Both type 1 and type 2 diabetes can lead to altered retinal microvascular function and diabetic retinopathy. Insulin signaling may also play a role in this process, and mice lacking insulin receptors in endothelial cells are protected from retinal neovascularization. To define the role of diabetes in retinal function, we compared insulin signaling in the retinal vasculature of mouse models of type 1 (streptozotocin-induced) and type 2 diabetes (ob/ob). In streptozotocin mice, in both retina and liver, insulin receptor (IR) and insulin receptor substrate (IRS)-2 protein and tyrosine phosphorylation were increased by insulin, while IRS-1 protein and its phosphorylation were maintained. By contrast, in ob/ob mice, there was marked down-regulation of IR, IRS-1, and IRS-2 protein and phosphorylation in liver; these were maintained or increased in retina. In both mice, Phosphatidylinositol 3,4,5-trisphosphate generation by acute insulin stimulation was enhanced in retina and endothelial cells. On the other hand, protein levels and phosphorylation of PDK1 and Akt were decreased in retina of both mice. Interestingly, phosphorylation of p38 mitogen-activated protein kinase and ERK1 were responsive to insulin in retina of both mice but were unresponsive in liver. HIF-1α and vascular endothelial growth factor were increased and endothelial nitric-oxide synthase was decreased in retina. These observations indicate that, in both insulin-resistant and insulin-deficient diabetic states, there are alterations in insulin signaling, such as impaired PDK/Akt responses and enhanced mitogen-activated protein kinases responses that could contribute to the retinopathy. Furthermore, insulin signaling in retinal endothelial cells is differentially altered in diabetes and is also differentially regulated from insulin signaling in classical target tissues such as liver.

One of the major long-term complications of both type 1 (insulin-deficient) and type 2 (insulin-resistant) diabetes is proliferative retinopathy, which is characterized by increased neovascularization and neuronal degeneration in the retina. Clinical studies have indicated that hyperglycemia and poor metabolic control are important factors in the development of diabetic retinopathy (1, 2). Multiple mechanisms have been implicated, including relative hypoxia in retina, resulting in the induction of vascular endothelial growth factor (VEGF)1 and other vascular mediators that stimulate proliferation of retinal endothelial cells in preretinal area (3–7).

Although the risk of diabetic retinopathy over the long term is correlated with the degree of metabolic control (8), several clinical studies (9–11) have demonstrated that insulin may also play a role and that intensive insulin therapy may cause a transient worsening of retinopathy in some individuals, even when compared with treatment with oral hypoglycemic agents. This is supported by our previous observation that mice with a vascular endothelial cell specific knock-out of the insulin receptor (VENIRKO) are protected from retinal neovascularization (7). Furthermore, in general, rates of development of retinopathy are somewhat lower in patients with insulin-resistant type 2 diabetes than in those with insulin-deficient type 1 diabetes (8, 12).

Insulin action at a molecular level is created by a complex signaling network using alternative or complementary pathways and multiple molecular isoforms of key signaling molecules (13). Insulin and insulin-like growth factor 1 receptors almost ubiquitously expressed are present in endothelial cells and other cells of the retina (14). How insulin signaling in these tissues might be altered in diabetes, as compared with classical target tissues, i.e. liver, muscle, and fat, is unknown.

To define the potential role of these pathways in development of diabetic retinopathy, we have assessed retinal insulin signaling in type 1 (streptozotocin (STZ)-induced: insulin-deficient hyperglycemic model) and type 2 (ob/ob: hyperglycemic insulin-resistant model) diabetic model mice. We find that circulating insulin can stimulate the insulin signaling cascade in retinal tissue primarily in endothelial cells and that these pathways are differentially altered in insulin-resistant and insulin-deficient diabetes, as compared with peripheral tissues. We also find that hyperglycemia in both types of diabetes is associated with increased expression of hypoxia-inducible factor (HIF)-1α and VEGF. Thus, the combination of hyperglycemia and altered insulin activation of the MAP kinase pathway in retinal endothelial cells and VEGF induction may contribute to altered retinal vascular function in diabetes and development of diabetic retinopathy.

