**Regulation of Insulin-Like Growth Factor I Receptors on Vascular Smooth Muscle Cells by Growth Factors and Phorbol Esters**

John J. Ververis, Li Ku, and Patrick Delafontaine

Insulin-like growth factor I (IGF I) is an important mitogen for vascular smooth muscle cells. To characterize regulation of vascular IGF I receptors, we performed radioligand displacement experiments using rat aortic smooth muscle cells (RASMs). Serum deprivation for 48 hours caused a 40% decrease in IGF I receptor number. Exposure of quiescent RASMs to platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), or angiotensin II (Ang II) caused a 1.5-2.0-fold increase in IGF I receptors per cell. After FGF exposure, there was a marked increase in the mitogenic response to IGF I. IGF I downregulated its receptors in the presence of platelet-poor plasma. Stimulation of protein kinase C (PKC) by exposure of quiescent RASMs to phorbol 12-myristate 13-acetate caused a biphasic response in IGF I binding: there was a 42% decrease in receptor number at 45 minutes and a 238% increase at 24 hours. To determine the role of PKC in growth factor–induced regulation of IGF I receptors, we downregulated PKC by exposing RASMs to phorbol 12,13-dibutyrate (PDBu) for 48 hours. PDGF- and FGF- but not Ang II–mediated upregulation of IGF I receptors was completely inhibited in PDBu-treated cells. Thus, acute PKC activation by phorbol esters inhibits IGF I binding, whereas chronic PKC activation increases IGF I binding. PDGF and FGF but not Ang II regulate vascular IGF I receptors through a PKC-dependent pathway. These data provide new insights into the regulation of vascular smooth muscle cell IGF I receptors in vitro and are of potential importance in characterizing vascular proliferative responses in vivo. (Circulation Research 1993;72:1285–1292)

**KEY WORDS**  • insulin-like growth factor I  • phorbol esters  • vascular smooth muscle cells  • platelet-derived growth factor  • angiotensin II  • fibroblast growth factor

A variety of peptidic growth factors have been shown to be important mitogens for vascular smooth muscle cells (VSMCs) in culture. These include competence growth factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) and progression factors such as insulin-like growth factor I (IGF I). Studies in BALB/c3T3 fibroblasts have clearly shown that competence factors stimulate entry of cells into the G1 phase of the cell cycle; however, progression through S phase and subsequent cellular division requires the presence of progression factors, of which IGF I is the most potent. The unique role of IGF I in the control of VSMC growth has been demonstrated by Clemmons,5 most notably in experiments using an anti–IGF I antibody to inhibit PDGF-induced VSMC DNA synthesis.6 This group has demonstrated that PDGF may stimulate immunoreactive IGF I release from VSMCs,5 and we have shown that this effect occurs at the mRNA level.6 Because VSMCs synthesize and secrete IGF I,7 this mitogen may then function in an autocrine/paracrine manner in addition to its role as a circulating growth factor. Other potentially important mitogens for VSMCs include the potent vasoactive agonist angiotensin II (Ang II).8 We have previously shown that Ang II also regulates IGF I mRNA levels and IGF I release from quiescent VSMCs in vitro.9 Thus, both PDGF and Ang II regulate VSMC IGF I expression.

IGF I mediates its effects after binding to a specific heterotetrameric cell-surface receptor that possesses intrinsic tyrosine kinase activity.10 Prior data have demonstrated that PDGF and FGF exposure increases 125I–IGF I binding to VSMCs.11 The main purpose of this study was to characterize regulation of VSMC IGF I receptors by growth factors. Furthermore, because activation of protein kinase C (PKC) is thought to play an important role in the cellular response to mitogens,12 we proposed to characterize the role of PKC in the regulation of VSMC IGF I receptors. Our findings demonstrate that IGF I binding to VSMCs is regulated by competence growth factors, by Ang II, and by phorbol esters. Upregulation of IGF I receptors is accompanied by increased responsiveness to IGF I. The effect of PDGF and FGF to increase cellular IGF I binding sites is distinct from that induced by Ang II, because the effect is PKC dependent and is therefore inhibited after PKC downregulation. These findings provide new insights into mechanisms regulating VSMC IGF I binding.

