Thrombin Regulates Insulin-Like Growth Factor-1 Receptor Transcription in Vascular Smooth Muscle Characterization of the Signaling Pathway

Jie Du,* Marijke Brink,* Tao Peng, Bianca Mottironi, Patrick Delafontaine

Abstract—We have previously demonstrated that thrombin upregulation of insulin-like growth factor-1 receptor (IGF-1R) is essential for thrombin-induced mitogenic signaling. To characterize the mechanisms involved, we studied transcription of the IGF-1R gene in rat aortic smooth muscle cells. Thrombin markedly increased IGF-1R mRNA levels, peaking at 3 hours (112±7% above control). This effect was mimicked by the hexapeptide SFFLRN (that functions as a tethered ligand) and was blocked by the thrombin inhibitor hirudin. Nuclear run-on assays indicated that thrombin stimulated IGF-1R gene transcription by 2.1-fold, and this was confirmed with the use of actinomycin D. Thrombin-mediated upregulation of IGF-1R mRNA and protein levels was protein kinase C independent but was completely inhibited by the protein tyrosine kinase inhibitor genistein and the antioxidants N-acetyl-L-cysteine and pyrrolidinedithiocarbamate, suggesting the involvement of reactive oxygen species. The thrombin-induced increase in IGF-1R mRNA was inhibitable by diphenyleneiodonium chloride but not by other inhibitors of cellular oxidase systems, suggesting that NAD(P)H oxidase was necessary for the increase. Furthermore, inhibitors of the epidermal growth factor receptor kinase, Janus kinase-2 kinase, and Src kinase did not block the effect. Thus, thrombin transcriptionally regulates the IGF-1R gene via a redox-sensitive protein tyrosine kinase–dependent pathway that does not require protein kinase C activation. In view of our prior data indicating that IGF-1R density is a critical determinant of vascular smooth muscle cell growth, our findings have particular relevance to understanding mechanisms whereby growth factors such as thrombin regulate vascular proliferation in vivo. (Circ Res. 2001;88:1044-1052.)

Key Words: growth factors ■ signal transduction ■ gene regulation

The serine proteinase thrombin has multiple physiological effects. It is a procoagulant proinflammatory molecule and is a mitogen for vascular smooth muscle cells (VSMCs). Thrombin activates its receptor by a unique mechanism involving cleavage of the N-terminus of the receptor, generating a new N-terminus, which, in turn, acts as a tethered ligand. Cellular responses induced by thrombin are, in most cases, due to activation of protease-activated receptor (PAR)-1. PAR-1 is a proteolytically activated 7-transmembrane–spanning receptor that has been linked to a variety of cellular pathways, including hydrolysis of phosphoinositides, calcium mobilization, and activation of heterotrimeric G proteins, protein tyrosine kinases (PTKs), and monomeric G proteins. G-protein–coupled receptors, including the thrombin receptor, do not possess intrinsic tyrosine kinase activity. Using thrombin receptor–activating peptides and PTK inhibitors, some investigators have demonstrated that along with activation of its G-protein–coupled receptor, thrombin also requires PTK activity, possibly of a receptor type, for its mitogenic effect. Thus, thrombin has been shown to activate Src and c-Jun N-terminal kinase in VSMCs. Recently, evidence has been obtained indicating that thrombin exerts some of its effects by transactivating the epidermal growth factor (EGF) receptor.

Insulin-like growth factor-1 (IGF-1) is a critical regulator of cell growth and differentiation. Its effects are mediated by the IGF-1 receptor (IGF-1R), a membrane tyrosine kinase. Evidence from a variety of studies using different cell types has shown that the IGF-1R is at a convergence point in the control of cell growth. Thus, a functional IGF-1/IGF-1R autocrine loop is required for the mitogenic effects of growth factors, such as platelet-derived growth factor, EGF, angiotensin II (Ang II), and cell transformation. Furthermore, the IGF-1R has been implicated in tumorigenesis.

