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Insulin-Like Growth Factor Binding Protein Production in Bovine Retinal Endothelial Cells

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Retinopathy is the most frequent microangiopathic complication in diabetes. Many circulating hormones and locally produced mitogenic factors have been involved. Bovine retinal endothelial cells (BRECs) were cultured to investigate if insulin, insulin-like growth factors (IGFs), IGF binding proteins (IGFBPs), and a chronic high-glucose condition could control endothelial cell growth. Specific IGF-I receptors with two binding sites with high (K_d 0.03 nmol/L) and low (K_d 1.3 nmol/L) affinity were found when analyzing families of displacement curves between IGF-I versus IGF-I and IGF-I versus insulin. However, IGFs failed to be mitogenic factors in these cells. This could be explained by an inhibitory effect due to the presence of specific IGFBPs with a molecular weight between 24 and 43 kd. Using Western blot and immunoblot analysis, Northern blot study, and specific radioimmunoassay (RIA), these IGFBPs have been identified as IGFBP-3, -2, -5, and -4. Insulin, which does not bind to IGFBPs, was a potent mitogenic factor in these cells at a high concentration (10 nmol/L), suggesting a cross-reaction to IGF-I receptor. These IGFBPs, except the 24-kd form (IGFBP-4), were modulated by both IGF-I and IGF-II, with a maximum effect at 100 and 10 nmol/L, respectively. This regulation on IGFBPs was IGF-I receptor–independent. In fact, (1) IGFBP mRNA levels were not modified after stimulation with 100 nmol/L IGF-I, (2) 100 nmol/L IGF plus an equimolar concentration of αIR3 did not affect IGFBP production, (3) Des(1-3)IGF-I had no effect on IGFBP modulation, whereas at 10 nmol/L it enhanced BREC thymidine cell incorporation, and (4) 100 nmol/L insulin, which at this concentration can cross-react with the IGF-I receptor, did not modify the IGFBP pattern. Chronic exposure (4 weeks) of BRECs to 25 mmol/L glucose had no effect on cell growth. However, after 3 weeks, we observed a decreased IGFBP detection, and addition of 100 nmol/L IGF-I did not change IGFBP levels and did not modify cell growth. Conversely, BRECs grown in regular medium for 4 weeks showed increased IGFBP production. In conclusion, we showed that conditions mimicking hyperinsulinemia, rather than high levels of IGFs, could regulate BREC growth and that the IGF-I analog, Des(1-3), even with reduced affinity for IGFBPs but in part capable of binding to IGFBP-3, significantly stimulated BRECs growth only at 10 nmol/L. IGF actions are modulated by locally produced endothelial IGFBPs, and in turn, these endothelial IGFBPs are regulated, via an IGF-I receptor–independent mechanism, by the presence of IGFs. The autoregulatory IGF system together with the direct glucose modulation of IGFBPs could contribute in diabetic subjects to the retinal endothelial cell growth and metabolism through local changes in IGF bioavailability.

RETINOPATHY is the commonest microangiopathic complication of diabetes. The most important risk factors for development of this angiopathy are the degree of glucometabolic control and the duration of the disease.[1] Retinal ischemia has been proposed to represent the stimulus that initiates the angiogenesis,[2] as a way of revascularizing the ischemic areas. This leads to formation of new aberrant vasculature on the surface of the retina (proliferative retinopathy). The factors responsible for the development of this retinal neovascularization remain to be established. However, numerous circulating hormones and locally produced mitogenic factors including transforming growth factor beta, platelet-derived growth factor, basic fibroblast growth factor (bFGF), and insulin-like growth factor-I (IGF-I) have been demonstrated to interact in cultured vascular endothelial cells[3]; more recently, vascular endothelial

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cell growth factor has been suggested to be implicated in proliferative retinopathy.[4] In particular, a number of studies have focused on the relationship between IGF-I and diabetic retinopathy. Conflicting results have been reported concerning increased IGF-I concentrations in the serum of diabetic patients,[5] whereas increased IGF-I levels in the vitreous of diabetic patients with proliferative retinopathy have been observed to correlate positively with serum concentrations in diabetic patients but not in normal subjects.[6] More recent studies reported an increase of vitreous levels of IGFs and IGF binding proteins (IGFBP-2 and -3) in neovascularization of the eye in diabetic subjects.[7,8] An altered distribution of IGF-I binding to IGFBPs in plasma from diabetic patients with advanced retinopathy has also been demonstrated.[9] Moreover, IGF-I has been reported to stimulate, approximately to the same extent as bFGF (the most potent endothelial mitogenic factor), retinal angiogenesis after intravitreal application in the rabbit, suggesting an angiogenic potential complementary role of both IGF-I and bFGF in the pathogenesis of proliferative diabetic retinopathy.[10] Additional in vitro data support a role for IGF-I in retinal endothelial cell growth,[11] showing the importance of retinal endothelial cells as a source of IGF-I, with emphasis on the role of the retinal vasculature in the control of autocrine production of growth factors. Several groups have demonstrated the presence of insulin and IGF-I receptors on endothelial cells.[12] Bovine retinal endothelial cells (BRECs) have specific IGF-I receptors and respond to IGF-I and insulin with an increase of thymidine incorporation into DNA,[13] suggesting also for insulin a potential role in retinal endothelial cell growth control either by binding to its own receptor or cross-reacting with the IGF-I receptor.[14] In this way, the hyperinsulinemic status in both type I and type II diabetic patients could be a critical condition for the development of diabetic retinopathy. Other studies have shown a chemotactic effect of IGF-I on endothelial cells[15] and the pivotal role of IGF-I during regulation of the proliferative response of wounded-cell endothelial monolayers.[16] Furthermore, physiological studies suggest that the actions of IGF-I and IGF-II are in part modulated by a family of structurally related proteins, the IGFBPs.[17,18] Six distinct IGFBPs have been identified and cloned.[19] They transport IGFs[20] and modulate cellular IGF availability depending on the nature of the IGFBPs and the experimental conditions,[21] and alter IGF receptor binding.[22] Vascular endothelial cells have been demonstrated to produce IGFBPs.[23-25] The objectives of this study were to identify the eventual presence of different forms of IGFBPs secreted into the conditioned medium (CM) by BRECs in long-term culture[26] and to determine the effects that these IGFBPs could potentially have on modulating IGF actions. Since endocrine factors do not explain all of the alterations in the diabetic retina, we studied the effect of chronic high-glucose cell culture mimicking the diabetic biological milieu on IGFBP production and endothelial cell growth.

MATERIALS AND METHODS

Cell Cultures

BRECs were isolated and cultured as previously described.[26] The growth medium consisted of Coon's modified Ham F12 medium containing 10% Nu-serum IV (Collaborative Research, Bedford, MA), 1% Ultroser G (IBF Biotechnics, Savage, MD), 100 U/mL penicillin, and 100 μg/mL streptomycin. The glucose concentration in this culture medium was 11 mmol/L. Cells were cultured in plastic 25-cm^2 culture flasks (Corning, Corning, NY), except for [^{3}H]thymidine incorporation, where we used six multiwell plates (Corning). All experiments were performed using cell culture passages 3 to 10. During the prolonged cell culture experimental protocol (4 weeks), BRECs were grown under the following experimental conditions: (1) normal culture medium, (2) high-glucose culture medium (25 mmol/L), and (3) 14 mmol/L mannitol + 11 mmol/L glucose culture medium to match the osmolarity of high-glucose medium. BRECs were also grown for 4 to 6 days, through one passage, on bovine serum albumin (BSA) previously incubated for 10 weeks in phosphate-buffered saline (PBS) in the absence or presence of 0.5 mol/L glucose to obtain nonglycated or glycated BSA with formation of advanced glycosylation end products (AGEs).

[^{3}H]Thymidine Incorporation

[^{3}H]thymidine incorporation was performed using a method previously described.[27] Briefly, BRECs were plated in six multiwell plates in regular medium. When subconfluent, the cells were maintained for 48 hours in Coon's modified Ham F-12 supplemented with 0.1% Nu-serum and then incubated 24 hours with the same medium with or without different concentrations of the growth factors and antibody to be tested: bFGF (Sigma Chemical, St Louis, MO), insulin (Sigma), IGF-I (Calbiochem, La Jolla, CA), IGF-II (Calbiochem), Des(1-3)IGF-I (Peninsula, Belmont, CA), or αIR3 (kind gift from Dr M. Maggi). Finally, the cells were pulsed for 4 hours with 2 μCi/mL methyl-[^{3}H]thymidine (Amersham International, Little Chalfont, UK).

