

# Protein Kinase C- $\delta$ Mediates Neuronal Apoptosis in the Retinas of Diabetic Rats via the Akt Signaling Pathway

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**OBJECTIVE**—Protein kinase C (PKC)- $\delta$ , an upstream regulator of the Akt survival pathway, contributes to cellular dysfunction in the pathogenesis of diabetes. Herein, we examined the role of PKC- $\delta$  in neuronal apoptosis through Akt in the retinas of diabetic rats.

**RESEARCH DESIGN AND METHODS**—We used retinas from 24- and 35-week-old male Otsuka Long-Evans Tokushima fatty (OLETF) diabetic and Long-Evans Tokushima Otsuka (LETO) nondiabetic rats. To assess whether PKC- $\delta$  affects Akt signaling and cell death in OLETF rat retinas, we examined 1) PKC- $\delta$  activity and apoptosis; 2) protein levels of phosphatidylinositol 3-kinase (PI 3-kinase) p85, heat shock protein 90 (HSP90), and protein phosphatase 2A (PP2A); 3) Akt phosphorylation; and 4) Akt binding to HSP90 or PP2A in LETO and OLETF retinas in the presence or absence of rottlerin, a highly specific PKC- $\delta$  inhibitor, or small interfering RNAs (siRNAs) for PKC- $\delta$  and HSP90.

**RESULTS**—In OLETF retinas from 35-week-old rats, ganglion cell death, PKC- $\delta$  and PP2A activity, and Akt-PP2A binding were significantly increased and Akt phosphorylation and Akt-HSP90 binding were decreased compared with retinas from 24-week-old OLETF and LETO rats. Rottlerin and PKC- $\delta$  siRNA abrogated these effects in OLETF retinas from 35-week-old rats. HSP90 siRNA significantly increased ganglion cell death and Akt-PP2A complexes and markedly decreased HSP90-Akt binding and Akt phosphorylation in LETO retinas from 35-week-old rats compared with those from nontreated LETO rats.

**CONCLUSIONS**—PKC- $\delta$  activation contributes to neuro-retinal apoptosis in diabetic rats by inhibiting Akt-mediated signaling pathways. *Diabetes* 57:2181–2190, 2008

**P**rotein kinase C (PKC)- $\delta$ , a ubiquitously expressed isoform of the novel PKC subfamily, mediates an anti-apoptotic signaling cascade through the phosphatidylinositol 3-kinase (PI 3-kinase)-mediated survival pathway (1,2) and also promotes apoptosis by interfering with Akt signaling (3–5).

Akt is a downstream target of PI 3-kinase that plays an integral role in cell survival. Dysregulation of Akt is

frequently observed in diseases such as cancers and diabetes (6–8). PI 3-kinase activates Akt through the phosphorylation of two key regulatory residues, Thr308 and Ser473, on Akt. Phosphorylation of both residues is necessary for full activation of Akt and subsequent regulation of many PI 3-kinase-mediated biological responses (9,10).

Protein phosphatase 2A (PP2A), a major cellular serine/threonine phosphatase, regulates the phosphorylation state of cellular proteins in various pathological conditions (11–13). Recently, it has been reported that PP2A is involved in the regulation of cell proliferation and survival through its ability to dephosphorylate Akt (11–15). Furthermore, heat shock protein 90 (HSP90) counteracts the effect of PP2A in cells through direct binding to Akt, protecting Akt from PP2A-mediated dephosphorylation and thus functioning as a positive regulator of Akt signaling (13,15,16). Of note, numerous reports have suggested that Akt- or HSP-mediated cytoprotection is regulated by PKC (1,5,13,17,18).

Otsuka Long-Evans Tokushima fatty (OLETF) rats are a genetic animal model of late onset of hyperglycemia. Animals spontaneously develop type 2 diabetes and exhibit hyperglycemia and insulin resistance at 20–40 weeks of age (19–21). Recently, we reported that PKC- $\delta$  activation is involved in neuronal apoptosis in 35-week OLETF rat retinas (22); however, a direct association between PKC- $\delta$  and Akt was not defined.

Therefore, we examined effects of PKC- $\delta$  on Akt-mediated survival pathways and neuronal apoptosis in the retinas of diabetic OLETF rats.

## RESEARCH DESIGN AND METHODS

Six-week-old male OLETF and Long-Evans Tokushima Otsuka (LETO) rats were obtained from the Otsuka Pharmaceutical Tokushima Research Institute (Tokushima, Japan). We used 8 LETO and 8 OLETF rats at 24 weeks of age and 28 LETO and 28 OLETF rats at 35 weeks of age. Rats were housed in groups of three animals and supplied with water and food ad libitum under ambient temperature conditions ( $22 \pm 2^\circ\text{C}$ ) and a 12-h light/dark cycle, in accordance with the protocol of the institutional review board. We randomly selected five LETO and five OLETF rats at 24 and 35 weeks of age and measured their body weights and blood glucose levels. Blood samples were obtained by tail snipping after a 2-h fasting period. Blood glucose levels were measured using the SureStep (LifeScan, Milpitas, CA).

**Intravitreal injection.** Rats were anesthetized with an intraperitoneal injection of 50 mg/kg sodium pentobarbital followed by topical application of 0.5% proparacaine to the eye. For intravitreal injection, a 30-gauge needle was inserted into the vitreous 2 mm posterior to the limbus through the pars plana using a microscope, without damaging the lens and the retina. Injections were covered by the institutional animal care and use committee of Gyeongsang National University.

Rottlerin (Sigma, St Louis, MO), a highly specific PKC- $\delta$  inhibitor, was dissolved in 0.5% dimethyl sulfoxide (DMSO), and 3  $\mu\text{l}$  rottlerin (5  $\mu\text{mol/l}$ ) was used for intravitreal injection into the right eye of 35-week-old LETO and OLETF rats. DMSO (3  $\mu\text{l}$ ) was injected into the left vitreous as a control. All rats were killed 1 day after the injection.

