In 15-month-old db/db mice, signs of diabetic retinopathy, including blood-retinal barrier breakdown, loss of pericytes, neuro-retinal apoptosis, glial reactivation, and proliferation of blood vessels, were evident. These changes in the diabetic retina were associated with increased expression of aldose reductase (AR). To further understand the role of AR in the pathogenesis of diabetic retinopathy, we generated db/db mice with an AR null mutation (AR−/−/−db/db). AR deficiency led to fewer retinal blood vessels with IgG leakage, suggesting that AR may contribute to blood-retinal barrier breakdown. AR deficiency also prevented diabetes-induced reduction of platelet/endothelial cell adhesion molecule-1 expression and increased expression of vascular endothelial growth factor, which may have contributed to blood-retinal barrier breakdown. In addition, long-term diabetes-induced neuro-retinal stress and apoptosis and proliferation of blood vessels were less prominent in AR−/−/−db/db mice. These findings indicate that AR is responsible for the early events in the pathogenesis of diabetic retinopathy, leading to a cascade of retinal lesions, including blood-retinal barrier breakdown, loss of pericytes, neuro-retinal apoptosis, glial reactivation, and neovascularization. *Diabetes* 54:3119–3125, 2005

Diabetic retinopathy is one of the major causes of blindness in the world. The hallmarks of this disease include basement membrane thickening, loss of pericytes, microaneurysms, blood-retinal barrier breakdown, and neovascularization (1). Metabolic and biochemical changes, such as increased flux of glucose through the polycl and hexosamine pathways, activation of protein kinase C, and increased advanced glycation end product formation (2), have been implicated in the pathogenesis of diabetes complications, including diabetic retinopathy. Among these proposed pathogenic mechanisms, the polycl pathway model has received the most scrutiny. Aldose reductase (AR) is the first enzyme in the polycl pathway, converting excess glucose to sorbitol, which is then further metabolized to fructose by sorbitol dehydrogenase. The involvement of AR in diabetic retinopathy was suggested by increased expression of AR in the retinas of the diabetic animals (3). Direct evidence was provided by experiments showing that administration of AR inhibitors to diabetic rats prevented basement membrane thickening, pericyte loss, and microaneurysms in their retinal capillaries (4). However, the role of AR in the development of these retinal pathologies is not clear.

Several growth factors that have angiogenic effects and show altered levels of expression under diabetic conditions have been implicated in the pathogenesis of diabetic retinopathy (5). Vascular endothelial growth factor (VEGF) is one such growth factor that is highly expressed under diabetic conditions and thought to induce neovascularization and blood-retinal barrier breakdown (6). Induction of VEGF expression and vascular leakage that may lead to neovascularization can be prevented by the AR inhibitor fidarestat (7). The new vessels formed are more fragile and leaky (8), having the tendency to rupture, leading to more widespread neovascularization, massive hemorrhage, and loss of vision. Thus, AR appears to be involved in the early events of diabetic retinopathy, and blocking its activity with AR inhibitor would be an effective means to prevent the disease.

