Diabetes is associated with abnormal angiogenesis. Increased angiogenesis contributes to severe forms of diabetic retinopathy, but angiogenesis is decreased in response to myocardial ischemia in diabetic patients. We evaluated the direct effect of hyperglycemia on angiogenesis in the chicken chorioallantoic membrane assay, a model of active neoangiogenesis. Hyperglycemia, lasting up to 7 days, was induced in 7-day-old chick embryos by a single intravitellus glucose injection. Control embryos received either water (volumic control) or mannitol (osmotic control). Hyperglycemia decreased angiogenesis in this model from the 5th day on. The pattern and expression level of the main vascular growth factors’ genes were not altered by hyperglycemia, as assessed by in situ hybridization and semiquantitative RT-PCR. As early as 2 days after hyperglycemia was induced, an increased apoptosis of endothelial cells and pericytes was detected by transferase-mediated deoxyuridine triphosphate nick-end labeling assay and electron microscopy. In the meantime, endothelial cell proliferation was decreased, as assessed by incorporation of bromo-deoxyuridine. Hyperglycemia can therefore impair angiogenesis without altering the expression level of vascular growth factors through induction of apoptosis and decreased proliferation of endothelial cells. Diabetes 53:752–761, 2004

Alterations in structure and function of both micro- and macrovessels cause most diabetes-associated morbidity and mortality. Microvessel pathology is characteristically involved in the pathogenesis of diabetic retinopathy, neuropathy, and nephropathy. In the retina, the process begins in isolated capillaries that become acellular and nonperfused, extends to groups of capillaries, and ultimately advances centripetally to involve the arterioles and their side branches (1). Both endothelial cells and pericytes are involved through a complex set of cellular and molecular abnormalities (2). In addition to the cellular defects, functional vascular abnormalities involving retinal blood flow, vascular permeability, and rheological perturbation have been well documented (3). When capillary occlusion is extensive, it is responsible for retinal ischemia, which triggers an angiogenic response. In contrast, the arteriogenic response triggered by myocardial or lower limb ischemia is decreased in diabetic patients (4). This dual response to ischemia in diabetic patients has been called “the diabetic paradox” (5). It therefore remains unclear whether hyperglycemia has a direct stimulatory or an inhibitory role on ongoing angiogenesis and arteriogenesis.

Angiogenic response to tissue ischemia recapitulates many aspects of embryonic vessel development (6). Ischemia, via hypoxia-inducible transcription factors (HIF-1α and -2α), triggers a coordinated response of angiogenesis and arteriogenesis by inducing the expression of growth factors, such as the vascular endothelial growth factor (VEGF), the angiopoietins, and the transforming growth factor-β1 (TGF-β1) and their receptors, and by decreasing expression of natural inhibitors (e.g., thrombospondin-1, pigment epithelium-derived factor).

Few animal models have been developed to study the effects of hyperglycemia on ongoing physiological angiogenesis. In a murine model of short-term in vitro culture of embryos, it has been shown that hyperglycemia affects VEGF/VEGF receptor signaling pathways, causing arrest of vascular development at the primary capillary plexus stage in the yolk sac (7).

The chicken chorioallantoic membrane (CAM) assay is a well-established model of angiogenesis that can be used to test pro- and antiangiogenic conditions and molecules (8–10). The allantois is an extraembryonic membrane, composed of endoderm and mesoderm, in which primitive blood vessels begin to take shape on day 3 of incubation. Primitive vessels continue to proliferate into an arteriovenous system until day 8, and the vascular system attains its final arrangement on day 18, just before hatching. The main function of CAM is to mediate gas exchanges with the outer environment.

The CAM model has several advantages over mammalian models. The vascular system of CAM is directly accessible to observation and experimentation, and there are no metabolic or hormonal influences from the mother. In addition, it is a more physiological model than in vitro models because vascularization of CAMs is subject to regulations through fluxes, pressure, shear stress, growth factors, and so on. Furthermore, in contrast to mammalian models of embryonic development, there is no external (i.e., maternal) influence.

In this study, we used the CAM model to study the direct effects of hyperglycemia on physiological embryonic an-
gluconeogenesis, specifically on vascular cell apoptosis and proliferation and on expression of vascular growth factors.