Binding Experiments

Binding experiments were performed on BRECs in suspension. Cells were first washed with EGTA 4 mmol/L in Ca^{2+}- and Mg^{2+}-free HBSS and then treated with low concentrations of trypsin (0.01% in Ca^{2+}- and Mg^{2+}-free PBS) for 10 minutes. The action of the enzyme was then stopped by addition of 0.2% soybean trypsin inhibitor (Sigma Chemical) in Ca^{2+}- and Mg^{2+}-free PBS. Receptor binding studies were performed as previously described.[28] Briefly, the cell suspension obtained was pooled, centrifuged, and resuspended in the binding buffer (HEPES 100 mmol/L, NaCl 120 mmol/L, KCl 5 mmol/L, $MgSO_4$ 1.3 mmol/L, EDTA 1 mmol/L, glucose 10 mmol/L, $NaC_2H_3O_2$ 15 mmol/L, and BSA 1%, pH 7.6) at a concentration of 5 to 6 $\times$ 10^6 cells/mL. Binding experiments were performed in the presence of increasing concentrations of labeled ligand (1 to 4 $\times$ 10^5 cpm/mL human [^{125}I]IGF-I; Amersham, Milan, Italy) without unlabeled ligands or with a fixed concentration (4 $\times$ 10^5 cpm/mL) of labeled ligand in the presence of increasing concentrations (details are reported in the results) of unlabeled peptides or αIR3 antibody for 4 hours at 15°C (final volume, 0.4 mL). The reaction was stopped adding 400 μL cold binding buffer. The cells were collected and separated from the binding buffer by centrifugation at 8,000 $\times$ g for 2 minutes in a Beckman microfuge (Beckman, Palo Alto, CA); the tips were excised and counted in a gamma counter at 80% efficency. All experiments were performed in duplicate at least three times on separate batches of cells. Homologous and heterologous displacement curves were analyzed using the LIGAND program.[29]

Collection of CM

Cells were seeded in 25-cm^2 tissue culture flasks at a density of 35 $\times$ $10^3/cm^2$ in regular medium. One day later, the cells were washed with PBS and grown for 2 days in 1.5 mL serum-free culture medium. CM was then harvested and stored at −80°C in polypropylene tubes (Nalgene; Nalge/Sybron, Rochester, NY) already treated as previously described[30] to reduce nonspecific binding of proteins to the tubes. The tubes were incubated at 37°C for 3 hours in serum-free medium containing 1 mg/mL BSA (Sigma) and 20 mmol/L HEPES, pH 7.2, and completely dried at room temperature. After collection of the CM, the

cell number was determined with a hemocytometer, and the volume of CM analyzed was adjusted accordingly.

Western Ligand Blotting

The procedure for IGFBP identification was performed according to the method of Hossenlop et al.[31] Briefly, 100 μL CM along with prestained molecular-weight marker proteins (Bio-Rad, Richmond, CA) underwent sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (12% gel, 18 × 12 cm) in nonreducing conditions. The electrophoresed proteins were then blotted onto a nitrocellulose membrane at 10 V for 16 hours using a transblotting cell apparatus (Bio-Rad). The nitrocellulose membrane (Sartorius, Gottingen, Germany) was prewashed with buffers containing detergents and incubated for 2 hours at room temperature with 1×10^6 cpm [^{125}I]IGF-II (Amersham). The membrane was then washed and dried; radioactive bands were visualized by autoradiography after 3 days' exposure of the film at −80°C.

Immunoblotting and Radioimmunoassay

CM derived from BRECs was electrophoresed and blotted onto a nitrocellulose membrane as described for Western ligand blotting. Polyclonal antibodies against IGFBP-2, -4, and -5 were purchased from UBI (Lake Placid, NY), and goat antibody immunoglobulin (Ig)G conjugate with alkaline phosphatase was obtained from Sigma. The nitrocellulose membrane was incubated at 4°C with a 1:400 dilution of each antibody in PBS containing 3% nonfat dry milk. The nitrocellulose membrane was rinsed three times with PBS-3% milk and incubated at 25°C for 3 hours with antirabbit IgG-alkaline phosphatase conjugate in PBS-3% milk. Then, the membrane was washed once with 0.1 mol/L Tris hydrochloride buffer, pH 9.5, containing 0.1 mol/L NaCl and 5 mmol/L $MgCl_2$ and then incubated with bromochloroindole phosphate-Nitro Blue Tetrazolium substrate solution. The reaction was then stopped with PBS containing 20 mmol/L EDTA.

IGFBP-3, -4, and -5 radioimmunoassays (RIAs) were performed in CM of BRECs as previously reported.[32-33]

Northern Blot Analysis

Total RNA was prepared from BRECs using the guanidium thiocyanate-phenol-chloroform extraction method.[34] Total RNA (20/lane) was loaded onto 1% agarose formaldehyde gel and run at 80 V for 3.5 hours in MOPS (3-morpholinopropanesulfonic acid). The gel was then stained with ethidium bromide to ensure that ribosomal RNAs were intact and that equal amounts of RNA were loaded. The gel was incubated with 50 mmol/L NaOH/10 mmol/L NaCl for 10 minutes, neutralized with 1 mol/L ammonium acetate, and blotted onto GeneScreen (Du Pont-NEN, Boston, MA). The gels were then hybridized with complementary DNA probes for IGFBP-1 to -6 obtained from plasmid human IGFBPs as a kind gift from Dr Shunichi Shimazaki (JCR Biopharmaceuticals, San Diego, CA). All probes were labeled using the Prime-H random primer kit (Stratagene, La Jolla, CA). Each blot was hybridized with 2×10^6 cpm/mL probe in 50% formamide, 3.6X SSPE (1X SSPE is 0.5 mol/L NaCl, 5 mmol/L EDTA, and 50 mmol/L sodium phosphate, pH 6.8), 1% SDS, 10% dextran sulfate, and 5X Denhardt solution at 42°C overnight. The blot was washed in 2X SSPE-0.2% SDS at room temperature followed by 0.1X SSPE-0.2% SDS at 60°C and then autoradiographed.

Statistical Analysis

Results are expressed as the mean ± SD or SE. Statistical significance was calculated using a paired *t* test. Experiments were repeated at least three times, each time being consistent.

RESULTS

Binding Studies

The binding reaction of [^{125}I]IGF-I to BRECs was dependent on the time and temperature of incubation. The experiments were performed for different incubation times (after 4, 8, and 12 hours) and at three different temperatures (4°, 15°, and 37°C). Maximal specific binding was obtained after 4 hours at 15°C (data not shown). Accordingly, incubation for all experiments was performed at 15°C for 4 hours. To characterize the binding of [^{125}I]IGF-I to BRECs, homologous and heterologous displacement curves between IGF-I and insulin were constructed. Simultaneous mathematical analysis of competition curves was highly suggestive of the presence of two classes of IGF-I binding sites. Indeed, in six separate experiments, the fit for the two-site model appeared consistently better than that for the one-site model ($P < .009$). Figure 1 graphically represents a typical competition curve and the predicted relationship for the two-site model. The IGF-I binding site bound with high affinity for IGF-I, whereas insulin apparently did not bind to this site until 10^{-6} mol/L. Figure 1 (inset) depicts a typical experiment showing the nonlinear relationship for the IGF-I ligand, suggesting the presence of two different classes of binding sites for IGF-I: one with high affinity ($K_d = 0.036 \pm 0.015$ nmol/L, mean ± SE; six experiments) and low capacity (2E-12 ± 0.97 fmol/million cells) and the other with lower affinity (1.30 ± 0.57 nmol/L) and higher capacity (34.4E-12 ± 3.17 fmol/million cells).