The role of AR in diabetes-induced blood-retinal barrier breakdown is not clear. Another AR inhibitor, sorbinil, was shown to normalize the aberrant expression of integrin, one of the cell adhesion molecules, in galactosemice rats (9). Integrin is known to promote adhesion of leukocytes to capillaries, which may contribute to blood-retinal barrier breakdown (10). Decreased expression of another adhesion molecule, platelet/endothelial cell adhesion molecule-1 (PECAM-1), has also been implicated in causing vascular permeability (11). In addition, it was shown that astrocytes in the retina express AR (12) and that administration of the AR inhibitor toresst to diabetic dogs or rats both prevented diabetes-induced gliosis in the retinas (13) and attenuated the upregulation of glial fibrillary acidic protein (GFAP) (14). Because glial cells in the retina are tightly associated with retinal capillaries (15), it is likely that impairment in glial-vascular cell interactions may lead to blood-retinal barrier breakdown and diabetic retinopathy (16).
Because the specificity of the AR inhibitors is not clear and drug availability in different cell types in the retina is difficult to determine, we decided to study the effect of AR null mutation on diabetic retinopathy. We have developed and characterized the AR gene knockout mice (17). In this report the AR null mutation (AR<sup>−/−</sup>) was introduced into genetically predisposed diabetic C57BL/KsJ-db/db (db/db) mice. These mice carry a mutation in the leptin receptor gene, and they are a model for obesity-induced type 2 diabetes (18). They develop hyperglycemia starting at ~8 weeks of age as a result of excessive food consumption (19). They have been shown to develop diabetes complications, such as neuropathy (20) and nephropathy (21). In addition, they also show early signs of diabetic retinopathy, such as thickening of capillary basement membrane at 22 weeks (22) and loss of pericytes in retinas at 26 weeks, followed by endothelial cell loss at 34 weeks (23). More recently, increased microvascular flow in the retinas of 18-week-old db/db mice was also demonstrated by determining the erythrocyte flow velocity (24). However, the mechanism of these microvascular changes is not clear. The present study shows that AR deficiency prevents pericyte and neuronal loss, blood-retinal barrier breakdown, blood vessel proliferation, and glial reactivation found in the retinas of 15-month-old db/db mice, indicating that diabetes-induced expression of AR plays a key role in the pathogenesis of diabetic retinopathy.

**RESEARCH DESIGN AND METHODS**

The AR null mutant mice (17) in C57BL/6J genetic background were mated with C57BL/KsJ db/n mice (The Jackson Laboratories, Bar Harbor, ME). The AR<sup>−/−</sup> db/db offspring were backcrossed with C57BL/KsJ db/n for five generations. Then, sibling matings generated AR<sup>+/−</sup> db/n, AR<sup>+/+</sup> db/n, AR<sup>−/−</sup> db/n, and AR<sup>−/−</sup> db/db mice. The mice were killed by cervical dislocation at 15 months old according the approved protocol of The University of Hong Kong Committee on the use of animals for teaching and research. The eyes were immediately enucleated and fixed in 4% paraformaldehyde overnight at 4°C. They were then dehydrated with a graded series of ethanol and embedded in paraffin wax. Serial sections 7 μm thick were prepared for immunohistochemistry.

**Immunohistochemistry.** Paraffin sections of the retinas were deparaffinized in xylene and rehydrated with a graded series of ethanol. After washing, sections were blocked with 0.3% hydrogen peroxide for 15 min. The sections were then blocked for 1 h with 1.5% normal goat serum—for subsequent application of CAM4, GFAP, AR, VEGF, and α-SMA antibodies. The sections were blocked with 0.3% hydrogen peroxide for 15 min. The sections were blocked with 0.3% hydrogen peroxide for 15 min. The sections were then incubated with diluted primary antibodies overnight at 4°C. The primary antibodies and their concentrations were rabbit anti-PCEM (1:400; PharMingen, San Diego, CA), rabbit anti-GFAP (1:200; Dako, Carpinteria, CA), rabbit anti-AR (1:1,500), rabbit anti-VEGF (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-nitrotyrosine (1:200 Santa Cruz Biotechnology), rabbit anti-S-100 (1:500; Dako), rabbit anti-PCNA (1:500; Santa Cruz Biotechnology), rabbit anti–cleaved caspase-3 (1:200; Cell Signaling, St. Louis, MO), mouse anti–α-SMA (1:200; Sigma Aldrich, St. Louis, MO), and mouse anti-PAR (1:200; Alexis, Lausen, Switzerland). Immunoreactivity was detected with biotinylated goat anti-rabbit, goat anti-tau, or Mouse On Mouse biotinated mouse anti-mouse IgG secondary antibodies and the avidin-biotin-peroxidase complex (Vector Laboratories). An immunoreactive signal was developed, using dianminobenidine as a substrate (Zymed Laboratories, San Francisco, CA) for 2 min. Photomicrographs were taken with an Olympus IX71 microscope system. All histological and immunohistochemical samples were coded and examined and graded in a blinded fashion.

