

# Angiopoietin-2 Causes Pericyte Dropout in the Normal Retina

## Evidence for Involvement in Diabetic Retinopathy

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**Pericyte loss is an early pathologic feature of diabetic retinopathy, consistently present in retinæ of diabetic humans and animals. Because pericyte recruitment and endothelial cell survival are controlled, in part, by the angiopoietin/Tie2 ligand/receptor system, we studied the expression of angiopoietin-2 and -1 in relation to the evolution of pericyte loss in diabetic rat retinæ, using quantitative retinal morphometry, and in retinæ from mice with heterozygous angiopoietin deficiency (Ang-2 LacZ knock-in mice). Finally, recombinant angiopoietin-2 was injected into eyes of nondiabetic rats, and pericyte numbers were quantitated in retinal capillaries. Angiopoietin-1 protein was present in the normal maturing retina and was upregulated 2.5-fold in diabetic retinæ over 3 months of diabetes. In contrast, angiopoietin-2 protein was consistently upregulated more than 30-fold in the retinæ of diabetic rats, preceding the onset of pericyte loss. Heterozygous angiopoietin-2 deficiency completely prevented diabetes-induced pericyte loss and reduced the number of acellular capillary segments. Injection of angiopoietin-2 into the eyes of normal rats induced a dose-dependent pericyte loss. These data show that upregulation of angiopoietin-2 plays a critical role in the loss of pericytes in the diabetic retina. *Diabetes* 53:1104–1110, 2004**

**D**iabetic retinopathy is one of the major microvascular complications in both type 1 and type 2 diabetes. Proliferative diabetic retinopathy is the leading cause of blindness among adults of working age (1–4).

Studies on retinal capillaries revealed that the loss of pericytes is the first morphologic change in a diabetic

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Ang, angiopoietin; PAS, periodic acid Schiff base; PDGF, platelet-derived growth factor; STZ, streptozotocin; VEGF, vascular endothelial growth factor. © 2004 by the American Diabetes Association.

retina. Endothelial cells subsequently disappear, leaving behind acellular capillaries, which are no longer perfused (5,6). In human diabetic retinæ, focal areas are observed in which endothelial cells proliferate, forming microaneurysms. Similarly, increased endothelial cell numbers are found in diabetic rodent retinæ. Capillary nonperfusion can subsequently stimulate proliferative retinopathy, which is characterized by the growth of new vessels from the retina into the vitreous.

The developing retina appears particularly dependent on pericytes, because a defective recruitment affects the retina more than other tissues (7). If pericyte recruitment is blocked by genetic or pharmacological manipulation during the postnatal period of vessel development, profound aberrations in vessel patterning and endothelial hyperplasia are observed (8,9). If the gene dosage of platelet-derived growth factor (PDGF)-B, one important pericyte-recruiting factor, is reduced by half, a moderate increase in acellular capillary formation occurs. These data suggest that pericytes provide vessel stability and control of endothelial proliferation (10).

Apart from PDGF-B, several other molecules are crucial for the timely and coordinated recruitment of pericytes to the vascular wall during vessel development. New factors have been identified that modulate the vascular response by affecting endothelial cells and secondarily also pericytes. The activity of the endothelial receptor tyrosine kinase, Tie-2, is differentially regulated by the two ligands, angiopoietin (Ang)-1 and Ang-2. According to gene-targeting experiments *in vivo*, Ang-1 signaling via Tie-2 is involved in capillary sprouting, endothelial cell survival, and vascular remodeling (11–14). Importantly, Ang-1 has also been implicated in the stabilization of vessels by recruiting pericytes and smooth muscle cells to the vessel wall. Ang-2 can antagonize the effect of Ang-1-stimulated Tie-2 activity by inhibiting the autophosphorylation of Tie-2 induced by Ang-1 (15). Several lines of evidence suggest that Ang-2 in combination with vascular endothelial growth factor (VEGF) leads to sprouting angiogenesis while Ang-2 in the absence of growth-promoting signals renders vessels susceptible to regression (16–19).

Based on the distinct functions of Ang-1 and Ang-2, we hypothesized that the Ang/Tie-2 system is involved in pericyte loss in the diabetic retina. Therefore, we investigated the temporal expression pattern of Ang-1 and Ang-2 relative to pericyte loss in experimental diabetic retinopathy of the rat (20,21). To confirm the regulation of Ang-2

TABLE 1

Metabolic and physical parameters of the nondiabetic and diabetic rats, studied prior (3 weeks) and after (3 months) the onset of pericyte dropout

	Nondiabetic		Diabetic	
	3 weeks	3 months	3 weeks	3 months
Blood glucose (mmol/l)	5.5 ± 0.5	5.4 ± 0.4	27.4 ± 3.1*	29.0 ± 3.7*
HbA <sub>1c</sub> (%)	4.5 ± 0.3	4.6 ± 0.6	13.4 ± 2.5*	18.0 ± 1.5*
Body weight (g)	490 ± 28	605 ± 27	300 ± 38*	331 ± 50*

\* $P < 0.05$  or less vs. nondiabetic animals.

in diabetes, we also used a diabetic mouse model with a reporter gene under control of the Ang-2 promoter (Ang-2 LacZ knock-in) (22). We investigated whether Ang-2 can induce pericyte loss by injecting the recombinant protein into the eyes of normal rodents. Finally, we studied pericyte loss and endothelial damage in the diabetic Ang-2 LacZ knock-in mouse model. These experiments aimed to identify the Ang-Tie system as contributor to diabetes-induced pericyte loss.

## RESEARCH DESIGN AND METHODS

Experiments performed in this study adhered to the Association for Research in Vision and Ophthalmology (ARVO) statement for the "Use of Animals in Ophthalmic and Vision Research."

Six-week-old male Wistar rats (Charles River, Sulzfeld, Germany) were rendered diabetic by intravenous streptozotocin (STZ) injection of 65 mg/kg body wt (Boehringer Mannheim, Mannheim, Germany). Glucose levels and body weight were monitored consecutively, and glycated hemoglobin was determined by affinity chromatography (Glyc Affin; Isolab, Akron, OH).

