Characterization of Multiple Signaling Pathways of Insulin in the Regulation of Vascular Endothelial Growth Factor Expression in Vascular Cells and Angiogenesis

The effects of insulin on vascular endothelial growth factor (VEGF) expression in cultured vascular cells and in angiogenesis were characterized. Insulin increased VEGF mRNA levels in mouse aortic smooth muscle cells from $10^{-9}$ to $10^{-7}$ x with an initial peak of 3.7-fold increases at 1 h and a second peak of 2.8-fold after 12 h. The first peak of VEGF expression was inhibited by LY294002, an inhibitor of phosphatidylinositol (PI) 3-kinase, and by the overexpression of dominant negative forms of p85 subunit of PI 3-kinase or Akt. Inhibitors of MEK kinase, PD98059, or overexpression of dominant negative forms of Ras were ineffective. In contrast, the chronic effect of insulin on VEGF expression was partially inhibited by both LY294002 or PD98059 as well as by the overexpression of dominant negatives of PI 3-kinase or Ras. The importance of PI 3-kinase-Akt pathway on VEGF expression was confirmed in mouse aortic smooth muscle cells isolated from insulin receptor substrate −1 knockout (IRS-1 KO) mice that showed parallel reductions of 48–49% in insulin-stimulated VEGF expression and PI 3-kinase-Akt activation. Insulin-induced activation of PI 3-kinase-Akt on hypoxia-induced VEGF expression and neovascularization was reduced by 40% in the retina of neonatal hypoxia model using IRS-1−/− mice. Thus, unlike other cells, insulin can regulate VEGF expression by both IRS-1/PI 3-kinase-Akt cascade and Ras-MAPK pathways in aortic smooth muscle cells. The in vivo results provide direct evidence that insulin can modulate hypoxia-induced angiogenesis via reduction in VEGF expression in vivo.

Vascular abnormalities and pathologies are commonly associated with diabetes or insulin-resistant states (1). Loss of endothelial function and poor arterial collateral formation are frequently observed in multiple tissues and contribute to the morbidity and mortality of patients with diabetes (2, 3). The major cell types involved in these changes include endothelial cells (EC), vascular smooth muscle cells, and capillary pericytes. The dysfunction of the vasculature is likely associated with the loss of the direct action of insulin on vascular cells that have been shown to be insulin-responsive (4, 5).

Insulin can induce a host of effects on vascular cells, including traditional metabolic actions such as glucose transport and utilization (6, 7), protein synthesis, and cellular proliferation (8–10). In addition, insulin also has vascular-specific actions that are particular to vascular tissues; for example, the activation and the induction of endothelial nitric-oxide synthase (eNOS) in EC (5, 11) and the regulation of blood flow (12). We have reported that insulin can stimulate several signaling cascades in EC and vascular smooth muscle cells similar to other insulin-sensitive cells after the activation of insulin receptor via its tyrosine kinase β subunit (IRβ) by phosphorylating both insulin receptor substrate-1 and -2 (IRS-1 and IRS-2) (13). Tyrosine-phosphorylated IRS-1 and -2 interact with several downstream cellular proteins containing the Src homology 2 domain including the p85 regulatory subunit of PI 3-kinase and activate its p110 catalytic subunit resulting in the formation of several phosphorylated complexes and the activation of Akt kinase (13). Utilization of IRS-PI 3-kinase-Akt pathways by insulin has been shown in EC to mediate the activation and expression of eNOS (5, 13, 14). In contrast, the mitogenic actions of insulin in EC and ASMC growth are mostly stimulated by the activation of Ras-MAPK cascade (10). We have also reported that in both micro- and macrovessels from insulin-resistant rodents there is a selective inhibition of insulin-induced activation of the IRS-PI 3-kinase-Akt pathway, whereas the Ras-MAPK pathway can be fully activated (13).