**IGG extravasations.** Sections were prepared as described above and blocked with 2% horse serum for 1 h. They were then incubated with Mouse On Mouse biotinated anti-mouse IgG secondary antibody (Vector Laboratories) and avidin-biotin-peroxidase complex. The positive immunoreactivity of IgG was detected by exposing the sections to the substrate, dianminobenidine, for 2 min.

**RESULTS**

**AR deficiency did not affect body weight and blood glucose levels in db/db mice.** Mice of different genotypes were generated as described in the RESEARCH DESIGN AND METHODS section. Before killing the animals at 15 months old, the body weight and blood glucose level for each animal were measured. The body weights of the AR<sup>+/−</sup> db/db (39.28 ± 0.54 g) and AR<sup>−/−</sup> db/db (31.15 ± 1.5 g) mice were significantly heavier than that of the AR<sup>−/−</sup> db/m (27.52 ± 0.54 g) mice (n = 4 in all three groups, P < 0.05 for both comparisons). Blood glucose levels were also elevated significantly in both AR<sup>−/−</sup> db/db (28.08 ± 1.25 mmol/l) and AR<sup>−/−</sup> db/m (26.52 ± 2.25 mmol/l) mice compared with that of AR<sup>−/−</sup> db/n (9.48 ± 1.09 mmol/l) mice (n = 4 in all three groups, P < 0.001 for both comparisons).

**AR expression is increased in the retina of db/db mice.** To understand the role of AR in diabetic retinopathy in mice, the locations of its gene expression in the retinas of the 15-month-old mice were determined (Fig. 1). In the retinas of the AR<sup>−/−</sup> db/n mice, AR immunoreactivity was present in astrocytes, Müller cells, retinal ganglion cells, and the neurons in the inner nuclear layer (Fig. 1A). In general, the intensity of AR immunoreactivity per cell and the number of AR-immunoreactive cells in all areas of the retina were increased in the AR<sup>−/−</sup> db/db mice compared with that of the AR<sup>−/−</sup> db/m mice (Fig. 1B). As expected, the retinas from the AR<sup>−/−</sup> db/db mice did not show any AR immunoreactivity (Fig. 1C and D). In the blood vessels of the retina, AR was mainly present in the pericytes but not in the endothelial cells (Fig. 1D and E), and the expression level of AR in these cells was higher in the db/db mice than in the db/n mice.

**AR deficiency protects against diabetes-induced pericyte loss.** To assess pericyte loss, retinal sections were stained with antibody against α-SMA, which specifically marks pericytes in the capillaries. The number of α-SMA–positive cells in the inner nuclear layer of the retinas was counted and normalized against the area of the inner nuclear layer (Fig. 2A). The retinas of AR<sup>−/−</sup> db/db mice showed a >25% decrease in pericyte density compared with that of the AR<sup>−/−</sup> db/n mice (Fig. 2B). There was no decrease in pericyte density in the retinas of AR<sup>−/−</sup> db/db mice.

**AR deficiency reduced diabetes-induced oxidative stress in the retinal neurons.** In the retinas of the AR<sup>−/−</sup> db/db mice, formation of nitrotyrosine, an oxidative-nitrosative stress marker (28), was increased in retinal ganglion cells and in the neurons in inner and outer nuclear layers.
as well as in Müller cells (Fig. 3A2, indicated by arrows) compared with that of the AR\(^{+/+}\) db/m mice (Fig. 3A1). The Müller cells and their processes seem to accumulate much less nitrotyrosine. In the retinas of the AR\(^{-/-}\) db/db mice, there was even less nitrotyrosine accumulation in these cell types (Fig. 3A3). Perhaps as a consequence of increased nitrosative stress that might lead to DNA damage, the level of PAR, the product of PAR polymerase (PARP) that is activated by DNA strand breaks (29), was increased in the retinal ganglion cells and neurons in the inner nuclear layer in the retinas of the AR\(^{+/+}\) db/db mice (indicated by arrows) (Fig. 3B1 and B2). The retinas of the AR\(^{-/-}\) db/db mice showed no increased level of PAR (Fig. 3B3).