MATERIALS AND METHODS

Animals—8–10-week-old male obese hyperglycemic mice (c57B/6J ob/ob) and their lean matched controls (ob/+ ) were purchased from Jackson Laboratory (Bar Harbor, ME). For the generation of the STZ mouse model, 8-week-old c57B/6J mice were given STZ (Sigma), 180 mg/kg as a single intraperitoneal injection, and studied 7 days later.

1 The abbreviations used are: VEGF, vascular endothelial growth factor; STZ, streptozotocin; HIF, hypoxia-inducible factor; ERK, extraacellular signal-regulated protein kinase; PDK, 3-phosphoinositide-dependent kinase; MAP, mitogen-activated protein; MAPK, MAP kinase; eNOS, endothelial nitric-oxide synthase; PIP3, phosphatidylinositol 3,4,5-trisphosphate; IR, insulin receptor; IRS, insulin receptor substrate; PI, phosphatidylinositol.

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after blood sugars were elevated more than 250 mg/dl. All mice were fed standard rodent chow and water ad libitum. Food was withdrawn 16 h before the experiments. Mice were anesthetized with 100 mg/kg of sodium pentobarbital injected intraperitoneally. Following loss of pedal and corneal reflexes, 5 units of regular human insulin or its diluent was injected into the inferior vena cava. The retina and liver were excised 5 min after insulin or its diluent was injected and frozen in liquid nitrogen.

**Immunoprecipitation and Western Blotting**—Frozen tissues were homogenized in T-PER Tissue Protein Extraction Reagent (Pierce) with Halt Protease Inhibitor Mixture (Pierce), and cytoplasmic or nuclear fractions were isolated. Equal amounts of the protein supernatant were subjected to immunoprecipitation for 2 h using the indicated antibodies, followed by adding of protein A-Sepharose for another 1 h. The samples were processed for SDS-PAGE electrophoresis and Western blotting as described previously (15). Retinal protein was isolated in pools from three mice. At least three independent experiments were performed for each condition.

**Antibodies**—Rabbit polyclonal anti-insulin receptor antibody, rabbit polyclonal anti-IRS-1 antibody, rabbit polyclonal anti-IRS-2 antibody, rabbit polyclonal anti-HIF-1α antibody, and mouse monoclonal anti-VEGF antibody were purchased from Santa Cruz Biotecnology, Inc., Santa Cruz, CA. Rabbit polyclonal anti-phospho-3-phosphoinositide-dependent kinase 1 (PDK1) (Ser-241) antibody, rabbit polyclonal anti-phospho-Akt (Ser-473) antibody, rabbit polyclonal anti-phospho-p38-dependent kinase 1 (PDK1) (Ser-241) antibody, and mouse monoclonal anti-phospho extra-cellular signal-regulated protein kinase 1 (ERK1) (Thr-202/Tyr-204) were purchased from Cell Signaling Technology, Inc., Beverly, MA. Mouse monoclonal anti-endothelial nitric-oxide synthase (eNOS) antibody was purchased from BD Transduction Laboratories, Franklin Lakes, NJ. Mouse monoclonal anti-phosphotyrosine antibody (4G10) was purchased from Upstate Biotechnology, Lake Placid, NY.

**Immunohistochemistry for Phosphatidylinositol 3,4,5-Trisphosphate (PIP3) and Vascular Marker**—PIP3, and a vascular marker, Fluorescein Griffonia (Bandeiraea) Simplicifolia Lectin 1 (Isolect B4), were assessed by immunohistochemical analysis of the retinas. After a PBS equilibration, 6-μm retinal frozen sections were incubated with 3% normal goat serum in 2.5% Triton X-100/PBS for 2 h at room temperature. Incubations with a primary antibody to PIP3 (Echelon, Salt Lake City, UT) were performed with 1/50 dilution in blocking solution (3% normal goat serum in 2.5% Triton X-100/PBS) for overnight at 4 °C. After six washes with PBS, slides were incubated with a secondary antibody conjugated to immunofluorescent dyes (Alexa 546 for red fluorescence: Molecular Probes, Inc., Eugene, OR) with 1/200 dilution and fluorescein-conjugated Lectin B4 (Vector Laboratories, Inc., Burlingame, CA) with 1/100 dilution in blocking solution for 2 h at room temperature. The stained sections were then washed, mounted using a SlowFade kit (Molecular Probes, Inc.), and examined with a fluorescent microscope.