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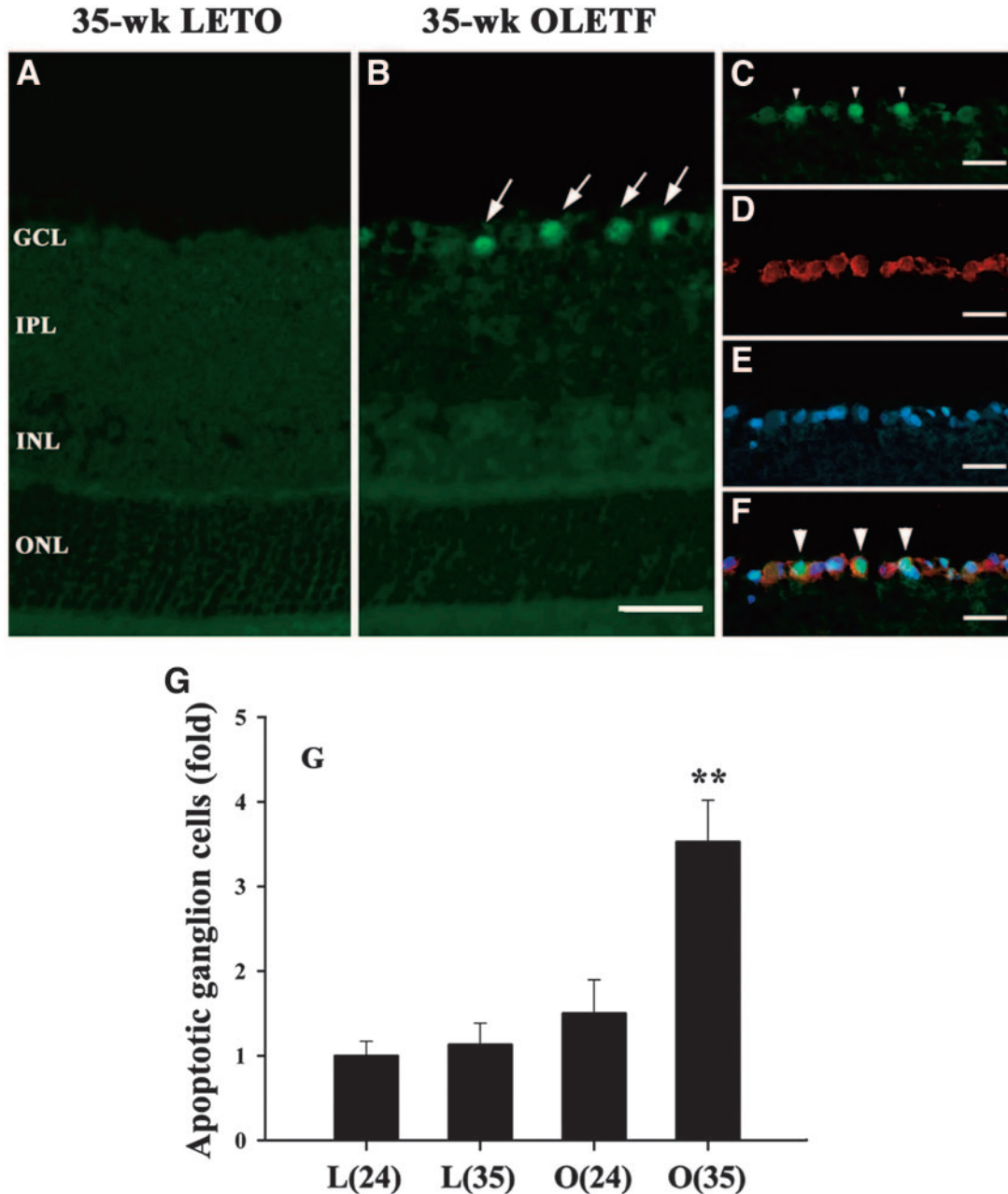
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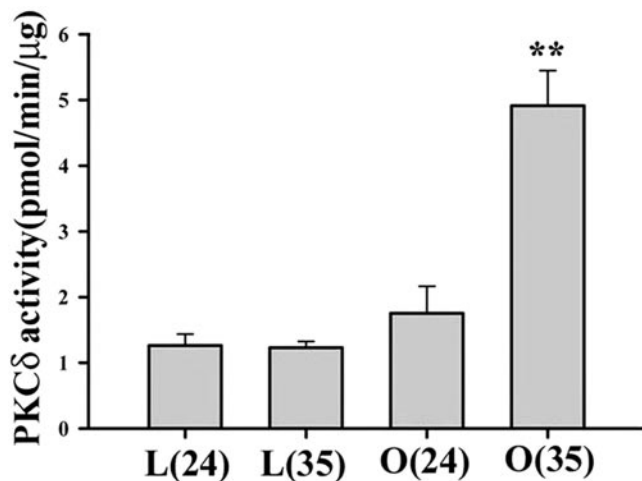
For PKC- $\delta$  and HSP90 gene silencing, we used commercially available small interfering RNAs (siRNAs) from Dharmacon (ON-TARGET plus Duplex J-080142-05-0050 for Rat PRKCD and J-102259-01-0020 for Rat Hspcb; Dharmacon, Chicago). The sense and antisense strands of the PKC- $\delta$  and HSP90 siRNAs were as follows: PKC- $\delta$ , 5'-GCAACGCUGCCAUCUAAUU-3' (sense) and 5'-PUUAUGG AUGCAGCGUUGCUU-3' (antisense); HSP90, 5'-GCUUUGAGGUGGUAUACAU UU-3' (sense) and 5'-PAUGUAUACCACCUCUAAAGCUU-3' (antisense). siRNAs were completely dissolved in RNase-free distilled water (Dharmacon) at a final concentration of 500  $\mu$ mol/l before injection. To assess the effects of PKC- $\delta$  and HSP90 siRNAs on retinas, 1 and 3  $\mu$ l siRNAs, each at a concentration of 500  $\mu$ mol/l, were intravitreally injected into the right eye of OLETF and LETO rats at 35 weeks. Control rats received 1 and 3  $\mu$ l distilled water into the left eye. Rats were killed at 1, 2, and 5 days after the injection, and the effects of siRNAs on

PKC- $\delta$  and HSP90 were determined by immunoblotting. Data are representative of four independent experiments.

**Antibodies.** Mouse monoclonal antibodies against PKC- $\delta$ , PI 3-kinase p85 (regulatory subunit), HSP90, and Akt; goat polyclonal anti-Thy-1 antibody; and rabbit polyclonal anti-PP2A (catalytic subunit, 36 kDa) and anti-Akt antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against PP2B and phospho-Akt were obtained from BD Biosciences (San Jose, CA) and Cell Signaling (Danvers, MA), and mouse monoclonal anti- $\alpha$ -tubulin antibody was purchased from Sigma. Horseradish peroxidase-conjugated secondary antibodies were purchased from Pierce (Rockford, IL). Cy 3-conjugated donkey anti-rabbit and anti-mouse IgGs and Alexa Fluor 405-conjugated chicken anti-goat IgG were obtained from Amersham Biosciences (Piscataway, NJ) and Invitrogen (Carlsbad, CA), respectively.



**FIG. 1.** Ganglion cell apoptosis in retinas of LETO and OLETF rats at 24 and 35 weeks. The TUNEL assay was performed after Thy-1 immunostaining, a specific ganglion cell marker, and then sections were stained with the nuclear marker DAPI. **A** and **B–F**: Representative images of 35-week LETO and OLETF retinas. The arrows indicate TUNEL-positive ganglion cells in 35-week-old OLETF rats (**B**). The arrowheads in **F** show the codistribution of TUNEL-positive signals (small arrowheads in **C**) and Thy-1-positive ganglion cells in 35-week OLETF retinas. The number of co-positive cells was counted and the fold changes are presented as the means  $\pm$  SE ( $n = 4$ ) (**G**).  $**P < 0.01$  compared with 24-week LETO and the other groups. INL, inner nuclear layer; IPL, inner plexiform layer; L (24) and L (35), 24- and 35-week LETO retinas, respectively; O (24) and O (35), 24- and 35-week OLETF retinas, respectively; ONL, outer nuclear layer. Bars, 12.5  $\mu$ m. (Please see <http://dx.doi.org/10.2337/db07-1431> for a high-quality digital representation of this figure.)



**FIG. 2.** PKC- $\delta$  activity in retinas of LETO and OLETF rats at 24 and 35 weeks. A PKC activity assay was performed using PKC- $\delta$  immune complexes and the SignaTECT PKC assay system. [ $\gamma$ - $^{32}$ P]ATP-labeled PKC- $\delta$  was measured by scintillation counter. Data are the means  $\pm$  SE ( $n = 4$ ). \*\* $P < 0.01$  compared with 24-week LETO and the other groups. L (24) and L (35), 24- and 35-week LETO retinas, respectively; O (24) and O (35), 24- and 35-week OLETF retinas, respectively.