Mice with the reporter gene LacZ under control of the Ang-2 promoter (Ang-2 LacZ knock-in) (22) have been recently developed, and were kindly provided by Regeneron Pharmaceuticals (Tarrytown, NJ). Wild-type litters were used as controls. Stable diabetes was induced in 6-week-old animals weighing  $24 \pm 3$  g by STZ (150 mg/kg i.p.). Monitoring of glycemia was performed the same way as for diabetic and nondiabetic rats. Diabetic mice and age-matched controls were maintained for 6 weeks to analyze changes of Ang-2 expression under hyperglycemic conditions and for 26 weeks to analyze changes in pericyte numbers and formation of acellular capillaries (see below).

**Retinal digest preparation.** To determine the time of onset of pericyte loss, eyes were obtained from diabetic and nondiabetic rats ( $n = 5$  for each group) at monthly intervals. One group of five nondiabetic animals was investigated at the beginning of the study. Retinae were obtained after enucleation of the eyes from the animals under deep anesthesia and immediately fixed in 4% buffered formalin. Retinal vascular preparations were performed using a pepsin-trypsin digestion technique as previously described (20,21). Briefly, a combined pepsin (5% pepsin in 0.2% hydrochloric acid for 1.5 h) trypsin (2.5% in 0.2 mol/l Tris for 15–30 min) digestion was used to isolate the retinal vasculature. Subsequently, the samples were stained with periodic acid Schiff base (PAS). The total number of pericytes was counted in 10 randomly selected fields of the retina using an image analyzing system (CUE-2; Olympus Optical, Hamburg, Germany), and their numbers were normalized to the relative capillary density (numbers of cells per  $\text{mm}^2$  of capillary area). Because significant pericyte loss was found to occur after 2 months of diabetes, eyes were obtained from rats after 3 weeks of diabetes, from age-matched nondiabetic controls, from diabetic rats after 3 months of diabetes, and from age-matched normal controls. Eyes were embedded in OCT (Miles, IL), and sections (6  $\mu\text{m}$ ) were cut on a cryostat at  $-20^\circ\text{C}$  and placed on poly-L-lysine-coated slides.

Retinal digest preparations ( $n = 4$ ) of 26-week-diabetic Ang-2 LacZ<sup>+/-</sup> mice and nondiabetic controls were performed likewise, and pericyte numbers and acellular capillaries were analyzed in PAS-stained samples, as described above.

**Immunoblotting for angiopoietins.** To identify hyperglycemia-mediated regulation of Ang-1 and Ang-2 in the retina, protein extracts from the four experimental groups were used for Western blot analysis. Briefly, retinal protein extracts were subjected to SDS-polyacrylamide-gel electrophoresis under reducing conditions, and after transfer to nitrocellulose filters, incubated with antibodies against Ang-1 (affinity-purified goat polyclonal antibody against the amino terminus of human Ang-1; 0.2  $\mu\text{g}/\text{ml}$ , SC 6319; St. Cruz

Biotechnology, Heidelberg, Germany) and Ang-2 (affinity-purified goat antibody against mouse Ang-2; 0.2  $\mu\text{g}/\text{ml}$ ; SC 7017; St. Cruz Biotechnology). Filters were developed using the enhanced chemiluminescence Western blotting detection system (Amersham, Braunschweig, Germany), and immunocomplexes were quantified using digital copies and quantitative analytical software (Analysis; Olympus Optical, Hamburg, Germany). Filters were reprobbed using a mouse monoclonal anti-actin antibody (0.1  $\mu\text{g}/\text{ml}$ ; C4, Boehringer Mannheim) and developed as described above. The data were used to normalize angiopoietin levels to protein loading.

**Ang-2 expression in diabetic Ang-2 LacZ knock-in mice.** Eyes from 6-week diabetic Ang-2 LacZ knock-in mice and age-matched nondiabetic controls ( $n = 5$  retinae/group) were removed under deep anesthesia and stained for LacZ and lectin to visualize Ang-2 expression in relation to the retinal vasculature according to published protocols (23). Briefly, eyes from nondiabetic and diabetic Ang-2 LacZ knock-in mice were fixed in a solution containing 0.2% glutaraldehyde and 1.5% formaldehyde, and X-gal staining was done. Retinae were isolated from eyes and postfixed in 4% buffered formalin, permeabilized with 1% BSA and 0.5% Triton-X-100, followed by staining with TRITC-conjugated isolectin (Sigma L-5264, 1:50) at  $4^\circ\text{C}$  overnight. The numbers of Ang-2-expressing cells were assessed by counting LacZ-positive cells per microscopic field in the superficial and the deep layers of the retina. An average of 10 randomly selected microscopic fields was counted per retina at  $40\times$  magnification. The numbers of cells were recorded in the inner retinal layers (ganglion cell layer and nerve fiber layer), with microscopic focus on large vessels as a reference, and in the inner nuclear layer with the deep capillary layer in focus. Additionally, eyes stained for LacZ were obtained, and vertical cryostat sections were performed as described (22).

**Intravitreal injection of Ang-2.** Normal male Wistar rats (age 6 weeks; Charles River, Sulzbach, Germany) were used. Ten microliters containing 0.330  $\mu\text{g}$  (four animals) and 1  $\mu\text{g}$  (four animals) of recombinant Ang-2 (R&D, Wiesbaden, Germany) were injected intravitreally into the left eye under deep anesthesia (Avertin). Sterile PBS was injected into the right eye for control. Eyes were obtained from the deeply anesthetized animals after 1 week, retinae were subjected to digestion preparation as described above, and the capillary network was evaluated for the numbers of pericytes per capillary area.