Effect of Insulin and Growth Factors on DNA Synthesis in BRECs

The effect of insulin, IGFs, and bFGF on [^{3}H]thymidine incorporation into DNA was measured in our cell line (Fig 2). In the nonconfluent growing state of BRECs, insulin appeared to be as potent as bFGF to stimulate cell growth. In fact, addition of 100 nmol/L insulin and bFGF produced approximately a fivefold significant ($P < .001$) increase of [^{3}H]thymidine incorporation, compared with the approximately sevenfold increase obtained by bFGF. BRECs were not responsive to the same concentrations of IGF-I and IGF-II. Increasing concentrations

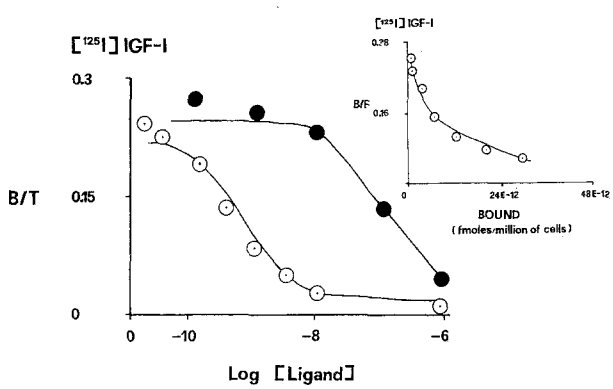

Fig 1. Typical self- and cross-displacement curves between IGF-I (◉) and insulin (●) obtained in BRECs in suspension cells. The results shown are derived from computer analysis of 6 different experiments. The curves were generated by the program LIGAND (SCAGRF). Ordinate: B/T, bound to total ratio for [^{125}I]IGF-I. Abscissa: [Ligand], the concentration of ligand used. In homologous competition curves, the concentration of the tracer was progressively reduced to optimally characterize the high-affinity region of the binding curves. Inset: Scatchard analysis of equilibrium binding curve for [^{125}I]IGF-I in BRECs. Ordinate: B/F, bound to free ratio. Abscissa: concentration of bound ligand. The curvilinear relationship indicates the presence of 2 binding sites for IGF-I on BRECs. Parameters were estimated using the computer program LIGAND.

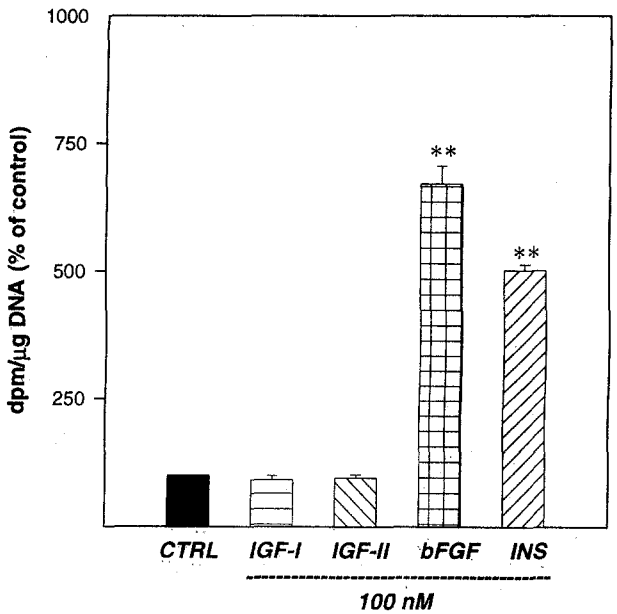

Fig 2. Effect of IGF-I, IGF-II, bFGF, and insulin on [^{3}H]thymidine incorporation in BRECs. Data are expressed in dpm/µg DNA (% of control) as the mean ± SD. **P < .001 v control. Values were obtained from 4 separate experiments.

of insulin (1 to 1,000 nmol/L) induced a dose-dependent [^{3}H]thymidine incorporation into DNA in BRECs (Fig 3A). The response was significant after addition of 10 nmol/L insulin, and it reached the maximum effect at 1,000 nmol/L. To establish if this effect was mediated through the insulin or the IGF-I receptor, we used the monoclonal antibody αIR3, which is specific against the IGF-I receptor. We observed that addition of 100 nmol/L insulin together with an equimolar concentration of αIR3 produced about a twofold reduction of DNA synthesis in our cellular system (Fig 3A) compared with the effect reached with the same insulin concentration in absence of the antibody. Figure 3B shows the action of αIR3 on [^{125}I]IGF-I displacement on BRECs. Increasing concentrations of the antibody specifically inhibited IGF-I binding with an ID_{50} for the displacement of [^{125}I]IGF-I of about 0.6 nmol/L. Insulin was able to displace [^{125}I]IGF-I only starting from 10 nmol/L.

Identification of IGFBPs Released From BREC Culture in CM

To assess whether BRECs in culture produced IGFBPs, we analyzed the CM by [^{125}I]IGF-II Western ligand blot. Figure 4 shows three separate bands of migration of IGFBPs after SDS-PAGE of medium samples obtained from BRECs in long-term culture. Medium from unstimulated BRECs contained IGFBPs with an apparent molecular weight of 43 to 24 kd. In particular, the ligand blot showed a strong presence of the lower band of about 24 kd and another two bands (the highest of about 34 kd and an additional central weak band of about 30 kd). The pattern of IGFBP production was not modified after collection of CM at different cell culture passages (from 3 to 26, data not presented). By Western immunoblot analysis, the 34- and 24-kd bands were identified as IGFBP-2 and IGFBP-4, respectively (Fig 5). IGFBP-1 and IGFBP-5 antibodies did not show positive signals. We could not characterize the 30-kd band, because the other commercially available antibodies did not recognize cow IGFBPs. Instead, using a specific RIA, we could detect a small amount of IGFBP-3 (5.17 ± 1.95 ng/mL) not revealed by the ligand blot, and using a newly developed RIA, 3.05 ± 1.77 ng/mL IGFBP-5 was found in four samples of BREC CM (Table 1, control). Thus, the 30-kd protein present in the ligand blot study could be IGFBP-5.

Regulation of BREC IGFBPs

IGFBP modulation by growth factors involved in BREC growth was investigated. The Western ligand blot (Fig 6) and densitometric quantification (Table 2) are presented. IGF-I (100 nmol/L) dramatically increased the levels of IGFBP-2 and IGFBP-5 in CM from BRECs after 24 hours of stimulation. This effect was not observed for IGFBP-4. With addition of 100 nmol/L bFGF and insulin, there was no apparent increase in IGFBP levels with respect to controls. In particular, the IGFBP

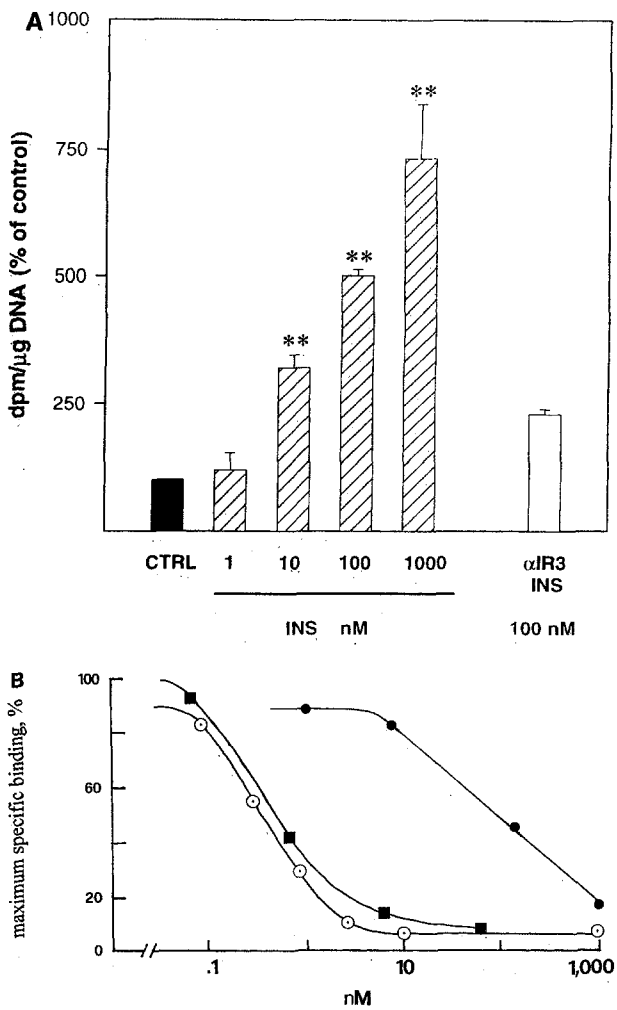

Fig 3. (A) Effect of different concentrations (1 to 1,000 nmol/L) of insulin and αIR3 (100 nmol/L) + insulin (100 nmol/L) on [^{3}H]thymidine incorporation in BRECs. Data are expressed in dpm/µg DNA (% of control) as the mean ± SD. **P < .001 v control. Values were obtained from 3 different experiments. (B) [^{125}I]IGF-I binding to BREC cultures. Binding data are expressed as a percent of maximum specific binding. Experimental points are the mean of triplicate determinations. (◉) IGF-I, (●) insulin, (■) αIR3.