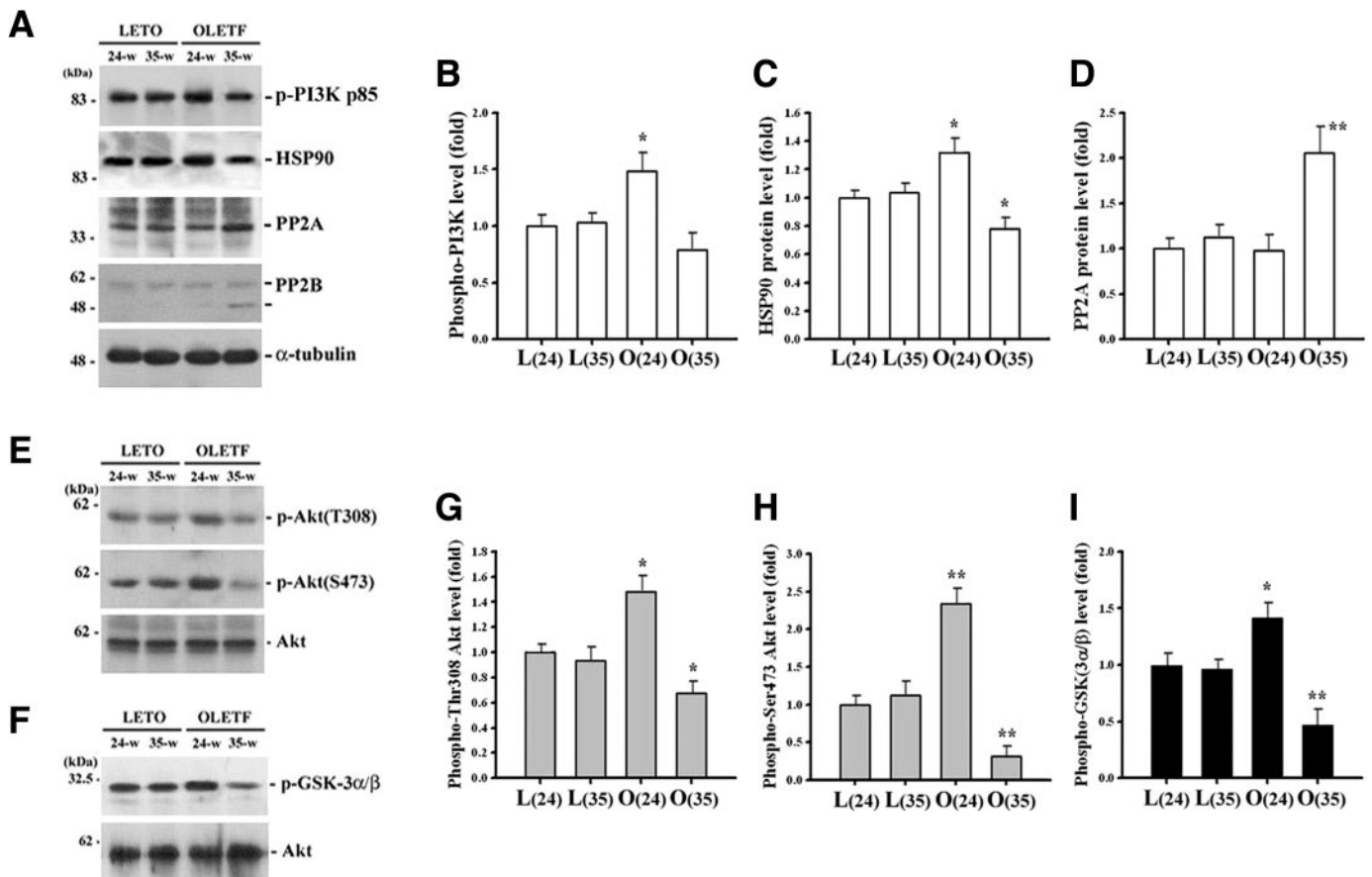
**Immunoblot analysis.** Retinal protein extraction and immunoblot analysis were performed as described previously (23). Total protein (30  $\mu$ g) from LETO and OLETF rat retinas was subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. Antibody incubations and washing were performed on

the membranes, and the immunoreactive proteins were visualized using an enhanced chemiluminescent kit (Amersham Biosciences). Each membrane was then stripped and reblotted with anti- $\alpha$ -tubulin antibody as a control. Data are representative of four independent experiments. The fold changes in protein levels are indicated below the blots in each figure.

**Immunoprecipitation.** Immunoprecipitations were performed as described previously (24). Pre-cleared immune complexes were collected using protein-G/A-agarose beads and washed with radioimmunoprecipitation assay buffer (50 mmol/l Tris-HCl [pH 8.0], 150 mmol/l NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Nonidet P-40) containing protease inhibitors. SDS-PAGE sample buffer was added to the beads, and final fractions were subjected to immunoblot analysis. All immunoblots were reprobed with the immunoprecipitating antibody to account for loading differences in protein levels, and each reciprocal analysis was performed. Data are representative of three to four independent experiments.

**PKC- $\delta$  kinase assay.** We performed PKC- $\delta$  kinase assay using the SignaTECT PKC Assay System (Promega, Madison, WI) according to the manufacturer's protocol, as described previously (23). Briefly, PKC- $\delta$  immune complexes were collected using protein G/A-agarose beads, and then the beads were resuspended in 20  $\mu$ l kinase reaction buffer (25 mmol/l Tris-HCl [pH 7.5], 5 mmol/l  $\beta$ -glycerol phosphate, 2 mmol/l dithiothreitol, 0.1 mmol/l sodium orthovanadate, 10 mmol/l MgCl<sub>2</sub>, and 0.5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP [3,000 Ci/mmol]). Kinase activity was determined using a scintillation counter. Data are representative of four independent experiments.

**PP2A phosphatase assay.** PP2A activity was determined using a PP2A-IP phosphatase assay kit (catalog no. D-001810-01-20; Upstate, Temecula, CA) according to the manufacturer's protocol. Total protein (300  $\mu$ g) from retinas was incubated with 4  $\mu$ g anti-PP2A-C (catalytic subunit) antibody and 40  $\mu$ l protein A-agarose beads for 2 h at 4°C with constant rocking. The immune complexes were washed three times in Tris-buffered saline and once with Ser/Thr assay buffer (50 mmol/l Tris-HCl [pH 7.0] and 100  $\mu$ mol/l CaCl<sub>2</sub>). The phosphatase reaction was initiated by the addition of 60  $\mu$ l phosphopeptide substrate (final



**FIG. 3.** Protein levels of PI 3-kinase, HSP90, PP2A, PP2B, phospho-Akt (Thr308) and -Akt (Ser473), and phospho-GSK in retinas of LETO and OLETF rats at 24 and 35 weeks. A: Representative immunoblots of PI 3-kinase, HSP90, PP2A, and PP2B. B–D: Fold changes in these protein levels. E, F, and G–I: Representative immunoblots of phospho-Akt and -GSK and the corresponding fold changes. The immunoblots were stripped and reprobed with anti- $\alpha$ -tubulin or -Akt antibodies. Data are means  $\pm$  SE ( $n = 4$ ). \* $P < 0.05$  and \*\* $P < 0.01$  compared with 24-week LETO and the other groups. L (24) and L (35), 24- and 35-week LETO retinas, respectively; O (24) and O (35), 24- and 35-week OLETF retinas, respectively.

reaction concentration of 750  $\mu\text{mol/l}$ ) and allowed to proceed for 30 min in a shaking incubator. The reaction mixture was centrifuged briefly, and the supernatant was transferred to a 96-well microplate. Malachite green phosphate detection solution was added to each well and allowed to develop for 15 min at room temperature. Free phosphate was quantified by measuring the absorbance of each well at 650 nm using a microplate reader. Data are representative of four independent experiments.

**Akt kinase assay.** Akt activity was measured using a nonradioactive Akt kinase assay kit (Cell Signaling) according to the manufacturer's protocol without any modification. Akt immune complexes from total protein (300  $\mu\text{g}$ ) from retinas were incubated with recombinant glycogen synthase kinase (GSK)-3 $\alpha/\beta$  fusion protein (30 kDa). Phosphorylation of GSK-3 was measured by immunoblotting using anti-phospho-GSK-3 $\alpha/\beta$  (Ser21/9) antibody. Data represent the results of four independent experiments.

**Immunohistochemistry.** The preparation of frozen retinal sections and immunohistochemical staining were performed as described previously (24). After blocking, retinal sections were incubated with primary antibodies against HSP90, PP2A, and phospho-Akt (Ser473) and biotinylated secondary antibodies. The sections were washed in PBS, incubated with an avidin-biotinylated horseradish peroxidase complex (ABC; Vector Laboratories, Burlingame, CA), and developed using 0.025% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma)/0.003%  $\text{H}_2\text{O}_2$  in PBS.