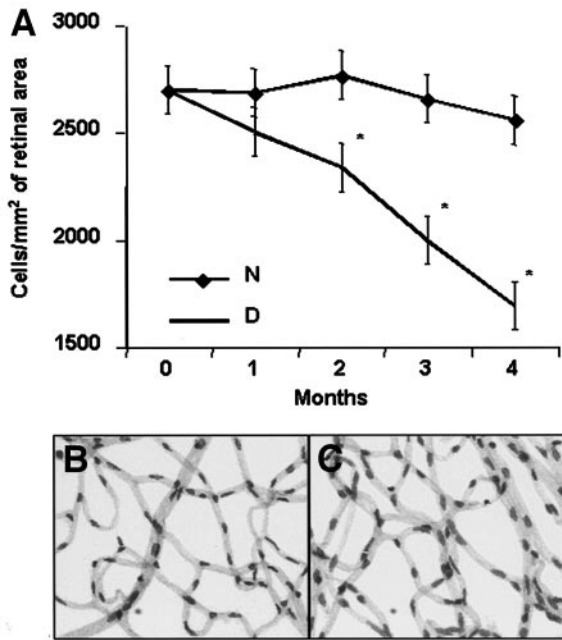
**Pericyte loss and acellular capillaries in diabetic Ang-2 LacZ knock-in mice.**

Diabetes was induced as described above. After 26 weeks of stable hyperglycemia, eyes were obtained from the following groups: nondiabetic wild-type mice ( $n = 6$ ), diabetic wild-type mice ( $n = 6$ ), nondiabetic Ang-2 LacZ knock-in mice ( $n = 4$ ), and diabetic Ang-2 LacZ knock-in mice ( $n = 4$ ). Eyes were obtained from the deeply anesthetized animals, retinae were subjected to digestion preparation as described above, and the capillary network was evaluated for the numbers of pericytes and acellular capillaries using previously described quantitation methods.

**Statistical analysis.** Results are given as means  $\pm$  SD. For statistical analysis, ANOVA and the Bonferroni multiple comparison test were used (Instat; GraphPad, San Diego, CA).

## RESULTS

We determined the onset and magnitude of pericyte loss in retinal capillaries over a period of 3 months of diabetes, using quantitative retinal morphometry. Table 1 shows body weight, blood glucose, and HbA<sub>1c</sub> of the experimental groups. STZ-induced diabetic rats had a more than threefold increase of blood glucose and increased levels of glycated hemoglobin (threefold increase at 3 weeks, more than fourfold increase at 3 months). Diabetic animals gained much less weight (+10.3% over basal) than the

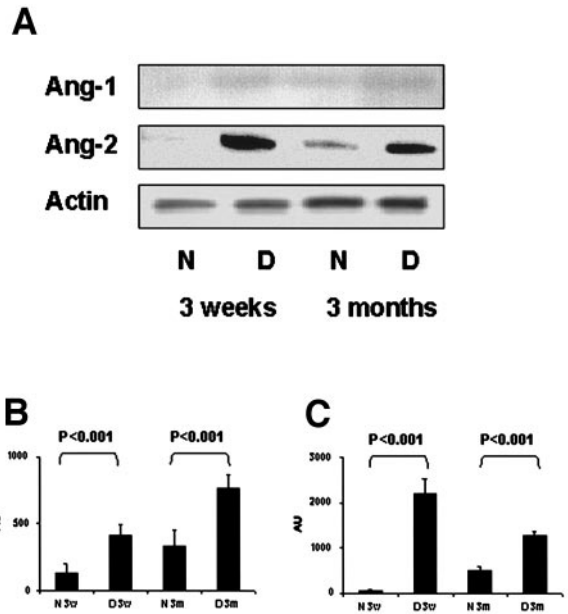


**FIG. 1. A:** Quantitative morphometry of pericytes in STZ-diabetic rats of short duration (D) compared with controls (N). Pericytes were identified in retinal digest preparations by morphologic criteria (shape, staining intensity with PAS, relative position in the capillary, and additional staining with a polyclonal anti-vitronectin antibody (rb 69) (21), and quantitated morphometrically by computer-based image analysis. The number of pericytes was normalized to the area of capillaries in which they were counted. \* $P < 0.05$  or less versus nondiabetic. Representative examples of retinal digest preparations of nondiabetic (B) and diabetic (C) rats after 3 months of diabetes are shown. Acellular capillaries are not different between nondiabetic and diabetic groups, and the differences between pericyte numbers are subject to quantitative analysis. B and C: PAS-stained retinal digest preparations; original magnification 400 $\times$ .

nondiabetic controls (+23.5%). After 2 months of diabetes duration, the numbers of pericytes became significantly different between diabetic ( $2,342 \pm 104$  pericytes/ $\text{mm}^2$  of capillary area) and nondiabetic animals ( $2,769 \pm 99$  pericytes/ $\text{mm}^2$  of capillary area;  $P < 0.001$ ) (Fig. 1), indicating an early onset of pericyte loss in the diabetic rat model. Based on these data, subsequent experiments were performed using retinae obtained at a time point before (i.e., 3 weeks after diabetes induction) and after (i.e., 3 months after diabetes induction) the onset of pericyte loss.

**Ang-1 and -2 expression in the diabetic retina.** Next, the effect of hyperglycemia on Ang-1 and Ang-2 expression relative to the initiation of pericyte dropout was evaluated by immunoblotting. Ang-1 was present in nondiabetic retinae both at 3 weeks and 3 months. In diabetic retinae, Ang-1 increased 2.5-fold at 3 weeks and remained elevated over nondiabetic controls at 3 months, indicating a persistent upregulation by chronic hyperglycemia. Ang-2 protein expression in nondiabetic animals was very low at 3 weeks and low at 3 months. In contrast, in diabetic rats at 3 weeks, Ang-2 protein was upregulated 37-fold compared with nondiabetic levels and remained 2.5-fold elevated over nondiabetic controls at 3 months of diabetes (Fig. 2). These data demonstrate that Ang-2 is predominantly and strongly upregulated by hyperglycemia and that Ang-2 upregulation precedes the onset of pericyte loss in the diabetic retina.

