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Vascular Endothelial Growth Factor Is Induced by Long-Term High Glucose Concentration and Up-Regulated by Acute Glucose Deprivation in Cultured Bovine Retinal Pigmented Epithelial Cells

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Vascular endothelial growth factor (VEGF) is closely correlated to diabetic retinopathy. Its basal production in three types of cultured retinal cells (endothelial cells, pericytes and retinal pigment epithelial cells; RPE) was examined. RPE production of VEGF was markedly higher than the rest of the cells. VEGF production in RPE was significantly elevated by 10-day, but not by 1- or 3-day exposure to 16.5 mM glucose compared to a 5.5 mM glucose group. Transient deterioration of diabetic retinopathy is frequently observed during rapid correction of glycemic control. To determine whether VEGF is up-regulated following a sharp drop in the glucose concentration or not, we examined the changes in VEGF production in RPE before and after a sudden drop in the glucose concentration. VEGF production was significantly increased by a glucose concentration decrease from 5.5 to 0.5 mM, but not by a decrease from 33 or 16.5 to 5.5 mM. These findings suggest that up-regulation of VEGF may contribute to the development of diabetic retinopathy and its worsening by hypoglycemia.

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor (VPF), is an endothelial-selective potent angiogenic factor and also a potent stimulator of vascular permeability which was identified by Ferrarra et al. (1) and Senger et al. (2). Its amino acid sequence is 18–20% homologous with platelet-derived growth factor (PDGF) (3). Four isoforms of VEGF are produced from a single gene by alternate splicing (4). The four isoforms have 121, 165, 189, and 206 amino acids, respectively, after removal of the signal peptide, and the former two are released from the producing cells, while the latter two bind to the extracellular matrix (5). VEGF is secreted by both various tumor cell lines (6) and normal cultured cells (3). Its expression is mainly up-regulated by hypoxia (7) but also by epidermal growth factor (8), estrogens (9, 10), prostaglandin E_1 and E_2 (11), transforming growth factor-β (12), mutant p53 (13) and cobalt (14).

There is much evidence of a close correlation between VEGF levels and intraocular angiogenic diseases, especially diabetic proliferative retinopathy (PDR) (15–18), and VEGF, in addition to insulin-like growth factor-I (IGF-I) and basic fibroblast growth factor (bFGF), is considered to be one of the most important factors affecting the progression of diabetic retinopathy (19). However, it is not clear whether or not VEGF is up-regulated by a high glucose concentration in retinal cells. At the same time, although various types of retinal cells reportedly secrete VEGF (16, 20, 21), there are no reports comparing VEGF production concurrently in different retinal cell types.

Recently, a rapid and large drop in the glucose concentration was reported to stimulate VEGF mRNA expression in multicellular tumor spheroids of rat glioma cells (22). Although strict glycemic control is undoubtedly associated with a reduced risk of diabetic retinopathy, much evidence also suggests that accomplishing this goal too rapidly may induce progression (though transiently in most cases) of diabetic retinopathy (23–25). The results of some prospective controlled clinical trials (26, 27) also revealed a transient worsening of retinopathy at the beginning of intensive

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treatment and confirmed the importance of good glycemic control (28), although the mechanism remains obscure (28).

Based on the above, we examined basal VEGF production of three different types of bovine retinal cells: retinal endothelial cells (REC), retinal pericytes (RPC) and retinal pigment epithelial cells (RPE). Moreover, to clarify whether or not VEGF is induced by a high glucose concentration and/or a sharp drop in the glucose concentration, we examined VEGF production in cultured RPE at various glucose concentrations and after a sharp drop in the concentration.