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Materials and Methods

Cell Culture

VSMCs were isolated from rat thoracic aorta as described previously.\textsuperscript{13,14} Cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 2 mM glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin. They were incubated at 37°C in a humidified atmosphere of 95% air–5% CO\textsubscript{2} and were passaged twice a week by harvesting with trypsin–versene and seeding at a 1:8 ratio in 75-cm\textsuperscript{2} flasks. For experiments, cells between passage levels 5 and 15 were seeded into 100-mm 12-well or 24-well cluster dishes.

To determine the effects of serum deprivation, 80–90% confluent rat aortic smooth muscle cells (RASMs) were washed three times in serum-free medium (SFM) and incubated in SFM for 48 hours or maintained in medium with 10% calf serum. To determine the effects of agonists on IGF I binding, 80–90% confluent RASMs were quiesced by serum deprivation for 48 hours and then exposed to fresh SFM in the presence or absence of 10 ng/ml PDGF-BB, 5 ng/ml basic FGF (bFGF), 100 nM Ang II, 20 ng/ml recombinant human IGF I (rhIGF I), or 100 nM phorbol 12-myristate 13-acetate (PMA) for various times before binding studies. For some experiments, quiescent RASMs were exposed to fresh SFM containing 1% platelet-poor plasma in the presence or absence of 20 ng/ml rhIGF I for 24 hours. To determine the effects of agonists in PKC-downregulated cells, 200 nM phorbol 12,13-dibutyrate (PDBu) was added during the latter half of the 48-hour period of serum deprivation and maintained during the 24-hour exposure to the agonist. All incubations before binding assays were performed at 37°C.

Radio ligand Binding Assay

Cells were washed three times with phosphate-buffered saline (PBS) and suspended by incubation with PBS and 25 mM EDTA at 37°C for 60 minutes. After two washes and centrifugation, cells were resuspended in binding buffer (20 mM HEPES, 120 mM NaCl, 5 mM KCl, 1.2 mM MgSO\textsubscript{4}, 10 mM NaHCO\textsubscript{3}, 1.3 mM CaCl\textsubscript{2}, 1.2 mM KH\textsubscript{2}PO\textsubscript{4}, and 0.25% bovine serum albumin, pH 7.4) at a final dilution of 3.0–4.5×10\textsuperscript{5} cells/100 μl. Binding assays were performed by incubating 100 μl cell suspension with 100 μl binding buffer containing 10\textsuperscript{–10} M 125I–IGF I (2,300–3,100 Ci/mmol) and increasing concentrations of unlabeled IGF I (0–2×10\textsuperscript{–7} M) for 90 minutes at 20°C. On-rate experiments were performed by incubating cells with 10\textsuperscript{–10} M 125I–IGF I in the presence or absence of 2×10\textsuperscript{–7} M unlabeled IGF I for various times. Bound and free radioactivity was separated by rapid filtration through a GF/C filter (Whatman Inc., Clifton, N.J.) preincubated with 1% gelatin, 10% horse serum, and 1 μM insulin. Filters were counted in an automated gamma counter with 80% efficiency. To demonstrate that results obtained using cell suspensions were representative of binding to cell monolayers, additional binding experiments were performed using cells grown in 12-well plates. These were quiesced for 48 hours, exposed to 5 ng/ml bFGF in fresh SFM for 24 hours, washed, and then exposed to 10\textsuperscript{–10} M 125I–IGF I and increasing concentrations of unlabeled IGF I for 90 minutes at 20°C. Cells were then washed in ice-cold binding buffer and solubilized in 2N NaOH before counting. All assays were performed in duplicate for each experimental point. Data were analyzed using the LIGAND program.

