Insulin and Insulin-like Growth Factor-I Induce Vascular Endothelial Growth Factor mRNA Expression via Different Signaling Pathways

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In this study we have investigated the molecular mechanisms of insulin and insulin-like growth factor-I (IGF-I) action on vascular endothelial growth factor (VEGF) gene expression. Treatment with insulin or IGF-I for 4 h increased the abundance of VEGF mRNA in NIH3T3 fibroblasts expressing either the human insulin receptor (NIH-IR) or the human IGF-I receptor (NIH-IGFR) by 6- and 8-fold, respectively. The same elevated levels of mRNA were maintained after 24 h of stimulation with insulin, whereas IGF-I treatment further increased VEGF mRNA expression to 12-fold after 24 h. Pre-incubation with the phosphatidylinositol 3-kinase inhibitor wortmannin abolished the effect of insulin on VEGF mRNA expression in NIH-IR cells but did not modify the IGF-I-induced VEGF mRNA expression in NIH-IGFR cells. Blocking mitogen-activated protein kinase activation with the MEK inhibitor PD98059 abolished the effect of IGF-I on VEGF mRNA expression in NIH-IGFR cells but had no effect on insulin-induced VEGF mRNA expression in NIH-IR cells. Expression of a constitutively active PKB in NIH-IR cells induced the expression of VEGF mRNA, which was not further modified by insulin treatment. We conclude that VEGF induction by insulin and IGF-I occurs via different signaling pathways, the former involving phosphatidylinositol 3-kinase/protein kinase B and the latter involving MEK/mitogen-activated protein kinase.

Angiogenesis, the development of new blood vessels by sprouting from pre-existing endothelium, is a significant component of a wide variety of biological processes including embryonic vascular development and differentiation, wound healing, and organ regeneration (1). However, many diseases are driven by persistent unregulated angiogenesis. In arthritis, new capillary blood vessels invade the joint, destroying cartilage (2), and tumor growth and metastasis are angiogenesis-dependent (3, 4). Diabetic retinopathy is characterized by progressive alterations in the retinal microvasculature, leading to the formation of areas of retinal non-perfusion, increased vasopermeability, and the pathologic intra-ocular proliferation of retinal vessels. Increased vasopermeability and uncontrolled neovascularization can result in severe and permanent visual loss in diabetic patients (5).

A variety of growth factors are associated with angiogenesis, including tumor necrosis factor, transforming growth factor β, and basic fibroblast growth factor. However, many of these factors are believed to induce angiogenesis indirectly (1, 6).

Vascular endothelial growth factor (VEGF) is a unique potent angiogenic factor that stimulates capillary formation in vivo and has direct mitogenic actions that are restricted to endothelial cells (7, 8). Human VEGF has at least four structurally related isoforms, VEGF121, VEGF165, VEGF189, and VEGF206 resulting from alternative splicing of the VEGF gene (9, 10). Of these, VEGF165 has the most potent biological activity and is the most abundant subtype in vivo (11). VEGF121 is also well expressed in many normal and pathological tissues; however, its biological activity has been shown to be 10–100-fold weaker than that of VEGF165 (11).

VEGF is secreted by many cell types, and its expression is regulated by a number of growth factors and cytokines. For example, interleukin 1-β (12), platelet-derived growth factor (PDGF), and transforming growth factor β (13) stimulate VEGF production by smooth muscle cells. Furthermore, stimulation of NIH3T3 fibroblasts by PDGF also results in the induction of the VEGF gene in a Ras- and Raf-dependent manner (14). However, the precise molecular mechanisms involved in the induction of VEGF expression by growth factors remains poorly understood.

Insulin plays a central role in regulating metabolic pathways associated with energy storage and utilization but also with cell growth control (15). A perturbation of normal insulin-induced metabolic responses is central to the pathology of type 2 diabetes (16). Following ligand binding, the insulin receptor kinase is activated, leading to the phosphorylation of intracellular proteins including IRS-1, IRS-2, and Shc. These initial events lead to the stimulation of multiple signaling cascades that mediate the cellular responses to insulin (16, 17). Insulin promotes the transcription of a variety of genes, including those encoding the glucose transporter GLUT1 and VEGF, by inducing the hypoxia-inducible factor HIF-1α/ARNT (18).