RESEARCH DESIGN AND METHODS

Fertilized White Leghorn chicken eggs were incubated at 37.5°C in a humidified atmosphere (~60% relative humidity). By day 3 postincubation, 2 ml albumin was aspirated through a small hole pricked in the shell and a 2-3 cm² window was opened in the shell. The window was sealed with adhesive tape.

Hyperglycemia was induced by a single, intravitellus injection of 5 mg/g whole egg glucose by day 8 postincubation. This age was chosen because it corresponds to the end of organogenesis. When injected earlier, such as on day 6 postincubation, or at a higher dosage, glucose was lethal (>90% deaths by day 8 postinjection; data not shown). Control embryos received either mannitol (osmotic control) or water (volumetric control).

Glucose (Fluka, Buchs, Switzerland) was injected as a 30% (wt/vol) solution in water (5 mg/g egg). Mannitol (Fluka) was injected as a 20% (wt/vol) solution in water (2 mg/g egg). Water-injected embryos received a 1-ml injection of sterile water. In some experiments, water-injected embryos were compared with embryos incubated in the same conditions but left uninjected (untreated embryos). Blood glucose levels were measured on blood samples taken from a CAM vessel using a One Touch Reflectance meter (Lifescan, Milpitas, CA)

Plasma osmolarity. Plasma osmolarity was measured using a freezing point osmometer (Roehling, Berlin, Germany) on 50- or 100-μl plasma samples in duplicate. In water-injected embryos, plasma osmolarity was 275 ± 6 mOsm/l (n = 10) by day 7 postinjection. In embryos treated with 2 mg/g mannitol, plasma osmolarity was 288 ± 4 mOsm/l (n = 5), similar to that observed in glucose-treated embryos with plasma glucose levels of 12 mmol/l (286 ± 3 mOsm/l; n = 3).

Plasma insulin levels. Plasma insulin was assayed as previously described using a guinea pig anti-porcine antibody and chicken insulin as the standard and tracer (11) in triplicate on pools of 900 μl plasma obtained from 1-3 embryos.

Evaluation of CAM vessels. Embryos were injected through a CAM vessel with 10-100 μl of 20 mg/ml 70-kDa fluoresceinated dextran (Sigma, St. Louis, MO) in PBS. The vessels were observed with a MZ FLIII Leica stereomicroscope, and digitalized pictures were taken with a CoolSnap digital camera (Roper Scientific, Trenton, NJ). Based on the centripetal ordering method of microvascular mapping of the first two orders of pre- and postcapillary microvessels, with the capillaries serving as the initial point of reference (12), first- and second-order centripetal blood vessels (Fig. 3A) were manually counted, as previously described (10), on a quarter of the digitalized picture (i.e., in a zone consisting of ~50 first-order vessels and 20 second-order vessels). All observations were made blinded of the treatment group and postinjection day.

Histology. Tissues were fixed for 2 h in 4% paraformaldehyde and processed for paraffin embedding. Next 7-μm thick sections were cut, as previously described (13). Endothelial cells were stained using biotin-labeled Sambucus nigra lectin (1/300; Vector, Burlingame, CA) (14). For the study of apoptosis, CAMs were fixed overnight in 4% paraformaldehyde at 4°C, dehydrated in graded alcohol, cleared in xylene, and embedded in paraffin. For the study of proliferation by the evaluation of bromo-deoxyuridine (BrdU) incorporation (see below), CAMs were fixed in 3% acetic acid in absolute ethanol for 1 h, dehydrated, cleared in xylene, and embedded in paraffin. Analyses of histological samples were performed by two independent observers who were blinded to groups and time after treatment.

Islets of Langerhans were stained using guinea pig anti-porcine insulin (1/300; Dako, Glostrup, Denmark) and rabbit anti-glucagon 1/2,000 (ICN) primary antibodies, biotinylated goat anti-rabbit immunoglobulin 1/200 secondary antibodies (Vector), a Vectastain ABC kit (Vector), and diaminobenzidine.