Measurement of DNA and Protein Synthesis

Cells cultured in 24-well plates were quiesced by serum deprivation for 48 hours as described above and exposed to agonists in the presence of 1 μCi/ml [3H]thymidine or [3H]leucine for 24 hours. To determine the effects of agonists after PKC downregulation, cells were first exposed to 200 nM PDBu for 48 hours as described above. To determine whether bFGF exposure altered the mitogenic response to IGF I, quiescent RASMs exposed to 5 ng/ml bFGF for 24 hours were then exposed to fresh SFM in the presence of increasing concentrations of rhIGF I (0–50 ng/ml) and 1 μCi/ml [3H]thymidine. After agonist exposure, cells were washed three times with ice-cold PBS, incubated for 15 minutes with 10% trichloroacetic acid on ice, washed two times with ice-cold 95% ethanol, and allowed to air-dry. Cellular radioactivity was extracted by incubation with 0.4N NaOH and counted by liquid scintillation spectrophotometry. All experiments were performed in triplicate for each experimental point.

Cross-linking of 125I–IGF I to VSMC Monolayers

To confirm our results obtained using suspension binding assays and to determine whether 125I–IGF I bound to cell-surface IGF binding proteins, quiescent RASM monolayers were incubated in the presence or absence of 10 ng/ml PDGF-BB, 5 ng/ml bFGF, or 100 nM Ang II for 24 hours. Cells were then exposed to 10\textsuperscript{–10} M 125I–IGF I and increasing concentrations of unlabeled IGF I or insulin for 90 minutes at 20°C, and ligand–receptor complexes were covalently cross-linked with disuccinimidyl suberate (final concentration, 0.5 mM) for 10 minutes at room temperature. The reaction was quenched with 10 mM Tris HCl/25 mM EDTA for 5 minutes, and cell lysates were analyzed by reducing polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli\textsuperscript{15} in 4–15% gradient gels. Gels were stained, dried, and autoradiographed for 2–4 days.

Materials

rhIGF I was kindly provided by Dr. H.P. Guler, CIBA-GEIGY Corp., Summit, N.J., and 125I–IGF I was furnished by the Yerkes RIA Laboratory, Emory University, Atlanta, Ga. [3H]Thymidine (20 Ci/mmol) and [3H]leucine (5 Ci/mmol) were obtained from DuPont–New England Nuclear, Boston. PDGF-BB and bFGF were purchased from Collaborative Research Inc., Bedford, Mass. Ang II, PDBu, PMA, gelatin, horse serum, and insulin were purchased from Sigma Chemical Co., St. Louis, Mo.

Statistical Analysis

Data are expressed as mean±SEM. Statistical differences were calculated using Student’s t test, and a value of p<0.05 was considered significant.

Results

IGF I Binding to RASMs

Initial experiments were performed to determine the rate of association of IGF I with its receptor on RASMs.
binding parameters, we measured displacement of $^{125}$I-IGF I from its vascular receptor by increasing concentrations of unlabeled ligand. A representative homologous displacement experiment is shown in Figure 1B. Scatchard analysis of the binding data indicated one class of high-affinity binding sites (Figure 1C).

### Regulation of IGF I Receptors by Serum

To determine the relation between growth state and IGF I binding, we measured IGF I receptor number and affinity in proliferating RASMs exposed to 10% calf serum and in RASMs quiesced by exposure to SFM for 48 hours. Serum deprivation caused a 40% decrease in receptor number ($B_{\text{max}}$ for 10% calf serum, 23.8±2.1 fmol/10^5 cells; $B_{\text{max}}$ for SFM, 14.4±2.0 fmol/10^5 cells; $n=5$; $p<0.01$). This decrease in receptor number in quiescent cells was accompanied by a significant increase in binding affinity ($K_d$ for 10% calf serum, 18.2±2.3 nM; $K_d$ for SFM, 9.5±0.7 nM; $n=5$; $p<0.025$).