MATERIALS AND METHODS

Cell culture. Bovine eyes were obtained within 2 h of death. Primary cultures of REC, RPC (25) and RPE (21) were established concurrently as previously described with slight modifications. Briefly, the globes were bisected circumferentially 3 mm posterior to the limbus and the vitreous was removed. After incubation with 0.5% collagenase (Wako Pure Chemical Industries, Tokyo, Japan) for 20 min (37°C), the retina was homogenized and filtered using nylon mesh. The flowthrough between 75 and 50 μm sieves was collected and resuspended in Dulbecco's modified Eagle's medium (DMEM, Gibco Laboratories, Grand Island, NY) containing 5.5 mM glucose, supplemented with 20% fetal calf serum (FCS, Gibco) for RPC, and with 10% horse serum (Bio Whittaker, Walkersville, MA), 150 mg/l endothelial cell growth supplement (ICN Biochemicals, Cleveland, OH) and 5 IU/l heparin (Novo Nordisk, Copenhagen, Denmark) for REC. The remaining eyecups were incubated with 0.25% trypsin in 0.02% EDTA (Gibco) for 15 min (37°C). Removed RPE was grown in DMEM supplemented with 10% FCS and 1% guinea pig serum (IBL, Gunma, Japan). RPC and REC were seeded into 35-mm fibronectin-coated dishes (Corning, New York, NY), while RPE was transferred to 35-mm collagen-coated dishes (Corning). All types of cells were incubated in 5% CO_2 under the same temperature (37°C) and humidity (95%), and their media were changed every 2 days. At confluence, cells were subcultured using 0.25% trypsin in 0.02% EDTA. Cells at the third passage were used for each experiment. Each type of cell was identified immunohistochemically by specific antibodies [Factor VIII-related antibody (Zymed, South San Francisco, CA) for REC, monoclonal 3G5 antibody (a generous gift from Prof Hiromitsu Nakauchi, Department of Immunology, University of Tsukuba, Tsukuba, Japan) for RPC and anti-cytokeratin antibody (Sigma Chemical Co., St. Louis, MO) for RPE].

Basal VEGF production in each cell type. Unstimulated VEGF secretion by REC, RPC and RPE was determined. Each type of cell was re-plated in 35-mm collagen-coated dishes (5 dishes for each type) and grown until confluency under the same conditions as above. After 48 h of incubation, all the conditioned media (1.5 ml per dish) were collected, clarified by centrifugation and stored at −70°C or below until assay for VEGF.

VEGF production at high glucose concentrations. RPE was re-plated in four plastic plates, each containing six non-coated wells (Becton Dickinson Labware, Lincoln Park, NJ) and grown to confluency. The RPE medium described above but with different concentrations of D-glucose (5.5, 11 and 16.5 mM) or mannitol (11 mM plus 5.5 mM D-glucose) was added to each plate. The medium (1.5 ml per well) was exchanged daily, and the conditioned medium on the 1st, 3rd and 10th days was collected and stored as above for assay.

VEGF production by RPE on acute drop in glucose concentration. Eight non-coated six-well plates with a confluent monolayer of RPE were prepared as above. Figure 1 shows the time courses and changes in culture conditions in each group. Group A, the control group, was maintained at the physiological glucose concentration of 5.5 mM. After confluency, groups B–F were exposed to various concentrations of D-glucose or mannitol (group B, 16.5 mM glucose; group C, 33 mM glucose; group D, 5.5 mM glucose plus 27.5 mM mannitol; and groups E, F and G, 5.5 mM glucose) for 10 days. After this adaptation period, the glucose concentration in each medium was decreased suddenly to various degrees (to 5.5 mM glucose for groups B–D, to 2.75 mM glucose for group E, to 0.5 mM glucose for group F and to 0.5 mM glucose plus 5.0

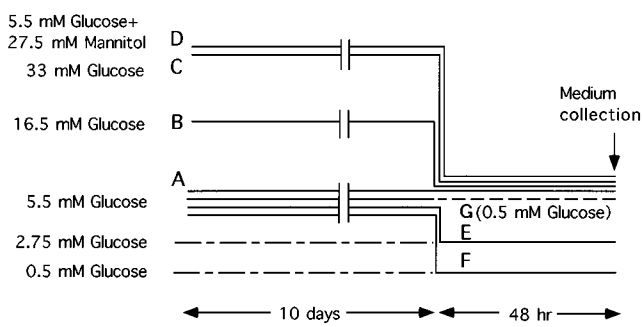

FIG. 1. Schematic diagram of time course and change in glucose/mannitol concentrations in media of each group.

mM mannitol for group G). After 48 h of incubation following this sudden change in the glucose concentration, the conditioned media of all groups were collected and stored as above.