**Regulation of Ang-2 in diabetic Ang-2 LacZ knock-in mice.** We used a mouse model in which Ang-2 expression



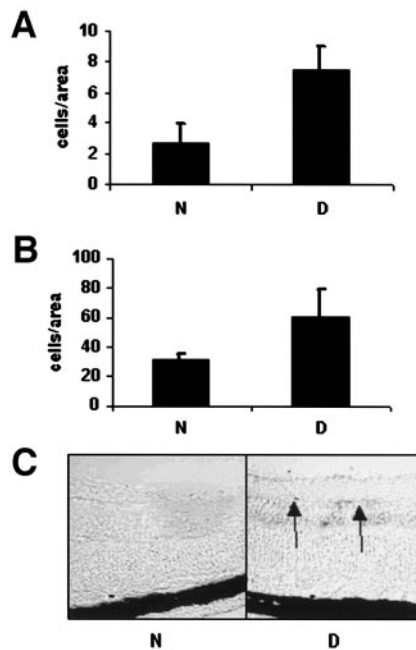
**FIG. 2. A:** Representative immunoblots of retinal protein extracts against Ang-1 and Ang-2 in nondiabetic and diabetic rat retina confirming the marked upregulation of Ang-2 at 3 weeks of diabetes (second lane), i.e., preceding pericyte loss. Actin: control for equal lane loading. **B:** Quantitation of immunocomplexes of Ang-1 expression in retinal extracts of nondiabetic (N) and diabetic (D) rats at 3 weeks (3w) and 3 months (3m). Results are given as means  $\pm$  SD from three independent experiments. AU, arbitrary units: ratio of Ang-1 signal normalized to the actin signal of the respective samples. **C:** Quantitation of Ang-2 expression. The groups tested and the procedure are as described under B.

is visualized by a LacZ reporter construct to confirm the effect of hyperglycemia on Ang-2 expression. Previously, these mice had been shown to express Ang-2 in horizontal cells of the inner nuclear layer of the adult retina and in few cells of unclear allocation in the ganglion cell layer (24). The numbers of retinal cells with detectable LacZ expression were compared between mice with 6-week STZ-induced diabetes and age-matched nondiabetic control mice. The metabolic data of the two groups are given in Table 2. In nondiabetic animals (age 12 weeks), a total of 34 cells per microscopic field expressing Ang-2 was identified, of which 8% ( $2.7 \pm 1.2$  cells/area) were in the ganglion cell layer (Fig. 3), while the majority of cells were found in the outer capillary plexus, i.e., in the outer margin of the inner nuclear layer ( $31.25 \pm 4.18$  cells/area). There was a 2.8-fold increase of LacZ-positive cells in the ganglion cell layer ( $7.5 \pm 1.61$  cells/area;  $P < 0.0001$  vs. nondiabetic group) and a 2.0-fold increase in the inner nuclear layer ( $60.87 \pm 18.35$  cells/area;  $P < 0.0001$  vs. nondiabetic group) of the 6-week diabetic mice. Beside expression in the known locations of the retina, Ang-2 was

**TABLE 2**  
Metabolic and physical parameters of the Ang-2 LacZ mice at 6 weeks

	Nondiabetic	Diabetic
Body weight (g)	24.4 $\pm$ 2.2	19.5 $\pm$ 2.1
Blood glucose (mmol/l)	8.9 $\pm$ 1.3	33.3 $\pm$ 4.5
HbA <sub>1</sub> (%)	4.3 $\pm$ 0.3	11.7 $\pm$ 0.7

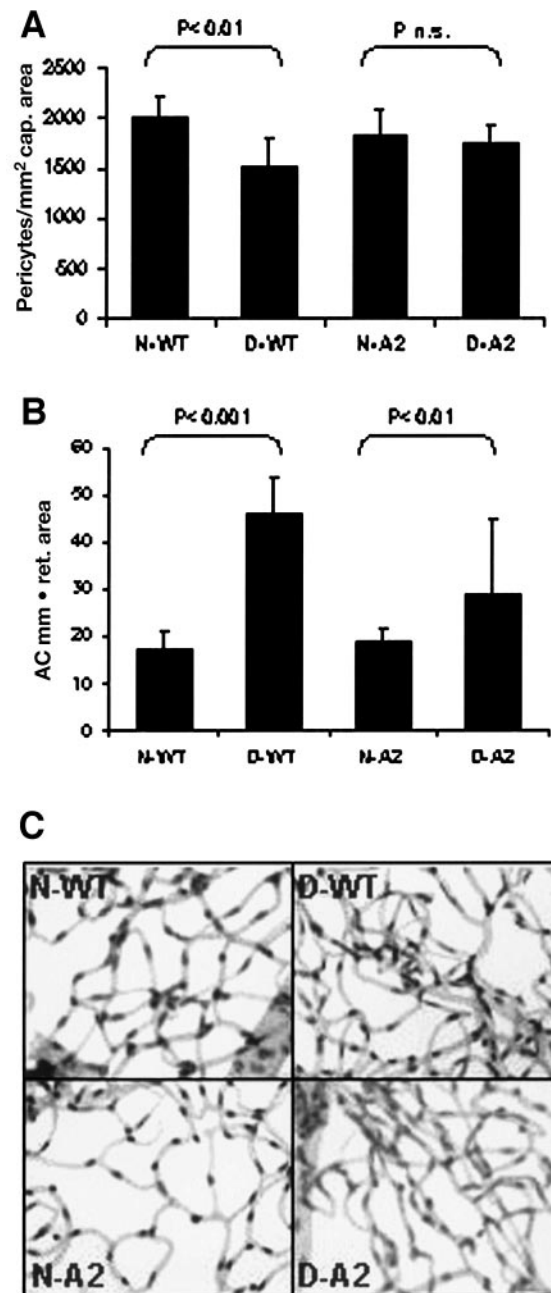
All parameters were significantly ( $P < 0.05$  or less) elevated in diabetic Ang-2 LacZ mice.