The vascular-specific action of insulin may be attributable to the direct action of insulin or mediated through the growth factors and cytokines of which the expression is regulated by insulin (4). Recently, insulin has been reported to increase the expression of vascular endothelial growth factor (VEGF) in multiple cell types including epithelial cells (15), fibroblasts (16), and transformed cells (17). VEGF is an angiogenic factor that plays key roles both in the maintenance of vascular homeostasis (18) and in pathological angiogenesis such as cancer metastasis (19) and diabetic retinopathy (20). This regulation could be clinically relevant because intensive insulin treatment is associated with worsening of diabetic retinopathy and is related to VEGF expression (15). Insulin-induced VEGF expression is associated with the loss of the direct action of insulin on vascular cells that have been shown to be insulin-responsive (4, 5).

1 The abbreviations used are: EC, endothelial cells; VEGF, vascular endothelial growth factor; ASMC, mouse aortic smooth muscle cells; PI, phosphatidylinositol; IRS, insulin receptor substrate; MAP, mitogen-activated protein; MAPK, MAP kinase; WT, wild type; DMEM, Dulbecco’s minimal essential medium; MOPS, 4-morpholinepropanesulfonic acid; RT, reverse transcriptase; eNOS, endothelial nitric-oxide synthase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; HIF-1α, hypoxia-inducible factor-1α.
expression has been reported to be mediated through the activation of phosphorylidyinositol (PI) 3-kinase/Akt that is downstream to the insulin receptor. It has been reported by multiple groups that the activation of PI 3-kinase/Akt pathway can up-regulate the expression of hypoxia-inducible factor-1α (HIF-1α) and HIF-1α/aryl hydrocarbon nuclear translocator (15, 16, 21, 22), and then increases VEGF expression through a direct interaction with VEGF promoter (15). However, this action may be cell type-dependent because Takahashi and colleagues (23) clearly showed that activation of Akt by insulin and IGF-1 can enhance VEGF expression independent of HIF in C2C12 myoblasts.

The involvement of MEK/MAPK pathway in insulin-induced VEGF expression has not been reported. Fukuda et al. (24) reported that increases of p44/42 MAPK induced the expression of HIF-1α and VEGF in colon cancer cells; however, this effect appears to be related to the overexpression of IGF receptor in transformed cells because it is not seen in other cell types such as cultured retinal pigment epithelial cells or in insulin-infused retinal tissue in vivo (15). In this study, we have made a novel finding showing that insulin stimulation resulted in a bi-phasic up-regulation of VEGF expression in ASMc and further characterized the potential signaling pathways utilized by insulin to induce this expression. Our data suggested that the activation of both PI 3-kinase/Akt and p44/42 MAPK pathways is required in this regulation with PI 3-kinase/Akt activation being responsible for short term stimulation, whereas both pathways are essential for the long term up-regulation of VEGF. The physiological significance of these effects is also discussed in an in vivo hypoxia-induced retinal angiogenesis model in IRS-1-/- null mice.

EXPERIMENTAL PROCEDURES

Materials—Human recombinant insulin (human R) was purchased from Lilly. Monoclonal antibody against phosphotyrosine (aPY) and polyclonal antibodies against p85 subunit of PI 3-kinase were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Polyclonal antibodies against rat IRS-1 (JD229) and IRS-2 (JD110) were raised as described previously (25). Polyclonal antibody against human insulin receptor β subunit and rabbit anti-mouse antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against p44/42 MAPK kinase, threonine/tyrosine-phosphorylated MAP kinase, and serine-phosphorylated Akt (PKB) were from New England Biolabs (Beverly, MA). Donkey anti-rabbit Ig horseradish peroxidase–linked antibody, enhanced chemiluminescence (ECL) kit, 125I-labeled protein A, and protein A-Sepharose 6MB were from Amersham Biosciences. 32P-labeled rabbit anti-Akt antibodies, and protein A-Sepharose 6MB were from Amersham Biosciences.