Insulin-like growth factor-I (IGF-I) is a homologue of insulin and shares many signaling components and cellular responses with insulin itself (19). IGF-I is also able to induce the expres-

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1 The abbreviations used are: VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; IGF, insulin-like growth factor; IGFRI, IGF receptor; IR, insulin receptor; PKB, protein kinase B; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; PI, phosphatidylinositol; MAP, mitogen-activated protein kinase; MOPS, 4-morpholinopropanesulfonic acid; HUVEC, human umbilical vein endothelial cells; TBS, Tris-buffered saline; ARNT, aryl hydrocarbon receptor nuclear translocator.
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sion of different genes, including that encoding VEGF (18). Indeed, IGF-I increases the expression of VEGF mRNA and production of VEGF protein by COLO 205 colon carcinoma cells (20) and enhances expression of VEGF in osteoblasts (21). Furthermore, IGF-I levels are shown to be elevated in the vitreous of patients with proliferative diabetic retinopathy (22). Although many of the effects of insulin and IGF-I are similar, these proteins have differing effects in vivo, and it is therefore important to define the molecular basis for this.

Considering the key role of VEGF in controlling angiogenesis (23), we wished to identify the growth factor-activated signaling pathways involved in controlling VEGF gene expression. In this study we have used NIH3T3 fibroblasts expressing equal numbers of either the insulin (NIH-IR) or IGF-I (NIH-IGFR) receptors, allowing for direct comparison of signaling by these two polypeptides. Having determined that VEGF expression is regulated in these cells, we have sought to more clearly define the signaling pathways used by insulin and IGF-I to modulate VEGF gene expression.

EXPERIMENTAL PROCEDURES

Materials—VEGF<sub>165</sub> cDNA was a gift from J. Plouet (CNRS-Lab. Biologie Moleculaire, Toulouse, France). PKBmyr was a gift from B. Hemmings (F. Miescher Inst., Basel, Switzerland). Monoclonal anti–hemagglutinin 12CA5 antibody and the phospho-specific PKB (Ser473) antibody were obtained as described in Filippa et al. (24). Culture media were from Life Technologies, Inc. Reagents for SDS-PAGE were purchased from Bio-Rad. Unless otherwise stated, all chemicals were from Sigma.

Cell Culture—NIH3T3 fibroblasts expressing either human insulin receptor or human IGF-I receptor were described in Tartare et al. (25). Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% v/v bovine calf serum (HyClone), 100 units/ml penicillin, 100 units/ml streptomycin in a 37 °C, 5% CO2 atmosphere. To inhibit PI Sigma.

were from Life Technologies, Inc. Reagents for SDS-PAGE were pur-

ishment, BioWhittaker) and were used between passages 2 and 4. Assay—

nM IGF-I for the times indicated. RNA was extracted, and 20 ng/ml PDGF for the times indicated. RNA was extracted, and 20 μg of RNA samples were resolved by formaldehyde agarose gel electrophoresis in MOPS buffer, transferred to a polyvinylidene difluoride membrane. Membranes were blocked for 1 h in TBS containing 4% w/v bovine serum albumin and then incubated either with a monoclonal antibody to VEGF (Pharmingen, San Diego, CA), with the phospho-specific PKB (Ser473) antibody, or with an antibody to phospho-phytolated MAP kinase (New England Biolabs, Beverly, MA) as indicated. After extensive washing in TBS containing 0.1% w/v Triton X-100, detection was performed with horseradish peroxidase-conjugated anti–mouse antibody and ECL Western blotting detection reagents (Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