AR deficiency inhibited diabetes-induced changes in PECAM-1 and VEGF expressions. Immunostaining of retinal sections showed that the expression of PECAM-1 was localized in the vascular endothelial cells (Fig. 4AI–A3) and not in the pericytes surrounding the endothelial cells. The immunoreactivity of PECAM-1 was decreased in the retinas of the AR\(^{+/+}\) db/db mice (Fig. 4A2) compared with those of the AR\(^{+/+}\) db/m mice (Fig. 4A1). No decrease was observed in the retinas of AR\(^{-/-}\) db/db mice (Fig. 4A3).

Immunostaining of VEGF was found in the astrocytes, Müller cells, retinal ganglion cells, and neurons in the inner nuclear layer (Fig. 4B1–3). Only the endfeet of Müller cells around the capillaries in inner nuclear layer, but not the radial processes in both plexiform layers of Müller cells, were stained with VEGF (Fig. 4B2, indicated by arrows). The expression of VEGF was increased in the retinas of the AR\(^{+/+}\) db/db mice (Fig. 4B2) but not in the AR\(^{-/-}\) db/db mice (Fig. 4B3), indicating that AR deficiency suppressed hyperglycemia-induced induction of this gene.

Diabetes-induced GFAP expression in Müller cells and astrocytes was attenuated by AR deficiency. To determine the diabetes-induced glial response in the retinas, expression of GFAP, a marker for astrocytes (14) and reactive Müller cells after eye injury (30), was determined. In the retinas of AR\(^{+/+}\) db/m mice, GFAP expression was observed in astrocytes in the inner limiting membrane and

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**Figures**

**Fig. 1.** Immunohistochemical staining of AR. AR immunoreactivity was localized on retinal sections of AR\(^{+/+}\) db/m (A and D), AR\(^{+/+}\) db/db (B and E), and AR\(^{-/-}\) db/db (C and F) mice. AR expression was shown in fibers in inner limiting membrane, Müller cells, and neuronal cells in both retinal ganglion cell and the inner nuclear layer (INL) (A–B) and pericytes (D–E) in retinas (arrows). The retinas of AR\(^{-/-}\) db/db are also shown (C and F). \(n = 4\) retinas per group. Magnification \(\times 63\). INL, inner nuclear layer; ONL, outer nuclear layer.

**Fig. 2.** Pericyte density. Pericyte density was determined on retinal sections by counting the number of \(\alpha\)-SMA–immunoreactive cells (arrows) per area of inner nuclear layer (INL) of the retinas (A). Magnification \(\times 100\). The density change between the retinas of these mice is shown in B.

**Fig. 3.** Immunohistochemical staining of nitrotyrosine and PAR. Nitrotyrosine (A) and PAR (B) staining on retinal sections of AR\(^{+/+}\) db/m (A1 and B1), AR\(^{+/+}\) db/db (A2 and B2), and AR\(^{-/-}\) db/db (A3 and B3) mice are shown. Increased nitrotyrosine (A2, arrows) and PAR (B2, arrows) immunoreactivities were observed in retinas of AR\(^{-/-}\) db/db mice when compared with those of AR\(^{+/+}\) db/m and AR\(^{-/-}\) db/db mice. \(n = 4\) retinas per group. Magnification \(\times 63\). INL, inner nuclear layer; ONL, outer nuclear layer; RGC, retinal ganglion cell.
Müller cells with their cell bodies residing in the inner nuclear layer (Fig. 5A1). In the AR\(^{+/−}\) db/db mice, more processes of the GFAP-stained Müller cells were observed in both inner and outer plexiform layers (indicated by arrows). Their locations confirmed that the processes are from Müller cells (Fig. 5A2). In addition, more GFAP immunoreactivity in the retinas of AR\(^{+/−}\) db/db mice was observed in the inner limiting membrane (indicated by arrows), where the astrocytes reside, compared with that of AR\(^{+/+}\) db/m mice (Fig. 5A1). The induction of GFAP expression in Müller cells and astrocytes in the retinas of AR\(^{−/−}\) db/db mice was not as high as in those of AR\(^{+/+}\) db/db mice (Fig. 5A3).