To confirm ganglion cell-specific expression of HSP90, PP2A, and phospho-Akt, double-immunofluorescent staining was performed with Thy-1, a ganglion cell marker, as described previously (24). Briefly, retinal sections were incubated in a mixture of primary antibodies, rinsed in PBS, incubated in a mixture of secondary antibodies, and then wet-mounted. Images were obtained using a BH-2 Olympus microscope (Melville, NY) at a point that was  $\sim 0.8$ –1 mm from the optic nerve head. Data are representative of three independent experiments.

**Cell death assay.** Cell death was determined using a Terminal dUTP transferase nick end labeling (TUNEL) assay (In Situ Cell Death Detection kit; Roche, Mannheim, Germany) according to the manufacturer's protocol, as described previously (25). To assess ganglion cell death, the TUNEL assay was performed after Thy-1 immunofluorescent staining on retinal sections or flat mounts. All sections were stained with the nuclear marker 40,6-diamidino-2-phenylindole dihydrochloride (DAPI; Invitrogen) before being wet-mounted. Whole retinal preparation and flat-mounting were carried out as described previously (26). TUNEL-positive images were observed using a confocal microscope (Axioplan2 Imaging; Zeiss). The number of cells that had co-positive signal of TUNEL and Thy-1 was quantified using the Soft Imaging System (Soft Imaging System; Münster, Germany). Data are representative of four independent experiments from different retinal sections or flat mounts.

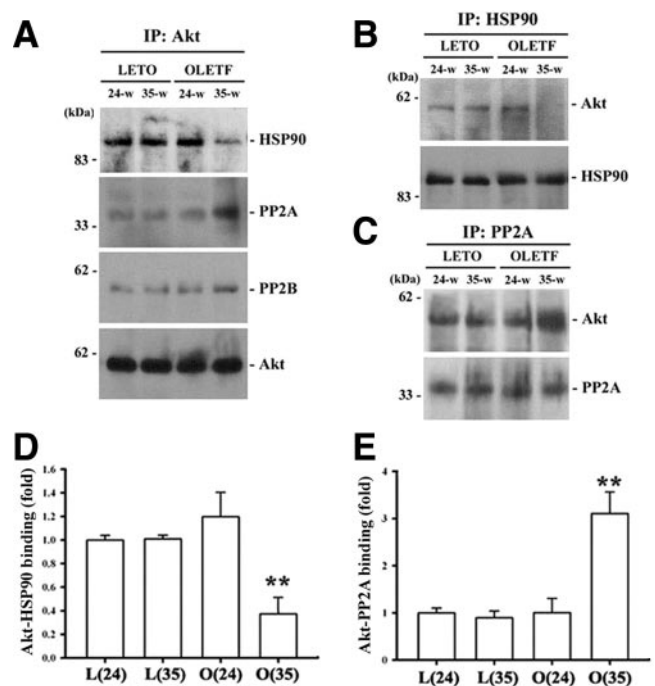
**Data analysis.** Densitometric analysis of immunoblots was performed using SigmaGel 1.0 (Jandel Scientific, Erkrath, Germany) and SigmaPlot 4.0 (SPSS, Chicago). All data are presented as means  $\pm$  SE. Statistical significance was determined using one-way ANOVA followed by a Tukey post hoc test (SAS Institute, Cary, NC) and the Mann-Whitney  $U$  test (SPSS).  $P < 0.05$  was considered to be statistically significant.

## RESULTS

During the course of this study, OLETF rats gained weight faster than the control LETO rats. The mean body weights of OLETF and LETO rats at 24 weeks were  $688 \pm 10.5$  and  $471 \pm 8.2$  g, respectively, and the difference in weight was increased significantly at 35 weeks ( $732 \pm 20.1$  and  $520 \pm 11.2$  g, respectively;  $P < 0.05$ ,  $n = 5$ ). Blood glucose levels in OLETF and LETO rats were  $14.5 \pm 0.5$  and  $6.2 \pm 0.3$  mmol/l ( $P < 0.05$ ;  $n = 5$ , respectively) at 24 weeks and  $21.6 \pm 1.12$  and  $6.6 \pm 0.5$  mmol/l ( $P < 0.05$ ;  $n = 5$ , respectively) at 35 weeks. OLETF rats exhibited a steady increase in glucose levels from week 10, whereas LETO rats sustained normoglycemia throughout the period of study (data not shown).

The number of TUNEL-positive ganglion cells in 35-week-old OLETF rats was significantly higher (3.5-fold;  $P < 0.01$ ;  $n = 4$ ) than in 24-week-old LETO rats (Fig. 1G). There were no significant differences between 24- or 35-week-old LETO and 24-week-old OLETF rats.

PKC- $\delta$  activity was significantly higher (4.9-fold;  $P < 0.01$ ;  $n = 4$ ) in 35-week OLETF retinas than 24-week LETO retinas (Fig. 2). There were no significant differences between 24- or 35-week-old LETO and 24-week-old OLETF