Determination of PI 3-Kinase Activity—Cells were stimulated with 100 nm insulin or 10 nm IGF-I for 15 min at 37 °C as indicated and solubilized for 40 min at 4 °C in 50 mM HEPES, pH 7.5, 150 mM NaCl, 100 mM Na<sub>P</sub>O<sub>4</sub>, 2 mM sodium orthovanadate, 100 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 100 IU/ml apronitin, 20 μM leupeptin, and 1% (v/v) Triton X-100 (TAT buffer) for 15 min at 4 °C. The lysates were clarified by centrifugation at 15,000 rpm for 15 min at 4 °C, then samples were resolved by SDS-PAGE and transferred to a nylon mem-

brane (Immobilon-P, Millipore Corp., Bedford, Mass.). Membranes were blocked for 1 h in TBS (10 nm Tris-HCl, 140 mM NaCl, pH 7.4) containing 4% w/v bovine serum albumin and then incubated either with a monoclonal antibody to VEGF (Pharmingen, San Diego, CA), with the phospho-specific PKB (Ser473) antibody, or with an antibody to phospho-phytolated MAP kinase (New England Biolabs, Beverly, MA) as indicated. After extensive washing in TBS containing 0.1% w/v Triton X-100, detection was performed with horseradish peroxidase-conjugated anti–mouse antibody and ECL Western blotting detection reagents (Amersham Pharmacia Biotech), according to the manufacturer’s instructions.

RESULTS

To determine whether insulin and/or IGF-I were able to induce VEGF production in NIH3T3 fibroblasts, cells expressing either the human insulin receptor (NIH-IR) or IGF-I receptor (NIH-IGFR) (25) were treated with insulin or IGF-I, respectively, for various times. Expression of VEGF was then determined by Northern blotting. As shown in Fig. 1, incubation with insulin for 4 h increased the abundance of VEGF mRNA in NIH-IR cells by 6-fold over that in untreated cells (panel A). These levels of mRNA expression were maintained after 24 h of insulin treatment and decreased within 48 h. Similarly, treatment of NIH-IGFR cells with IGF-I for 4 h led to an 8-fold increase in VEGF mRNA accumulation (Fig. 1, panel B). However, treatment with IGF-I for 24 and 48 h further increased VEGF mRNA expression up to 12-fold greater than basal levels. Thus, both insulin and IGF-I stimulate VEGF mRNA expression in NIH-IR or NIH-IGFR fibroblasts, respectively, allowing use of these cells to study the mechanisms underlying these effects.

We next wished to determine whether insulin or IGF-I increased the stability of VEGF mRNA. NIH-IR or NIH-IGFR cells were treated with insulin or IGF-I, respectively, for 4 h, then transcription was terminated by the addition of actinomycin D. Following inhibition of transcription the loss of insulin- or IGF-I-induced VEGF mRNA was rapid and not significantly different from that observed in the untreated cells. The failure of both growth factors to increase the half-life of VEGF mRNA suggests that insulin and IGF-I increase transcription of the VEGF gene in these cells.

To determine whether new protein synthesis was involved in insulin- or IGF-I-induced VEGF mRNA transcription, cells were incubated with cycloheximide before growth factor treat-
The addition of cycloheximide alone led to a 2-fold enhancement of VEGF mRNA, thus, substantially less than that observed in insulin- or IGF-I-treated cells. After treatment with cycloheximide, both insulin (Fig. 3, panel A) and IGF-I (Fig. 3, panel B) induced an increase in VEGF mRNA expression similar to that observed in the absence of cycloheximide. Thus, although inhibition of protein synthesis by cycloheximide alone led to a slight increase in VEGF mRNA expression, the stimulation by either insulin or IGF-I was retained. These data suggest that the stimulation of VEGF mRNA expression by insulin and IGF-I is not induced by increased synthesis of a regulatory protein but rather via the acute activation of intracellular signaling pathways.

We compared also GLUT1 mRNA expression in NIH-IR versus NIH-IGFR cells. The expression of GLUT1 mRNA increased after 4 h of stimulation with either insulin (Fig. 3, panel A) or IGF-I (Fig. 3, panel B), respectively, and decreased after 24 h as described previously (18). Thus, unlike VEGF, the time course of stimulation of GLUT1 gene expression is similar for both insulin and IGF-I in NIH3T3 fibroblasts, suggesting that the observed differences were not due to differences in the cell line used.