Determination of VEGF levels in cultured media. The VEGF concentrations of the conditioned media were measured by our previously described highly sensitive enzyme-linked immunosorbent assay (ELISA) using rabbit anti-human VEGF/VPF polyclonal antibody for both capture (solid phase) and secondary (enzyme-labeled) antibodies (29). This immunoassay detects all known isoforms of VEGF molecules. The limit of detection for the assay, defined as +2SD above the zero standard, is 1 pg/ml. The intra-assay coefficient of variation was less than 6.1%. All the conditioned media were assayed after being concentrated to one-fourth of its original volume by vacuum centrifugation. Neither of these concentrating processes nor glucose in the media affected the results of the VEGF assay (data not shown). All the measurements were corrected by the protein content in each dish or well according to the method of Lowry et al. (30).

Statistical analysis. All the results are expressed as the mean ± SD and analysis of variance (ANOVA) was used for statistical analysis. Differences with a p value of less than 0.05 were considered significant.

RESULTS

Basal VEGF production in each cell type. Among the three types of cells studied, RPE produced markedly more (166.7 ± 45.3 ng/g protein, $p < 0.0001$) VEGF compared to REC (4.0 ± 0.4) or RPC (3.6 ± 0.4). There were no significant differences in the VEGF concentrations between the REC and RPC groups.

VEGF production in RPE at high glucose concentrations (*Fig. 2*). None of the groups showed a significant change in the VEGF concentration compared to the control group (5.5 mM glucose) after 1 or 3 days of incubation (data not shown). However, after 10 days of incubation, the VEGF concentration in the 16.5 mM glucose group (231.0 ± 33.0 ng/g protein, $p < 0.01$) was significantly elevated compared to the control group (173.4 ± 36.7) incubated for the same period. The 11 mM glucose group showed increased VEGF secretion compared to the control group after a 10-day incubation, but the increase was not significant. The high osmolality group (5.5 mM glucose plus 11.0 mM mannitol) did not show any significant change compared to the control group throughout the study period.

VEGF induction in RPE following acute drop in glucose concentration (*Fig. 3*). The VEGF concentration of the conditioned medium was not changed by a sudden decrease in the glucose or mannitol concentration from high (16.5 mM or 33 mM) to physiological (5.5 mM) (groups B–D) concentrations. However, it increased in the three groups (E, F and G) in which the glucose concentration was decreased from physiological (5.5 mM) to low (2.75 mM or 0.5 mM) concentrations, although only the values in group F (258.7 ± 41.5 ng/g protein, $p < 0.01$) and group G (249.1 ± 46.9, $p < 0.01$) were significantly different from that in the control group (group A) (164.8 ± 24.1).

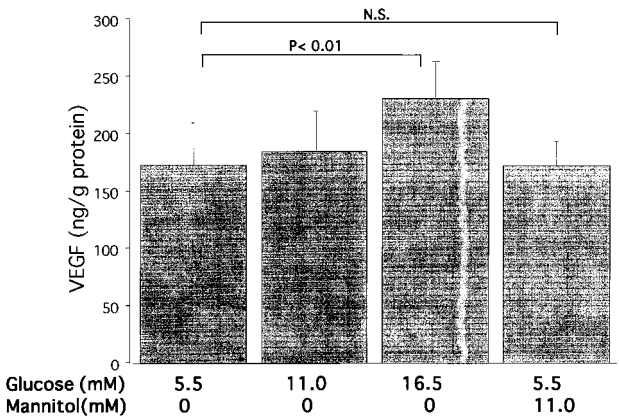

FIG. 2. VEGF production by RPE after 10-day incubation under various concentrations of glucose or mannitol (n = 6, mean ± SD).

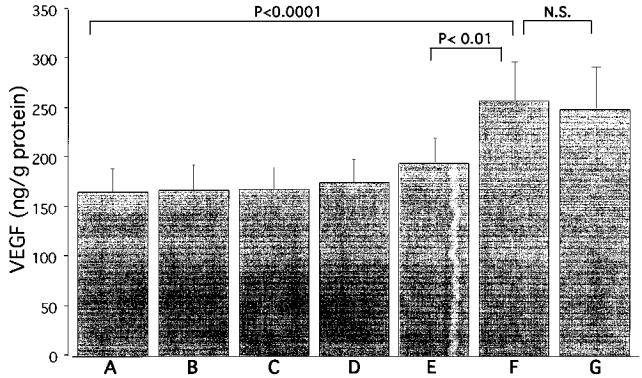

FIG. 3. VEGF production on RPE by 48-h incubation after sudden drop in glucose or mannitol concentration (n = 6, mean ± SD).