**FIG. 3.** Quantitation of LacZ-positive cells in the superficial (A) and the deep (B) capillary network of the retina in nondiabetic (N) and diabetic (D) Ang-2 LacZ mice. \* $P < 0.001$  vs. N. C: Representative vertical cryostat sections of a nondiabetic (N) and a diabetic (D) Ang-2LacZ knock-in mouse. Ang-2 regulation was assessed after 6 weeks of hyperglycemia. Arrows point to the expression of Ang-2 in diabetic animals, representing capillaries that traverse the retina in the inner plexiform layer. Original magnification 200 $\times$ ; LacZ staining.

also noticed in capillaries traversing from the ganglion cell to the inner nuclear layer of the retina, as depicted in Fig. 3. These data confirmed the data obtained in the STZ-diabetic rat model and are consistent with the hypothesis that chronic hyperglycemia upregulates Ang-2 in the retina. **Retinae of diabetic Ang-2 LacZ<sup>+/-</sup> mice are protected from diabetes-induced pericyte dropout.** Because both chronic hyperglycemia and pharmacological Ang-2 application to the eye induce pericyte dropout, we asked whether mice with reduced Ang-2 expression were protected from pericyte dropout and from subsequent consequences such as formation of acellular capillaries. Thus, we studied pericyte dropout and the formation of acellular capillaries in retinae of Ang-2 LacZ knock-in mice that had had diabetes for 26 weeks. Metabolic data of the mice are given in Table 3. Despite a >3.5-fold higher level of glycated hemoglobin and a 4.0-fold increase in glucose levels, pericyte dropout was absent in the diabetic Ang-2 LacZ knock-in mice, while a 25% loss of pericytes was observed in diabetic wild-type litters compared with nondiabetic wild-type controls (diabetic Ang-2 LacZ knock-in mice  $1,762 \pm 180$  pericytes/ $\text{mm}^2$  of capillary area, nondiabetic Ang-2 LacZ knock-in mice  $1,835 \pm 248$  pericytes/ $\text{mm}^2$ ; NS) (Fig. 4A). A significant increase in the number of acellular capillaries was observed in diabetic Ang-2 LacZ knock-in mice despite the absence of pericyte loss (Fig. 4B). This increase was significantly less pronounced compared with the increase in formation of acellular capillaries in diabetic wild-type litters.

**Intravitreal injection of Ang2.** To investigate whether increased intraocular concentrations of Ang-2 can induce pericyte dropout, recombinant Ang-2 was injected intra-



**FIG. 4.** Pericyte numbers (A) and acellular capillaries (B) in nondiabetic (N) and diabetic (D) retinae from mice with a heterozygous deletion of the Ang-2 gene by knock in of a LacZ reporter construct (22). Pericyte numbers and acellular capillaries were quantitated as described. Representative retinal digests of nondiabetic (N) and diabetic (D) wild-type (WT) and Ang-2 LacZ knock-in mice are shown. The qualitative presence of pericytes and of acellular capillaries in the diabetic Ang-2 LacZ knock-in mice can be appreciated. Original magnification 400 $\times$ ; PAS staining.

vitreally into 6-week-old nondiabetic rats. Retinae were isolated 1 week after the injection and subjected to retinal digest preparation and morphometry. Qualitatively, no signs of inflammation were observed in any of the treated animal eyes, suggesting that the pharmacological modulation of the cross-talk between capillary cells was not induced by inflammatory cells. The injection of 330 ng Ang-2 induced a 13% reduction and the injection of 1  $\mu\text{g}$  Ang-2 a 22% reduction in pericyte numbers in the nondia-

TABLE 3  
Metabolic and physical parameters of the Ang-2 LacZ mice and wild-type litters at 26 weeks of diabetes

	Nondiabetic wild-type	Diabetic wild-type	Nondiabetic Ang-2 LacZ	Diabetic Ang-2 LacZ
Body weight (g)	36.4 ± 5.2	27.3 ± 3.9*	33.3 ± 4.0	19.6 ± 5.8†
Blood glucose (mmol/l)	8.1 ± 1.9	29.2 ± 8.3*	8.8 ± 2.1	33.33 ± 4.1†
HbA <sub>1c</sub> (%)	4.4 ± 0.5	14.9 ± 1.6*	4.2 ± 0.2	14.59 ± 1.9†

\**P* < 0.05 or less vs. nondiabetic wild-type mice; †*P* < 0.05 or less vs. nondiabetic Ang-2 LacZ mice.

betic rat retinae after a week (control 2,871 ± 550 cells/mm<sup>2</sup> of capillary area; 330 ng Ang-2 2,490 ± 380 cells/mm<sup>2</sup> of capillary area; *P* < 0.01 vs. control; 1 μg Ang-2 2,260 ± 430 cells/mm<sup>2</sup> of capillary area; *P* < 0.001 vs. control; Fig. 5). These quantitative changes in pericyte coverage were not associated with endothelial cell proliferation, as assessed by quantitative morphometry (not shown). Importantly, Ang-2 injection did not affect the diameter or the regularity of retinal capillaries (capillary width of PBS-injected retinae: 7.29 ± 0.81 μm vs. Ang-2 1 μg 6.97 ± 0.69 μm; NS). Figure 5B and C illustrate the regularity of capillaries after Ang-2 injection. We did not observe qualitative changes; particularly, there was neither indication of sprouting and new vessel formation nor that of vascular regression.

DISCUSSION

Our experimental data suggest that chronic hyperglycemia upregulates Ang-2 in the diabetic retina and that Ang-2 upregulation is causally involved in the pathogenesis of pericyte loss in diabetic retinopathy. Thus, these data add the novel as yet unappreciated aspect that Ang-2 upregulation contributes to the pathogenesis of pericyte loss by a mechanism of active elimination.