We have demonstrated that a functional IGF-1/IGF-1R pathway is essential for thrombin stimulation of VSMC mitogenesis. Thrombin upregulates VSMC IGF-1R, and
inhibition of this effect using antisense phosphorothioate oligonucleotides inhibits thrombin-induced cellular proliferation. In addition, use of neutralizing anti–IGF-1 antibodies inhibits the mitogenic effects of thrombin. In the present study, we explored mechanisms and signaling pathways mediating the ability of thrombin to upregulate IGF-1R. Our findings indicate that thrombin regulates IGF-1R gene transcription via stimulation of its classic receptor, PAR-1, and that the signaling pathway is redox sensitive, requiring NAD(P)H oxidase and tyrosine kinase activity. These findings have important implications for understanding the mechanisms by which thrombin promotes cellular growth responses in vivo.

Materials and Methods

Cell Culture
VSMCs were isolated from rat thoracic aorta and grown as described. 27 For experiments, cells from passages 5 to 16 were seeded into 100-mm dishes, grown to 80% to 90% confluence, and reached inhibition, genistein (60 

\( \mu \text{mol/L} \)), was applied at 1 hour before exposing the cells to thrombin. To analyze the specific kinases involved, cells were pretreated with AG490 (10 to 50 

\( \mu \text{mol/L} \)), PP1 (10 to 100 

\( \mu \text{mol/L} \)), PP2 (10 

\( \mu \text{mol/L} \)), or a combination of CAT and SOD for 1 hour before growth factor stimulation. To determine the effects of PTK inhibition, genistein (60 

\( \mu \text{mol/L} \)) was applied 1 hour before exposing the cells to thrombin. To analyze the specific kinases involved, cells were pretreated with AG490 (10 to 50 

\( \mu \text{mol/L} \)), AG1478 (10 to 50 

\( \mu \text{mol/L} \)), PP1 (10 

\( \mu \text{mol/L} \)), or PP2 (1 

\( \mu \text{mol/L} \)) (all from Calbiochem).  

Inhibition of PKC
To determine the effects of protein kinase C (PKC) downregulation, phorbol 12,13-dibutyrate (PDBu, 200 

\( \mu \text{mol/L} \)) was added during the second 24-hour period of serum deprivation and maintained during the period of exposure to the agonist. A PKC inhibitor, bisindolylmaleimide (50 

\( \mu \text{mol/L} \)) or chelerythrine (3 

\( \mu \text{mol/L} \)), was applied at time 0 after 48-hour serum deprivation, immediately before the cells were exposed to the agonist. Concentrations of PKC inhibitors were at least 3 times the IC\( _{50} \) values for these compounds. 28

IGF-1R mRNA Levels
Control and inhibitor-treated cells were exposed to SFM without or with thrombin or SFPLRN for 3 hours, total RNA was extracted with the use of TRI-reagent (Molecular Research Center), and IGF-1R mRNA levels were determined by solution hybridization/RNase protection assays with a radiolabeled antisense probe transcribed from a rat IGF-1R cDNA. 29 The full-length probe is 251 bp, and the protected fragment is 195 bp. GAPDH or 18S riboprobes were included as a control, giving 133- and 158-bp protected bands, respectively. Autoradiograms of protected bands were quantified by densitometry.

Radioligand Binding Assays, Immunoblotting, and Measurement of DNA Synthesis
To determine the effect of thrombin on IGF-1R number and binding affinity, quiescent VSMCs were exposed to SFM without or with thrombin or SFPLRN for 24 hours. The dependence of thrombin upregulation of IGF-1R on PKC and ROS was determined by coincubating the cells with thrombin and inhibitors for 24 hours before the binding assays. Binding assays were performed and analyzed as described previously. 29

To analyze the effect of kinase inhibitors on IGF-1R protein expression and DNA synthesis, quiescent VSMCs were incubated with an inhibitor for 1 hour before the administration of growth factors. After 16 hours, proteins were collected for analysis of IGF-1R by Western blotting. 30 DNA synthesis was measured at 16 hours by adding \(^{3} \text{H} \) thymidine (1 

\( \mu \text{Ci/mL} \)) during the last 4 hours of incubation. Samples were processed as described previously. 29