DISCUSSION

VEGF has been reported to be secreted in REC (via an autocrine pathway) (16), RPC (31) and RPE (21); however, its main source of supply on the retina is still unknown. Although an immunohistochemical study using postmortem normal human eyes (20) or *in situ* hybridization using a primate ocular neovascular model (17) demonstrated a wide distribution of VEGF on the retina, especially in glial cells, neither of these materials was diabetic in origin. Our findings showed that basal VEGF production in RPE was much higher than in REC and RPC (considered to be derived from glial cells). This difference was not considered to be due to the cell-type-specific patterns of VEGF isoform expression because our assay detects both of its two secreted isoforms.

The retinal pigment epithelium is the tenth and deepest layer of the retina, and is situated just in front of Bruch's membrane. Its role in the development of diabetic retinopathy has been underestimated because of its location. However, RPE produces mitogenic factors for retinal microvascular cells (32) including bFGF (33), that has a synergistic effect with VEGF (34), and evidence that RPE contributes to the PDR membrane has been obtained by ultrastructural investigation (35). Furthermore, Hiscott et al. (36) histochemically showed in PDR patients that RPE is contained (5–20%) in combined traction rhegmatogenous retinal detachment membrane but rarely in simple traction retinal detachment membrane. These observations indicate that RPE migrates through the retinal break to access the PDR membrane and could contribute to its progression by secreting some angiogenic factors. In fact, the PDGF A chain was confirmed to be expressed in RPE of the PDR membranes (37). Half-maximal stimulation of endothelial cell growth was obtained at 100–150 pg/ml of VEGF (3), which is approximately the same as the concentration range of the basal RPE conditioned media obtained in our study. Our findings suggest that the up-regulated RPE-derived VEGF could contribute to the deterioration of PDR, possibly with bFGF in a synergistic manner (33, 34). Moreover, the retinal pigment epithelium is known to form an outer blood-retinal barrier, and its breakdown is known to be the earliest pathological change in the diabetic retina (38). In the diabetic rat model, this breakdown is due to alteration of membrane permeability rather than to a loss of tight junctions (39). RPE-derived VEGF may also be involved in this process through autocrine pathways, because VEGF/VPF is also known to markedly increase vascular permeability, as its name indicates.

PDGF (40) is reported to be up-regulated by acute (24 h) high glucose stimulation in cultured cells, but this is considered to depend mainly on high osmolality, and is not a glucose-specific response. Unlike PDGF, VEGF showed osmolality-independent, glucose-specific up-regulation, but required chronic exposure to a high glucose environment. The mechanism of osmolarity-independent up-regulation by chronic high glucose concentrations is not yet clear. In macrophages,

the secretion of PDGF (41), tumor necrosis factor and interleukin-1 (42) is stimulated by advanced glycation endproducts (AGE) via AGE receptors. Thus, AGE is also speculated to be involved in the production of VEGF on RPE. Most factors which induce VEGF are reported only as increased mRNA expression (7, 9–13, 43); therefore, we cannot compare the degree of induction of our results with other reported factors.