Several mechanisms have been proposed to explain pericyte loss in the diabetic retina. One hypothesis refers to the accumulation of toxic intracellular products such as sorbitol or advanced glycation end products (AGEs)

(25,26). The enzyme aldose reductase, which converts glucose to sorbitol, was thought to be only expressed in retinal pericytes, but other studies have shown expression in a variety of cell types including endothelial cells (27). Although this leaves the relative importance of the aldose reductase pathway for diabetic retinopathy undecided, its role in pericyte dropout is presumably not primary. AGEs are found to accumulate in pericytes of diabetic animals, but the time course of this accumulation is inconsistent with the time course of pericyte loss, suggesting that AGE accumulation may not be causal (26). AGEs can accumulate in pericytes when injected into nondiabetic animals. However, this may be more attributable to the documented propensity of pericytes for phagocytosis, rather than a specific mechanism of diabetes-induced damage (28). Further data suggesting a selective damage of pericytes in the diabetic retina include differential susceptibility to exposure to modified proteins, such as oxidized LDL (29), and the selective activation of proapoptotic pathways in response to glucose exposure (30). Rather than a passive destruction of pericytes, our data are consistent with the alternative hypothesis for diabetic pericyte loss, i.e., that pericyte loss in the diabetic retina is part of a process that is initiated by the active elimination of pericytes involving Ang-2.

There are three possible sources of Ang-2. In the normal retina Ang-2 is expressed in horizontal cells and in some undefined cell types of the ganglion cell layer (24). The expression of Ang-2 in microvascular endothelial cell has also been described (31). Moreover, in vitro data suggest that Müller cells are increasing Ang-2 expression under hyperglycemic conditions (32). Although we failed to assess the precise localization by immunological means of cells that contribute to the overall upregulation of Ang-2 in the diabetic retina in rats, our data suggest that cells providing Ang-2 become more numerous in diabetes. Moreover, our studies in the diabetic Ang-2 LacZ knock-in mouse indicate that vascular cells can become a source of Ang-2 under hyperglycemic conditions.

The (up)regulation of Ang-2 by hyperglycemia has not yet been reported. Factors described so far to be involved in the regulation of Ang-2 are hypoxia, tissue ischemia and growth factors such as VEGF (33–35). However, the specific mechanisms involved in the early upregulation of Ang-2 in the diabetic retina are unclear. Retinae from diabetic rats in which we found increased expression of Ang-2 protein do not yet develop significant numbers of acellular capillaries during the first 3 months, putting hypoxia as a possible inducer of Ang-2 expression into perspective. Recent data have shown that hyperglycemia-induced mitochondrial overproduction of reactive oxygen species leads to the inhibition of the glycolytic pathway,

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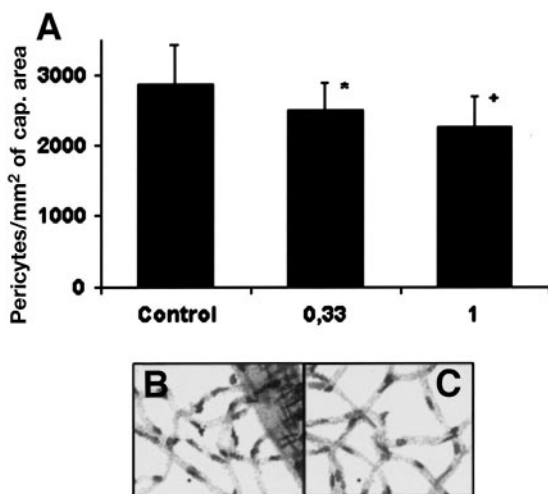


FIG. 5. A: Effect of intravitreal injection of PBS (1 μl) and 330 ng and 1 μg recombinant Ang-2 on pericyte numbers in nondiabetic rats (*n* = 4 in each group), evaluated by quantitative retinal morphometry 7 days after injection. \**P* < 0.05 and +*P* < 0.01 vs. control. Representative photomicrographs of a retinal digest preparation of a rat injected with PBS (B) and a corresponding retinal preparation of a rat injected with 1 μg recombinant Ang-2 (C). Note the absence of capillary dilatation or other abnormalities. PAS staining, original magnification 400×.

and the excess flow of substrates through biochemical pathways, which have fructose-6-phosphate and glyceraldehyde-3-phosphate as their starting points. Modifications of gene transcription and of protein production can be the result, possibly including increased transcription of Ang-2 (36–38).

Developmental studies have shown that the interaction of Ang-1 with Tie-2 leads to vessel maturation and stabilization via recruitment of pericytes and smooth muscle cells (11–15). Ang-1 is part of a system that keeps pericytes in place and renders vessels refractory to proliferative or regressive signals. Ang-2 has been invoked (no functional data as proof are available) as a natural antagonist of Ang-1 in cyclic physiologic remodelling via inhibition of Tie-2 phosphorylation (15). The vascular response to Ang-2 is context dependent, as Ang-2 can also induce rather than inhibit autophosphorylation of Tie-2 after prolonged exposure (22). Endogenous Ang-2, while expressed at low level or even absent in the adult retina, is strongly upregulated in experimental proliferative retinopathy, and exogenous administration of Ang-2 in a model of transient vessel formation (in which the endothelial cell remains VEGF dependent for survival), leads to endothelial cell proliferation and sprouting angiogenesis (39–41). Thus, Ang-2 can act as an angiogenic factor in the retina, provided that it encounters VEGF, and a sensitized retinal vasculature. Our study adds a new aspect to the complexity of the Ang-Tie system, i.e., that Ang-2 induces pericyte dropout in the mature retina without concomitant acute endothelial cell changes such as endothelial proliferation or irregular shaping of capillaries. Intraretinal sprouting angiogenesis is absent in our model systems of experimental diabetic retinopathy in rodents. Thus, Ang-2 upregulation in the early diabetic retina does not elicit an angiogenic response, despite loss of pericytes and presence of VEGF upregulation (10,42). Important information on the role of pericytes in general, and specifically in the retina, evolve from developmental studies. It is believed that the recruitment of pericytes and smooth muscle cells and the deposition of new extracellular matrix determines the transition from an immature to a mature capillary network (19). Previous data suggested that recruitment of pericytes to the retinal vessels marks the end of a window of plasticity, during which endothelial cell functions are modulated by the presence or absence of growth factors (43). New data, however, challenge this concept and demonstrate that pericytes are present in the vicinity of the sprouting tip (44). Which molecular signals mark the final maturation of retinal vessels remains unclear. More importantly, with regard to the pathogenesis of diabetic retinopathy, which develops almost exclusively in the mature retina, pericytes are crucially involved in promoting the survival of retinal capillaries. Active disruption of the cellular cross-talk between endothelial cells and pericytes leads to aberrant remodelling, while completion of vascular coverage with pericytes makes the endothelial cells refractory to the modulation by growth factors (8,9,45). It is not known which the relevant factors are, by which endothelial cells in the developing retina differ from those in the adult retina, but when a pericyte deficit persists into adulthood in retinal capillaries, a moderate but significant increase in acellular capillaries becomes apparent (10). Moreover, a dose-dependent effect of vas-