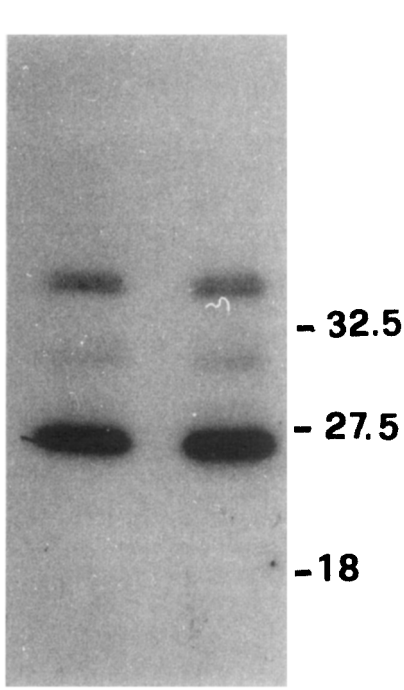

Fig 4. [^{125}I]IGF-II Western ligand blot of BREC CM harvested after a 48-hour serum-free culture. The autoradiogram is the result of a 3-day film exposure. Indicative Molecular weight in kd is shown at right. A single representative experiment is shown.

dose-response autoradiogram after addition of IGF-I is clearly shown in Fig 7A, and the densitometric analysis is reported in Fig 7B. The effect on IGFBP production was significant at 10 nmol/L IGF-I in all bands except for IGFBP-4, and it reached the maximum stimulation with 100 nmol/L IGF-I ($P < .001$). Besides, the presence of 10 nmol/L IGF-I determined the appearance on the autoradiogram of two new bands of migration: one of about 32 kd and a weaker one of about 43 kd that,

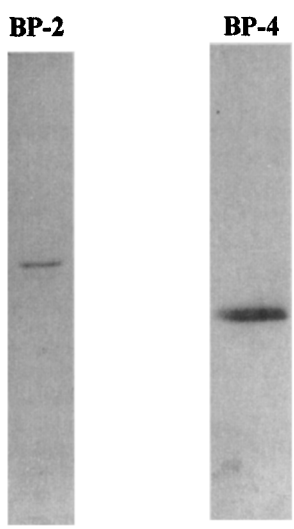

Fig 5. Immunoblot analysis of BREC CM with IGFBP-2 and IGFBP-4 antisera. Antisera were anti-bovine IGFBP-2 and anti-human IGFBP-4 both at 1:400 dilution. The 34- and 24-kd bands were identified as IGFBP-2 and IGFBP-4, respectively.

Table 1. CM Levels (ng/mL) of IGFBP-3, -4, and -5 in the Basal Condition and After Addition of 100 nmol/L IGF-I

	Control	IGF-I
IGFBP-3	5.17 ± 1.95	12.11 ± 1.60†
IGFBP-5	3.05 ± 1.77	11.20 ± 3.45*
IGFBP-4	19.05 ± 5.13	18.58 ± 0.53

NOTE. Data are the mean ± SD for 4 separate experiments.
*$P < .01$.
†$P < .005$.

following RIA analysis, could be IGFBP-3. Moreover, RIA analysis confirmed that addition of 100 nmol/L IGF-I dramatically increases the level of IGFBP-3, and to a lesser extent but still significantly, IGFBP-5. The presence of IGFBP-4 is instead confirmed to be unaffected by IGF-I (Table 1), as observed in the ligand blot study. To determine whether the increased production of medium IGFBP levels reflected changes in steady-state protein production or an alternative mechanism, we used the αIR3 antibody (Fig 8A, lane c). Densitometric quantification of this autoradiogram (Table 3) showed that addition of 100 nmol/L blocking antibody against the IGF-I receptor had no effect on the IGF-I–induced IGFBP increase in BREC CM, suggesting an IGF-I receptor–independent mechanism for IGFBP production. αIR3 alone was unable to induce any IGFBP secretion (Fig 8A, lane b). We also studied the action of IGF-II on IGFBP production. Figure 8A (lanes d to g) shows the IGFBP ligand blot profile after IGF-II treatment in a dose-response study, and the respective densitometric analysis is reported in Fig 8B. IGF-II, compared with IGF-I, significantly stimulated IGFBP (except the IGFBP-4) release at a concentration of 0.1 nmol/L, reaching the maximum effect with 10 nmol/L, suggesting that IGF-II showed higher affinity in inducing IGFBP production in CM from BRECs. In addition, albeit at a lower concentration (0.1 nmol/L) than IGF-I, IGF-II

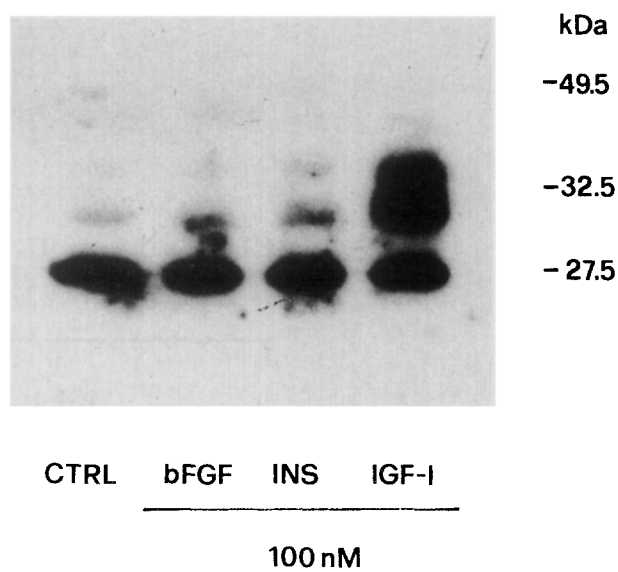

Fig 6. [^{125}I]IGF-II Western ligand blot of BREC CM harvested after a 48-hour serum-free culture in the presence (24-hour stimulation) of 100 nmol/L bFGF, insulin, and IGF-I. The autoradiogram is the result of a 3-day film exposure. Indicative molecular weight in kd is shown at right. A single representative experiment is shown.

Table 2. Densitometric Quantification of Western Ligand Blot Analysis of Fig 6

	43 kd	34 kd	32 kd	30 kd	24 kd
Control	ND	164 ± 4	ND	181 ± 7	244 ± 7
bFGF	ND	163 ± 5	ND	200 ± 10	244 ± 8
Insulin	ND	180 ± 7	ND	200 ± 12	242 ± 9
IGF-I	ND	244 ± 4†	244 ± 7†	246 ± 6*	245 ± 7

NOTE. Data are the mean ± SD obtained from triplicate determinations.

*$P < .05$.

†$P < .001$.

Table 3. Densitometric Evaluation of Western Ligand Blot Analysis of Fig 8 Only for Lanes a, b, c, and h

	43 kd	34 kd	32 kd	30 kd	24 kd
Control	ND	129 ± 6	ND	129 ± 5	189 ± 14
αIR3	ND	129 ± 7	ND	134 ± 11	187 ± 15
IGF-I + αIR3	ND	202 ± 11*	208 ± 10*	196 ± 12*	211 ± 13
IGF-II + αIR3	ND	190 ± 8*	198 ± 4*	192 ± 8*	205 ± 13

NOTE. Data are the mean ± SD obtained from triplicate determinations.

*$P < .001$.

significantly stimulated the new band of migration of 32 kd, whereas IGFBP-3 was apparently unaffected. The use of the monoclonal antibody against the IGF-I receptor did not modify (densitometric evaluation in Table 3) the IGF-II pattern of IGFBP production (Fig 8A, lane h), suggesting also for IGF-II an IGF-I receptor–independent mechanism of IGFBP modulation. To better characterize this mechanism, we used the IGF-I analog, Des(1-3)IGF-I, which binds and activates the IGF-I receptor with high affinity[32] but has a reduced affinity for IGFBPs.[33] Figure 9 shows the effect of different concentrations of Des(1-3)IGF-I on [^{3}H]thymidine incorporation into DNA. BRECs responded significantly to the analog at a concentration of 10 nmol/L, and maximum stimulation was apparently achieved at 100 nmol/L, with no significant difference if compared against insulin used at the same concentration. The IGFBP pattern observed with Western ligand blotting after addition of increasing concentrations of Des(1-3)IGF-I is shown in Fig 9. The presence of high concentrations of Des(1-3)IGF-I apparently did not change the IGFBP bands detected.