In situ hybridization. In situ hybridization was performed as previously described on paraffin sections (13). The antisense and sense probes used, cEPAS, cVEGF, and Quek-I, have been previously described (15,16). Probes for chicken-angiopoietin-1, chicken-angiopoietin-2 (17,19), and their receptor-tyrosine kinase, were obtained commercially (Promega, Madison, WI). In situ hybridization was performed using the Dig EasyHyb kit (Roche). Hybridized sections were counterstained with hematoxylin or with 4′-diamidino-2-phenylindole (DAPI). Apoptotic cells were stained using the transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) technique. Sections were mounted using 5 μg/ml proteinase K (Roche, Basel, Switzerland) for 8 min at room temperature. The TUNEL reaction was performed for 1 h at 37°C in a humidified chamber, using 0.5 nmol biotin-labeled deoxyuridine triphosphate (Enzo, New York, NY) and 12.5 units of recombinant terminal-deoxyribo- nucleotide-transferase (Invitrogen, Cergy Pontoise, France).

Proliferation. BrdU (10 μmol/l, Roche) was laid onto the CAM in two siliconized rings, 25 μl each, overnight, as previously described (9). Detection was performed using the BrdU detection kit (Roche) according to the manufacturer’s instructions, with the anti-BrdU antibody at a 1:50 dilution.

FIG. 1. Blood glucose levels in chicken embryos. Whole-blood glucose levels were measured on blood samples taken from a CAM vessel, 1-7 days (D1-D7) after a single, intravitellus injection of glucose (●), mannitol (□), or water (□) at day 8 postincubation. Results are shown as means ± SD. *P = 2 × 10⁻³; **P < 10⁻⁴ for the day-to-day comparison between glucose-injected and control embryos.

FIG. 2. Weight of embryos. Embryos were weighed 1-7 days (D1-D7) after a single, intravitellus injection of glucose (●), mannitol (□), or water (□) at day 8 postincubation. Results are shown as means ± SD. *P < 10⁻³; **P < 10⁻⁴.
Induction of hyperglycemia in chicken embryos. As shown in Fig. 1, blood glucose levels were not different between water- and mannitol-injected embryos throughout the period of observation, up to 7 days postinjection. By day 7 postinjection, blood glucose levels were similar in water-injected and untreated embryos (5.2 ± 1.2 mmol/l [n = 63] and 5.6 ± 0.8 mmol/l [n = 12]). Hyperglycemia was induced in embryos injected with glucose (Fig. 1). Blood glucose levels were significantly higher in the glucose group than in the water and mannitol groups throughout the whole period of observation (glucose effect $P < 10^{-4}$ vs. water- or mannitol-injected embryos, by ANOVA). Although blood glucose values decreased throughout the period of observation in glucose-injected embryos, they were significantly higher in glucose-injected embryos as compared with control embryos at every day postinjection ($P = 2.10^{-4}$ at day 1 and $P < 10^{-4}$ at days 2–7 postinjection) (Fig. 1). However, because blood glucose levels varied to some extent in glucose-injected embryos, with some having blood glucose levels similar to those observed in mannitol- or water-injected embryos, only en-
bryos with a blood glucose level >10 mmol/l were considered as hyperglycemic in the following experiments. Considering only those embryos, the blood glucose levels of glucose-injected embryos were as follows: day 1: 15 ± 6.8 mmol/l (n = 8/12); day 2: 12.4 ± 2.5 mmol/l (n = 42/74); day 3: 12.4 ± 2.0 mmol/l (n = 36/57); day 4: 12.3 ± 2.0 mmol/l (n = 22/59); day 5: 13.1 ± 3.2 mmol/l (n = 29/75); day 6: 12.4 ± 3.3 mmol/l (n = 45/167); and day 7: 12.2 ± 3.1 mmol/l (n = 39/152).

**Plasma insulin levels and morphology of the pancreas.** Plasma insulin levels were measured by day 7 postinjection in pools of plasma obtained from one to three embryos. The level of plasma insulin was 17 ± 1 μU/ml in water-injected embryos (n = 2); plasma glucose level 8.3 mmol/l, 17 ± 2 μU/ml in mannitol-injected embryos (n = 4); plasma glucose level 8.7 mmol/l, and 31 ± 5 μU/ml in glucose-injected embryos (n = 4; plasma glucose level 14.7 mmol/l) (P = 0.04).

After staining pancreatic tissue sections with anti-insulin and anti-glucagon antibodies, absolute numbers and relative proportions of pancreatic α- and β-cells were found to be highly variable from one section to the other in all the studied groups. At least four sections were evaluated per pancreas (range four to eight). No difference was seen among the different groups by day 7 postinjection (n = 6 in each group) with regards to absolute numbers of α- and β-cells or relative proportions.