To determine whether VEGF mRNA induction by insulin and IGF-I led to increased VEGF secretion by the cells, we analyzed the level of VEGF in culture supernatants of insulin- and IGF-I-treated cells with an endothelial cell proliferation assay. Medium from NIH-IR cells treated with insulin for 4 h increased the number of HUVEC by 1.4-fold, as shown in Fig. 4. The same increase was observed when HUVEC were incubated with conditioned medium from cells treated with insulin for 24 h. Supernatants of NIH-IGFR cells treated for 4 or for 24 h with IGF-I increased the number of HUVEC by 1.3- and 1.6-fold, respectively (Fig. 4, panel A). Insulin and IGF-I alone or media obtained from untreated NIH-IR and NIH-IGFR cells did not significantly increase proliferation of HUVEC. Furthermore, when VEGF was immunoprecipitated from media obtained from NIH-IR cells treated with insulin for 4 or from NIH-IGFR treated for 24 h with IGF-I, the proliferative effects of these media were abolished (Fig. 4, panel A).

To further study the expression of VEGF protein by these cells we made lysates of insulin- or IGF-I-treated NIH-IR and NIH-IGFR, respectively, and analyzed them for VEGF protein expression by Western blotting. After 4 h of insulin treatment in NIH-IR, the amount of VEGF increased by 4-fold compared with basal levels. The same increase was observed when cells were stimulated with insulin for 24 h (Fig. 4, panel B). In NIH-IGFR cells treated for 4 or for 24 h with IGF-I, the VEGF protein level was increased by 3- and 7-fold, respectively (Fig. 4, panel B). Interestingly, the time courses for VEGF expression correlate well with the expression of VEGF mRNA induced by each ligand (compare with Fig. 3). These observations demonstrate that the stimulatory effect of insulin and IGF-I on VEGF gene expression in NIH fibroblasts leads to the production and secretion of active VEGF protein under these conditions.
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**A**

| VEGF mRNA | GLUT1 mRNA | 28 S |
|-----------|------------|------|
| Insulin (hours) | Cycloheximide | - | - | + | + | + |

**B**

| VEGF mRNA | GLUT1 mRNA | 28 S |
|-----------|------------|------|
| IGF-I (hours) | Cycloheximide | - | - | + | + | + |

**Fig. 3.** Induction of VEGF mRNA by insulin and IGF-I is not dependent on new protein synthesis. Cells were incubated with 100 nM insulin (panel A) or 10 nM IGF-I (panel B) in the absence or presence of 10 μg/ml cycloheximide for the indicated times before isolation of total RNA, which was resolved by agarose electrophoresis and hybridized to a cDNA probe for the VEGF_165_. After autoradiography, membranes were stripped and rehybridized to a labeled fragment of the cDNA for GLUT1. 28 S ribosomal RNA is shown as a loading control.

To more clearly define the signaling mechanisms involved in the insulin- and IGF-I-stimulated VEGF mRNA expression, we examined the role of two key enzymes involved in growth factor signaling, namely PI 3-kinase and MAP kinase. To this end, cells were preincubated with either the selective PI 3-kinase inhibitor wortmannin or the MEK inhibitor PD98059 before growth factor treatment. As shown in Fig. 5, insulin treatment led to a robust activation of IRS-1-associated PI 3-kinase in NIH-IR cells and, similarly, activation of PKB (Fig. 5, panels A and B). Preincubation with wortmannin completely blocked the activation of PKB by insulin. Insulin also led to the stimulation of MAP kinase, which could be completely prevented by pretreatment with PD98059 (Fig. 5, panel C).

In an almost identical fashion, in NIH-IGFR cells IGF-I stimulated PI 3-kinase and, consequently, PKB activity, in a wortmannin-sensitive manner (Fig. 5, panels D and E). IGF-I also stimulated MAP kinase activity, which could be blocked by pre-treatment with PD98059 (Fig. 5, panel F). When PKB activities were measured by kinase assays, insulin- and IGF-I-treatment led to 3.8- and 4.2-fold increases in activity in NIH-IR and NIH-IGFR cells, respectively (data not shown). Therefore, the extent of the stimulation of PKB by insulin and IGF-I was very similar.

As shown in Fig. 6, pre-incubation of NIH-IR cells with PD98059 to prevent MAP kinase activation did not affect the stimulation of VEGF mRNA expression by insulin (Fig. 6, panel A) despite MAP kinase activity being completely inhibited in these cells (Fig. 5, panel C). When PI 3-kinase was inhibited with wortmannin, however, the stimulation of VEGF mRNA expression by insulin was almost completely abolished (Fig. 6, panel B). Insulin-induced GLUT1 mRNA expression was also inhibited by wortmannin, as previously demonstrated (28), and unmodified by PD98059 (Fig. 6, panel C).