Northern Blot Studies

To further characterize IGFBP production and regulation in BREC CM, Northern blot analysis was performed using ^{32}P-labeled cDNA probes for human IGFBPs. Figure 10 shows Northern blot studies of total RNA prepared from BRECs cultured in standard growth medium and after treatment with 100 nmol/L IGF-I. In basal conditions, BRECs showed the presence of mRNA for IGFBP-2, -4, and -5. A single hybridizing band of about 2.5, 2.2, and 6 kb in length was detected in each gel, respectively. No positive signals were found for

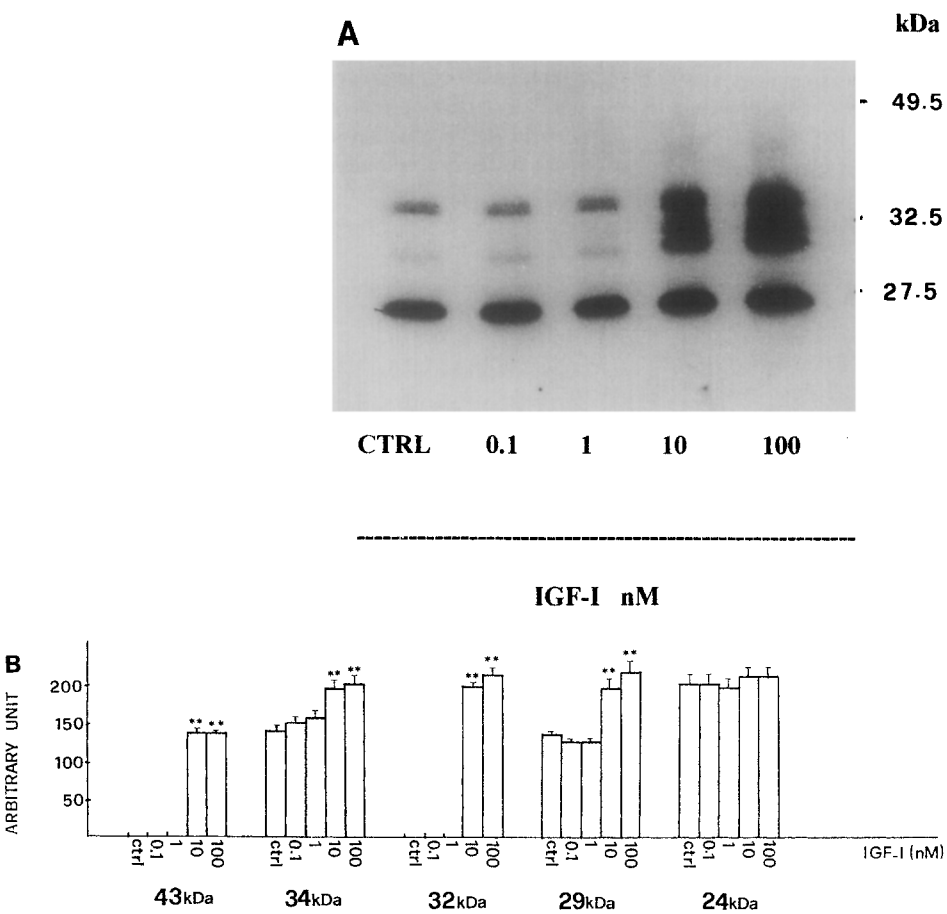

Fig 7. (A) [^{125}I]IGF-II Western ligand blot of BREC CM harvested after a 48-hour serum-free culture in the presence (24-hour stimulation) of increasing concentrations of IGF-I (0.1 to 100 nmol/L). The autoradiogram is the result of a 3-day film exposure. Indicative molecular weight in kd is shown at right. A single representative experiment is shown. (B) Densitometric quantification of IGF-I dose-response effect on IGFBP release from BREC CM. *$P < .05$, **$P < .001$: *v* control.

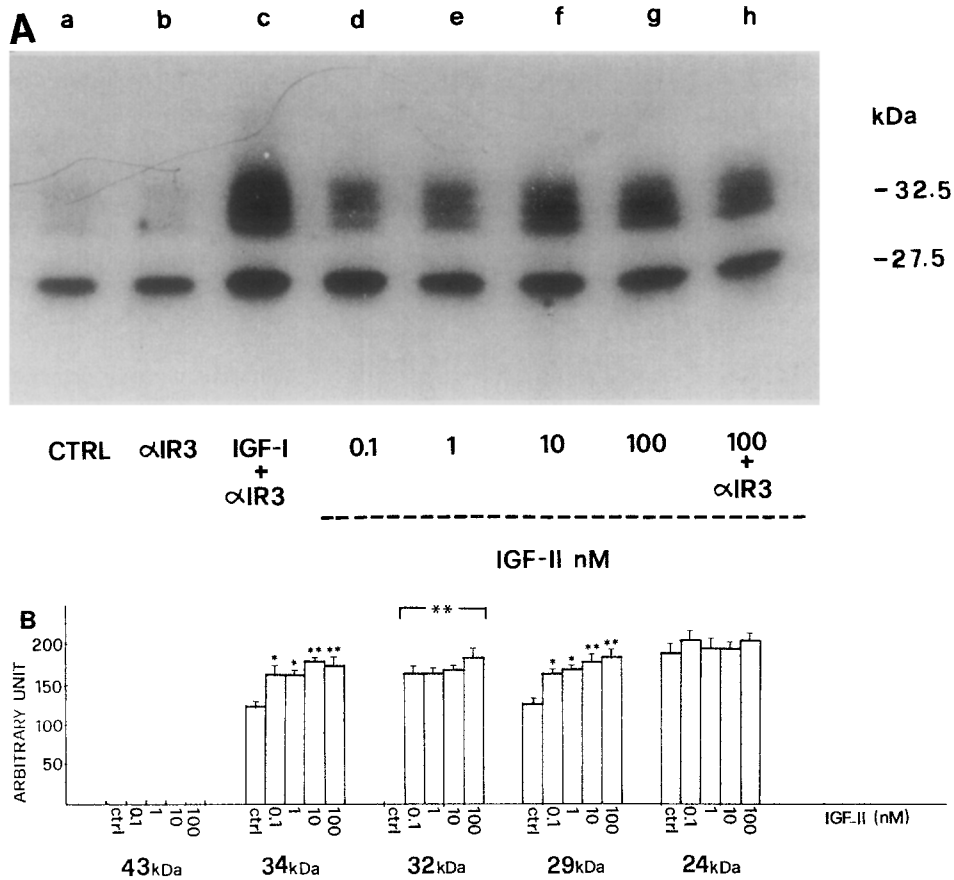

Fig 8. (A) [^{125}I]IGF-II Western ligand blot of BREC CM harvested after a 48-hour serum-free culture in the presence (24-hour stimulation) of 100 nmol/L αIR3 (lane b), 100 nmol/L IGF-I + 100 nmol/L αIR3 (lane c), increasing concentrations of IGF-II (0.1 to 100 nmol/L, lanes d to g), and 100 nmol/L IGF-II + 100 nmol/L αIR3 (lane h). The autoradiogram is the result of a 3-day film exposure. Indicative molecular weight in kd is shown at right. A representative experiment is shown. (B) Densitometric quantification of IGF-II dose-response effect on IGFBP release. *$P < .05$, **$P < .001$: *v* control.

IGFBP-1 and -6 mRNA, whereas the absence of mRNA for IGFBP-3 was due to the lack of cross-reactivity of human IGFBP-3 probe with bovine mRNA. When the cells were treated with high-dose IGF-I, mRNA for IGFBP-1 and -6 was still undetectable and the other mRNAs did not show a significant change in their levels compared with the standard growth medium. This suggests that the modulation of IGFBP concentrations in BREC CM after addition of IGF-I could be unrelated to events modulating the transcriptional levels of these proteins.