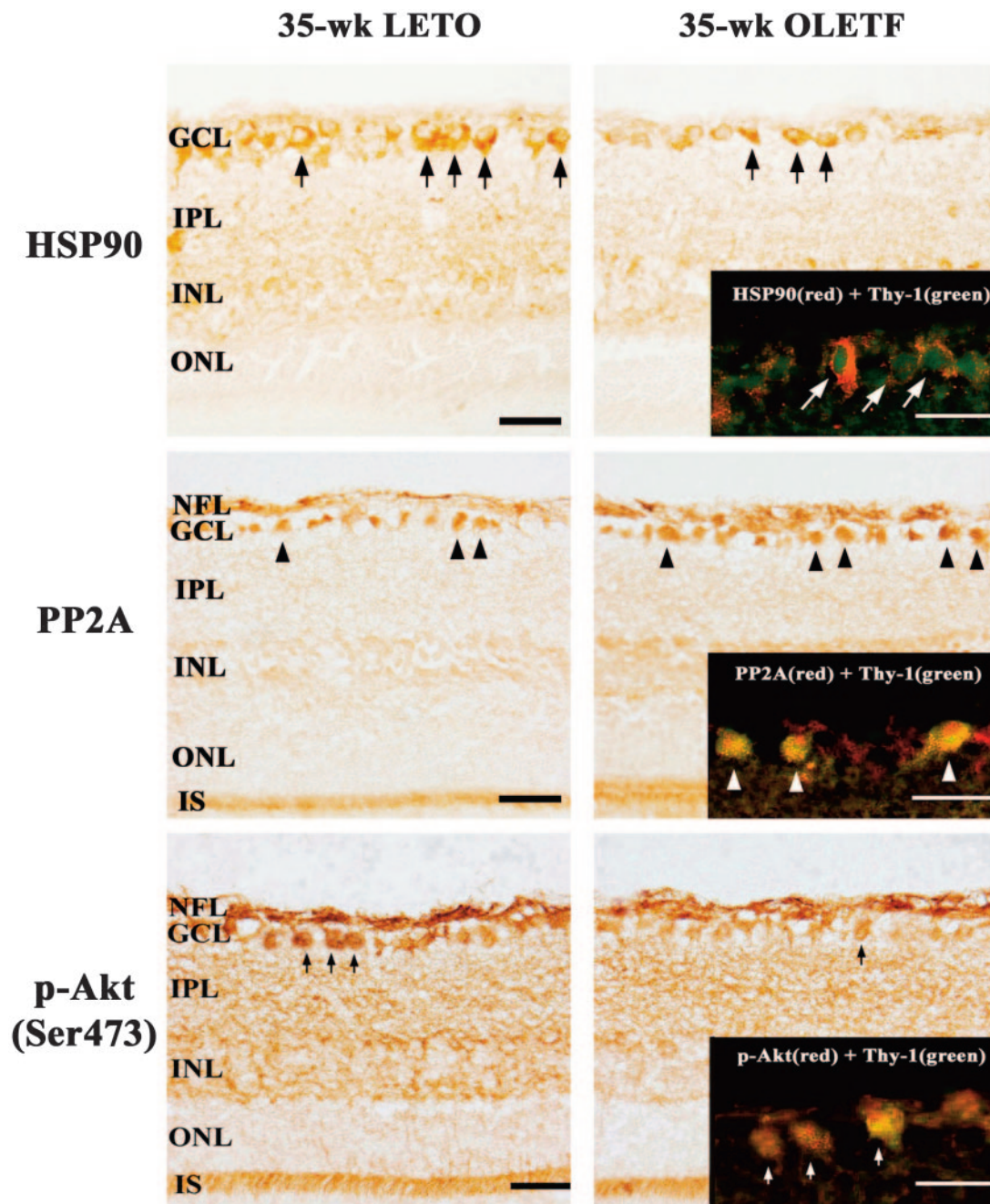


**FIG. 4.** The associations with Akt and HSP90, PP2A, and PP2B in retinas of LETO and OLETF rats at 24 and 35 weeks. Akt, HSP90, and PP2A immune complexes were subjected to immunoblot analysis (A–C). Data are representative blots of four independent experiments. The blots were reprobed with the immunoprecipitating antibody to account for loading differences. The immunoblot data were quantified using densitometry and the fold changes in Akt-HSP90 and Akt-PP2A binding are presented in D and E. Data are means  $\pm$  SE ( $n = 4$ ).  $**P < 0.01$  compared with 24-week LETO and the other groups. IP, immunoprecipitation; L (24) and L (35), 24- and 35-week LETO retinas, respectively; O (24) and O (35), 24- and 35-week OLETF retinas, respectively.

rats. PKC- $\delta$  protein levels were similar in all groups (data not shown).

The protein levels of PI 3-kinase p85 and HSP90 were increased in 24-week OLETF retinas compared with LETO retinas (Fig. 3A–C); however, HSP90 levels were lower in 35-week OLETF retinas than in LETO retinas, and there was no significant difference between PI 3-kinase levels in both age-groups of OLETF rats. The levels of PP2A (catalytic subunit) and cleaved PP2B (48 kDa) were not significantly different in 24-week-old LETO and OLETF rats but were greatly increased in retinas from 35-week-old OLETF rats (Fig. 3D). Phospho-Akt (Thr308) and -Akt (Ser473) levels were increased (1.4- and 2.3-fold;  $P < 0.05$  and 0.01, respectively;  $n = 4$ ) in 24-week OLETF retinas compared with LETO retinas and decreased significantly (1.7- and 2.5-fold;  $P < 0.05$  and 0.01, respectively;  $n = 4$ ) in 35-week OLETF retinas (Fig. 3E–H). Akt activity, based on phospho-GSK-3 $\alpha/\beta$  level (30 kDa), was significantly lower (twofold;  $P < 0.01$ ;  $n = 4$ ) in 35-week-old OLETF rats than 24-week-old LETO rats (Fig. 3I).

To assess whether PKC- $\delta$  affects the association of Akt with its binding partners, we subjected Akt immune complexes to immunoblot analysis using anti-HSP90, -PP2A, and -PP2B antibodies (Fig. 4). Akt binding to HSP90 or PP2A was similar in 24-week LETO and OLETF retinas; however, in 35-week OLETF retinas, this association was significantly decreased or increased more than threefold ( $P < 0.01$ ;  $n = 4$ ), respectively, compared with 24-week-old LETO rats. Neither PI 3-kinase binding to HSP90 nor PP2A or HSP90 binding to PP2A was detectable in all



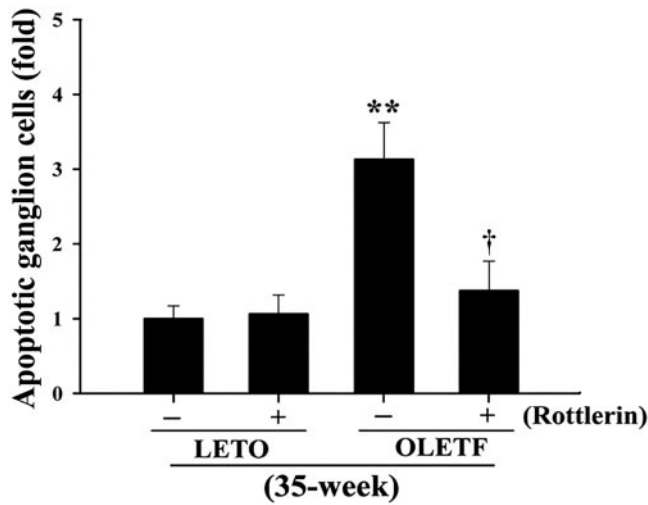
**FIG. 5.** Distribution of HSP90, PP2A, and phospho-Akt (Ser473) in retinas of LETO and OLETF rats at 35 weeks. Their positive signals in the GCL are indicated by arrows, arrowheads, and small arrows, respectively. *Insets* show enlarged images of ganglion cells co-labeled with these proteins and Thy-1, a specific ganglion cell marker. Data are representative of three independent experiments. INL, inner nuclear layer; IPL, inner plexiform layer; IS, inner segment layer; ONL, outer nuclear layer; OPL, outer plexiform layer. Bars, 12.5  $\mu\text{m}$ . (Please see <http://dx.doi.org/10.2337/db07-1431> for a high-quality digital representation of this figure.)

groups, there were no differences in PI 3-kinase binding to PKC- $\delta$  among groups, and PKC- $\delta$ -PP2A binding appeared only in 35-week OLETF rat retinas (data not shown).

HSP90 immunoreactivity was specific only in the ganglion cell layer (GCL), and PP2A- and phospho-Akt (Ser473) signals were positive in the nerve fiber layer (NFL), the inner segment layer, and the GCL in 35-week LETO and OLETF retinas (Fig. 5). HSP90 and phospho-Akt signals in the GCL (Fig. 5, large arrows and arrowheads) were decreased and PP2A signals (Fig. 5, small arrows) were increased in 35-week-old OLETF rats compared with LETO rats. By double-immunostaining with Thy-1 of HSP90, PP2A, and phospho-Akt (Ser473),

we confirmed that these positive signals colocalized to ganglion cells (Fig. 5, *right panels, insets*). PKC- $\delta$  immunoreactivity was also observed throughout the retina, including the GCL, and there was no significant difference among 24- and 35-week-old LETO and OLETF rats (data not shown).

In a previous study, we found that PKC- $\delta$  activity was greatly increased (4.9-fold;  $P < 0.01$ ;  $n = 4$ ) in 35-week OLETF retinas compared with LETO retinas, and 5  $\mu\text{mol/l}$  rottlerin abrogated this effect (22). In this study, 5  $\mu\text{mol/l}$  rottlerin treatment also significantly decreased ganglion cell death in 35-week OLETF retinas (2.4-fold;  $P < 0.05$ ;  $n = 4$ ) compared with DMSO-treated OLETF retinas (Fig.