### Regulation of IGF I Receptors by Growth Factors

To determine whether growth factors regulate IGF I receptors on VSMCs, we exposed quiescent RASMs to SFM in the presence or absence of 10 ng/ml PDGF-BB, 5 ng/ml bFGF, and 100 ng/ml Ang II for 24 hours and performed radioligand displacement experiments. As shown in Table 1, PDGF, bFGF, and Ang II caused a highly significant increase in IGF I receptor number on quiescent VSMCs, averaging 74%, 65%, and 58%, respectively. These growth factors did not significantly alter IGF I binding affinity. To confirm that this pattern of regulation was representative of anchorage-dependent cultures, additional experiments were performed by measuring binding of $^{125}$I-IGF I to cell monolayers after bFGF exposure. These experiments demonstrated

### Table 1. Regulation of Insulin-Like Growth Factor I Receptors on Quiescent Vascular Smooth Muscle Cells by Growth Factors

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>$K_d$ (nM)</th>
<th>$B_{\text{max}}$ (fmol/10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bFGF (n=6)</td>
<td>5.2±1.0</td>
<td>8.7±1.4</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 ng/ml bFGF</td>
<td>5.8±1.1</td>
<td>14.4±2.3*</td>
</tr>
<tr>
<td>PDGF-BB (n=6)</td>
<td>3.1±0.5</td>
<td>5.6±1.5</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ng/ml PDGF-BB</td>
<td>3.5±0.6</td>
<td>9.8±2.9†</td>
</tr>
<tr>
<td>Ang II (n=8)</td>
<td>5.6±1.3</td>
<td>5.3±1.2</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 nM Ang II</td>
<td>4.6±1.0</td>
<td>8.3±2.2*</td>
</tr>
<tr>
<td>rhIGF I (n=4)</td>
<td>10.1±0.9</td>
<td>11.4±1.2</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 ng/ml rhIGF I</td>
<td>8.4±2.4</td>
<td>11.1±2.1</td>
</tr>
<tr>
<td>PPP (n=4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1% PPP</td>
<td>24.2±5.3</td>
<td>53.2±13.4</td>
</tr>
<tr>
<td>1% PPP/20 ng/ml rhIGF I</td>
<td>12.2±2.8§</td>
<td>25.5±6.3§</td>
</tr>
</tbody>
</table>

$K_d$, equilibrium dissociation constant; $B_{\text{max}}$, receptor number; bFGF, basic fibroblast growth factor; PDGF-BB, platelet-derived growth factor-BB; Ang II, angiotensin II; rhIGF I, recombinant human insulin-like growth factor I; PPP, platelet-poor plasma. Binding data were analyzed using the LIGAND program and are presented as mean±SEM.

* $p<0.025$, † $p<0.05$ vs. corresponding control value; § $p<0.025$, $\& p<0.05$ vs. 1% PPP.

A representative on-rate experiment is shown in Figure 1A and demonstrates that equilibrium binding was reached after 60 minutes at 20°C. To determine IGF I