In contrast, pre-treatment of NIH-IGFR cells with PD98059 abolished the IGF-I-induced expression of VEGF mRNA (Fig. 7, panel A), whereas wortmannin had no effect (Fig. 7, panel B). As with insulin, IGF-I-induced GLUT1 mRNA expression was blocked in the presence of wortmannin, whereas PD98059 had no effect (Fig. 7, panel C).

Taken together, these data strongly suggest that different pathways exist for the stimulation of VEGF mRNA expression by insulin and IGF-I. Although insulin requires the stimulation of PI 3-kinase activity to induce the expression of VEGF mRNA, IGF-I does not. However, insulin does not require the activation of MAP kinase to increase VEGF mRNA expression, whereas MAP kinase activity is critical for that induced by IGF-I. In contrast, both insulin and IGF-I use the same PI 3-kinase-dependent pathway to activate GLUT1 gene expression.

To further confirm that the differences observed between the effects of insulin and IGF-I on VEGF mRNA expression were not due to differences between the cell lines, we examined signaling by a different growth factor, PDGF, in these two cell lines. It has been demonstrated previously that the stimulation of VEGF mRNA expression by PDGF occurs via protein kinase Cα (29). Consistent with this, we observed an almost identical stimulation of VEGF mRNA expression by PDGF in the two cell types that was unaffected by either wortmannin or PD98059 (Fig. 8).

To investigate the role of other proteins downstream of PI 3-kinase in insulin-dependent VEGF gene regulation, we used the selective inhibitor of p70S6K rapamycin, to determine its possible involvement. Rapamycin had no effect either on insulin-
or on IGF-I-induced VEGF mRNA expression (data not shown).

Next we examined the involvement of PKB in this process. To this end, we transiently transfected NIH-IR cells with a constitutively active form of PKB (PKBmyr). The expression of the constitutively active enzyme induced a strong increase in VEGF mRNA in the absence of insulin that was not further enhanced by insulin treatment (Fig. 9, panel A). Similar results were obtained for GLUT1 mRNA, which was strongly induced by PKBmyr expression and not further stimulated by insulin (Fig. 9, panel A). We confirmed that the PKBmyr was expressed and constitutively phosphorylated in transfected cells by Western blotting with antibodies to HA epitope and phospho-Ser473 PKB, respectively (Fig. 9, panel B).

These results indicate that the activation of PKB alone is sufficient to induce the VEGF gene expression in NIH3T3 cells and so could mediate insulin effect on VEGF expression. As IGF-I-induced VEGF expression is independent of PI 3-kinase, this pathway cannot be involved. Indeed, we have shown that this effect occurs via MAP kinase activation.

**DISCUSSION**

Angiogenesis is a fundamental process required for organ development and differentiation during embryogenesis as well as for wound healing and reproductive functions in adults (1, 30). However, a variety of disorders are driven by unregulated angiogenesis including tumor development, rheumatoid arthritis, and proliferative diabetic retinopathy (2, 5, 31). The work of several laboratories in recent years has demonstrated the pivotal role of VEGF in the regulation of both normal and pathological angiogenesis (32). Hypoxia is probably the best-characterized regulator of VEGF expression apparently inducing transcription of the VEGF mRNA via binding of the hypoxia-inducible transcription factor HIF-1 to a binding site located in the VEGF promoter (33–36). Induction of VEGF by hypoxia has been reported to involve different mechanisms, including either a Src/Raf/MAP kinase pathway (37) or a PI 3-kinase/PKB pathway (38). VEGF expression by cells is also regulated by a plethora of
external factors, including cytokines and growth factors such as bFGF, PDGF, tumor necrosis factor α, transforming growth factor-β, interleukin-1β, and interleukin-6 (32). However, the exact mechanism involved in growth factor regulation of VEGF expression remains unclear.