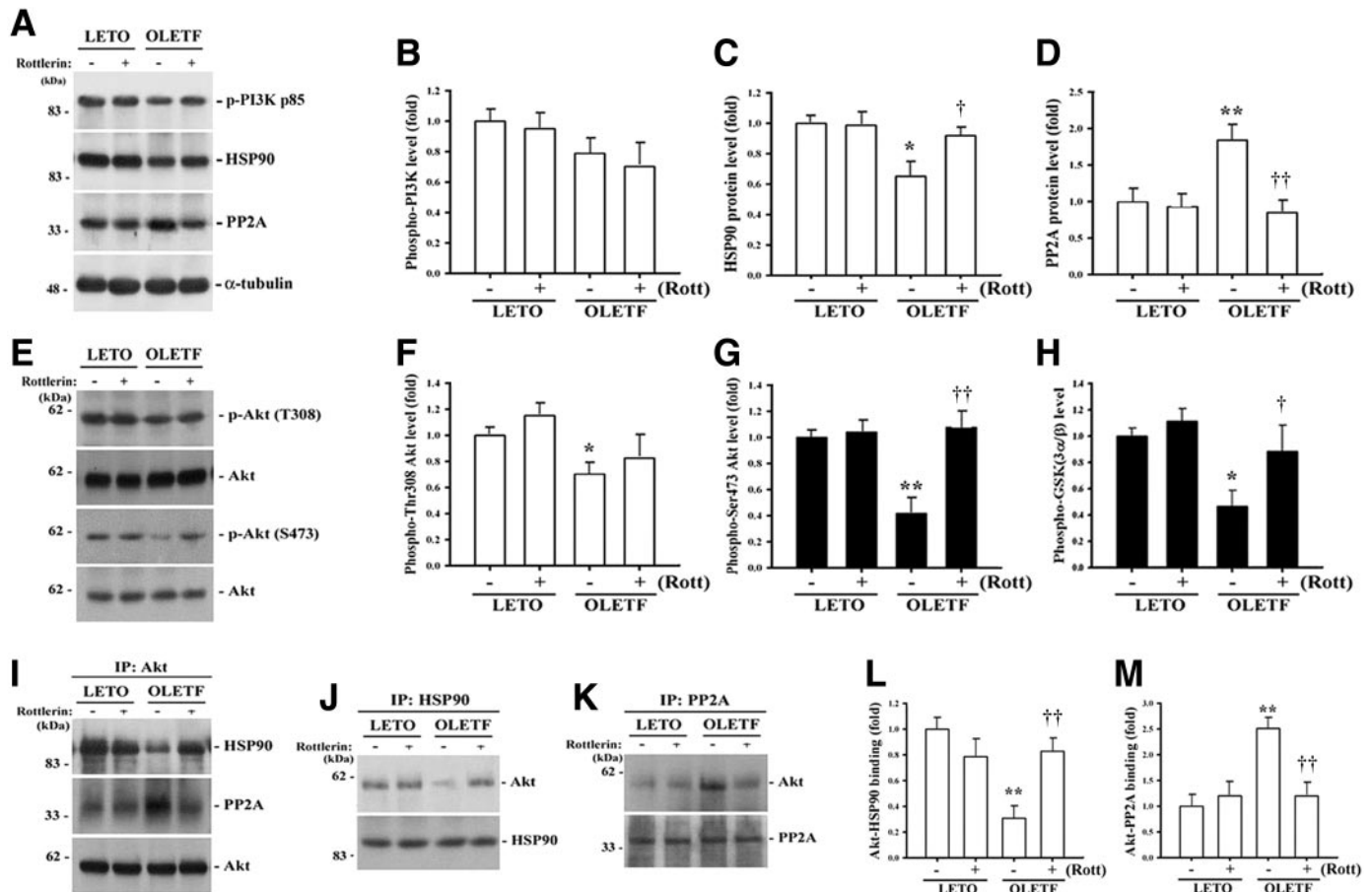


**FIG. 6.** The effects of rottlerin treatment on ganglion cell death in retinas of LETO and OLETF rats at 35 weeks. The effects of rottlerin (5  $\mu$ mol/l; 3  $\mu$ l) were examined 24 h after an intravitreal injection into the right eye of rats. As a control, 0.5% DMSO (3  $\mu$ l) was introduced into the left vitreous. Data (fold changes) are means  $\pm$  SE ( $n = 4$ ). \*\* $P < 0.01$  compared with DMSO-treated LETO and the other groups; † $P < 0.05$  compared with DMSO- and rottlerin-treated OLETF retinas.

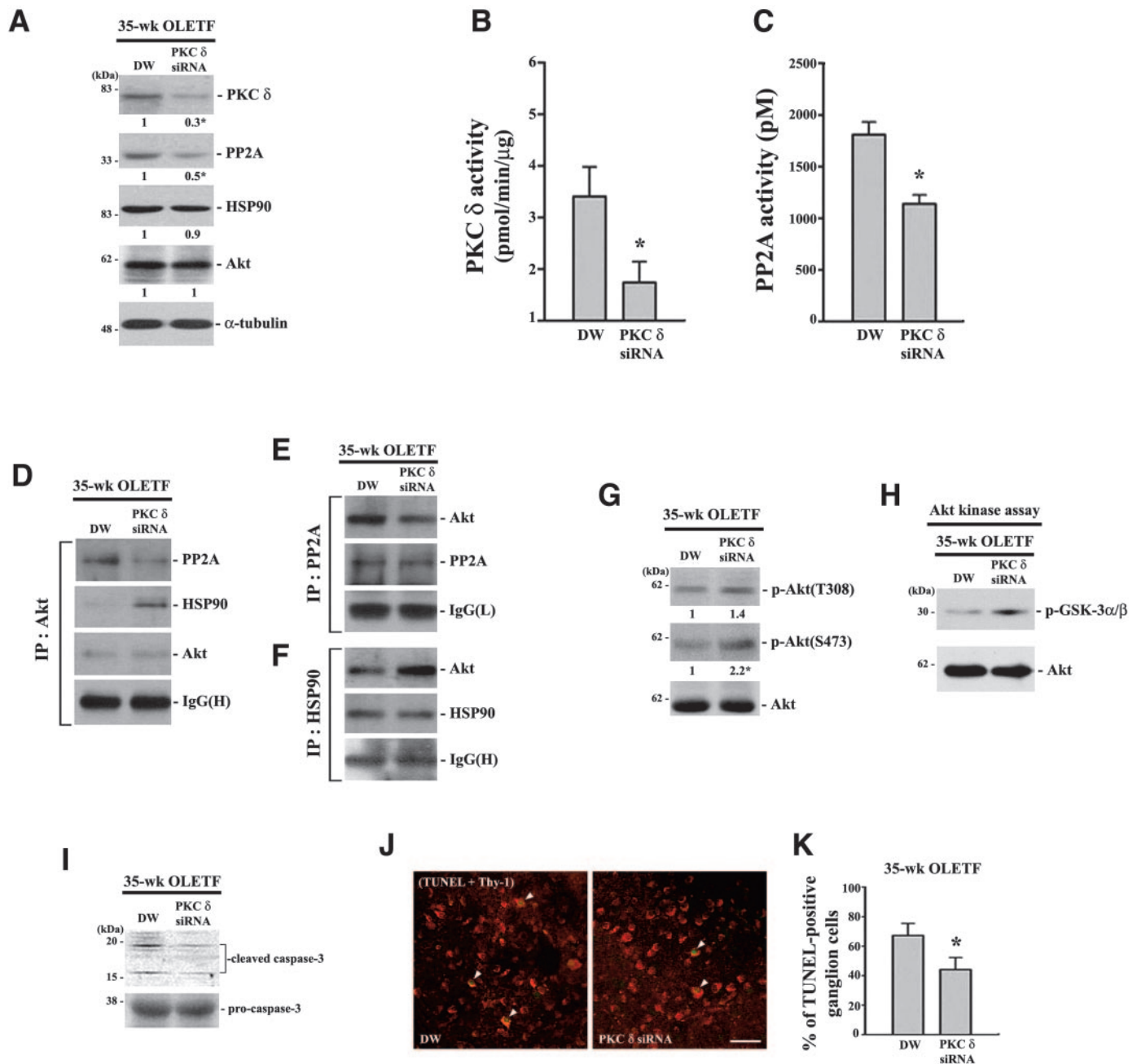
6). However, rottlerin had no effect on PKC- $\delta$  activity or cell death in LETO rats (data not shown).