**Diabetes-induced proliferation of Müller cells was reduced by AR deficiency.** To identify cells that are induced to proliferate by hyperglycemia, retinal sections were stained with antibody against PCNA, a marker for proliferating cells. The PCNA-positive cells were found mainly in the inner nuclear layer (Fig. 5B1), and they were shown to be Müller cells by staining the adjacent sections with S-100 antibody, a marker for Müller cells (Fig. 5B2). The number of PCNA-positive cells and the intensity of PCNA immunoreactivity was increased in the retinal ganglion cells and neurons in the inner nuclear layer, but not in the outer nuclear layer in the AR\(^{+/−}\) db/db mice (Fig. 5C2), compared with those of the AR\(^{+/−}\) db/m mice (Fig. 5C1). There was less PCNA immunoreactivity in the Müller cells of the AR\(^{−/−}\) db/db mice (Fig. 5C3).

**AR deficiency protects against diabetes-induced blood-retinal barrier leakage.** To detect blood-retinal barrier breakdown, mouse IgG extravasation in the retinal sections of the mice was examined, and the results are shown in Fig. 6. The majority of IgG-positive blood vessels were present in the inner nuclear layer. Staining of IgG detected outside the lumen of vessels suggests leakage as a consequence of blood-retinal barrier breakdown (31). In the AR\(^{+/−}\) db/m mice, IgG staining was located within the lumen of blood vessels and showed no signs of leakage out of the blood vessels (Fig. 6A). In the AR\(^{+/+}\) db/db mice, a
AR was observed in the retinas of the AR mice (Fig. 6A). A recent report showed that mice diabetic for 14 weeks from streptozotocin injection exhibited neuronal cell death in the retinal ganglion cell layer and shrinkage of the retinas (32). No abnormal vascular change was observed in these mice, presumably because of the short duration of the experiment. It is difficult to maintain the streptozotocin-treated mice for prolonged period. The 6-month-old mice, which develop type 2 diabetes when they are 8 weeks old, have been shown to exhibit early features of diabetic retinopathy, such as pericyte and endothelial cell loss (23), basement membrane thickening (22), and increased blood flow (24). In this report, we demonstrate that the retinas of the 15-month-old mice showed pericyte loss, breakdown of blood-retinal barrier, apoptosis of neuronal cells, glial reactivation, and even a feature of more advanced diabetic retinopathy: the proliferation of retinal capillaries. All of these hyperglycemia-induced changes were attenuated by AR null mutation, indicating that AR plays an important role in the pathogenesis of this disease. However, the mechanism is still unclear.

Increased flux of glucose through the polyol pathway is thought to increase osmotic and oxidative stress, activate protein kinase C, and increase advanced glycation end products (33). Depending on the cell type, one or more of these mechanisms may contribute to hyperglycemia-induced lesions. In the mouse retina, AR is expressed in the astrocytes, Müller cells, retinal ganglion cells, neurons in the inner nuclear layer, and capillary pericytes. In the diabetic mice, AR expression is increased in all of these cells, further exacerbating the toxic effect of hyperglycemia.

Pericyte loss is a hallmark of early diabetic retinopathy in human and animal models. Pericytes cultured in high glucose medium were shown to have decreased glutathione level and activation of caspase-3, leading to apoptosis (34). Treatment with an AR inhibitor or caspase-3 inhibitor was able to prevent such changes, suggesting that AR activity causes oxidative stress that activates the caspase-dependent apoptotic pathway. The pericytes wrap themselves around the vascular wall, making multiple contacts with the endothelial cells. These two types of cells communicate with each other through gap junctions. It is likely that pericyte loss would lead to endothelial cell death. This is supported by the observation that there was endothelial cell loss in mice with a null mutation in the platelet-derived growth factor gene, which is expressed in pericytes but not in endothelial cells, suggesting a close communication between these two types of cells (27). Therefore, although the endothelial cells have very low levels of AR, they are still sensitive to polyol pathway-mediated glucose toxicity.