cular responsiveness to pericyte deficiency exists according to a recent study showing responsive retinal neovascularization when the deficit in coverage of developing vessels by pericytes exceeds 50% of the retinal vessels (23).

Pericyte recruitment to capillaries in the developing retina is initially PDGF-receptor  $\beta$  dependent, while capillaries from older mice become resistant to PDGF-B depletion (9). From these data, it emerges that pericytes in the developing retina control sprouting and vessel remodelling, while in the adult/matured retina, they may serve as survival-supporting cells for endothelial cells. In this context, diabetes-induced Ang-2 upregulation may play a significant role in the vascular response to the hyperglycemic challenge. While the concomitant moderate upregulation of Ang-1 suggest an antipermeability and additional endothelial cell survival-promoting response, Ang-2 is considered as an initiator of pericyte elimination possibly facilitating repair of endothelial cells, but with the parallel casualty of the survival-promoting effect of pericytes.

The model of pericyte elimination by hyperglycemia-induced growth factor upregulation is supported by our data from diabetic heterozygous Ang-2 LacZ knock-in mice, in which Ang-2 gene dosage is reduced by 50%. We observed a complete prevention of diabetic pericyte loss in these animals, suggesting both a causal relationship between Ang-2 and diabetic pericyte dropout and a dose dependency of this effect. Despite the preservation of pericytes, the formation of acellular capillaries was significantly affected, indicating that additional hyperglycemia-associated mechanisms further contribute to diabetic endothelial damage. Evidence for the existence of such mechanisms has been provided by showing that endothelial cells, in contrast to smooth muscle cells, express Glut-1, which, in case of hyperglycemia, is not downregulated, leading to an increased flux of substrates through glycolysis and to increased production of mitochondrial reactive oxygen species (36). Mitochondrial overproduction of reactive oxygen species induces poly-ADP ribosylation through ROS-induced activation of poly(ADP-ribose)polymerase, leading to the inhibition of a critical enzyme of the glycolytic pathway. The resulting metabolic bloc results in the upbuilding of two important intermediates, fructose 6-phosphate and glyceraldehyde-3-phosphate, which give rise to the activation of different biochemical pathways involved in the pathogenesis of diabetic complications (46). The relative contribution of these pathways to endothelial damage in the absence of pericyte loss can be concluded from the experiments in diabetic Ang-2 LacZ mice.

In conclusion, our data suggest that Ang-2 modulates pericyte coverage of capillaries in the adult retina and that pericyte loss in early diabetic retinopathy is the result of an elimination process initiated by the upregulation of Ang-2. Despite pericyte preservation, acellular capillaries are not prevented, indicating that a complex interaction of vascular cells exist in which the survival of the endothelial lineage of the capillary has the highest priority.

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REFERENCES

1. Thylefors B, Negrel AD, Pararajasegaram R, Dadzie KY: Global data on blindness. *Bull World Health Organ* 73:115–121, 1995
2. Moss SE, Klein R, Klein BE: Ten-year incidence of visual loss in a diabetic population. *Ophthalmology* 101:1061–1070, 1994
3. Klein R, Klein BE, Moss SE, Davis MD, DeMets DL: The Wisconsin Epidemiologic Study of Diabetic Retinopathy. II. Prevalence and risk of diabetic retinopathy when age at diagnosis is less than 30 years. *Arch Ophthalmol* 102:520–526, 1984
4. Klein R, Klein BE, Moss SE, Davis MD, DeMets DL: The Wisconsin Epidemiologic Study of Diabetic Retinopathy. III. Prevalence and risk of diabetic retinopathy when age at diagnosis is 30 or more years. *Arch Ophthalmol* 102:527–532, 1984
5. Kohner EM, Henkind P: Correlation of fluorescein angiogram and retinal digest in diabetic retinopathy. *Am J Ophthalmol* 69:403–414, 1970
6. Bresnick GH, Engerman R, Davis MD, de Venecia G, Myers FL: Patterns of ischemia in diabetic retinopathy. *Trans Am Acad Ophthalmol Otolaryngol* 81:694–709, 1976
7. Klinghoffer RA, Muetting-Nelsen PF, Faerman A, Shani M, Soriano P: The two PDGF receptors maintain conserved signaling in vivo despite divergent embryological functions. *Mol Cell* 7:343–354, 2001
8. Lindblom P, Gerhardt H, Liebner S, Abramsson A, Enge M, Hellstrom M, Backstrom G, Fredriksson S, Landegren U, Nystrom HC, Bergstrom G, Dejana E, Ostman A, Lindahl P, Betsholtz C: Endothelial PDGF-B retention is required for proper investment of pericytes in the microvessel wall. *Genes Dev* 17:1835–1840, 2003
9. Uemura A, Ogawa M, Hirashima M, Fujiwara T, Koyama S, Takagi H, Honda Y, Wiegand SJ, Yancopoulos GD, Nishikawa S: Recombinant angiopoietin-1 restores higher-order architecture of growing blood vessels in mice in the absence of mural cells. *J Clin Invest* 110:1619–1628, 2002
10. Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, Brownlee M, Deutsch U: Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 51:3107–3112, 2002
11. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD: Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87:1171–1180, 1996
12. Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD: Isolation of angiopoietin-1, a ligand for the TIE2 receptor, by secretion-trap expression cloning. *Cell* 87:1161–1169, 1996
13. Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y: Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376:70–74, 1995
14. Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W: Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr Biol* 8:529–532, 1998
15. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277:55–60, 1997
16. Hanahan D: Signaling vascular morphogenesis and maintenance. *Science* 277:48–50, 1997
17. Holash J, Wiegand SJ, Yancopoulos GD: New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 18:5356–5362, 1999
18. Carmeliet P: Angiogenesis in health and disease. *Nat Med* 9:653–660, 2003
19. Jain R: Molecular regulation of vessel maturation. *Nat Med* 9:685–693, 2003
20. Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M: Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc Natl Acad Sci U S A* 88:11555–11558, 1991
21. Hammes HP, Weiss A, Fuhrer D, Kramer HJ, Papavassilis C, Grimminger F: Acceleration of experimental diabetic retinopathy in the rat by omega-3 fatty acids. *Diabetologia* 39:251–255, 1996
22. Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD: Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by angiopoietin-1. *Dev Cell* 3:411–423, 2002
23. Enge M, Bjarnegard M, Gerhardt H, Gustafsson E, Kalén M, Asker N, Hammes HP, Shani M, Fässler R, Betsholtz C: Endothelium-specific