RNA Extraction and Northern Blot Analysis—Semen-starved cells were treated with insulin for appropriate times, and total RNA was isolated with the TriReagent kit (Molecular Research Center, Inc.) 20 μg of total RNA was resolved in 1% agarose gel in MOPS/formaldehyde buffer and transferred to nylon membrane (Hybond N, Amersham Biosciences) in 20 × SSC buffer, and membrane was UV cross-linked. The membrane was prehybridized for 2 h at 65 °C in a High Efficiency Hybridization Buffer. Rat VEGF cDNA (29) or 36B4 cDNA probes were labeled with [α-32P]dCTP using random priming DNA labeling kit (Amersham Biosciences) and purified with nick column (Amersham Biosciences). Radiolabeled probes (1 × 106 cpm/ml) were added to membrane in the prehybridization buffer, and hybridization was continued and washed three times in 0.5 × SSC and 5% SDS. VEGF or 36B4 bands were quantified using ImageQuant software (Molecular Dynamics) and PhosphorImager (Amersham Biosciences). VEGF values were obtained by subtracting background signal in each lane from the corresponding band signal in that lane. Normalization was done using 36B4 value derived in the same manner (29).

Transient Transfections and Measurement of VEGF Promoter Activity—Mouse pGL2 VEGF promoter-luciferase construct (pGL2-VEGF, 1.6 kb) was described previously (30) and kindly provided by Dr. Patricia A. D’Amore and Eric Yin-Shan Ng (Schein Eye Institute, Harvard Medical School, Boston). The cells in 6-well plates were grown to 50–70% confluence in 5% fetal bovine serum/DMEM and were transfected with 2.0 μg of Renilla luciferase control reporter (pRL-SV40, Promega Co., Madison, WI) for 24 h using the FuGENETM 6 transfection reagent (Roach Diagnostics) according to the manufacturer’s instructions. Cells were starved for 24 h in 0.2% bovine serum albumin serum-free DMEM before treatment with insulin (100 nM) for 16 h. The cells were then harvested in lysis buffer and frozen-thawed once, and insoluble cell debris was removed by centrifugation (15,000 × g, 10 min). Luciferase activity in the lysate was measured with Dual-luciferase reporter assay system (Promega Co., Madison, WI). VEGF promoter-luciferase activity was normalized by the Renilla luciferase activity.

Immunoprecipitation of Insulin Receptor and IRS-1 and IRS-2—After the addition of insulin or other reagents, cells were washed with ice-cold phosphate-buffered saline and broken with 1 ml of lysis buffer (1% NP-40, 150 mM NaCl, 50 mM Tris, pH 7.4, containing 10 mM sodium fluoride, 2 mM EDTA, 2 mM sodium orthovanadate, 10% (v/v) glycerol, 1% (w/v) Triton X-100, 2 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin). The lysate was then precipitated with antibodies (αIRS-2 or αIRβ subunit) followed by incubation with protein A-Sepharose 6MB. The beads were washed for 3 times with lysis buffer before treatment with Laemmli buffer and heated in boiling water for 4 min. The protein extracts were used for electrophoresis and immunoblotting studies as described below (25).

Immunoblotting—Immunoprecipitated fractions (as described above) were separated with SDS-PAGE and electrotransferred to nitrocellulose filter which was incubated with blocking buffer (phosphate-buffered saline (PBS), pH 7.4, containing 0.2% Tween 20) and 3% bovine serum albumin overnight at 4 °C. To detect protein tyrosine phosphorylation, the blot was first incubated with monoclonal antibody aPY, then with rabbit anti-mouse antibody (Dako, Carpinteria, CA), and finally with 125I-labeled protein A at room temperature. To detect protein levels of IR, IRS-1, IRS-2 protein, or p85 subunit of PI 3-kinase, blots were incubated with polyclonal αIRα, αIRβ-2, or αp85 antibodies, respectively, and then with 125I-labeled protein A. Blots were washed with PBST after each incubation, and 125I-labeled protein A-bound proteins were quantified using the PhosphorImager as described above. To detect ERK-1/2 protein level, tyrosine phosphorylation of ERK-1/2, or serine phosphorylation of Akt, total cell lysates (50 μg of protein) were subjected to SDS-PAGE and electrophoresing (2% (w/v) SDS and 10% (w/v) SDS-PAGE) and transferred to nitrocellulose membranes, which were incubated with antibodies against ERK-1/2, tyrosine-phosphorylated ERK-1/2, or phospho-(Ser-473)-Akt respectively, overnight at 4 °C. Then horseradish peroxidase-linked anti-rabbit Ig antibodies were added for 1 h at room temperature. The membranes were then washed with buffer (phosphate-buffered saline, 1% (w/v) Tween 20, and 10% (w/v) non-fat dry milk) for 1 h at room temperature. Finally, the level of serine-phosphorylated Akt was detected with the ECL™ kit and quantified with a densitometer with readings performed in the linear range.