Nuclear Run-On Transcriptional Analysis
These assays were performed as previously described, 31 with modifications. Briefly, quiescent VSMCs were exposed to SFM in the presence or absence of thrombin for 3 hours. Nuclei were isolated with the use of a sucrose gradient, and transcription was carried out with the use of 2 to 5 \( \times \) \( 10^{9} \) nucleotides in a 300-\( \mu \text{L} \) reaction mixture containing 33 mmol/L Tris-HCl, pH 8.0, 26% glycerol, 5.5 mmol/L MgCl\( _{2} \), 0.066 mmol/L EDTA, 140 mmol/L KCl, 0.1 mmol/L each ATP, GTP, and CTP, and 20 \( \mu \text{L} \) \((\text{full-length rat sequence})\) and plasmid without insert (pGEM3) were linearized, denatured, and applied to a nylon membrane with use of a Bio-Gel P-30 columns. Plasmids containing cDNAs for the IGF-1R (\(-610 \) to \(+200 \) bp relative to ATG), GAPDH (1.2-kb fragment), Ang II type 1 receptor (full-length rat sequence), and plasmid without insert (pGEM3) were hybridized in the same solution containing 1 to 5 \( \times \) \( 10^{5} \) cpm/mL of 0.2 mmol/L NaOH treated \(^{3} \text{P} \)-labeled nuclear RNA for 40 hours at 65°C. Membranes were washed twice in 2 \( \times \) SSC and 0.1% SDS at room temperature and then in 0.1\( \times \) SSC and 0.1% SDS for 2 hours at 65°C. Autoradiographic signals were quantified by densitometry.

Statistical Analysis
All experiments were performed at least 3 times. Data are expressed as mean\( \pm \)SE. Analysis of repeated measures was performed by ANOVA. Comparison between groups was performed by using a protected Tukey \( t \) test or Student \( t \) test when appropriate.

Materials
Thrombin, PDBu, CAT, allopurinol, and NAC were purchased from Sigma Chemical Co. Bisindolylmaleimide, chelerythrine, and genistein were from LC Laboratories, Alexis Corp. PDTC was from Fluka Chemical Corp. DPI, SOD, PP1, PP2, AG490, and AG1478 were from Calbiochem. TRI-reagent was from Molecular Research Center. The 18S riboprobe was from Ambion.

Results
Effects of Thrombin on IGF-1R mRNA Levels
Exposure of VSMCs to thrombin caused a marked increase in IGF-1R mRNA levels (Figure 1A). To assess the time course of induction in IGF-1R expression, IGF-1R and GAPDH mRNAs were measured after exposure to an agonist for 0 to 24 hours. Thrombin induction of IGF-1R mRNA peaked at 3 hours (2.1-fold increase, \( P<0.0001; n=8 \)), and levels were still elevated at 24 hours (\( P<0.01 \), Figure 1B). Thrombin caused a more delayed increase in GAPDH mRNA (\( P<0.01 \) at 24 hours). To determine whether the effect was mediated via proteolytic activation of the thrombin receptor, we mea-
sured IGF-1R mRNA after exposure to the hexapeptide SFFLRN, which acts as a tethered ligand after cleavage of the amino terminus of the thrombin receptor. SFFLRN increased IGF-1R mRNA by 47% ($P$, 0.001, $n$=6), mimicking the effect obtained with the parent agonist thrombin (Figure 1C).

To further demonstrate that the effect was mediated by a receptor-dependent mechanism, we blocked the binding of thrombin to its receptor with hirudin, which resulted in complete inhibition of the induction of IGF-1R mRNA ($P$, 0.01, Figure 1D).

**Effects of Thrombin on Newly Transcribed IGF-1R mRNA**

To determine whether the increase in IGF-1R mRNA was transcriptionally mediated, we performed in vitro transcription with the use of nuclei from VSMCs treated without or with thrombin for 3 hours. Hybridization of radiolabeled nuclear RNA probes to IGF-1R cDNA demonstrated a 2.1-fold increase in IGF-1R signal from thrombin-stimulated cells ($n$=4, $P<0.001$, $n=6$), mimicking the effect obtained with the parent agonist thrombin (Figure 1C). To further demonstrate that the effect was mediated by a receptor-dependent mechanism, we blocked the binding of thrombin to its receptor with hirudin, which resulted in complete inhibition of the induction of IGF-1R mRNA ($P<0.01$, Figure 1D).