**RESULTS**

**Physiological Examinations of ob/ob and STZ Mice—STZ diabetic mice were lean, hyperglycemic, and hypoinsulinemic. The mean body weight of STZ mice was 20.8 ± 0.4 g versus 29.1 ± 1.9 g. Fasting glucose levels were 268.0 ± 26.5 mg/dl and 96.4 ± 5.2 mg/dl, and fasting insulin levels were 0.88 ± 0.1 ng/ml versus 1.91 ± 0.2 ng/ml, respectively. ob/ob mice were obese, hyperglycemic, and hyperinsulinemic. The mean body weight of ob/ob mice was 47.8 ± 0.8 g as compared with ob/+ controls of 27.9 ± 1.0 g. Fasting glucose levels were 196.2 ± 6.4 mg/dl versus 99.1 ± 3.9 mg/dl, respectively, and fasting plasma insulin levels were 40.7 ± 1.5 ng/ml versus 1.66 ± 0.1 ng/ml.

**Protein Expression and Phosphorylation of IR, IRS-1, and IRS-2**—To determine the level of insulin receptor (IR), IRS-1 and IRS-2, homogenates of retina and liver of ob/ob, STZ, and their controls were subjected to immununoprecipitation and Western blotting with or without stimulation 5 min after injection of insulin into the inferior vena cava. As reported previously, IR expression in liver of ob/ob mice was decreased by 70% by Western blotting, and following intravenous insulin injection, tyrosine phosphorylation of IR was diminished by >95% (Fig. 1A). By contrast, IR expression and tyrosine phosphorylation were not altered in retina of the same mice (Fig. 1A). In STZ diabetic mice, IR expression was increased by ~75 and ~80% in both retina and liver, and tyrosine phosphorylation of IR in retina and liver of these mice was also increased by 50–60% (Fig. 1B). Thus, insulin receptors in retina undergo up-regulation in type 1 diabetes but appear to be protected from the down-regulation and desensitization in type 2 diabetics as observed in peripheral tissues. Expression of IRS-1 protein was decreased by ~40% in ob/ob liver (Fig. 2A) and was increased by 50% in STZ liver (Fig. 2B). By contrast, IRS-1 expression in retina of ob/ob and STZ mice was not altered (Fig. 2, A and B). Tyrosine phosphorylation of IRS-1 paralleled the changes in protein expression and was decreased in ob/ob liver (Fig. 2A), increased in STZ liver (Fig. 2B), but was not changed in retina of both ob/ob and STZ mice (Fig. 2, A and B). In agreement with previous studies, IRS-2 protein and phosphorylation were decreased by 40 and 90%, respectively, in liver of ob/ob mice. By contrast, IRS-2 protein was increased by ~50% in ob/ob retina (Fig. 3A), and tyrosine phosphorylation of IRS-2 was almost doubled in ob/ob retina only under basal conditions (Fig. 3A). In STZ diabetic mice, IRS-2 protein expression was increased by ~110 and ~150% in retina and liver, respectively (Fig. 3B). Tyrosine phosphorylation of IRS-2 was also increased by 60–80% in both tissues in this model of insulin-deficient diabetes (Fig. 3B).

**Immunolocalization of PIP3 in Retina**—To identify the cell type in retina responding to insulin stimulation, we assessed PI 3-kinase activation, as measured by accumulation of PIP3, using immunohistochemistry and compared this with the distribution of the vascular marker, lectin B-4 binding. Under the basal conditions, PIP3 was faintly observed, and this appeared co-localized to retinal endothelial cells as identified by lectin B-4 staining. Following 5 min of insulin stimulation, the PIP3 level clearly increased exclusively in retinal endothelial cells of controls, and this was further enhanced in ob/ob and STZ retinal endothelial cells (Fig. 4A). No stimulation of PIP3 was observed in retinal neurons at this time point. Thus, in contrast to the decrease in insulin signaling observed in liver of ob/ob mice, PIP3 generation in response to insulin is either preserved or enhanced in retina of ob/ob and STZ diabetic mice, and this occurs primarily in retinal vasculature.