Mouse Model of Retinal Neovascularization—The in vivo studies were performed in a model of hyperoxia-induced retinal neovascularization (21). Litters of 7-day-old mice (day 7) IRS-1−/− or 129/C57 IRS-1−/− mice and their nursing mothers were exposed to 75 ± 2% oxygen for 5 days. At P12, the mice were returned to ambient air. The use of these animals were approved by Joslin Institutional Animal Care and Use Committee.
Retinal Flat Mount—After induction of retinal neovascularization, eyes were enucleated at P17 after intracardiac perfusion of the mice with fluorescein-dextran in 4% paraformaldehyde as described previously (31). Retinas were isolated, flat-mounted with VECTA SHIELD (Vector Laboratories, Burlingame, CA), and photographed under a fluorescence microscope (Olympus AX70THE, Japan).

Quantification of Neovascularization—As described previously (31), mice at P17 (n = 5) were deeply anesthetized with (100 mg/kg) pentobarbital sodium (Abbott) and sacrificed by cardiac perfusion of 4% paraformaldehyde in phosphate-buffered saline. Eyes were enucleated and fixed in 4% paraformaldehyde overnight at 4 °C. Retinal sections were stained with periodic acid-Schiff reagent and hematoxylin. Ten intact sections of equal length, each 30 μm apart, were evaluated for a span of 300 μm. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section by a fully masked protocol. Images of VEGF or 36B4 bands were representative of three independent experiments and were quantified using ImageQuant software. VEGF mRNA levels were normalized to 36B3 mRNA levels. Data (mean ± S.D., n = 5) are expressed as relative to control, assigning a value of 100% to the control cells.

Insulin Resistance and VEGF mRNA Expression

Insulin Increases VEGF mRNA Transcription but Not Its Stability—To determine whether insulin had an effect on VEGF mRNA stability, ASMC was treated with or without insulin for 1 h followed by the addition of actinomycin D to inhibit transcription. Fig. 2A showed that VEGF mRNA levels decreased rapidly with a half-life of 1 h in both control and insulin-treated mouse ASMC, suggesting insulin did not alter VEGF mRNA stability. Incubation with insulin (100 nM for 1 h) was not able to increase VEGF mRNA level when RNA polymerase activity was inhibited by actinomycin D in the cells (Fig. 2B), suggesting the effect of insulin on VEGF gene expression was due to an increase in the rate of transcription. To confirm that the increases of VEGF mRNA levels were due to an effect on gene transcription, mouse VEGF promoter luciferase construct was introduced into mouse ASMC, and luciferase activity was assessed in cell lysates after insulin stimulation. As shown in Fig. 2C, insulin increased VEGF promoter activity significantly by 2.7-fold.

Fig. 1. Insulin regulates VEGF mRNA expression in mouse ASMC in a time-dependent (A) and concentration-dependent (B) manner. Cells were treated with 100 nM insulin for the indicated time (A) or treated with different concentrations of insulin for 1 h (B). Total RNA (20 μg) was resolved by agarose gel electrophoresis, transferred to nylon membranes, and hybridized to 32P-labeled rat VEGF 165 cDNA probe. Images of VEGF or 36B4 bands were representative of three independent experiments and were quantified using ImageQuant software. VEGF mRNA levels were normalized to 36B3 mRNA levels. Data (mean ± S.D., n = 5) are expressed as relative to control, assigning a value of 100% to the control cells.

RESULTS

Concentration- and Time-dependent Stimulation of VEGF mRNA Expression—Insulin (100 nM) increased expression of VEGF mRNA levels by 3.7-fold after 1 h (Fig. 1A). A decline from the maximum occurred after 3 h of incubation, reaching a nadir after 6 h at 160% of basal levels. Interestingly, VEGF mRNA levels rebounded to 2.8-fold of basal after 12–24 h of incubation with insulin. Insulin also stimulated VEGF mRNA expression in a concentration-dependent manner with significant increases at 1 nM (Fig. 1B), and the maximal level (~3.7-fold increase) was achieved at 100 nM of insulin.