### Figure 1. Graphs characterizing insulin-like growth factor I (IGF I) binding to vascular smooth muscle cells. Panel A: Representative on-rate experiment. Suspended rat aortic smooth muscle cells (3–4.5×10^5 cells/100 μl) were incubated with 10^-10 M $^{125}$I-IGF I in the presence or absence of 2×10^-7 M human recombinant IGF I for various times at 20°C, and free and bound counts were separated as described in “Materials and Methods.” Shown is specific binding, which was maximal by 60 minutes. Panel B: Homologous displacement of $^{125}$I-IGF I from its vascular receptor. Suspended rat aortic smooth muscle cells were incubated with 10^-10 M $^{125}$I-IGF I and increasing concentrations of human recombinant IGF I (0–2×10^-7 M) for 90 minutes at 20°C, and free and bound counts were separated as described in “Materials and Methods.” Data were analyzed using the LIGAND program. Panel C: Scatchard transformation of the data in panel B using the LIGAND program to obtain the best linear fit of the data points (least-squares method). One class of high-affinity binding sites was present with a $K_d$ of 5.6 nM and a receptor number ($B_{\text{max}}$) of 16.9 fmol/10^5 cells.
that bFGF (5 ng/ml for 24 hours) caused a similar increase in IGF I receptors per cell without altering binding affinity (Bmax for SFM, 12.6±1.5 fmol/10⁵ cells; Bmax for FGF, 30.3 fmol/10⁵ cells; n=4; p<0.05) (Kd for SFM, 5.5±1.5 nM; Kd for FGF, 7.2±1.8 nM; n=4; p=NS). To determine whether IGF I may regulate its own receptors, quiescent cells were exposed to SFM in the presence or absence of 20 ng/ml rhIGF I for 24 hours. Measurement of IGF I binding showed no change in either receptor number or affinity. To determine whether IGF I could downregulate its receptor in cells exposed to a growth stimulus, we incubated quiescent RASMs in SFM containing 1% platelet-poor plasma for 24 hours in the presence or absence of IGF I. As shown in Table 1, IGF I in the presence of 1% platelet-poor plasma caused a 50% decrease in Kd and a 52% decrease in IGF I receptor number per cell.

Role of PKC in Growth Factor–Induced Upregulation of IGF I Receptors

Because PKC is thought to play an important role in the response of cells to various growth factors, we determined the relation between PKC and growth factor–mediated regulation of IGF I receptors. Initial experiments were performed to assess the effects of PKC stimulation by incubating quiescent RASMs in the presence of 100 nM PMA for various times. As shown in Figure 2, PMA exposure caused a rapid early decrease in IGF I receptors at 45 minutes (Bmax for control cells, 2.5±0.01 fmol/10⁵ cells; Bmax for PMA-treated cells, 1.4±0.2 fmol/10⁵ cells; n=3; p<0.05). However, prolonged exposure to PMA caused a progressive increase in IGF I receptors (Bmax at 24 hours in control cells, 2.6±0.5 fmol/10⁵ cells; Bmax at 24 hours in PMA-treated cells, 8.1±0.6 fmol/10⁵; n=3; p<0.05). PMA treatment did not significantly alter IGF I binding affinity at any time point. Because prolonged exposure to phorbol esters is associated with decreases in cellular PKC activity,16–18 we downregulated PKC by exposing cells to PDBu for 48 hours and determined the effects of growth factors on IGF I receptor parameters. Exposure of cells to PDBu for 48 hours caused an expected increase in IGF I receptor number (control cells, 5.3±0.9 fmol/10⁵ cells; PDBu-treated cells, 8.0±1.7 fmol/10⁵ cells; 51% increase; n=14; p<0.05). IGF I binding affinity was not affected by PDBu exposure. As shown in Figure 3, the effect of PDGF and FGF to increase IGF I receptors on VSMCs was completely inhibited by prior treatment of cells with PDBu. However, the effect of Ang II to increase IGF I receptors was present even after PDBu treatment, indicating that this effect was mediated largely through a PKC-independent pathway.