**Growth curve of the embryos.** As shown in Fig. 2, the growth curves of water- and mannitol-injected embryos were similar. By day 7 postinjection, the weight of water-injected embryos (9.8 ± 1.7 g; n = 27) was similar to that of untreated embryos (9.1 ± 1 g; n = 12). The weight of glucose-injected, hyperglycemic embryos was decreased compared with that of mannitol- or water-injected embryos (group effect P < 10<sup>-4</sup> by two-way ANOVA). The weight of glucose-injected embryos became significantly lower than that of control embryos by day 2 postinjection, and the weight difference between glucose- and control-
injected embryos was maximal at day 7 postinjection. At day 7, the weight of glucose-injected embryos was $5.7 \pm 0.9 \, g (n = 24)$ vs. $9.8 \pm 1.7 \, g (n = 27)$ and $10.8 \pm 1 \, g (n = 18)$ for water- and mannitol-injected embryos, respectively ($P < 10^{-4}$).

**Assessment of CAM blood vessels.** CAM vessels were angiographically assessed after injection of 70-kDa fluoresceinated dextran. At least 5 embryos per group were evaluated (range 5–17) every day through day 7 postinjection after glucose or control injection. Hyperglycemia significantly decreased angiogenesis in the CAM, reaching $\sim 30\%$ from day 5 to day 7 (Fig. 3B). In glucose-injected animals, at day 5, the number of first- and second-order vessels was $67 \pm 30\% (n = 10)$ that of water-injected embryos, as compared with $100 \pm 16 (n = 9)$ and $93 \pm 15\% (n = 6)$ in water- and mannitol-injected embryos, respectively ($P = 0.05$, Kruskal-Wallis test). At day 6, the percent changed in glucose-injected embryos to $67 \pm 25\% (n = 11)$ vs. $100 \pm 9 (n = 14)$ and $88 \pm 14\% (n = 5)$ in water- and mannitol-injected embryos, respectively ($P = 0.005$). At day 7, the percent was $70 \pm 20\% (n = 11)$ in glucose-injected embryos vs. $100 \pm 16 (n = 13)$ and $88 \pm 12\% (n = 5)$ in water- and mannitol-injected embryos, respectively ($P = 0.0009$). Not only was the number of vessels reduced in glucose-injected embryos, but also their appearance was abnormal. Circulating microthrombi were sometimes observed, and thrombosis of arteries was frequently observed. Acapillary zones were frequently observed accompanied by leakage of the 70-kDa fluoresceinated dextran. (Fig. 3A).

**Lectin staining.** The average diameter of subectodermal arteries was measured after they were stained with *Sambucus nigra* agglutinin at days 2–4 postinjection (14) (Fig. 4A). At least 20 subectodermal arteries, corresponding to first- and second-order vessels on angiographies using fluoresceinated dextran, were evaluated per CAM. The diameter of arteries was measured on digitalized pictures (magnification ×250). Arbitrary units, corresponding to...
the number of pixels of the measured diameter, were used. The average diameter decreased, although not statistically significantly so, at day 3 after glucose injection ($P = 0.08$ for glucose versus water or mannitol; $n = 5$ in each group) and became significant by day 4 ($P = 0.01; n = 5$ in each group) (Fig. 4B).

**Apoptosis.** Apoptosis was evaluated on CAMs using TUNEL staining. Sections were evaluated for the presence of apoptotic cells in the endothelium of medium and big arteries, as it was not possible in the ectoderm to ascertain whether a stained cell was a capillary endothelial cell or an ectodermal cell (Fig. 5). Apoptotic endothelial cells were observed more frequently in glucose-injected embryos. By day 2 posttreatment, apoptotic cells were observed in two of six glucose-injected embryos compared with none in five in both water- and mannitol-injected embryos ($P = 0.09$). By day 3, the figures were five of five in glucose-injected, zero of five in water-injected, and two of five in mannitol-injected embryos ($P = 0.04$), and by day 4, were four of six in glucose-injected, one of five in water-injected, and none of five in mannitol-injected embryos ($P = 0.05$).

**Electron microscopy.** By day 3 after glucose injection, the microvascular ectodermal network appeared altered in glucose-injected embryos (Fig. 6). Occluded capillaries were observed. Indications suggesting that endothelial cells were undergoing an apoptotic process were observed in microvessels and in arteries, such as condensed nuclei and vacuolation of the cytoplasm.