Effect of PI 3-Kinase and MAP Kinase Pathway Inhibitors—Insulin stimulated the activation of p44/42 MAPK in a time-dependent manner with a peak at 10 min and declined to basal levels between 30 and 60 min (data not shown). Two signal transduction pathways involved in insulin-induced VEGF mRNA expression in mouse ASMC were examined using inhibitors of PI 3-kinase and MAP kinase pathways. Preincubation of mouse ASMC with LY292004 (30 μM), a PI 3-kinase inhibitor (5), decreased VEGF mRNA level in ASMC stimulated with insulin for 1 h by 70% (Fig. 3A). However, pretreatment of the cells with MEK inhibitor PD98059 (50 μM) (32) did not alter the short term effect of insulin on VEGF mRNA expression. Interestingly, both LY292004 and PD98059 inhibited 46 and 58%, respectively, of the VEGF mRNA levels induced by chronic insulin (12 h) incubation with ASMC cells (Fig. 3B). PD98059, even after 12 h, was still able to inhibit insulin-induced phosphorylation of p44/42 MAPK (10 min of stimulation) but did not
have any effects on insulin-stimulated Akt phosphorylation (data not shown).

The effect of IGF-1 on VEGF expression was also examined. IGF-1 (25 nM) increased VEGF mRNA levels significantly at 1 h by 1.7-fold. Addition of LY294002 appears to partly decrease this up-regulation. In addition, PD98059 also inhibited IGF-1 action on VEGF expression completely (Fig. 3).

To confirm the results derived from pharmacological inhibitors, we utilized adenovirus-mediated overexpression of dominant negative mutants of both PI 3-kinase and MAP kinase pathways to examine their effects on insulin-induced VEGF mRNA expression. Overexpression of the Src homology 2 domain deleted p85 subunit of PI 3-kinase (DN-p85) but not N17-Ras mutant (DN-Ras) (32) inhibited short term (1 h) effect of insulin on VEGF mRNA (Fig. 4A). However, both mutants partially inhibited the chronic effects (12 h) of insulin on VEGF mRNA expression (Fig. 4B), consistent with the conclusion that both activation of PI 3-kinase and MAPK pathways are involved, similar to the results from pharmacological inhibitors.

Selective Insulin Resistance in ASMC of IRS-1+/−/− Mice—To clarify the signaling pathway of the effect of insulin on VEGF mRNA expression in vascular cells, we cultured and characterized ASMC derived from the aorta of IRS-1+/−/− mice, a genetic model of insulin resistance (33). As shown in Fig. 5A, IRS-1+/−/− ASMC did not have IRS-1 expression detectable by immunoblotting. There were no differences between wild type and IRS-1+/−/− ASMC at insulin receptor levels as quantitated by immunoblotting after immunoprecipitation with antibodies to p85 subunit of insulin receptor (IR) (data not shown) or in the levels of the receptor tyrosine phosphorylation stimulated with insulin (100 nM) (Fig. 5A). Tyrosine phosphorylation of IRS proteins (IRS-1 and IRS-2) and their association with PI 3-kinase in wild type and IRS-1+/−/− ASMC were evaluated by immunoblotting with antibodies against phosphotyrosines and p85 subunit of PI 3-kinase, respectively, after immunoprecipitation with aIRS-1 and aIRS-2 antibodies. Only IRS-2 tyrosine phosphorylation was detectable in IRS-1+/−/− ASMC after the addition of insulin, whereas both IRS-1 and IRS-2 tyrosine phosphorylation were demonstrated in wild type cells (Fig. 5A). Insulin-stimulated association of PI 3-kinase to IRS proteins in
Insulin Resistance and VEGF mRNA Expression

Fig. 4. Modulation of insulin-induced VEGF mRNA expression by adenovirus-mediated overexpression of β-galactosidase (DN-p85), Akt-1 (DN-Akt), and c-Ras (DN-Ras). Cells were infected with adenovirus for 24 h before treating with or without 100 nM insulin for 1 h (A) or 12 h (B). VEGF mRNA levels were normalized to 36B4 mRNA levels. Data (mean ± S.D., n = 3) are expressed as relative to control, assigning a value of 100% to the control cells (adenovirus-β-galactosidase-transfected cells).