IGF-I is a potent mitogen that induces tumor growth and promotes the transformed phenotype (39, 40). Furthermore, elevated IGF-I levels are strongly linked to proliferative diabetic retinopathy (22, 41). Overexpression of the insulin receptor has also been reported in tumors (42–44), and chronic hyperglycemia and hyperinsulinemia are linked to diabetic micro- and macrovascular complications (5, 45, 46). Both insulin and IGF-I have been shown to induce VEGF mRNA expression in different cell systems (20, 21, 47, 48). Once again the molecular mechanisms underlying these effects are not clear.

In this work we have tried to clarify the signaling pathways involved in insulin and IGF-I induction of VEGF expression. Despite the high degree of similarity in structure and substrate specificity, the insulin and the IGF-I receptors do not appear to have redundant functions in vivo (49, 50). However, the biochemical and biological comparison of the two receptors is complicated by the cross-reactivity of the two ligands for the two receptors (51, 52) and by the formation of heterodimers when the receptors are expressed in the same cells (53). To circumvent these problems, we have compared the effect of insulin and IGF-I in NIH3T3 fibroblasts expressing comparable numbers of either human insulin or IGF-I receptors (25). We used NIH3T3 fibroblasts because they are a well established model that has been used to study the regulation of VEGF expression in the past (14, 29, 38, 54). Both insulin and IGF-I induced VEGF mRNA expression in NIH3T3 fibroblasts. However, although both polypeptides led to similar increases after 4 h of treatment, at later time points insulin-induced VEGF mRNA expression leveled off and then began to fall within 48 h treatment. In contrast, IGF-I-induced VEGF mRNA expression continued to rise at later time points and, therefore, appeared to be a more sustained response. These differences not only suggested different regulatory mechanisms but indicated that these two polypeptides could have different angiogenic potentials in the same cell type by inducing VEGF secretion in a more or less sustained manner. Both insulin and IGF-I induce VEGF expression in retinal cells. However, increased angiogenesis in proliferative diabetic retinopathy appears to correlate with increased circulating IGF-I rather than insulin levels, as diabetic subjects with decreased IGF-I serum concentrations are less prone to the development of proliferative diabetic retinopathy (22). It is tempting to speculate that a more sustained induction of VEGF expression by IGF-I than insulin could contribute to this effect.

As previously demonstrated in other cell lines (18, 20, 21, 47, 48), we have shown that both insulin and IGF-I are able to induce the expression of VEGF mRNA and to increase the level of VEGF protein in supernatants of NIH3T3 cells. This occurs by increasing the rate of VEGF mRNA transcription without modifying its stability or requiring new protein synthesis. We have subsequently analyzed the role of the signal-transducing molecules PI 3-kinase and MAP kinase, which are known to be involved in both insulin and IGF-I signaling (16). We observed that inhibition of PI 3-kinase with wortmannin did not modify the effect of IGF-I on VEGF expression, whereas the effect of insulin was almost completely abolished. In contrast, blocking MAP kinase activation prevented IGF-I- but not insulin-induced VEGF expression. These results suggest a pivotal role for PI 3-kinase in mediating the effect of insulin on VEGF gene regulation, whereas IGF-I signaling to VEGF gene expression occurs via a PI 3-kinase independent pathway requiring MAP kinase activation. This is consistent with several reports indicating that insulin preferentially activates an IRS/PI 3-kinase pathway to modulate its metabolic effects, DNA synthesis, and cell proliferation (50, 55, 56). In contrast, the IGF-I receptor appears to mediate more effective phosphorylation of Shc, its association with Grb2, and consequent activation of the MAP kinase pathway (50). This selectivity may partly explain the essential role of the latter enzyme in IGF-I, but not insulin-induced VEGF expression. However, IGF-I can also use PI3-kinase-dependent pathways to regulate other genes, as we find that both
insulin- and IGF-I-induced GLUT-1 mRNA expression is dependent on PI 3-kinase activation in these cells. This is consistent with data previously obtained in other cell types (28, 55).

PKB is a key downstream effector of PI 3-kinase-mediated effects. PKB is activated by at least two different kinase activities, PDK1 and PDK2; the latter may be comprising PDK1 and an activating peptide (57, 58). This occurs following recruitment of both PKB and PDK to the cell membrane via association with the PI 3-kinase product PI 3,4,5-trisphosphate (59). We have demonstrated that PKB may be involved in insulin regulation of VEGF mRNA expression, as the expression of a constitutively active PKB (27) potently induces VEGF expression in NIH-IR fibroblasts and no further induction is observed following insulin treatment. Insulin-induced GLUT-1 mRNA expression has previously been shown to be dependent on PKB activation (28). As with VEGF mRNA, expression of PKBmyr strongly induced GLUT-1 mRNA in NIH-IR, consistent with both these genes being regulated via PKB.