**Protein Levels and Phosphorylation of PDK1 and Akt—Akt (protein kinase B) is one of the key downstream targets of PI 3-kinase. Phosphorylation of Akt is mainly regulated by PDK1 and Akt phosphorylation in retina was reduced in both ob/ob and STZ diabetic mice by 90% in liver of ob/ob mice and by 80% in liver of STZ diabetic mice (Fig. 4, B and C). Surprisingly, although PIP3 levels were increased, both PDK1 and Akt phosphorylation in retina were reduced in both ob/ob mice by 60 and 70%, and in STZ diabetic mice by 90 and 80%, respectively (Fig. 4, B and C). These contradictory observations can be explained by a reduction of PDK1 and Akt protein levels by 70–80% in the retinas of ob/ob and STZ diabetic mice (Fig. 4, B and C). Thus, it seems that the retinal tissue is more susceptible to the diabetic status in terms of PDK1 and Akt protein expression or stability than classical insulin-sensitive tissues such as liver.

**Phosphorylation of p38 and ERK1—Whereas the components of signaling downstream of PI 3-kinase were impaired in retina of ob/ob and STZ diabetic mice, basal phosphorylation, which reflects activation (16) of both p38 MAPK and ERK1, were increased by ~50% in ob/ob retina (Fig. 5A). Activation of both these kinase was also enhanced in liver of these mice (Fig. 5A). In STZ diabetes, basal phosphorylation of p38 was even more dramatically increased 3-fold in retina, and this was paralleled by a 130% increase in liver (Fig. 5B). Phosphorylation of ERK1 was also increased by 50–100% in both retina and
**Fig. 1.** Insulin receptor protein expression and its tyrosine phosphorylation in retina and liver of ob/ob (A) and STZ (B) mice as compared with their controls. Retina and liver proteins from these mice were isolated and subjected to immunoblotting (IB) with αIR (top panels) or immunoprecipitation (IP) with αIR followed by immunoblotting with 4G10 antibody (bottom panels). Asterisks indicate *p < 0.05* for difference. The bar graphs show data quantification by NIH image 163. Data are the mean ± S.E. of at least three independent experiments using 12–16 animals for each group and are expressed as relative to control values, which were set at 100%.
Fig. 2. IRS-1 protein expression and its tyrosine phosphorylation in retina and liver of ob/ob (A) and STZ (B) mice as compared with their controls. Retina and liver proteins from these mice were isolated and subjected to immunoblotting (IB) with αIRS-1 (top panels) or immunoprecipitation (IP) with αIRS-1 followed by immunoblotting with 4G10 antibody (bottom panels). Asterisks indicate differences significant at \( p < 0.05 \). Data are the mean ± S.E. of at least three independent experiments using 12–16 animals for each group and are expressed as relative to control values, which were set at 100%.
FIG. 3. IRS-2 protein expression and its tyrosine phosphorylation in retina and liver of ob/ob (A) and STZ (B) mice as compared with their controls. Retina and liver proteins from these mice were isolated and subjected to immunoblotting with aIRS-2 (top panels) or immunoprecipitation with aIRS-2 followed by immunoblotting with 4G10 antibody (bottom panels). Asterisks indicate differences significant at \( p < 0.05 \). Data are the mean ± S.E. of at least three independent experiments using 12–16 animals for each group and are expressed as relative to control values, which were set at 100%.
Interestingly, p38 and ERK1 phosphorylation were still insulin-responsive in retinal tissues of both ob/ob and STZ mice, despite the diabetic state, whereas in liver, basal levels were high, but there was not further insulin response (Fig. 5, A and B).

Protein Expression of HIF-1α, VEGF, and eNOS—Since hyperglycemia and diabetic state can produce a relative cellular hypoxia, we examined the levels of HIF-1α, VEGF, and eNOS protein expression in retina of ob/ob and STZ mice. HIF-1α expression was increased by 7–9-fold in both ob/ob and STZ retina. VEGF expression in retina was also increased by 3–4-fold in both ob/ob and STZ mice. By contrast, eNOS expression was decreased by −60% in ob/ob and decreased by 50% in STZ retina (Fig. 6). None of these changed at the protein level in acute response to insulin.

