysis, which led to retinal degeneration in WBN/Kob rats.

Total calcium concentration were increased in retina from WBN/Kob rats at five weeks and gradually increased until 48 weeks. A positive relationship between increased calcium content and activation of calpain was observed during retinal degeneration in WBN/Kob rats. Another possible candidate protease for retinal degeneration in WBN/Kob rat was the recently discovered calpain 10, which is a third ubiquitous calpain in tissues, along with calpains 1 and 2. The Rt88, a retina-specific calpain 3 splice variant, also was a possible candidate protease.

Our results indicated that the expression of calpain 10 mRNA was only slightly down-regulated in male and female WBN/Kob rats, but the expression patterns of calpain 10 proteins were different in male and female WBN/Kob and age-matched Wistar rats. These changes were associated with retinal degeneration in WBN/Kob rats.

To our knowledge, this is the first documentation that mRNA expression levels for retina-specific calpains such as Rt88, Rt88', and Rt90 are changed in retinal disease. Our study results indicated that mRNAs for Rt88, Rt88', and Rt90 were markedly down-regulated in WBN/Kob rats. Mutations in muscle-specific calpain 3 were previously found to cause limb girdle muscular dystrophy type 2A in man, due to loss in proteolytic activity against a potential substrate (20). Taken together, a decrease of Rt88 activity may cause retinal degeneration. In vivo measurement of Rt88 proteolytic activity is difficult, since Rt88 is susceptible to autolysis (3). Further studies are needed to measure the proteolytic activities of Rt88, Rt88', and Rt90 and to determine their relationship to retinal degeneration.

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Azuma is an employee of Senju Pharmaceutical Co., Ltd., a company that may have commercial interest in the results of this research and technology. This potential conflict of interest has been reviewed and managed by the OHSU Conflict of Interest in Research Committee.

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