**Figure 1.** Effect of thrombin and SFFLRN on IGF-1R mRNA levels. A, Representative solution hybridization autoradiogram. Total RNA from thrombin-treated VSMCs (3 hours) was hybridized to IGF-1R and GAPDH riboprobes and RNAse-digested, and protected bands were detected after sequential gel electrophoresis. B, Densitometric analysis of time course of changes in IGF-1R. Shown is the percent increase in mRNA in thrombin-exposed cells over levels in cells in SFM. C, Solution hybridization analysis showing the effect of SFFLRN (3 hours) on IGF-1R mRNA (3 representative experiments). D, Solution hybridization analysis showing the effect of the antithrombin agent hirudin (representative experiment).

gave a significant 2-fold decrease in signal intensity ($n$=4, $P<0.025$), consistent with a prior study indicating that thrombin decreases Ang II receptor expression. Vector alone gave a minimal signal. To confirm that thrombin induces IGF-1R gene transcription, we inhibited RNA synthesis with actinomycin D, resulting in complete loss of the upregulation of IGF-1R mRNA (Figures 3A and 3B) and protein (Figure 3C) levels by thrombin.

**Effect of PKC Downregulation on Thrombin-Induced IGF-1R Expression**

PKC activation is an important signaling pathway for the cellular effects of multiple mitogens. To define the PKC dependence of thrombin regulation of IGF-1R, we measured IGF-1R mRNA levels after 24 hours of preincubation with the phorbol ester PDBu. Chronic exposure to PDBu has been shown to downregulate PKC activity in VSMCs by 80% to 90%. After preexposure to PDBu, thrombin still increased IGF-1R mRNA (1.7-fold increase, $P<0.025$; $n=6$) and binding (1.5-fold increase, $P<0.05$; $n=4$) levels (Figure 4A). To further define the PKC dependence of the effect, we used 2 structurally unrelated inhibitors of PKC (Figure 4B). Thrombin still increased IGF-1R mRNA by 2.0-fold in the presence of bisindolylmaleimide ($n=5$, $P<0.05$) or chelerythrine ($n=5$, $P<0.05$). A small increase in IGF-1R mRNA was observed with the inhibitors alone ($P=NS$). Furthermore, thrombin upregulated the IGF-1R number by 1.8-fold in the presence of bisindolylmaleimide ($n=4$, $P<0.05$) and by 1.5-fold in the presence of chelerythrine ($n=4$, $P<0.025$), without altering IGF-1R binding affinity ($K_d$) (not shown).

**Effect of Inhibition of ROS on Thrombin-Induced IGF-1R Expression**

We have previously shown that Ang II induces IGF-1R transcription in VSMCs through a redox-sensitive pathway.
zymes, such as NAD(P)H oxidase (Figure 6). DPI alone had negligible effects on IGF-1R mRNA levels in the absence of thrombin. No inhibition was observed with allopurinol, which is an inhibitor of xanthine oxidase (data not shown), with CAT, which blocks the production of the reactive oxygen intermediate H$_2$O$_2$, with superoxide dismutase, which blocks the reactive oxygen intermediate O$_2^-$, or with a combination of CAT and SOD (Figure 6). Taken together, these results indicate that the NAD(P)H oxidase system is involved in the response to thrombin.