Long-Term Effects of High Glucose Levels in the Medium on IGFBP Production and Cell Growth

To assess whether IGFBPs from BRECs undergo changes during continuous cell culture at regular (11 mmol/L) and high-glucose (25 mmol/L) status, we analyzed, by Western ligand blot, CM obtained from BRECs cultured for 4 weeks for up to 6 passages. The pattern of IGFBP levels was examined every week. The results are reported in Fig 10, and were statistically analyzed after densitometric quantification (Table 4). BREC CM harvested after 4 weeks' culture in standard conditions (Fig 10, lanes a and b) showed that prolonged cell culture produced an increase of IGFBPs, except for IGFBP-4. Prolonged cell culture also stimulated the appearance of IGFBP-3. This effect was already observed after 2 weeks of culture (data not shown). When the cells were grown for 4 weeks in the presence of 25 mmol/L glucose (Fig 11, lanes c to f), we detected an increase of IGFBP-3, -5, and -2 and 32-kd band production in CM of BRECs after 2 weeks (lane d), but the pattern of this IGFBP production decreased after 3 weeks to the levels of the first week of culture, and it did not change after 4 weeks (lanes e and f). Addition of 100 nmol/L IGF-I to the cells grown in high-glucose conditions for 1 (lane g) or 2 (not shown) weeks showed the already observed response in terms of IGFBP production, with an increase of IGFBP-3, -2, -5, and the 30-kd band; the same concentration of IGF-I added after 4 weeks of high-glucose culture was unable to produce any modification of IGFBP production (lane h). The effect of 25 mmol/L glucose on IGFBP modulation was not mimicked by 11 mmol/L glucose plus 14 mmol/L mannitol, suggesting that this effect was not due to the increased osmolarity of the culture medium; moreover, the lack of IGFBP modulation was also observed when IGFBP production was investigated in the presence of only BSA or glycated BSA with formation of AGEs (data not shown).

The effect of a prolonged high-glucose condition on thymidine incorporation in the BREC line was also studied. For this purpose, two populations of BRECs were chronically grown in medium containing 25 mmol/L (high-glucose growth) and 11 mmol/L (regular growth) glucose. When cellular growth was compared at 11 mmol/L and 25 mmol/L glucose after 2 (time of maximum IGFBP production) and 4 weeks of culture, no significant difference between the two growth populations was observed, and addition of 100 nmmol/L IGF-I during the fourth week of high-glucose culture (time of minimum IGFBP production) did not modify BREC growth (data not presented). This could be due to the unmodified presence of the inhibitory effect

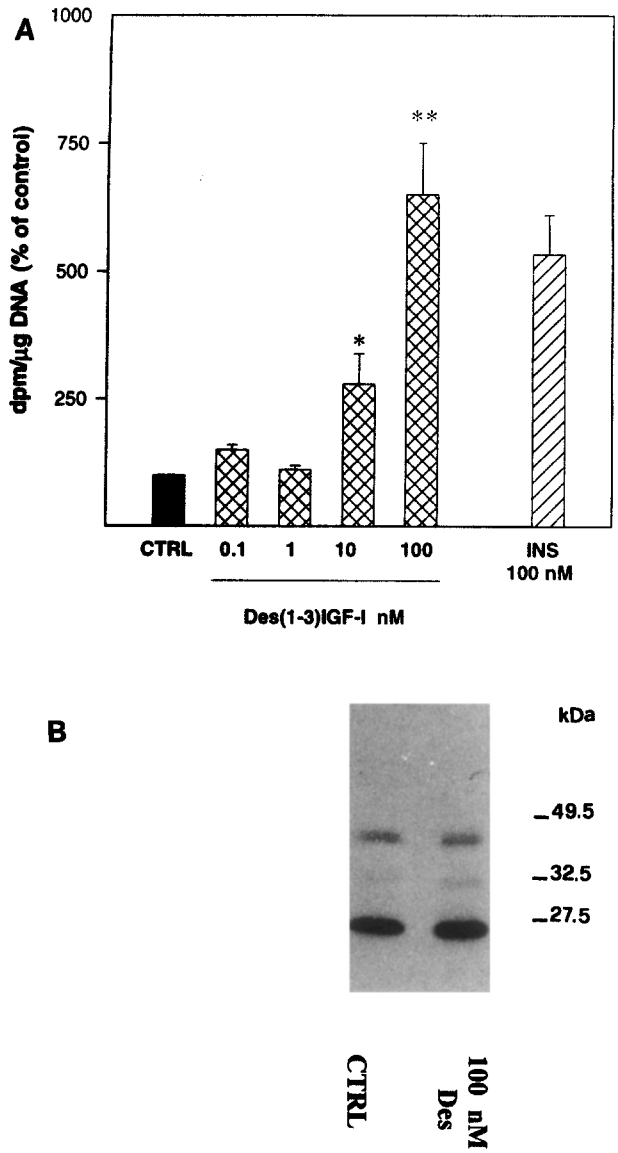

Fig 9. Effect of Des(1-3)IGF-I at increasing concentrations (0.1 to 100 nmol/L) on [^{3}H]thymidine incorporation (A) and IGFBP release (B) by BRECs. Data are expressed in dpm/µg DNA (% of control) as the mean ± SD. $^{**}P < .001$, $^{*}P < .05$: *v* control. The data were obtained from 3 different experiments.

of IGFBP-4. Moreover, since the lowest level of IGFBPs in the high-glucose cell culture was at least the same or higher than IGFBP levels in basal conditions (Table 4), the blocking effect of IGFBPs in high glucose on IGF actions was probably conserved.

DISCUSSION

Several lines of evidence suggest that a physiological role exists for IGF-I in retinal endothelial cells during diabetic retinopathy that includes a mitogenic effect. Moreover, the demonstration of direct IGF-I secretion from endothelial cells[11] and the presence of IGFBPs in the CM of endothelial cell cultures[25] suggest an important role for endothelial cells in controlling IGF-I autocrine and paracrine actions. In the present study, we demonstrated the presence of IGFBPs in CM from BRECs in long-term culture and their modulation by IGFs and by chronic high-glucose experimental conditions. To assess whether BRECs released IGFBPs during cell culture, we analyzed the CM by Western ligand blotting, and with this method in nonstimulated conditions, we detected three different bands of IGFBPs with a molecular weight of approximately 24, 30, and 34 kd. We identified the 24-kd band as IGFBP-4 and the 34-kd band as IGFBP-2. Moreover, using a more sensitive RIA,[33] we also detected IGFBP-5 (probably the 30-kd band) and IGFBP-3, which were not detected by Western ligand blot analysis.

Previous reports[12] demonstrated the presence of the IGF-I receptor in BRECs. The investigators showed one single class of IGF-I receptors on BRECs, constructing the IGF-I binding curves on cells directly on plastic plates. Mathematical analysis of our binding studies of families of competition curves between [^{125}I]IGF-I and IGF-I or insulin on BRECs in suspen-

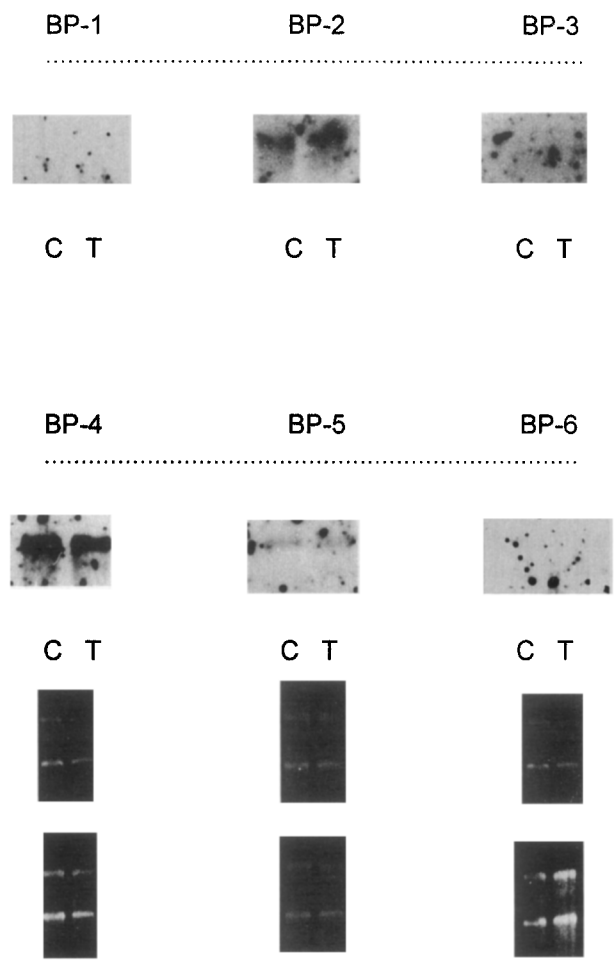

Fig 10. Effect of 100 nmol/L IGF-I (T) on steady-state (C) mRNA levels for IGFBP-1 to IGFBP-6. Total RNA (20 µg) from BRECs was electrophoresed, transferred to a nylon membrane, and hybridized with ^{32}P-labeled cDNA human probes for IGFBPs. The size of hybridizing bands was estimated from the relative migration rates of 18S and 28S ribosomal RNAs and is indicated at right in each Northern blot. At the bottom of the Northern blot, ethidium bromide staining is reported. The autoradiograms are the result of a 1-day exposure at −80°C.