DISCUSSION

Hyperglycemia and the metabolic alterations associated with diabetes are essential causative factors in the development of diabetic retinopathy (1, 2). The relative roles of insulin deficiency and insulin resistance present in both type 1 and type 2 diabetic patients may also contribute to increase the risk.
of proliferative diabetic retinopathy (12, 17), although the exact relationships is less clear.

Although the risk of progression in diabetic retinopathy over the long term is related to the degree of glycemic control (8), several clinical studies have demonstrated that intensive insulin therapy may cause a transient worsening of retinopathy (9–11). We have reported recently that mice lacking IR or insulin-like growth factor 1 receptors in vascular endothelial cells are protected from development of retinal neovascularization following a relative hypoxic status (7). While vascular endothelial cells do not appear to be an important insulin target in terms of glucose utilization in whole body (18), these cells do possess insulin receptors and are highly susceptible to stress conditions such as hypoxia and hyperglycemia (19).

Alterations in the early steps of insulin signaling have been recognized as an important component of many insulin-resistant states. Decreased insulin binding, decreased receptor kinase activity, decreased IRS-1 and -2 protein, and decreased IRS-1/2-associated PI 3-kinase activity have all been demonstrated in ob/ob mice and other models of type 2 diabetes (15, 20–24). Other alterations in these same steps of insulin receptors have been described in insulin-deficient states, including increased insulin binding, increased phosphorylation of IRS-1/2, increased IRS-1/2-associated PI 3-kinase activity, and decreased phosphorylation of Akt (25, 26). The present study demonstrates that the early steps of insulin signaling in retina are regulated in both insulin-resistant and insulin-deficient diabetes and that this regulation is quite different from that in a classical target tissue, i.e. liver. Thus, IR and IRS-1 protein and tyrosine phosphorylation are maintained, and IRS-2 and phosphorylation are actually increased in retina of ob/ob mice, whereas these are all down-regulated in liver of the same mice. We also found maintained levels of IRS-1 protein and IRS-1 phosphorylation in STZ retina and increased levels of both IR and IRS-2 protein and phosphorylation in this model. A 1.5-fold increase in IRS proteins and phosphorylation has also been observed in neurons of STZ diabetic rats (27) and type 1 diabetic subjects (28). In peripheral tissues, such as liver, the major mechanism of down-regulation of IR and IRS proteins in the hyperinsulinemic state is accelerated rates of degradation of these proteins (29–31). Clearly, this mechanism does not exist to the same extent in the retina. This is not simply due to be blood retinal barrier effect, since most of the insulin signaling proteins studied are derived from retinal endothelial cells, and these are clearly responding to peripheral insulin injection. Thus, retinal endothelium, and perhaps other endothelial beds, possess mechanisms that protect against down-regulation of IR or IRS proteins.

In ob/ob and STZ retina, the increased insulin signaling results in increased levels of PIP3 in retinal endothelial cells as shown by immunohistochemistry. This immunohistochemical approach has also proved useful for insulin signaling in other tissues including brain (32–34). In retina, PIP3 is increased upon insulin stimulation only in endothelial cells that can be visualized by co-staining with the vascular marker, lectin B-4. Although the blood-retinal barrier might prevent insulin from acting on neuronal cells in retina, it seems likely that this was not observed due to the acute nature (5 min) of the stimulus. In other studies by our laboratory2 and others (33), insulin stim-