Rottlerin did not significantly affect PI 3-kinase p85 and phospho-Akt (Thr308) protein levels, although it modestly increased HSP90 levels in 35-week OLETF retinas (Fig. 7A–C). Furthermore, rottlerin greatly decreased PP2A protein levels (2.5-fold;  $P < 0.01$ ;  $n = 4$ ; Fig. 7D) and increased phospho-Akt (Ser473) levels (2.8-fold;  $P < 0.01$ ;  $n = 4$ ; Fig. 7E–G). Rottlerin also blocked the decrease in Akt activity in 35-week OLETF retinas (Fig. 7H). Akt binding to HSP90 or PP2A was significantly increased or decreased, respectively, (2.7- and 2-fold;  $P < 0.01$ , respectively;  $n = 4$ ; Fig. 7I–M) in rottlerin-treated 35-week OLETF retinas compared with the untreated group.

We next examined the effect of PKC- $\delta$  and HSP90 siRNA treatment on 35-week OLETF and LETO rat retinas. Three microliters siRNA significantly reduced PKC- $\delta$  and HSP90 protein expression in OLETF and LETO rats 1 day ( $30.2 \pm 1.1$  and  $11.1 \pm 0.8\%$ ;  $P < 0.05$ , respectively;  $n = 4$ ) and 2 days ( $70 \pm 2.1$  and  $50.1 \pm 1.8\%$ ;  $P < 0.01$  and  $0.05$ , respectively;  $n = 4$ ) after the injection, whereas 1  $\mu$ l siRNA did not show these effects. No effects were shown at 5 days after siRNA injection (data not shown). Distilled water treatment did not show any effect on PKC- $\delta$  and HSP90 protein expression in the retinas. Figures 8 and 9



**FIG. 7.** The effects of rottlerin treatment on protein levels of PI 3-kinase, HSP90, PP2A, and Akt; Akt activity; and the associations with Akt and HSP90 or PP2A in retinas of LETO and OLETF rats at 35 weeks. Immunoblot data are representative of four independent experiments (A and E). B–D and F–H: Fold changes in protein levels after rottlerin treatment. Akt, HSP90, and PP2A immune complexes were subjected to immunoblot analysis using the indicated antibodies (I–K). The immunoblots were reprobed with the immunoprecipitating antibody to account for loading differences. Data are representative blots of four independent experiments. L and M: Fold changes in Akt-HSP90 and Akt-PP2A binding after rottlerin treatment. Data are means  $\pm$  SE ( $n = 4$ ). \* $P < 0.05$  and \*\* $P < 0.01$  compared with DMSO-treated LETO and the other groups; † $P < 0.05$  and †† $P < 0.01$  compared with DMSO- and rottlerin-treated OLETF retinas. IP, immunoprecipitation; Rott, rottlerin; L (24) and L (35), 24- and 35-week LETO retinas, respectively; O (24) and O (35), 24- and 35-week OLETF retinas, respectively.



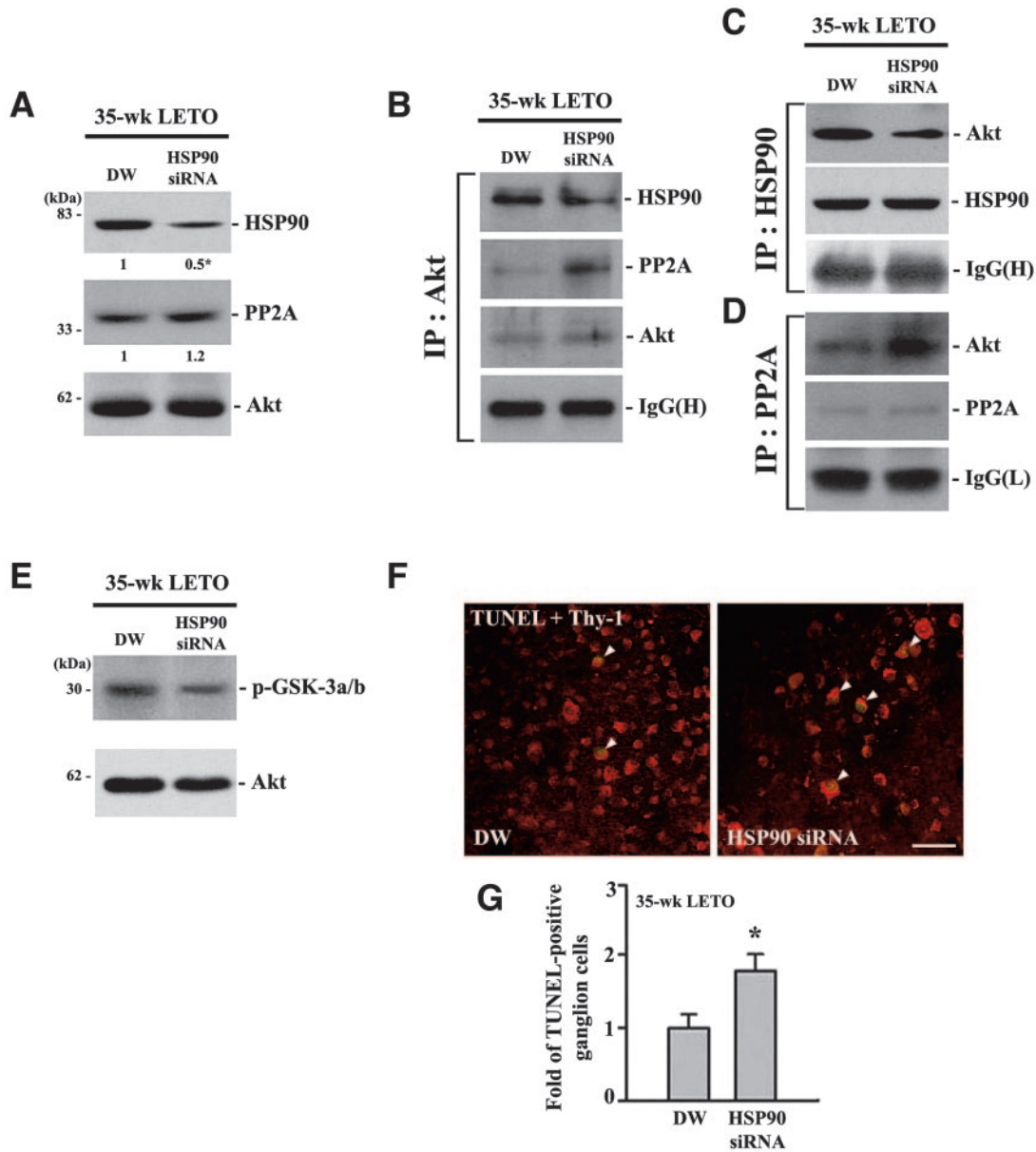
**FIG. 8.** Changes in Akt signaling and cell death in retinas of 35-week-old OLETF rats after PKC- $\delta$  knockdown using RNA interference. **A:** Representative immunoblots of PKC- $\delta$ , PP2A, HSP90, and Akt, and the fold changes in these protein levels are indicated below the blots. **B** and **C:** Results from PKC- $\delta$  kinase and PP2A phosphatase assays. **D–F:** Representative immunoblots of Akt, HSP90, and PP2A immune complexes. The immunoblots were reprobbed with the immunoprecipitating antibodies to account for loading differences. **G** and **H:** Representative immunoblots of phospho-Akts and the result from Akt kinase assay using a recombinant GSK-3 $\alpha/\beta$  fusion protein and the corresponding fold changes are indicated below the blots. **I:** A representative immunoblot of cleaved caspase-3. The immunoblots were stripped and reprobbed with anti- $\alpha$ -tubulin, -Akt, and -pro-caspase-3 antibodies. Data are representative images of four independent experiments. **J:** Result of the TUNEL assay performed after Thy-1 immunostaining in flat mounts of 35-week OLETF rat retinas. The number of their co-positive cells was counted (**K**). Data are means  $\pm$  SE ( $n = 4$ ). \* $P < 0.05$  compared with distilled water- and PKC- $\delta$  siRNA-treated 35-week OLETF retinas. DW, RNase-free distilled water; IP, immunoprecipitation. Bars, 12.5  $\mu$ m. (Please see <http://dx.doi.org/10.2337/db07-1431> for a high-quality digital representation of this figure.)

show data at 2 days after 3- $\mu$ l siRNA (500  $\mu$ mol/l) injection. PKC- $\delta$  siRNA significantly decreased PP2A protein levels and phosphatase activity and Akt-PP2A binding in 35-week OLETF retinas (Fig. 8A–C), whereas it modestly increased Akt-HSP90 binding (Fig. 8D–F). PKC- $\delta$  silencing also significantly increased phospho-Akt (Ser473) levels and Akt activity but did not affect phospho-Akt (Thr308) levels (Fig. 8G and H). We also observed that ganglion cell death in 35-week OLETF retinas was blocked by PKC- $\delta$

siRNA treatment (Fig. 8I–K). Additionally, we found that HSP90-silenced LETO retinas show significant decreases in Akt-HSP90 binding and Akt activity and increases in Akt-PP2A binding and ganglion cell death (Fig. 9A–G).