Table 4. Densitometric Quantification of Western Ligand Blot Shown in Fig 11

	43 kd	34 kd	32 kd	30 kd	24 kd
Control					
1 wk	ND	85 ± 5	ND	86 ± 7	218 ± 13
4 wk (*v* 1 wk)	117 ± 5†	165 ± 12†	190 ± 10†	195 ± 11†	221 ± 10
Glucose 1 wk *v* control 1 wk	ND	175 ± 10†	ND	146 ± 5†	239 ± 4
Glucose 2 wk *v* glucose 1 wk	124 ± 6†	194 ± 11†	190 ± 10†	205 ± 11†	245 ± 1
Glucose 3 wk *v* glucose 2 wk	ND†	152 ± 9*	ND†	143 ± 8†	239 ± 5
Glucose 4 wk	ND	141 ± 9	ND	132 ± 8	239 ± 4
IGF-I					
1 wk	136 ± 8	172 ± 12	176 ± 10	187 ± 10	245 ± 5
4 wk (*v* 1 wk)	ND†	132 ± 8*	ND†	143 ± 4*	236 ± 9

NOTE. Data are the mean ± SD obtained from triplicate determinations.
*$P < .05$.
†$P < .001$.

sion cells showed that the best fit of our experimental data is a two–binding site model for IGF-I, in harmony with what is described for most cell types.[17,28] As suggested for other cell lines releasing IGFBPs,[37,38] the presence of IGF carrier proteins released into the media during the binding incubation or attached to the cell membranes could modulate the binding of the IGF-I tracer to cell receptors. For this purpose, binding studies in monolayers have been performed (data not shown). In these studies, we found a lower amount of [^{125}I]IGF-I bound in "tracer" concentrations as compared with experiments performed in suspension cells. However, addition of increasing concentrations of unlabeled IGF-I led to a paradoxical increase in bound counts due to the movement of [^{125}I]IGF-I bound to the IGFBPs to the IGF-I receptors.[39] This effect was maximal at nanomolar concentrations. The total displacement of [^{125}I]IGF-I from the IGF-I receptors was reached with 100 nmol/L IGF-I. The apparent discrepancy between our data and previous reports concerning IGF binding studies on endothelial cell monolayers could be explained by the higher affinity of IGF-I for IGFBPs as compared with IGF-I receptors, in this way hiding the higher affinity (0.036 nmol/L) IGF-I binding site. In fact, the affinity constant of IGF-I for BRECs reported by King et al (1.8 nmol/L) has the same value as our low-affinity second binding site (1.3 nmol/L). Using heterologous displacement curves between [^{125}I]IGF-I and insulin, which has no affinity for IGFBPs, we confirmed that binding sites identified on BRECs were specific IGF-I receptors since the K_d of insulin for this receptor was about 10 to 500 nmol/L, which is the reported affinity of insulin for the IGF-I receptor.[17,40,41]

[^{3}H]thymidine incorporation studies confirmed previous data[12,42] concerning the mitogenic effect of insulin on microvascular endothelial cells. This insulin action was comparable to the effect reached with an equimolar concentration of bFGF (100 nmol/L). As shown in the dose-response curve, a significant increase in [^{3}H]thymidine incorporation in BRECs is reached starting from 10 nmol/L insulin. An explanation for this pharmacological effect is probably the fact that undetectable insulin receptors were found working in the same experimental conditions of IGF-I binding studies on BRECs. It is possible that high concentrations of insulin (10 nmol/L) could bind to the

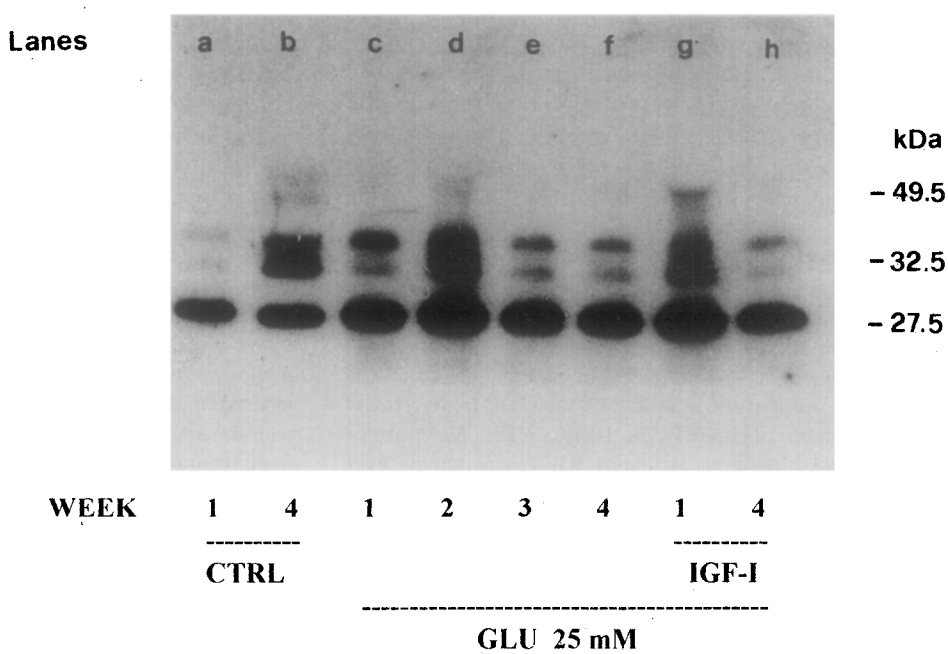

Fig 11. [^{125}I]IGF-II Western ligand blot of CM from BRECs cultured up to 4 weeks in high glucose without (lanes c to f) or with 24-hour stimulation (lanes g and h) of 100 nmol/L IGF-I. CM from cells grown in regular medium and harvested after 1 and 4 weeks (lanes a and b) was used as a control. The autoradiogram is the result of a 3-day film exposure. Indicative molecular weight in kd is shown at right. A single representative experiment is shown.