Effects of Growth Factors on DNA and Protein Synthesis

To determine the growth-promoting effects of agonist exposure, we measured [³H]leucine and [³H]thymidine incorporation in response to FGF, PDGF, and Ang II in RASMs at baseline and after preexposure to PDBu for 48 hours. As shown in Figure 4, these three growth factors caused a significant increase in [³H]thymidine incorporation in cells quiesced by serum deprivation for 48 hours. This increase averaged 32% for PDGF, 79% for FGF, and 28% for Ang II. PKC downregulation by exposure to 200 nM PDBu for 48 hours caused a 91% increase in [³H]thymidine incorporation (absence of PDBu, 11,078±1,121 cpm per dish; presence of PDBu, 20,281±1,761 cpm per dish; n=14; p<0.001). The effect of PDGF and FGF to increase DNA synthesis in RASMs was completely inhibited after preexposure of the cells to PDBu. However, the Ang II–induced in-
increase in DNA synthesis was preserved in PDBu-treated cells and averaged 26%. Measurements of [3H]leucine incorporation indicated that PDGF, FGF, and Ang II caused marked increases in protein synthesis. As shown in Figure 5, these averaged 130%, 78%, and 75%, respectively. Downregulation of PKC by PDBu treatment caused a 75% increase in [3H]leucine incorporation (absence of PDBu, 10,576±848 cpm per dish; presence of PDBu, 18,679±2,127 cpm per dish; n=14; p<0.0025). Contrary to their effects on DNA synthesis in PDBu-treated cells, the effects of PDGF and FGF to induce protein synthesis were maintained, although blunted, after PDBu treatment (71% and 54% increase in [3H]leucine incorporation, respectively). Likewise, the effect of Ang II to increase protein synthesis was blunted but still significant in PDBu-treated cells (25% increase). To determine whether growth factor–induced increases in IGF I receptor number were accompanied by increased mitogenic responsiveness to IGF I, we preincubated quiescent RASMs in the presence or absence of bFGF for 24 hours, removed the medium, and then measured [3H]thymidine incorporation for 24 hours in the presence of increasing doses of rhIGF I. As shown in Figure 6, the mitogenic response to IGF I in cells pretreated with bFGF was markedly increased when compared with control cells (n=3, p<0.025).

**Cross-linking of 125I–IGF I to VSMC Monolayers**

Binding of 125I–IGF I to other cell types has been found to be influenced by cell surface–associated and secreted binding proteins. To confirm our results obtained using radioligand binding to cell suspensions, we performed binding of 125I–IGF I to quiescent RASM monolayers, cross-linked the ligand, and analyzed solubilized proteins by reducing sodium dodecyl sulfate (SDS)–PAGE. As shown in Figure 7, the α chain of the IGF I receptor was clearly visible as a 140-kd band.

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**Figure 4.** Bar graph showing growth factor–induced changes in DNA synthesis at baseline and after protein kinase C downregulation. PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; Ang II, angiotensin II; PDBu, phorbol 12,13-dibutyrate. Quiescent rat aortic smooth muscle cells (−PDBu) and quiescent rat aortic smooth muscle cells after protein kinase C downregulation (+PDBu) were exposed to 10 ng/ml PDGF-BB, 5 ng/ml bFGF, or 100 nM Ang II for 24 hours in the presence of 1 μCi/ml [3H]thymidine, and incorporated counts were determined as described in “Materials and Methods.” Shown is the mean±SEM of triplicate determinations from six to eight separate experiments. *p<0.05 compared with control value.

**Figure 5.** Bar graph showing growth factor–induced changes in protein synthesis at baseline and after protein kinase C downregulation. PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; Ang II, angiotensin II; PDBu, phorbol 12,13-dibutyrate. Quiescent rat aortic smooth muscle cells (−PDBu) and quiescent rat aortic smooth muscle cells after protein kinase C downregulation (+PDBu) were exposed to 10 ng/ml PDGF-BB, 5 ng/ml bFGF, or 100 nM Ang II for 24 hours in the presence of 1 μCi/ml [3H]leucine, and incorporated counts were determined as described in “Materials and Methods.” Shown is the mean±SEM of triplicate determinations from six to seven separate experiments. **p<0.01 compared with control value; ***p<0.001 compared with control value.

**Figure 6.** Graph showing the effect of preincubation with fibroblast growth factor (FGF) on mitogenic response to insulin-like growth factor I (IGF I). Quiescent rat aortic smooth muscle cells were exposed to serum-free medium in the presence or absence of 5 ng/ml basic FGF for 24 hours. The medium was then replaced with fresh serum-free medium containing increasing concentrations of IGF I and 1 μCi/ml [3H]thymidine. After 24 hours, incorporated counts were determined as described in “Materials and Methods.” Shown is the mean±SEM of triplicate determinations from three separate experiments. The IGF I–induced increase in thymidine incorporation was significantly greater in cells preexposed to FGF than in cells not preexposed to FGF (p<0.025).
binding of $^{125}$I-IGF I to cell monolayers further confirmed these results. These showed increases in IGF I receptors analyzed by SDS-PAGE after exposure of cells to PDGF, FGF, and Ang II (Figure 7).