Fig. 5. Analysis of insulin receptor signaling on PI 3-kinase pathway in ASMC from wild type and IRS-1−/− mice. Cell lysate was processed as described under “Experimental Procedures” for immunoblotting, and the presence of IRS-1 was assessed using antibodies against IRS-1 (A). Serum-starved cells were treated with or without 100 nM insulin for 1 h (A). Cell lysate was immunoprecipitated with αIRS-1 and αIRS-2, or αIRβ (py-IR-β) antibodies followed by immuno- blotting with antibodies to α PY (py-IR-β and py-IRS-1/2) or a p85 subunit of PI-3 kinase (p85 subunit of PI-3K), respectively. Phosphorylation of Akt in WT and IRS-1−/− ASMC was examined in cells treated with or without 100 nM insulin for 20 min. 50 μg of protein in cell lysate was used for immunoblotting with phospho-specific Akt (Ser-473) antibodies (phospho-Akt). Results shown are representative of three experiments. Quantitation of the results are shown in B.

IRS-1−/− cells was ~45% that observed in wild type ASMC (Fig. 5A). To compare the downstream activation of PI 3-kinase pathway between wild type and IRS-1−/− ASMC, the level of Akt phosphorylation after stimulation by insulin for 20 min was characterized. Insulin increased Akt phosphorylation in both wild type and IRS-1−/− cells. However, the levels of Akt phosphorylation increased by insulin were consistently 2-fold greater in wild type cells than those observed in IRS-1−/− cells (Fig. 5A). Quantitation of the changes is displayed in Fig. 5B.

Insulin signaling via the MAP kinase pathway in IRS-1−/− ASMC cells was also determined by measuring threonine/tyrosine phosphorylation of ERK-1/2 after stimulation with 0, 2, and 100 nM insulin for 10 min. Insulin stimulated the phosphorylation of ERK-1/2 equally well in wild type and IRS-1−/− ASMC (Fig. 6A).

Effect of Insulin on VEGF mRNA Expression in ASMC of IRS-1−/− or Wild Type Mice—Because both PI 3-kinase and MAP kinase pathways are involved in insulin-induced VEGF gene expression in ASMC, we determined whether selective impairment of insulin signaling on the PI 3-kinase pathway in IRS-1−/− ASMC affected the action of insulin on VEGF mRNA expression. Again, insulin induced time-dependent increases in VEGF gene expression in both wild type and IRS-1−/− cells at 1, 3, and 12 h in a biphasic pattern. Although there was no significant difference in the basal levels of VEGF mRNA expressed in control and IRS-1−/− ASMC, the short term and chronic effects of insulin on VEGF mRNA expression were reduced at 1 and 12 h by 49% and 46%, respectively, in IRS-1−/− cells as compared with ASMC derived from wild type mice (Fig. 6B). Thus in ASMC, insulin can significantly increase VEGF expression both at short (1 h) and long term (12 h) via the PI 3-kinase-Akt pathway, and this effect is mediated in part by an IRS-1-dependent pathway.

Comparative Analysis of Hypoxia-induced Retinal Neovascularization in IRS-1−/− and Wild Type Mice—To determine whether these findings are applicable in vivo, we characterized the neovascularization response in the retina of neonatal wild type and IRS-1−/− mice, which were exposed to hyperoxia for 5 days and then returned to normal ambient O2 conditions. Fig. 7 depicted the flat mounts of the retina from wild type and IRS-1−/− mice, and vascular development was similar between these mice when the animals were exposed to ambient O2 levels without an initial treatment of hyperoxia at 75% O2 (Fig. 7, C and D).