**Effect of PTK Inhibition on Thrombin-Induced IGF-1R Expression**

Thrombin activates several PTKs, which may be important in its signaling cascade, including nonreceptor tyrosine kinases, such as Src$^{13}$ and focal adhesion kinase,$^{37}$ and receptor tyrosine kinases, such as the IGF-1 receptor$^{38}$ and the EGF receptor.$^{39}$ To study the role of PTK in thrombin-induced IGF-1R transcription, VSMCs were treated with genistein. The induction of IGF-1R mRNA by thrombin (2.8-fold increase, $P<0.00001$; $n=5$) was completely blocked by genistein (Figures 7A and 7B). Furthermore, the ability of SFFLRN to increase IGF-1R mRNA (1.5-fold increase, $P<0.001$; $n=6$) was inhibited by genistein (Figure 7C). We further confirmed the requirement of PTK in thrombin upregulation of IGF-1R by measuring IGF-1R numbers. In these experiments, thrombin upregulated the IGF-1R number by 1.8-fold ($n=4$, $P<0.01$), and genistein completely inhibited this effect (Figure 7D). These data demonstrate that PTKs are required for thrombin stimulation of IGF-1R expression.

To obtain insight into the specific PTKs involved, we studied the effects of inhibitors of receptor and nonreceptor kinases. Thrombin has been shown to transactivate the EGF receptor.$^{17}$ However, the selective EGF receptor kinase inhibitor AG1478 did not abolish the induction of IGF-1R mRNA or protein by thrombin at 10, 20, or 50 μmol/L (Figure 8), concentrations that have been shown effective in inhibiting Ang II–induced extracellular signal–regulated kinase activation in rat VSMCs.$^{46}$ As expected, the induction of IGF-1R mRNA and protein by EGF was inhibited by AG1478 (Figures 8A and 8B). Furthermore, the EGF-induced increase in [H]thymidine incorporation was significantly inhibited by 50 μmol/L AG1478 (Figure 8C), confirming activity of the drug.

The Janus kinase (JAK)-2 inhibitor AG490 had no effect on thrombin-induced increases of IGF-1R mRNA, protein, or DNA synthesis at doses of 10, 20, and 50 μmol/L. This inhibitor also failed to block induction of the IGF-1R by basic fibroblast growth factor (bFGF) (not shown) or EGF (Figures 8A and 8B). In a [H]thymidine incorporation assay, AG490 significantly reduced the thrombin- and EGF-induced increases in DNA synthesis ($P<0.01$), demonstrating that the compound was active.

Furthermore, we studied the effects of Src kinase inhibitors. In these experiments, the increase in IGF-1R mRNA levels was slightly enhanced after incubation with PP1 before thrombin treatment (Figure 8A). The same results were obtained with PP2 (not shown). bFGF significantly increased

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**Figure 3.** Actinomycin D blocks thrombin-induced IGF-1R expression. A, Representative solution hybridization autoradiogram from cells incubated with thrombin and actinomycin D. B, Densitometric analysis showing that actinomycin D inhibits thrombin-induced increases in IGF-1R mRNA at 3 hours ($n=8$). C, Radioligand binding assays indicating that actinomycin D inhibits thrombin-induced increases in IGF-1R protein at 24 hours ($n=2$).

To test whether the action of thrombin to increase the IGF-1R is mediated through ROS, we determined the inhibitory effect of the antioxidant PDTC (Figure 5A) or NAC (Figure 5B). Both compounds completely inhibited the induction of IGF-1R mRNA. The findings for NAC were confirmed in radioligand binding assays. Thrombin increased the IGF-1R number 1.8-fold at 24 hours ($n=6$, $P<0.01$), and this increase was inhibited by NAC (Figure 5B). Scatchard analysis indicated no significant changes in IGF-1R binding affinity: $K_i$ control, 4.7±0.1 nmol/L; $K_i$ thrombin, 5.5±0.4 nmol/L; $K_i$ NAC, 4.8±0.3 nmol/L; and $K_i$ NAC+thrombin, 7.1±1.0 nmol/L ($n=5$, $P=NS$). These findings strongly suggest that an oxidant signal was required for thrombin induction of IGF-1R transcription.

ROS-producing systems include various NADPH and NADH oxidases, xanthine oxidase, and NO synthase. We examined the effects of inhibitors of different oxidase systems on the thrombin-induced increase in IGF-1R. This increase was completely blocked by DPI at 100 μmol/L ($n=5$, $P<0.05$), an inhibitor of flavoprotein-containing en-
IGF-1R mRNA and protein, and this increase was not inhibited by PP1 (Figures 8A and 8B); however, PP1 significantly reduced bFGF-induced increases in DNA synthesis (Figure 8C).