**Proliferation.** The proliferation of endothelial cells was...
evaluated 2 and 3 days posttreatment in all conditions after overnight treatment with 25 μl BrdU (Fig. 7). Staining levels were classified as low and irregular or normal. By day 2 posttreatment, the proliferation was low and irregular in four of five glucose-injected embryos, and one of five of both water- and mannitol-injected embryos ($P < 0.01$); by day 3 the proportions were five of five, none of five, and two of five, respectively ($P = 0.01$).

**In situ hybridization.** Evaluation of the expression of mRNA was performed using in situ hybridization at days 1–4 postinjection. The expression level of the following mRNAs was evaluated on five embryos per group: VEGF and its receptor VEGF-R2, HIF-2α–EPAS-1, angiopoietin 1, angiopoietin 2, and the angiopoietin receptor, tie 2. No differences in the expression level were found for any of these probes (Figs. 8 and 9).

**Semiquantitative RT-PCR.** The expression level of the main vascular growth factor VEGF and FGF-2, angiopoietin-1, angiopoietin-2, and HIF-2α–EPAS was evaluated on the CAMs using a semiquantitative RT-PCR assay, on three to five samples per group taken at days 1–4 after glucose injection. As shown in Fig. 10, no consistent significant difference was observed for any of the studied growth factors at any time following treatment.

**DISCUSSION**

Although the effect of hyperglycemia on angiogenesis is often presented as a stimulatory one, on the basis of what is observed in the late phase of diabetic retinopathy, defective angiogenesis has been observed in different models such as wound healing (20,21), embryonic growth (7,22), and transplantation of avascular islets of Langerhans (23). In diabetic patients and in rodent models of diabetes, collateral vessel development, in response to coronary vessel occlusion, has been shown to be impaired by hyperglycemia (4). There are, however, few models to study the direct effect of hyperglycemia on physiological angiogenesis. In the mouse model, embryos from a diabetic mother or embryos cultured in vitro in a diabetic milieu show...
FIG. 10. Semiquantitative RT-PCR. The expression levels of mRNA coding for HIF-2α, EPAS-1, VEGF, angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), and FGF-2 were evaluated by semiquantitative RT-PCR, as described in RESEARCH DESIGN AND METHODS, in embryos injected with 5 mg/g egg glucose (■), 2 mg/g egg mannitol (□), or 1 ml water (○). Results are expressed as the percent (means ± SD) of the mean value in control, water-injected embryos. At any time point, three to five samples were studied in each group. No significant differences were observed between the glucose and control groups for any of the studied factors. Ethidium bromide-stained agarose gels are shown for each tested growth factor. For VEGF, the primers amplify all isoforms of VEGF; quantification was performed on the lowest molecular weight isoform, VEGF-122. A semiquantitative RT-PCR for FGF-2 performed 3 days posttreatment in embryos treated with glucose (G), water (H), or mannitol (M) (n = 3 in each group) is also shown.
impaired angiogenesis (7,22). In the model we used, a short-term, modest hyperglycemia without hypoinsuline-
mia was found to decrease embryonic angiogenesis in vivo. This effect was not mediated by alterations in the pattern or level of expression of vascular growth factors or their receptors. Instead, we found that hyperglycemia decreased the proliferation rate of endothelial cells and induced apoptosis of endothelial cells.

Compared with in vitro models (i.e., cell cultures of endothelial or vascular smooth muscle cells), the CAM model has the advantage of being subject to regulations involving growth factors, vascular flux, and blood pressure. Compared with mammalian models, there is no influence of factors coming from the mother. A possible therapeutic intervention may thus be through administration of antioxidant molecules or regulatory peptides (10).

In the present study, defective angiogenesis in response to high glucose was seen, even though we observed a normal level and pattern of VEGF expression as well as that of other major vascular growth factors and their receptors using morphological evaluation with in situ hybridization and/or semiquantitative RT-PCR.