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T. Kondo and C. Ronald Kahn, unpublished data.
Fig. 5. Phosphorylation of p38 and ERK-1 protein in retina and liver of ob/ob (A) and STZ (B) mice as compared with their controls. Retina and liver proteins from these mice were isolated and subjected to immunoblotting with α-phospho-p38 (top panels) and α-phospho-ERK1 (bottom panels). Asterisks indicate differences significant at \( p < 0.05 \). Data are the mean ± S.E. of at least three independent experiments using 12–16 animals for each group and are expressed as relative to control values, which were set at 100%.
ulation of PIP3 can be observed in hypothalamus following intravenous insulin injection, but this effect requires 15–30 min. In any case, PIP3 levels upon acute insulin stimulation in ob/ob and STZ retina are increased, suggesting that PI 3-kinase activity is increased in parallel with the phosphorylation of IRS proteins. Despite this up-regulation of IRS phosphorylation and PIP3 generation, phosphorylation of Akt is substantially impaired in retina and liver of ob/ob and STZ mice. Indeed, while ob/ob mice have obvious insulin resistance, STZ diabetic mice may have a mixture of insulin deficiency and insulin resistance due to the hyperglycemia and other metabolic alterations. Thus, there is a decrease in phosphorylation and stimulation of PDK1 in retina, but not in liver, of ob/ob and STZ mice. Indeed, while ob/ob mice have obvious insulin resistance, STZ diabetic mice may have a mixture of insulin deficiency and insulin resistance due to the hyperglycemia and other metabolic alterations. Thus, there is a decrease in phosphorylation and stimulation of PDK1 in retina, but not in liver, of ob/ob and STZ mice. Interestingly, protein levels of PDK1 and Akt in the retinal tissue of ob/ob and STZ mice were significantly decreased. These observations indicate that retinal tissue is more susceptible to the diabetic milieu in terms of PDK1 and Akt protein expression and/or stability. PDK1 activation depends on binding to PIP3 in the context of the plasma membrane. It is possible that PDK1 is mislocated and therefore misregulated in diabetic endothelial cells (35). It is also possible that the multiple PDK isoforms, such as PDK2, with select tissue distribution may contribute to the diversified actions of insulin signaling in different tissue (35).

The Ras-MAP kinase pathway is a central component of most growth factor signaling and is also involved in the regulation of gene transcription. The stress-induced p38 MAPK is phosphorylated by insulin, possibly through the MAP kinase kinase (MKK) 3/6 (36, 37). ERK1 is a major signaling molecule in Ras-MAP kinase pathway. Phosphorylation of p38 and ERK1 is increased upon insulin stimulation in retina of ob/ob or STZ mice, whereas these are not enhanced in ob/ob and STZ liver. Thus, there is a pathway of selective insulin resistance in endothelium with impaired PDK1 and Akt mediated signaling, but normal to increased ERK1 and p38 mediated signaling. This type of divergence, with enhanced insulin signaling via Shc and MAP kinase to the prenyltransferases, has also been suggested to play a role in peripheral vascular complications of diabetes (38, 39), and thus could be important in both micro- and macro-vascular complications in diabetes.

It has been shown previously that relative hypoxia can mimic hyperglycemic in terms of development of retinal neovascularization (40). Hypoxia regulates VEGF expression by enhancing transcription, and this occurs via binding of HIF-1α to a regulatory site in the VEGF promoter (41). VEGF then acts as a potent stimulator of both normal and abnormal vascular growth (42, 43). The induction of HIF-1α in retina of ob/ob and
STZ mice indicates that this tissue perceives a relative hypoxia stress. The fact that this is not simply the effect of hyperglycemia is indicated by the fact that HIF-1α expression does not change in the livers of these mice (data not shown). The induction of HIF-1α positively correlates with the increase in VEGF, and VEGF can directly stimulate retinal neovascularization. On the other hand, eNOS expression in retina of ob/ob and STZ mice is decreased. This reduction might reflect the decreased Akt phosphorylation, since Akt directly activates eNOS in endothelial cells (44) and could cause vascular dysfunctions such as decreased retinal blood flow.

Taken together, our data demonstrated that insulin signaling in retina of type 1 and type 2 diabetic mice are differentially regulated from signaling in peripheral tissues and may activate cell growth. The induction of vascular mediators, such as VEGF, also enhances the retinal neovascularization. Thus, these observations explain discrepancies in insulin resistance in vascular tissues versus other tissues and illustrate how the combination of hyperglycemia and altered insulin activation of the MAP kinase pathway in retinal endothelial cells and VEGF induction may contribute to altered retinal vascular function in diabetes and development of diabetic retinopathy.

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