**Discussion**

It has been demonstrated that the ability of thrombin to upregulate IGF-1R is a critical determinant of its mitogenic effects on VSMCs. Thus, use of IGF-1R gene–specific antisense oligonucleotides or of an anti–IGF-1 antibody blocks thrombin-induced DNA synthesis. In the present study, we demonstrate that the ability of thrombin to upregulate IGF-1R on VSMCs is mediated by transcriptional activation of the IGF-1R gene, resulting in a rapid increase in IGF-1R mRNA levels, with a peak at 3 hours that was maintained for at least 24 hours. The effect is transduced by the classic PAR-1, inasmuch as it is mimicked by the PAR-1–specific peptide SFFLRN. We further examined pathways whereby the G-protein–coupled receptor agonist thrombin increases expression of the tyrosine kinase IGF-1R.

A variety of signaling events are induced by thrombin. These include stimulation of PTK and PKC and activation of multiple intracellular kinases and phosphatases. Because PKC is postulated to be an important mediator of the effects of multiple growth factors, we assessed its role in thrombin regulation of IGF-1R mRNA. Chronic stimulation of PKC by incubation with PDBu results in marked downregulation of PKC activity. In this condition, thrombin caused a further significant 1.7-fold increase in IGF-1R mRNA and 1.5-fold increase in IGF-1R protein levels, suggesting that thrombin induces IGF-1R transcription via a PKC-independent pathway. To confirm this, we used 2 structurally unrelated PKC inhibitors, but in all cases, the stimulatory effect of thrombin on IGF-1R was retained. It is of note that atypical PKC isoforms (eg, PKCζ) lack a phorbol ester binding site and are not downregulated by PDBu treatment. However, the failure of 2 different PKC inhibitors (and in particular, chelerythrine, which blocks the catalytic domain) to block thrombin-induced increases in IGF-1R mRNA and protein argues against an involvement of PKC. Thus, the present
findings are consistent with a PKC-independent pathway whereby thrombin stimulates IGF-1R expression.

To further define the potential mechanisms involved in thrombin regulation of IGF-1R, we studied the role of oxidant stress. Our findings show that the antioxidants PDTC and NAC suppress thrombin stimulation of IGF-1R expression. Patterson et al have recently shown that thrombin induces the generation of ROS in VSMCs at least in part through activation of NAD(P)H oxidase and that blocking this enzyme with DPI inhibits thrombin-induced proliferation. Consistently, we have demonstrated in the present study that DPI blocks the induction of IGF-1R by thrombin. Thus, unlike Ang II, thrombin activation of NAD(P)H oxidase provides a mechanism whereby activation of the thrombin receptor could lead to IGF-1R transcription. Again, unlike Ang II, the ability of thrombin to upregulate IGF-1R was not blocked by CAT. SOD also did not block the ability of thrombin to regulate IGF receptors. This lack of effect may be due to ineffective scavenging of intracellular reactive oxygen intermediates, or other intermediates may play a role. 

Recent studies have shown that PAR-1 couples functionally to multiple heterotrimeric G proteins that activate the mitogen-activated protein kinase (MAPK) pathway via the small GTP-binding protein p21 ras . In various cell types, thrombin activation of the Ras/MAPK cascade requires non–receptor-dependent tyrosine kinase activity, such as Src or focal adhesion kinase, or receptor-dependent tyrosine kinase activity, eg, through the EGF receptor, which is transactivated by thrombin in rat 1 fibroblasts and in VSMCs. Although PKC activation provides a potential Ras-independent pathway whereby G-protein–coupled receptors may activate the MAPK pathway, our data using PDBu and PKC inhibitors argue strongly against the possibility that thrombin upregulation of IGF-1R uses PKC. To examine the potential role of PTK in the signal leading to IGF-1R transcription, we determined the effects of genistein. This tyrosine kinase inhibitor blocked the ability of thrombin to increase IGF-1R mRNA and protein levels. To determine which specific tyrosine kinases are involved, we determined the effect of Src, EGF receptor kinase, and JAK-2 kinase inhibitors. None of the inhibitors tested blocked IGF-1R induction by thrombin. JAK-2 is also not involved in IGF-1R induction by bFGF or EGF, indicating that JAK-2 is not required for IGF-1R regulation. However, growth factor–induced DNA synthesis was blocked by AG490, suggesting that JAK-2 is involved downstream from the IGF-1R. Taken together with our data using genistein, our results indicate that a kinase other than Src, EGF receptor, or JAK-2 must be involved in thrombin regulation of the IGF-1R.