The expression level of VEGF, the major vascular growth factor, has been found to be decreased by hyper-
glycemia in models where impaired angiogenesis was observed (7,20,21,24,25) and in fibroblasts isolated from diabetic embryos (26). The expression level of VEGF was found to be unaltered by hyperglycemia in other models (27,28) and increased at sites of aberrant angiogenesis in diabetic patients, particularly in the retina of diabetic patients with proliferative diabetic retinopathy (29). In the retina, the increased expression of VEGF was observed within 1 week after the induction of diabetes in animal models (30,31). However, a retinal gene expression profile using cDNA microarray found no significant increase of VEGF at days 3, 7, or 21 after the induction of hyperglycemia in a rat model (32). The direct effect of hyperglycemia on VEGF expression thus remains uncertain. The main regulators of VEGF expression are hypoglycemia and hypoxia, both acting through HIF nuclear factors. The main regulation of HIF-1α is at the protein level through a pathway involving the proline hydroxylases and von Hippel Lindau proteins (33). We have studied the expression level of HIF-2α−EPAS-1 mRNA rather than that of HIF-1α mRNA because in chicken embryo, expression of the HIF-2α−EPAS-1 gene is more extensively regulated than that of the HIF-1α (34). It has recently been shown that glucose can directly induce HIF-1α degradation via a proteasomal pathway (35) and that HIF-2α−EPAS-1 might be more involved in the response to hypoglycemia than to hypoxia (36). Hyperglycemia is thus expected to lead to decreased expression of VEGF through increased degradation of HIF proteins. However, there are many other pathways of HIF-1α regulation, including pathways involving insulin and IGF-I, and VEGF expression is also regulated by many cytokines and growth factors. In this regard, our model of hyperglycemia was characterized by hyperinsulinemia, rather than insulin deficiency, which is closer to the common state of insulin-treated diabetic patients than rodent models that are characterized by profound insulin deficiency. In our model, hyperinsulinemia may have counterbalanced the negative effects of hyperglycemia on HIF degradation (37,38). The influence of hyperglycemia on the expression of the angiopoietins has been studied only in a model of wound healing in mice, where a modest induction of angiopoietin 1 mRNA was observed, with no increase in the protein level, as compared with normal embryos (20). Angiopoietin 2 expression levels were similar in the early phase to those observed in control embryos (20). The effect of hyperglycemia on the expression of FGF-2 has been studied in rats (39). FGF-2 expression was increased in the retina, but not in the other tissues studied, and insulin treatment normalized the expression of FGF-2 in this model. Although the evidence from the literature suggests that the lack of significant alteration in the expression pattern of vascular growth factors we observed is plausible, our observations may have been hampered by insufficient statistical power. We can, however, exclude a major effect. Variations in other, unstudied growth factors might also have been involved.

Our results point to a hyperglycemia-induced defect in endothelial cell proliferation and an increase in apoptosis rather than to defects in the expression level of growth factors. Hyperglycemia-induced apoptosis has been observed in vitro on many endothelial cell types and in vivo in animal models of diabetes and the retinas of diabetic patients (40,41). The mechanisms by which hyperglycemia induces apoptosis are not fully characterized. It has been shown that in pericytes, but not in endothelial cells, apoptosis is the consequence of nuclear factor-κB activation (42). In vitro, the effector mechanism of increased apoptosis in endothelial cells has been shown to be the direct activation of caspases (41), an event that was prevented in vitro by incubation with an activator of AMP-kinase (43). It has been shown in vitro that hyperglycemia decreases endothelial cell proliferation (44) and that endothelial progenitor cells isolated from diabetic patients exhibit impaired proliferation, adhesion, and incorporation into vascular structure (45).

Our results indicate that short-term hyperglycemia can decrease angiogenesis without altering the expression of major vascular growth factors and their regulators. This antiangiogenic effect of hyperglycemia seems to be mediated by direct proapoptotic and antiproliferative effects of hyperglycemia on endothelial cells. Our results may account for some recent clinical observations. For example, in diabetic patients, collateral vessel development is impaired after coronary artery occlusion (4). Strict control of hyperglycemia, using insulin infusions, improves mortality in patients with type 2 diabetes by ~40% (46). Although an effect of insulin-glucose-potassium infusion on arrhythmic episodes has been hypothesized to account for this improved prognosis, improved collateral development through euglycemia and insulin infusion may have contributed to these results.

ACKNOWLEDGMENTS
During this work, E.L. was supported by a grant from l’Association Française des Diabétiques and Association Diabète Risque Vasculaire.

We thank Marie-Thérèse Morin and Françoise Mongiat for expert assistance with histology and electron microscopy, Nicole Rideau (INRA, Nouzilly) for performing the insulin assay, and Judith Favier for help with molecular biology.
REFERENCES