The reason for the temporary worsening of diabetic retinopathy by sudden correction of chronic hyperglycemia, which is frequently observed in patients with pre-existing retinopathy (27), is still controversial. Increasing levels of IGF-I (44) or acute normalization of increased retinal blood flow (45) are possible explanations. In the Oslo study (24), a higher frequency of hypoglycemia was shown in the patients with deteriorated retinopathy, suggesting that hypoglycemia unexpectedly accompanied by strict control is also responsible (26). Shweiki et al. (22) found that an acute drop in the glucose concentration is one of the up-regulatory stresses of VEGF induction, suggesting that tumor angiogenesis is induced by glucose deficiency. However, when we apply their hypothesis to diabetes *in vivo*, the falling glucose concentration in their study was too large from marked hyperglycemia (450 mg/dl, 25 mM) to life-threatening hypoglycemia (10 mg/dl, 0.55 mM). In our study, we divided this drop into two components to estimate their influences independently: 'from high to physiological' and 'from physiological to low'. Our results revealed that the latter induces VEGF production. Furthermore, the induction of VEGF by acute glucose deprivation (osmolality-independent) is not limited to multicellular spheroids of the tumor cell line but includes monolayer culture of non-tumor cells. Although the *in vitro* study using bovine materials and the length of the "adaptation period" was quite short and the exposure duration to medium containing a low concentration of glucose was relatively long, our findings imply that VEGF is up-regulated by acute glucose deficiency, mimicking hypoglycemia but not by acute normalization of high glucose concentrations, mimicking acute but careful correction of chronic hyperglycemia without producing hypoglycemia.

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REFERENCES

1. Ferrara, N., and Henzel, W. J. (1989) *Biochem. Biophys. Res. Commun.* **161,** 851–858.
2. Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S., and Dvorak, H. F. (1983) *Science* **219,** 983–985.
3. Ferrara, N., Houck, K., Jakeman, L., and Leung, D. W. (1992) *Endocr. Rev.* **13,** 18–32.
4. Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J. C., and Abraham, J. A. (1991) *J. Biol. Chem.* **266,** 11947–11954.
5. Houck, K. A., Leung, D. W., Rowland, A. M., Winer, J., and Ferrara, N. (1992) *J. Biol. Chem.* **267,** 26031–26037.
6. Kondo, S., Asano, M., and Suzuki, H. (1993) *Biochem. Biophys. Res. Commun.* **194,** 1234–1241.
7. Shweiki, D., Itin, A., Soffer, D., and Keshet, E. (1992) *Nature* **359,** 843–845.
8. Goldman, C. K., Kim, J., Wong, W. L., King, V., Brock, T., and Gillespie, G. Y. (1993) *Mol. Biol. Cell.* **4,** 121–133.
9. Charnock, J. D., Sharkey, A. M., Rajput, W. J., Burch, D., Schofield, J. P., Fountain, S. A., Boocock, C. A., and Smith, S. K. (1993) *Biol. Reprod.* **48,** 1120–1128.
10. Cullinan, B. K., and Koos, R. D. (1993) *Endocrinology* **133,** 829–837.
11. Harada, S., Nagy, J. A., Sullivan, K. A., Thomas, K. A., Endo, N., Rodan, G. A., and Rodan, S. B. (1994) *J. Clin. Invest.* **93,** 2490–2496.
12. Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., and Alitalo, K. (1994) *J. Biol. Chem.* **269,** 6271–6274.
13. Kieser, A., Weich, H. A., Brandner, G., Marme, D., and Kolch, W. (1994) *Oncogene* **9,** 963–969.
14. Ladoux, A., and Frelin, C. (1994) *Biochem. Biophys. Res. Commun.* **204,** 794–798.
15. Aiello, L. P., Avery, R. L., Arigg, P. G., Keyt, B. A., Jampel, H. D., Shah, S. T., Pasquale, L. R., Thieme, H., Iwamoto, M. A., Park, J. E., Nguyen, H. V., Aiello, L. M., Ferrara, N., and King, G. L. (1994) *N. Engl. J. Med.* **331,** 1480–1487.