To determine whether exposure of cells to growth factors altered their subsequent responsiveness to IGF I, we measured IGF I-induced DNA synthesis in cells preexposed to FGF for 24 hours. As shown in Figure 6, preexposure to FGF resulted in a marked increase in the mitogenic response to IGF I. Although FGF likely alters a variety of cellular processes, it is possible that this altered responsiveness to IGF I is partially mediated by FGF-induced increases in IGF I receptor number.

Limited data are available on regulation of VSMC IGF I receptors. Pfeifle and Ditshueneit25 have shown that $^{125}$I-IGF I binding to VSMCs is decreased in postconfluent versus preconfluent cells. The same group has shown that exposure of nonquiescent VSMC monolayers to PDGF or FGF caused a transient (<5-hour duration) increase in IGF I binding.1 However, relative effects on receptor number or affinity were not described. Short-term incubation of VSMCs with thyroid hormone has been found to increase $^{125}$I-IGF I binding.23 Clemmons et al24 have previously shown that PDGF modulates IGF I receptor number on BALB/c3T3 fibroblasts in the presence of platelet-poor plasma. Recently, bFGF in the presence of 1% fetal bovine serum has been shown to increase IGF I receptors on BC3H-1 myoblasts.25

Our experiments studying homologous regulation of the IGF I receptor indicate that IGF I downregulates its receptor on quiescent RASMs only in the presence of platelet-poor plasma. This effect is accompanied by an increase in binding affinity. It is possible that the lack of an effect of IGF I to downregulate its receptor on serum-deprived RASMs is due to the fact that in this condition IGF I receptors are already maximally downregulated. Other reports that IGF I downregulates its receptor have used nonquiescent cells.26,27 It is of note that Conover and Powell28 have shown that IGF binding protein 3 can block IGF I-induced receptor downregulation in bovine fibroblasts.

Because PKC has been proposed to play an important role in cellular signaling events in response to growth factors,12 we investigated its potential involvement in the regulatory effects of PDGF, FGF, and Ang II on IGF I binding to VSMCs. Exposure of quiescent VSMCs to PMA caused a biphasic response in IGF I binding. After an initial 42% decrease at 45 minutes, there was a gradual increase in IGF I receptor number per cell that averaged 238% after 24 hours. Exposure of VSMCs to PMA has been shown to cause rapid translocation of PKC activity from the cytosol to the cell membrane, with maximum increases in membranous PKC activity occurring at approximately 20–30 minutes.29 This is followed by a gradual decrease in membranous PKC activity. Our results showing that PMA exposure for 45 minutes decreases IGF I binding are consistent with an inhibitory effect of acute PKC activation on IGF I binding. In this regard, it is important to note that short-term (7-minute) exposure of IM-9 cells, a human B-lymphocyte line, to phorbol esters has been shown to stimulate IGF I receptor phosphorylation.30 Thus, a potential explanation for this acute effect is phorbol ester–induced phosphorylation of IGF I recep-
tors and subsequent internalization of the receptors. A prior study by Lammers et al.\(^1\) has shown that IGF I receptors on transfected NIH-3T3 cells are rapidly internalized after occupancy by their ligand. Chronic PKC stimulation, however, which is associated with a progressive decrease in total PKC activity in VSMCs, resulted in marked increases in IGF I receptors per cell. It is important to note that regulation of proteinoid receptors on VSMCs has also been shown to be PKC dependent; specifically, exposure of VSMCs to phorbol esters for ≥4 hours increased prostaglandin F\(_2\alpha\) and prostaglandin E\(_2\) binding.\(^2,3\)