IGF-I receptor.[40,41] In fact, we observed that the addition of equimolar concentrations (100 nmol/L) of insulin and of the monoclonal antibody αIR3 reduced the effect of insulin on [^{3}H]thymidine incorporation. Although other reports have been published concerning the specific antagonist effects of αIR3 in nonhuman IGF-I receptors,[43,44] we demonstrated that the monoclonal antibody competitively reacts with the bovine IGF-I receptor, blocking [^{125}I]IGF-I binding with an ID_{50} of about 1 nmol/L. Taken together, these data suggest that there probably is not a direct action of insulin on the insulin receptor, although demonstrated in BRECs,[42] and that, instead, the larger population of IGF-I receptors present on BRECs could modulate the largest part of insulin control on BREC growth. Surprisingly, IGFs did not have the same effect on cell proliferation. Differerent data have been published concerning BREC growth and IGFs. A proliferative effect of IGF-I was reported by King et al[13] and Grant et al.[11] On the contrary, recent reports showed, in agreement with our observations, a lack of a mitogenic effect of IGF-I on bovine retinal[46] and bone[47] endothelial cells in culture. It is possible that this difference is due to the harvesting and growth conditions of these cells. Moreover, we cannot completely exclude the possibility that a subpopulation of BRECs releasing larger amounts of IGFBPs, as compared with other reports,[48] could have been selected in our culture. In agreement with Moser et al,[25] we demonstrated that cultured bovine endothelial cells release IGFBP-2, IGFBP-4, and a small amount of IGFBP-3, and using a newly developed RIA,[33] we also demonstrated the presence of IGFBP-5 in bovine endothelial cells. Moreover, BRECs in culture have mRNA specific for IGFBP-2 and IGFBP-4, and an extremely faint band for IGFBP-5. Although the findings of this study that BRECs derived from CM contained material that cross-reacted in an IGFBP-5 RIA and that BRECs express low levels of IGFBP-5 message are consistent with the idea that BRECs in culture produce IGFBP-5, further studies are needed to determine whether intact IGFBP-5 is present in the CM and whether IGFBP-5 is produced by BRECs at concentrations sufficient to produce significant biological actions. However, in contrast to the report[25] of IGFBP-6 mRNA in large and microvessel bovine endothelial cells, we could not detect IGFBP-6 mRNA in our BRECs. This could probably be explained by the different types of bovine endothelial cells used as the experimental model. These IGFBPs have been demonstrated to have both a stimulatory and an inhibitory effect on IGF actions depending on the experimental conditions and the nature of the IGFBPs.[49] Therefore, the IGF unresponsiveness found in our cell line could be explained by the presence of large amounts of IGFBP-4, as also reported in several bovine endothelial cell lines,[45] which showed a uniformly inhibitory effect on IGF actions in several in vitro studies.[49] In contrast to insulin and bFGF, the presence of 100 nmol/L IGF-I and IGF-II affected the secretory pattern of IGFBPs in BRECs. The IGF-I dose-response curve showed that 10 nmol/L IGF-I increased the level of IGFBP-2 and the IGFBP-5 band and induced the appearance on the autoradiogram of two new bands of migration: one of about 32 kd, a probable glycosylated form of IGFBP-4,[25] and another one of about 43 kd, reaching a dramatic increase with 100 nmol/L IGF-I. This highest band of 43 kd, IGFBP-3 as identified by specific RIA, is mainly modulated by IGF-I and is reported to be the most potent reservoir and buffer for IGF-I and IGF-II actions.[50] We observed a more potent affinity on IGFBP release after stimulation with IGF-II. In fact, the dose-response curve shows that 0.1 nmol/L IGF-II was able to stimulate IGFBP production, increasing IGFBP-2 and -5 and inducing the presence of the 32-kd form. The maximum effect is reached at 10 nmol/L, but in terms of the total amount of IGFBP release, IGF-II seemed less potent than IGF-I. On the contrary, the IGF-I analog Des(1-3)IGF-I with reduced affinity for IGFBPs, albeit capable of significantly stimulating thymidine incorporation only at a concentration of 10 nmol/L, failed to modulate IGFBP release. These data confirm that IGFBPs produced by BRECs in basal conditions could be responsible for the lack of mitogenic effect of IGFs. It has been observed in several cell lines[51-54] that IGFBP production could be regulated by two distinct mechanisms, which include a transcriptional modulation of IGFBP availability mediated through the IGF-I receptor and an IGF-I receptor–independent release of IGF-induced IGFBPs. Our findings suggest that the IGF-associated IGFBP increase in BRECs is independent of IGF-I receptor interaction. This observation is supported by the following data: (1) IGFBP mRNA levels are not modified after addition of 100 nmol/L IGF-I, (2) insulin at high concentrations cross-reacts with the type I IGF receptor but has no effect on IGFBP secretion, (3) αIR3 did not block the IGF-induced increase in IGFBPs, and (4) Des(1-3)IGF-I, which binds with high affinity to the IGF-I receptor, was unable to modulate IGFBP production. This is not the first report concerning an IGF-I receptor–independent mechanism. Clemmons et al[55] showed that IGF-I stimulation of a 31-kd IGFBP band in human fetal fibroblasts depends more on IGF-I binding to the binding protein and less on IGF-I receptor association. In another study, Martin and Baxter[56] reported that IGF-I stimulated an increase of a 29- to 31-kd IGFBP band in neonatal human fibroblast medium, and the effect was not mimicked with a high concentration of insulin, consistent with a receptor-independent effect of IGF-I on this IGFBP. This IGF-I receptor–independent mechanism was observed also for other IGFBPs in human normal and transformed fibroblast cultures.[51] More recently, Neely and Rosenfeld[57] reported that IGF-I and IGF-II induce an increase of IGFBP-3 in human epidermal and fibroblast cells and that these changes do not require IGF binding to type I or type II IGF receptors. As clearly summarized by Bach and Rechler,[58] most of these IGFBPs have a putative heparin-binding sequence rich in basic amino acids that can bind them to glycosaminoglycans or to other negatively charged molecules of cell membranes and the extracellular matrix. Even if the mechanism that underlies IGF-IGFBP regulation remains unclear, we could hypothesize that cell/matrix-associated IGFBPs are released into the CM after binding with the IGFs. In this way, the adhesive IGFBPs could undergo molecular rearrangement and consequently detach from the sites involved in extracellular matrix and cell membrane binding. In fact, the 24-kd band (IGFBP-4), which is the only IGFBP without a heparin-binding sequence,[58] did not show a change in the band profile and level after addition of IGFs. Further, this IGF-I receptor–independent mechanism could also explain the different potency for IGFBP stimulation between IGF-I and IGF-II observed in the two dose-response curves: IGFBPs could be released proportionally to IGF concentrations

in the CM and proportionally to IGF affinity for IGFBPs. In agreement with this, IGF-II, which showed higher affinity than IGF-I for all IGFBPs,[59] was more potent in promoting IGFBP release; on the other hand, insulin and Des(1-3)IGF-I, which have no affinity for IGFBPs, were unable to modulate IGFBP secretion. However, the IGF-I analog, which has been reported to have six times more affinity than IGF-I for the IGF-I receptor,[35] in our cellular system stimulated thymidine incorporation only at a concentration of 10 nmol/L reaching its maximum effect at a dose of 100 nmol/L, whereas at the same concentration it was unable to stimulate IGFBP release. A possible explanation for this discrepancy could be the fact that, as previously reported,[52] Des(1-3)IGF-I at a concentration of 1 nmol/L (or higher) can bind to IGFBP-3 only slightly less than IGF-I.

Damage to the vessel wall caused by diverse mechanisms may lead to diabetic microangiopathy, and one of the most important risk factors is the degree of glucometabolic control. Hyperglycemia is the metabolic abnormality thought to be responsible for development of acellular vessels with subsequent capillary closure.[60,61] In the present study, we provide the first experimental evidence that prolonged cell culture with elevated glucose levels can affect IGFBP production by BRECs. BRECs grown for 4 weeks in normal medium showed, as recently demonstrated in other cellular systems,[62,63] a progressive increase of IGFBP content in the CM. In particular, the IGFBPs involved were IGFBP-3, -2, -5, and the 32-kd band. IGFBP-4 was unaffected. On the contrary, BRECs in prolonged high glucose showed a decrease of IGFBP-3, -2, -5, and the 32-kd band starting after 3 weeks of culture. Also in this case, the IGFBP-4 band was not modified. IGF-I addition to BREC CM after 4 weeks of cell culture did not modify the secretory pattern of IGFBPs, as compared with the stimulation of IGFBPs observed after only 1 week. The loss of the IGF-I response (involving IGFBP-3, -2, -5, and the 32-kd form) suggests that IGFBP production by BRECs could be affected by the decreased presence of IGFBPs on the cell/matrix surface caused by the presence of high levels of glucose. Even if a total decrease of IGFBP production (except IGFBP-4) was detected, this IGFBP reduction was not correlated with significant changes in thymidine incorporation, probably due to the persistent presence of the inhibitory effect exerted by IGFBP-4 and the fact that the minimum level of IGFBPs reached in CM during the high-glucose culture was the same as observed in the standard culture condition where the inhibitory effect of IGFBPs on IGF action was constantly present. No significant change in cell growth was observed during the first 2 weeks of culture, where a transient increase of IGFBPs was noted (data not shown), confirming the stable inhibitory role of IGFBPs in BREC growth. To identify the possible mechanisms through which high glucose could affect IGFBP production, we examined the effect of nonenzymatic glycation[64] on protein damage. BREC exposure to AGEs did not modify the IGFBP secretory pattern. Likewise, the role of osmotic damage can be ruled out, since our observations showed that equiosmolar concentrations of mannitol do not impair IGFBP production. Consequently, we cannot exclude the hypothesis that glucose could exert a direct toxic effect.

In conclusion, these experiments showed that high-dose insulin, rather than high levels of IGF-I or IGF-II, could play a role in controling BREC growth, probably cross-reacting with IGF-I receptors. However, although IGFs have been demonstrated to be potent mitogenic agents on retinal endothelial cells, based on our experimental data, we can assert instead that the biological actions of IGFs on BREC growth are modulated by the inhibitory presence of locally produced specific endothelial IGFBPs, and the IGF-I analog Des(1-3), with reduced affinity for IGFBPs, is able to stimulate BREC growth only at high doses. These local endothelial IGFBPs are regulated by the presence of IGFs through an IGF-I receptor–independent mechanism. This autoregulatory IGF system, together with the direct glucose modulation of IGFBP production demonstrated by the present data, could contribute to the retinal endothelial cell growth and metabolism in diabetic patients through local changes in IGF bioavailability.

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