- platelet-derived growth factor-B ablation mimics diabetic retinopathy. *EMBO J* 21:4307–4316, 2002
24. Hackett SF, Ozaki H, Strauss RW, Wahlin K, Suri C, Maisonpierre P, Yancopoulos G, Campochiaro PA: Angiopoietin 2 expression in the retina: upregulation during physiologic and pathologic neovascularization. *J Cell Physiol* 184:275–284, 2000
25. Hohman TC, Nishimura C, Robison WG Jr.: Aldose reductase and polyol in cultured pericytes of human retinal capillaries. *Exp Eye Res* 48:55–60, 1989
26. Stitt AW, Li YM, Gardiner TA, Bucala R, Archer DB, Vlassara H: Advanced glycation end products (AGEs) co-localize with AGE receptors in the retinal vasculature of diabetic and of AGE-infused rats. *Am J Pathol* 150:523–531, 1997
27. Vinore SA, Van Niel E, Swerdloff JL, Campochiaro PA: Electron microscopic immunocytochemical evidence for the mechanism of blood-retinal barrier breakdown in galactosemic rats and its association with aldose reductase expression and inhibition. *Exp Eye Res* 57:723–735, 1993
28. Sims DE: The pericyte: a review. *Tissue Cell* 18:153–174, 1986
29. Lyons TJ, Li W, Wojciechowski B, Wells-Knecht MC, Wells-Knecht KJ, Jenkins AJ: Aminoguanidine and the effects of modified LDL on cultured retinal capillary cells. *Invest Ophthalmol Vis Sci* 41:1176–1180, 2000
30. Romeo G, Liu WH, Asnaghi V, Kern TS, Lorenzi M: Activation of nuclear factor-κB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. *Diabetes* 51:2241–2248, 2002
31. Chi JT, Chang HY, Haraldsen G, Jahnsen FL, Troyanskaya OG, Chang DS, Wang Z, Rockson SG, van de Rijn M, Botstein D, Brown PO: Endothelial cell diversity revealed by global expression profiling. *Proc Natl Acad Sci U S A* 100:10623–28, 2003
32. Matsumura T, Hammes HP, Thornalley P, Edelstein D, Brownlee M: Hyperglycemia increases angiopoietin-2 expression in retinal Muller cells through superoxide-induced overproduction of a-oxoaldehyde age precursors (Abstract). *Diabetes* 49 (Suppl. 1):A55, 2000
33. Mandriota SJ, Pepper MS: Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ Res* 83:852–859, 1998
34. Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y: Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. *J Biol Chem* 274:15732–15739, 1999
35. Yuan HT, Yang SP, Woolf AS: Hypoxia upregulates angiopoietin-2, a Tie-2 ligand, in mouse mesangial cells. *Kidney Int* 58:1912–1919, 2000
36. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813–820, 2001
37. Du XL, Edelstein D, Rossetti L, Fantus IG, Goldberg H, Ziyadeh F, Wu J, Brownlee M: Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc Natl Acad Sci U S A* 97:12222–12226, 2000
38. Giardino I, Edelstein D, Brownlee M: Nonenzymatic glycosylation in vitro and in bovine endothelial cells alters basic fibroblast growth factor activity: a model for intracellular glycosylation in diabetes. *J Clin Invest* 94:110–117, 1994
39. Umeda N, Ozaki H, Hayashi H, Miyajima-Uchida H, Oshima K: Colocalization of Tie2, angiopoietin 2 and vascular endothelial growth factor in fibrovascular membrane from patients with retinopathy of prematurity. *Ophthalmic Res* 35:217–223, 2003
40. Hackett SF, Wiegand S, Yancopoulos G, Campochiaro PA: Angiopoietin-2 plays an important role in retinal angiogenesis. *J Cell Physiol* 192:182–187, 2002
41. Lobov IB, Brooks PC, Lang RA: Angiopoietin-2 displays VEGF-dependent modulation of capillary structure and endothelial cell survival in vivo. *Proc Natl Acad Sci U S A* 99:11205–11210, 2002
42. Hammes HP, Lin J, Bretzel RG, Brownlee M, Breier G: Upregulation of the VEGF/VEGF-receptor system in experimental background diabetic retinopathy of the rat. *Diabetes* 47:401–406, 1998
43. Benjamin LE, Hemo I, Keshet E: A plasticity window for blood vessel remodeling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* 125:1591–1598, 1998
44. Gerhardt H, Betsholtz C: Endothelial-pericyte interactions in angiogenesis. *Cell Tissue Res* 314:15–23, 2003
45. Benjamin LE, Golijanin D, Itin A, Podes D, Keshet E: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 103:159–165, 1999
46. Du X, Matsumura T, Edelstein D, Rossetti L, Zsengeller Z, Szabo C, Brownlee M: Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 112:1049–1057, 2003

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