Significant data have established that the IGF-1/IGF-1R autocrine loop is a critical regulator of the cell cycle. Thus, fibroblasts from mice expressing a null mutation for the
IGF-1R gene have a marked reduction in their growth rate in serum. Normal growth cannot be restored by exposure to other growth factors but can be corrected by expression of the IGF-1R. A functional IGF-1/IGF-1R pathway is essential for the growth response to other growth factors, such as EGF, platelet-derived growth factor, and thrombin. Furthermore, simian virus 40 T-antigen transformation of cells requires a functional IGF-1R. It is of note that the effects of growth factors on IGF-1R expression may be critical in allowing cells to subsequently respond to endogenously synthesized IGF-1. Thus, in VSMCs, Ang II–induced and thrombin-induced mitogenesis is blocked by antisense phosphorothioate oligonucleotides targeting the IGF-1R mRNA. Furthermore, in VSMCs transfected with an IGF-1R antisense expression plasmid, growth responses are markedly blunted. In these cells, as in cells exposed to antisense oligonucleotides targeting the IGF-1R mRNA, there is a reduction in IGF-1R density of ~50%. Therefore, relatively small changes in IGF-1R density appear to be critical for the regulation of VSMC growth. We have previously shown that thrombin doubles IGF-1R density on VSMCs and that this is a critical requirement for thrombin-induced DNA synthesis. Our present study has defined the signaling cascade involved. It is important to emphasize that the IGF-1R gene promoter lacks TATA or CAAT motifs and that transcription starts from a unique “initiator” sequence. It will be of paramount importance to determine how transcription factors interact with this receptor promoter and, specifically, to determine whether thrombin regulates transcription via a common cis-acting sequence.

In summary, we have demonstrated that thrombin upregulates IGF-1R via PAR-1–mediated transcriptional activation

Figure 7. PTK inhibitor blocks thrombin- and SFFLRN-induced upregulation of IGF-1R. A, Representative solution hybridization autoradiogram is shown. VSMCs were incubated with thrombin and genistein for 3 hours before RNA extraction and analysis. B, Densitometric analysis of IGF-1R mRNA is shown normalized for GAPDH (n=5). C, Genistein inhibits the ability of SFFLRN (3 hours) to increase IGF-1R mRNA. D, Radioligand binding studies performed after 24 hours of incubation with thrombin and genistein (n=4).

Figure 8. Effect of specific kinase inhibitors on growth factor–induced upregulation of IGF-1R mRNA (A), protein (B), and DNA synthesis (C). A, Densitometric analysis of IGF-1R mRNA levels after stimulation with thrombin, bFGF, or EGF in the presence or absence of the EGF receptor kinase inhibitor AG1478, the JAK-2 inhibitor AG490, or the Src kinase inhibitor PP1 (n=4 or 5). B, Representative Western blots. VSMCs were stimulated with thrombin (Thr), bFGF, or EGF in the presence or absence of AG1478, AG490, or PP1. C, Summary of data measuring [H]thymidine incorporation in response to agonists in the presence or absence of kinase inhibitors (n=4).
of the IGF-1R gene. Thrombin-dependent regulation of IGF-1R is mediated via a PKC-independent pathway that is redox sensitive and involves the activation of NAD(P)H oxidase. It requires a tyrosine kinase activity other than Src, EGF receptor, or JAK-2. In view of the critical role played by the IGF-1R in mediating the growth effects of thrombin, these findings are particularly relevant to understanding biochemical pathways required for thrombin stimulation of cellular proliferative responses.

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