To determine the effect of PDGF, FGF, and Ang II on IGF I binding in PKC-downregulated cells, we exposed VSMCs to these agonists after a 48-hour exposure to PDBu. This has previously been shown to decrease total VSMC PKC activity by 80–90%.\(^4\) As shown in Figure 3, the upregulation of IGF I receptors by PDGF and FGF was completely inhibited after PKC downregulation, whereas the effect of Ang II was maintained. These findings demonstrate that the effects of PDGF and FGF to increase IGF I receptors were dependent on PKC, whereas the Ang II–mediated increase in IGF I binding was PKC independent. It is important to note that Kawahara et al.\(^5\) have shown that there are significant differences in the PKC dependency of earlier signaling events between PDGF and Ang II in VSMCs. Specifically, acute exposure of VSMCs to 12-\(\alpha\)-tetradeoxyphorbol 13-acetate markedly blunts Ang II–induced phospholipase C activity and inositol phosphate formation but does not inhibit PDGF-stimulated phospholipase C activity. Our data provide further evidence for major differences in the PKC dependency of cellular responses to growth factors such as PDGF and vasoactive agonists such as Ang II.

Characterization of agonist-induced growth-promoting effects indicated that FGF- and PDGF-induced DNA synthesis was completely inhibited after PKC downregulation by PDBu, whereas Ang II maintained a small mitogenic effect. Our findings that PDGF-induced DNA synthesis was PKC dependent is in agreement with results from Kariya et al.\(^6\) and Takagi et al.\(^7\) However, our findings are at variance with those of Kihara et al.\(^8\) who found that PKC inhibition with polymyxin B suppressed FGF-induced but not PDGF-induced DNA synthesis in VSMCs. Interestingly, PDGF- and FGF-induced protein synthesis were not PKC dependent, since these growth factors still increased \([H]\)leucine incorporation into cells exposed to PDBu for 48 hours. Likewise, Ang II elicited a significant albeit blunted increase in \([H]\)leucine incorporation into PKC-downregulated cells.

Recently, binding studies have shown that homologous displacement of \(^{125}\)I-IGF I from fibroblasts is accompanied by a paradoxical initial increase in binding.\(^9\) This repartitioning effect has been ascribed to the presence of cell surface–associated and secreted IGF binding proteins but was not present in our cells (Figure 1B). To confirm that our radioligand binding data were specific for the IGF I receptor, we performed affinity cross-linking of \(^{125}\)I-IGF I to control or agonist-treated cell monolayers and analyzed cell lysates by reducing SDS-PAGE and autoradiography (Figure 7). The ligand-binding \(\alpha\)-subunit of the IGF I receptor was clearly visible as a 140-kd band. A second 240-kd band was consistent with cross-linked IGF I receptor dimers. The binding specificity pattern, i.e., displacement of binding by unlabeled IGF I and higher concentrations of unlabeled insulin, was typical of type I IGF receptors.\(^10\)Intensity of the 140- and 240-kd bands was increased after treatment of VSMCs with PDGF, bFGF, or Ang II. No lower molecular weight bands were seen, and since all IGF binding proteins described to date migrate at <50,000 \(M_r\), this confirmed the absence of cell surface–associated IGF binding proteins that may have interfered in our radioligand binding experiments.

In summary, we have shown that PDGF, FGF, and Ang II regulate IGF I receptors on quiescent VSMCs. IGF I downregulates its receptors on quiescent VSMCs only in the presence of platelet-poor plasma. Acute PKC stimulation by phorbol esters acutely decreases IGF I binding to VSMCs, whereas chronic PKC stimulation increases IGF I binding. The effects of PDGF and FGF to increase IGF I receptors on VSMCs are mediated by a PKC-dependent pathway, whereas the effect of Ang II to upregulate IGF I receptors is PKC independent. Upregulation of IGF I receptors on VSMCs by FGF is accompanied by increased mitogenic responsiveness to IGF I. These findings provide novel insights into mechanisms regulating IGF I receptors on VSMCs and will provide the basis for the study of the role of the IGF I receptor in vascular proliferative responses in vivo.

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