However, when the neonatal mice were initially exposed to 75% O2 for 5 days, and then returned to ambient O2 (20%), there was more avascular or sparsely vascularized areas in the
IRS-1\(^{-/-}\) mice as compared with the wild type (Fig. 7, A and B). This difference in the density of neovascularization was confirmed by analyzing the cross-sections of the retina from these two types of mice (Fig. 8, A and B). The arrangement and cellular content of cell layers in the neural retina appear to be similar in the IRS-1\(^{-/-}\)/H11002/H11002 and the wild type mice. However, the number of vascular cells extending beyond the internal limiting membrane or into the vitreous body were significantly less in IRS-1\(^{-/-}\)/H11002/H11002 mice. Quantitation revealed a 40% reduction in the number of nuclei beyond the internal limiting membrane of IRS-1\(^{-/-}\)/H11002/H11002 mice as compared with the wild type (Fig. 8 C).

Because previous studies using this neonatal hyperoxia-hypoxia model have shown that a major angiogenic mitogen in the retina in response to hypoxia is VEGF, we analyzed the expression of VEGF mRNA in the retina from the wild type and IRS-1\(^{-/-}\) mice by quantitative RT-PCR (29, 31). The results (Fig. 9) indicated that two isoforms of VEGF, VEGF\(_{164}\) and VEGF\(_{120}\), were expressed in retina of these mice. Statistical analysis of the VEGF mRNA levels showed a significant reduction of 25–30% in both VEGF\(_{164}\) and VEGF\(_{120}\) in the IRS-1\(^{-/-}\)/H11002/H11002 mice compared with wild type, consistent with the reduction of neovascularization in the IRS-1\(^{-/-}\)/H11002/H11002 mice.

**DISCUSSION**

The importance of studying the effect of insulin on VEGF expression is due to the well documented association among
endothelial abnormalities, vascular complications, and states of insulin resistance and diabetes (1, 3, 10). VEGF expression is clearly increased in the retina and renal glomeruli but decreased in the muscle, myocardium, and healing wounds of diabetic animals (34–37). In addition, we have reported previously that VEGF expression is also decreased in the myocardium of insulin-resistant animals and diabetic patients, because treatment with insulin normalized the levels of VEGF in all these tissues, suggesting that insulin may regulate VEGF expression in vivo (37). Indeed, several reports (13, 21, 22) have demonstrated that insulin can induce VEGF expression in fibroblasts and tumor cells. Our study has characterized the role for insulin in the regulation of VEGF expression in vascular smooth muscle cells and, furthermore, the underlying signaling mechanisms.

These data indicate that insulin can up-regulate VEGF expression in a physiological range from 1 to 20 nM (38) and suggested that this action is mediated via insulin receptor rather than IGF-1 receptor (8). We also showed that both insulin and IGF-1 can induce VEGF expression. This result is consistent with several previous reports (13, 21, 22) using nonvascular cells where both insulin and IGF-1 increased VEGF expression. Similar to nonvascular cells, the effect of insulin is to increase VEGF transcription rate without altering its half-life or degradation rates (Fig. 2).

In ASMC, insulin induced VEGF transcription rate in a biphasic nature (Fig. 1). This is a novel finding and clearly different from that observed in non-vascular cells (13, 21, 22). The rapid phase of the effect of insulin on insulin is mediated solely by the PI 3-kinase activation (Figs. 3 and 4), in accordance with the previous reports in fibroblasts using pharmacological inhibitors of PI 3-kinase (16). However, in ASMC, insulin has a second and sustained effect on VEGF expression that appears to be mediated by both PI 3-kinase and Ras/MAP kinase pathways (Figs. 3 and 4). This conclusion is supported by the effective inhibition of PI 3-kinase and Ras-MEK pathways using either inhibitors or dominant negative adenoviral mutants (Figs. 3 and 4). This action might be vascular specific because MEK kinase inhibitor failed to reduce insulin-induced VEGF expression in NIH3T3 fibroblasts (16). The delayed effect of insulin on VEGF expression is similar to the report of Fukuda et al. (24) showing the effects of IGF-1 after 6 h of stimulation. In addition, we also showed that the action of IGF-1 at 1 h is via MAPK but not PI 3-kinase, which again differed from the action of insulin (Fig. 3C). The effect of insulin on the phosphorylation of p44/42 MAP was maintained for less than 30 min (data not shown), yet inhibition of this pathway suppressed long term expression of VEGF (Figs. 3 and 4). These results suggest that the regulation of the late phase of VEGF expression might be a secondary effect of MAPK activation. The delayed effects of MAPK activation have been observed in other occasions such as in PDGF-BB-induced DNA replication in smooth muscle cells that occurred 12–18 h later following MAPK phosphorylation (39).