#### DISCUSSION

We have demonstrated here that PKC- $\delta$  activation is responsible for neuro-retinal apoptosis in diabetic OLETF rats via the inactivation of Akt.



**FIG. 9.** Changes in Akt signaling and cell death in retinas of 35-week-old LETO rats after HSP90 knockdown using RNA interference. **A:** Representative immunoblots of HSP90, PP2A, and Akt, and the fold changes in these protein levels are indicated below the blots. **B–D:** Representative immunoblots of Akt, HSP90, and PP2A immune complexes. The immunoblots were reprobed with the immunoprecipitating antibodies to account for loading differences. **E:** Result from Akt kinase assay using a recombinant GSK-3 $\alpha/\beta$  fusion protein. The corresponding fold change is indicated below the blot. Data are representative images of four independent experiments. **F:** Result of the TUNEL assay performed after Thy-1 immunostaining in flat mounts of 35-week LETO rat retinas. The number of their co-positive cells was counted (**G**). Data are means  $\pm$  SE ( $n = 4$ ). \* $P < 0.05$  compared with distilled water- and HSP90 siRNA-treated 35-week LETO retinas. DW, RNase-free distilled water; IP, immunoprecipitation. Bars, 12.5  $\mu$ m. (Please see <http://dx.doi.org/10.2337/db07-1431> for a high-quality digital representation of this figure.)

Previously, we found that PKC- $\delta$  acts as a selective mediator of neuronal apoptosis in the retinas of 35-week-old OLETF rats (22). In the current study, we demonstrated that apoptosis occurs only in ganglion cells of the 35-week OLETF retinas (Fig. 1) and that PKC- $\delta$  activity is greatly increased in 35-week OLETF retinas compared with 24- or 35-week LETO and 24-week OLETF retinas (Fig. 2). PKC- $\delta$  immunoreactivity was observed throughout the retina and was highest in the GCL group (data not shown). These results indicate that PKC- $\delta$  activation is involved in ganglion cell death in OLETF rat retinas.

The activity of Akt is regulated by its association with a variety of binding partners, and Akt binding to PP2A results in the dephosphorylation and inactivation of Akt,

consistent with our results. Moreover, HSP90 physically associates with Akt and disrupts the PP2A-Akt complex, stabilizing Akt activity (13,15,16). As expected, protein levels of PI 3-kinase, HSP90, and phospho-Akt and Akt activity were moderately increased in 24-week OLETF retinas compared with LETO retinas, but these were significantly decreased, with the exception of PI 3-kinase levels, in 35-week-old OLETF rats (Figs. 3 and 4). PI 3-kinase-Akt survival signals were differently regulated in OLETF rat retinas at 24 and 35 weeks. The significant reduction in these signaling components in 35-week-old OLETF rats may reflect the retinal damage associated with the pathological progression of diabetes, whereas these increases in 24-week-old OLETF rats may relate to func-



tional compensation for diabetes-induced cellular stress. Either PP2A or PP2B cleavage was specifically increased in 35-week OLETF retinas. Furthermore, Akt binding to HSP90 was greatly decreased and its association with PP2A was greatly increased in 35-week OLETF retinas compared with other groups. The levels of Akt-PP2B complexes were similar between LETO and 24-week OLETF retinas, and PI 3-kinase binding to HSP90 or PP2A was not detected in any of the groups (data not shown). Interestingly, the immunoreactivity of HSP90, PP2A, and phospho-Akt (Ser473) was altered specifically in ganglion cells of 35-week OLETF retinas compared with LETO retinas (Fig. 5), in agreement with the results of the TUNEL assay (Fig. 1). Taken together, our results suggest that Akt inactivation is due to the upregulation of PP2A rather than a PI 3-kinase-dependent pathway and that PP2A plays an important role in ganglion cell death in OLETF rat retinas.

PKC- $\delta$  inhibition or knockdown by rottlerin or siRNA treatment significantly abrogated not only PKC- $\delta$  activation and ganglion cell death but also PP2A activation and its association with Akt. PKC- $\delta$  inhibition also decreased HSP90, phospho-Akt (Ser473), and phospho-GSK levels and the association of Akt with HSP90 in 35-week OLETF retinas (Figs. 6–8). However, these PKC- $\delta$  downregulations had no significant effects on the levels of PI 3-kinase and phospho-Akt (Thr308) and PKC- $\delta$ -PI 3-kinase binding. Also, we found that HSP90 knockdown decreases Akt-HSP90 binding and Akt activity and increases Akt-PP2A binding and ganglion cell death in 35-week LETO retinas (Fig. 9A–G). Therefore, our findings indicate that PKC- $\delta$  regulates ganglion cell death in OLETF rats through upregulating PP2A that dephosphorylates phospho-Akt (Ser473), competing with Akt stabilization by HSP90. A recent study reported that PKC- $\delta$  enhances PP2A by its direct binding (27). Consistently, downregulation of PKC- $\delta$  by rottlerin or siRNA decreased both PP2A protein levels and phosphatase activity in 35-week OLETF rat retinas. Furthermore, PKC- $\delta$  also bound to PP2A in 35-week OLETF retinas (data not shown). Therefore, we suggest that PKC- $\delta$  directly acts on PP2A in the OLETF rat retina, resulting in retinal apoptosis.

PI 3-kinase-dependent activation of Akt is mediated by PKC- $\delta$ , which is required for cell survival in various cancer and immune cells (1,2,28). However, Zhong et al. suggested that PKC- $\delta$  downregulation suppresses apoptotic signals through a novel PI 3-kinase-independent survival pathway (29). As such, we found that PKC- $\delta$  inhibition did not affect PI 3-kinase levels, but it abrogated neuronal apoptosis, increasing Akt signaling including Akt disassociation from PP2A and phosphorylation of Akt on Ser473. Thus, it seems likely that PI 3-kinase-independent Akt pathway mediates PKC- $\delta$ -induced apoptosis during diabetes.

Rottlerin has virtually no effect on PKC- $\delta$  activity in cells in culture (30–33), whereas it specifically inhibits PKC- $\delta$  activity and PKC- $\delta$ -mediated apoptosis at concentrations of 3–6  $\mu\text{mol/l}$  (5,34,35), consistent with our results. In addition, rottlerin can inhibit many other kinases, including mitogen-activated protein kinase-activated protein kinase 2 (MAPKAP-K2) and p38MAPK (30,36), in a dose-dependent manner, whereas it has no significant effect on these kinases in OLETF rat retinas (data not shown). Therefore, this discrepancy may be due to differences in the doses of rottlerin used or in the physiological and pathological state of the animals.

In conclusion, our data suggest that PKC- $\delta$  mediates

neuronal death in retinas of diabetic rats via PP2A activation and Akt signaling inhibition. Ganglion cell death occurs early as an initial event in diabetic retinopathy, and the mechanism of this cell death is unknown. Therefore, our data provide new insights into the mechanism of diabetes-associated neuro-retinal damage, showing that specific PKC- $\delta$  inhibitors may have potential for therapeutic agents for the prevention of human diabetic retinopathy.

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