The increased number of retinal vessels leaking IgG in the AR−/− db/db mice indicates breakdown of the blood-
retinal barrier, a common sign of diabetic retinopathy in humans (35) and rats (36). AR deficiency prevented this blood-retinal barrier breakdown, indicating that AR contributes to the maintenance of the blood-retinal barrier. One possible mechanism causing blood-retinal barrier breakdown in diabetic mice is AR-mediated loss of pericytes and endothelial cells. We found that the expression of PECAM-1, which is primarily located in the endothelial cells, was decreased in the AR\(^{+/+}\) db/db mice and normal in the AR\(^{-/-}\) db/db mice, suggesting that AR may inhibit its expression via its toxic effect on the pericytes. Deficiency in PECAM-1 has been shown to delay the establishment of tight junctions in the endothelial cells to restore the blood-brain barrier after inflammatory insult (11), suggesting that AR may also cause blood-retinal barrier breakdown via the downregulation of PECAM-1.

Increased oxidative and nitrosative stress has been suggested to cause blood-retinal barrier breakdown in diabetic rats (37). Treatment with a peroxynitrite scavenger reduced both oxidative and nitrosative stress and attenuated blood-retinal barrier breakdown. This could be mediated by VEGF because increased VEGF has been shown to increase blood-retinal barrier permeability (38), and reducing oxidative stress normalized VEGF level and prevented blood-retinal barrier breakdown (37). In the db/db mice, increased nitrosative stress in their retinas, as indicated by an increased level of nitrotyrosine immunoreactivity, was reduced by AR deficiency, suggesting that AR activity could also contribute to blood-retinal barrier breakdown by increasing oxidative and nitrosative stress.

Another indication of increased oxidative or nitrosative stress is an increased level of PAR. Increased reactive free radicals would cause DNA damage, leading to the activation of PARP to synthesize more PAR. AR inhibitor has been shown to attenuate the diabetes-induced increase in nitrotyrosine and PAR (28). This is confirmed by our observation that AR\(^{-/-}\) db/db mice accumulated less nitrotyrosine and PAR than AR\(^{+/+}\) db/db mice. Increased oxidative stress and activation of PARP may have led to retinal degeneration in AR\(^{+/+}\) db/db mice. We found that there were also more cleaved caspase-3-positive cells in the retinal ganglion cell and inner nuclear layer of their retinas, indicating that they underwent apoptosis (39). These were correlated with increased PCNA expression, which was suggested to play a role in selective postischemic survival, in these cells (40). Photoreceptor cells in the outer nuclear layer of the retinas with very little expression of nitrotyrosine and PAR were also negative in cleaved caspase-3 immunostaining.

There were indications that the astrocytes and Müller cells were experiencing stress because the expression of GFAP, which is induced after retinal damage (30), was increased. Signs of increased apoptotic cells in the Müller cells in 15-month-old AR\(^{+/+}\) db/db mice was also shown by increased cleaved caspase-3 immunoreactivity. Furthermore, the level of PCNA, a marker for cell proliferation (41), was increased in the Müller cells. The expression of PCNA in the Müller cells was confirmed by the colocalization of PCNA and S-100, a Müller cell marker (42). The induction of GFAP expression and proliferation of glial cells, signs that the retina was undergoing degeneration (43), were not observed in the AR\(^{-/-}\) db/db mice, indicating that these hyperglycemia-induced pathological changes were mediated by the polyol pathway.

The retinas of the 15-month-old db/db mice had significantly more IgG-stained blood vessels than the nondiabetic db/m mice, suggesting the formation of new blood vessels. This represents a more advanced stage of diabetic retinopathy than previously reported for 14-week-old diabetic mice (32) or 26-month-old galactosemic mice (44). Because of the ease of genetic manipulation and availability of a large number of mutants, mice serve as a convenient animal model to study the pathogenesis of various diseases. Demonstration that they do develop early and more advanced features of diabetic retinopathy should make them an attractive model to study this disease. The key features of diabetic retinopathy in db/db mice, such as pericyte and endothelial cell loss, blood-retinal barrier breakdown, neuronal degeneration, glial reactivation, and neovascularization, were all attenuated by AR deficiency, indicating that aldose reductase plays an important role in the pathogenesis of this disease.

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REFERENCES