The results from the ASMC and the in vivo studies using IRS-1−/− mice provide the first physiological evidence that insulin and the expression and activation of IRS-1 contribute significantly to the expression of VEGF and hypoxia-induced neovascularization. This conclusion is supported by three lines of evidence. First, insulin-induced VEGF expression is decreased by 40–50% in ASMC cultured from IRS-1−/− mice in concordance with the loss of PI 3-kinase-Akt activation. Second, VEGF expression in the retina of IRS-1−/− mice is significantly lower than the retina of wild type. Third, retinal neovascularization in response to hypoxia is reduced by 40% in IRS-1−/− mice versus wild type mice in parallel with the VEGF expression. These studies establish, for the first time, the importance of insulin, IRS-1, and probably IRS-2 in the expression of VEGF and hypoxia-mediated neovascularization process in vivo. In addition, loss of IRS-1 may also impair the effects of VEGF on the target cells. It was recently reported that VEGF-R2 may recruit IRS-1 upon binding to VEGF, and this in turn will activate PI3 kinase. Inhibition of IRS-1 expression in cultured cells abolished VEGF-induced protein synthesis (40). The in vivo data using IRS-1−/− mice did not test the role of MEK pathway directly, because these mice only exhibit selective impairment of the PI 3-kinase-Akt pathway, whereas MAP kinase activation remained normal. However, there is retention of more than 50% of the basal VEGF expression in IRS-1−/− mice (Fig. 9) suggesting that MAPK also play substantial roles on insulin and IGF-1-regulated hypoxia-induced neovascularization.

The functional significance of the selective loss of the action of insulin on the PI 3-kinase-Akt pathway, but not in the ERK-1/2 MAP kinase pathway, is confirmed by the finding that ASMC isolated from IRS-1−/− mice responded normally to insulin in parallel with the VEGF expression. These data using IRS-1−/− mice did not test the role of MEK pathway directly, because these mice only exhibit selective impairment of the PI 3-kinase-Akt pathway, whereas MAP kinase activation remained normal. However, there is retention of more than 50% of the basal VEGF expression in IRS-1−/− mice (Fig. 9) suggesting that MAPK also play substantial roles on insulin and IGF-1-regulated hypoxia-induced neovascularization.

The effect of insulin on VEGF expression has been suggested as a potential explanation for the worsening of diabetic retinopathy at the initial period of intensive glycemic control in a subset of type 1 diabetic patients (15, 22, 41). However, consistent data to support an elevated retinal insulin level in insulin-treated group have not been documented. We have shown that improvement in glycemic control, which causes a decrease in insulin resistance in the vasculature, will enhance the activation of insulin of IRS-PI 3-kinase-Akt pathway and actions, such as VEGF and eNOS expression. Thus, the worsening of retinopathy attributed to intensive glycemic control could be indirectly due to improvements in insulin action on the
vascular cells but not due to the elevation of insulin levels. Thus, intensive glycemic control by an oral agent in type 2 diabetic patients, which can also improve the actions of insulin, may also enhance the initial period of worsening retinopathy.

In summary, these studies provide strong evidence that insulin can induce VEGF expression in vascular cells by activating PI 3-kinase-Akt and Ras/MAP kinase pathways. The in vitro studies have provided the first evidence that insulin plays a modulating role in retinal neovascularization in response to hypoxia. This pathway may be important in the vascular complications associated with insulin-resistant states, diabetes, and intensive glycemic control with insulin.

Acknowledgments—We thank Ron Burke and Aria Nikmo for the assistance in the preparation of this manuscript.

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Characterization of Multiple Signaling Pathways of Insulin in the Regulation of Vascular Endothelial Growth Factor Expression in Vascular Cells and Angiogenesis

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J. Biol. Chem. 2003, 278:31964-31971.
doi: 10.1074/jbc.M303314200 originally published online May 29, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M303314200

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