16. Adamis, A. P., Miller, J. W., Bernal, M. T., D'Amico, D. J., Folkman, J., Yeo, T. K., and Yeo, K. T. (1994) *Am. J. Ophthalmol.* **118,** 445–450.
17. Miller, J. W., Adamis, A. P., Shima, D. T., D'Amore, P. A., Moulton, R. S., O'Reilly, M. S., Folkman, J., Dvorak, H. F., Brown, L. F., Berse, B., Yeo, T. K., and Yeo, K. T. (1994) *Am. J. Pathol.* **145,** 574–584.
18. Pierce, E. A., Avery, R. L., Foley, E. D., Aiello, L. P., and Smith, E. H. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 905–909.
19. Barinaga, M. (1995) *Science* **267,** 452–453.
20. McGookin, E., Stopa, E., Kuo-LeBlanc, V., Baird, A., Gonzalez, A., Hanneken, A., and Streeten, B. (1992) *Invest. Ophthalmol. Vis. Sci.* **33,** 821. [ARVO abstracts]
21. Adamis, A. P., Shima, D. T., Yeo, K. T., Yeo, T. K., Brown, L. F., Berse, B., D'Amore, P. A., and Folkman, J. (1993) *Biochem. Biophys. Res. Commun.* **193,** 631–638.
22. Shweiki, D., Neeman, M., Itin, A., and Keshet, E. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 768–772.
23. Wolff, S. (1992) *Br. Med. J.* **304,** 505–506.
24. The Kroc Collaborative Study Group (1984) *N. Engl. J. Med.* **311,** 365–372.
25. Wang, P., Lau, J., and Chalmers, T. (1993) *Lancet* **341,** 1306–1309.
26. Dahl-Jorgensen, K., Brinchmann-Hansen, O., Hanssen, K., Sandvik, L., Aagensaes, F., and Aker Diabetes Group (1985) *Br. Med. J.* **290,** 811–815.
27. The Diabetes Control and Complication Trial Research Group (1993) *N. Engl. J. Med.* **329,** 977–986.
28. Clark, C., and Lee, D. (1995) *N. Engl. J. Med.* **332,** 1210–1217.
29. Hanatani, M., Tanaka, Y., Kondo, S., Omori, I., and Suzuki, H. (1995) *Biosci. Biotech. Biochem.* **59,** 1958–1959.
30. Lowry, O., Rosenbrough, N., Farr, A., and Randall, J. (1951) *J. Biol. Chem.* **194,** 265–275.
31. Plouet, J., Chollet, P., Moro, F., and Malecaze, F. (1993) *Invest. Ophthalmol. Vis. Sci.* **34,** 900. [ARVO abstracts]
32. Wong, H., Boulton, M., Clark, P., Bayly, M., and Marshall, J. (1987) *Eye* **1,** 754–756.
33. Bost, L., Aotaki-Keen, A., and Hjelmeland, L. (1992) *Exp. Eye Res.* **55,** 727–734.
34. Goto, F., Goto, K., Weindel, K., and Folkman, J. (1993) *Lab. Invest.* **69,** 508–517.
35. Hamilton, C., Chandler, D., Klintworth, G., and Machmer, R. (1982) *Am. J. Ophthalmol.* **94,** 479–488.
36. Hiscott, P., Gray, R., Grierson, I., and Gregor, Z. (1994) *Br. J. Ophthalmol.* **78,** 219–222.
37. Robbins, S., Mixon, R., Wilson, D., Hart, E., Robertson, J., Westra, I., Planck, S., and Rosenbaum, J. (1994) *Invest. Ophthalmol. Vis. Sci.* **35,** 3649–3663.
38. Waltam, S., Oestrich, C., Krupin, T., Hanish, S., Patzan, S., Santiago, J., and Kilo, C. (1978) *Diabetes* **27,** 85–87.
39. Caldwell, R., Slapnick, S., and McLaughlin, B. (1985) *Curr. Eye Res.* **4,** 215–227.
40. Mizutani, M., Okuda, Y., Yamaoka, T., Tsukahara, K., Isaka, M., Bannai, C., and Yamashita, K. (1992) *Biochem. Biophys. Res. Commun.* **187,** 664–669.
41. Kirstein, M., Brett, J., Radoff, S., Ogawa, S., Stern, D., and Vlassara, H. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 9010–9014.
42. Vlassara, H., Brownlee, M., Manogue, K., Dinarello, C., and Pasagian, A. (1988) *Science* **240,** 1546–1548.
43. Inagaki, N., Miura, T., Nagai, H., Ono, Y., and Koda, A. (1988) *Int. Arch. Allergy Appl. Immunol.* **87,** 254–259.
44. Tamborlane, M., Hintz, R. L., Bergman, M., Genel, M., Felig, P., and Sherwin, R. S. (1981) *N. Engl. J. Med.* **305,** 303–311.
45. Parving, H., Viberti, G., Keen, H., Christiansen, J., and Lassen, N. (1983) *Metabolism* **32,** 943–949.