As far as we are aware, we are the first to report the involvement of PKB in the induction of VEGF expression. PKB is involved in the regulation of a variety of other genes and mediates both positive effects of insulin on gene expression, such as induction of GLUT-1 mRNA and insulin-induced inhibition of genes such as IGFBP-1 (60). PKB may act directly by phosphorylating transcription factors, as in the case with the forkhead transcription factor (FKHR), where it leads to reduced transcription of the genes under its control (61). Alternatively, PKB may act indirectly, as is thought to occur in the induction of NFκB-regulated anti-apoptotic genes (62).

With respect to IGF-I-induced VEGF expression, it is tempting to speculate that the transcription factor HIF-1α may mediate this effect. Transcription from the VEGF promoter can be stimulated via HIF-1α in cells where MAP kinase is selectively activated (63, 64). Thus, because MAP kinase activation is essential for IGF-I-induced VEGF expression, HIF-1α is an obvious candidate. HIF-1α has also been implicated in insulin-induced VEGF expression (18). This could also be the case in our cell system, as it would require HIF-1α to be sensitive to both the PKB and MAP kinase pathways.

We believe that this is also the first report of insulin and IGF-I using different signaling pathways to regulate the expression of the same gene. As both mechanisms are activated by each growth factor, a key question is how and indeed why the cell should use them selectively in this way. This is particularly puzzling as the extent of stimulation induced by both insulin and IGF-I is very similar. Cross-talk between the PKB and MAP kinase signaling pathways has very recently been demonstrated, and it appears that phosphorylation of Raf, which lies upstream of MAP kinase, by PKB leads to its inhibition and, thus, down-regulates MAP kinase activity (65). In myocytes this mechanism appears to be specific for differentiated cells (66), and so it is likely that it will not operate in all cell types. This kind of cross-talk could be involved in insulin-induced VEGF expression where PKB mediated inhibition of the MAP kinase pathway following stimulation could leave VEGF expression reliant on a PKB dependent pathway. However, the real situation is likely to be more complicated as IGF-I also stimulates PKB activity in these cells but still uses a MAP kinase-dependent pathway to regulate VEGF expression, suggesting that it has not been suppressed in this way. It appears more probable that other unidentified signaling components are activated or inhibited specifically by insulin and IGF-I, which are also involved in VEGF expression.

While this paper was in review Nakae et al. (67) reported that in hepatocytes the transcription factor FKHR is differentially regulated by insulin and IGF-I. The phosphorylation of one threonine residue in particular (Thr-24) appears to be induced by insulin but not IGF-I. As this residue can be phosphorylated by PKB in vitro and PKB is also activated by IGF-I in these cells, the authors propose that a PKB-like kinase specifically activated by insulin may mediate this effect. This has obvious similarities to the work presented here where PKB can stimulate VEGF mRNA expression but is only necessary for that induced by insulin. Evidently a selectively insulin-stimulated, wortmannin-sensitive kinase with PKB-like substrate specificity could be at work in our cells, although there is no clear candidate for this role. Alternatively, we favor the explanation that there are subtle but effectual differences in the nature of the activation of these enzymes, such as subcellular localization and the presence of co-operating factors, which lend specificity to the signaling by insulin and IGF-I. However, both possibilities clearly merit further examination.

Our data suggest that one possible benefit of using different signaling pathways could be to allow one polypeptide to stimulate VEGF expression in a more sustained fashion than the other, as described above. Given the wide variety of exogenous factors known to regulate VEGF expression and angiogenesis, using different pathways would also allow for cross-talk from other factors to act differently on insulin- versus IGF-I-induced VEGF expression.

In summary, we have demonstrated that VEGF induction by insulin and IGF-I occurs via different signaling pathways, the former involving PI 3-kinase/PKB and the latter involving MAP kinase.

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