G-Protein Coupled and Tyrosine Kinase Receptors: Evidence that Activation of the Insulin-like Growth Factor I Receptor Is Required for Thrombin-induced Mitogenesis of Rat Aortic Smooth Muscle Cells

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Abstract

IGF I is an ubiquitous peptide that activates a membrane tyrosine kinase receptor and has autocrine/paracrine effects on vascular smooth muscle cells. Thrombin activates a G-protein coupled receptor and is also a mitogen for vascular smooth muscle cells. To assess the potential role of IGF I as a mediator of thrombin's effects, we characterized expression of IGF I and of its receptor on vascular smooth muscle cells exposed to thrombin. Thrombin dose-dependently decreased IGF I mRNA levels and caused a delayed decrease in IGF I secretion from vascular smooth muscle cells. This effect was mimicked by the hexapeptide SFFLRN (that functions as a tethered ligand) and was inhibited by hirudin. In contrast, thrombin doubled IGF I receptor density on vascular smooth muscle cells, without altering binding affinity (K_d). An anti–IGF I antiserum markedly reduced thrombin-induced DNA synthesis, whereas nonimmune serum and an anti–fibroblast growth factor antibody were without effect. Cell counts confirmed these results. Downregulation of IGF I receptors by antisense phosphorothioate oligonucleotides likewise markedly inhibited thrombin-induced DNA synthesis. These data demonstrate that a functional IGF I–IGF I receptor pathway is essential for thrombin-induced mitogenic signaling and support the concept of cross talk between G-protein coupled and tyrosine kinase receptors. (*J. Clin. Invest.* 1996. 97:139–145.) Key words: cell proliferation • gene expression • receptors • vasculature • antisense oligonucleotides

Introduction

There is evidence that vascular growth responses in pathophysiological conditions such as hypertension and after mechanical injury are modulated by a variety of growth factors (for reviews see references 1–3). Recently IGF I, a central regulator of developmental growth, has been proposed to be an important autocrine/paracrine factor for vascular smooth muscle cells (VSMC)[1] (4–13). Thus, PDGF- and angiotensin II–induced DNA synthesis in VSMC is inhibited by anti–IGF I antiserum (6, 14). The serine proteinase thrombin has a well-established role in the coagulation cascade (15). Thrombin also has proinflammatory effects (16–18) and is a mitogen for VSMC (19–21). The effects of thrombin are mediated by proteolytic activation of a G-protein coupled receptor (22). Thus, the amino terminus of the receptor that is exposed after cleavage functions as a tethered ligand.

The mechanisms whereby thrombin functions as a mitogen are poorly understood. Thrombin induces secretion of PDGF from endothelial cells (23) and VSMC (24, 25). Thrombin's mitogenic effect appears to be independent of inositol phosphate hydrolysis and calcium mobilization (26–29), and Weiss et al. (30) have suggested that the basic fibroblast growth factor (bFGF) receptor tyrosine kinase plays a role in thrombin-induced mitogenic signaling. The delayed onset of thrombin-induced maximal DNA synthesis (21) suggests that thrombin-stimulated growth is mediated by an autocrine pathway.

The purpose of this study was to determine whether thrombin regulates IGF I and its receptor on VSMC, and to assess the role of IGF I in thrombin-induced DNA synthesis. Our findings indicate that thrombin exerts opposing effects on this ligand-receptor system, resulting in a slow reduction in IGF I release and a marked upregulation of IGF I receptors. Use of a neutralizing anti–IGF I antiserum blocks thrombin-induced proliferation, as does IGF I receptor downregulation by specific antisense oligonucleotides (ODNs). These findings are consistent with a model whereby IGF I receptor upregulation is a primary component of the thrombin-induced mitogenic signaling cascade. Thus, increases in IGF I receptor availability are required for thrombin-induced DNA synthesis. They corroborate our prior observations demonstrating that IGF I receptor density on VSMC is a major determinant of growth responses of these cells (13, 31). The demonstration that the IGF I–IGF I receptor pathway has a critical role in thrombin-induced mitogenic responses in VSMC supports the concept of physiologically relevant interactions between G-protein coupled and tyrosine kinase receptors.

Methods

Cell culture. VSMC were isolated from rat thoracic aorta as described previously by Alexander et al. (32). Cells were grown in medium containing equal parts of Dulbecco's modified Eagle's medium (Gibco Laboratories, Grand Island, NY) and Ham's F12 (Gibco Laboratories), supplemented with 10% calf serum, 2 mM glutamine, 100

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1. *Abbreviations used in this paper:* αIGF I, anti–IGF I polyclonal antiserum; AS, antisense; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; M, mismatch; ODN(s), oligonucleotides(s); VSMC, vascular smooth muscle cell(s).

U/ml of penicillin, and 100 μg/ml of streptomycin. They were passaged twice a week by harvesting with trypsin versene and seeding at a 1:8 ratio in 75-cm^2 flasks. For experiments, cells between passage levels 5 and 15 were seeded into 100-mm dishes or 24- or 48-well cluster dishes. At 80% confluence, cells were quiesced for 24 h in serum-free medium containing antibiotics, glutamine, insulin (0.5 μM), transferrin (5 μg/ml), and ascorbate (0.2 mM) (hereafter designated as serum-free medium).

IGF I mRNA levels. To determine the effect of thrombin on IGF I mRNA levels, quiescent VSMC were exposed to human thrombin (0–5 U/ml, sp act 1,000 U/mg, Calbiochem-Novabiochem Corp., La Jolla, CA) for various times. For some experiments cells were exposed to the rat thrombin receptor-specific activation peptide SFFLRN (1, 10, and 100 μM). The peptide was synthesized by the Microchemical Facility, Emory University, and HPLC purified. In addition, some experiments were performed by incubating cells in the presence of the thrombin inhibitor hirudin alone (1 U/ml) or hirudin and thrombin. Total RNA was extracted from the cells using the guanidium isothiocyanate/cesium chloride method. RNA was quantitated by spectrophotometry, and solution hybridization/RNase protection assays were performed as described previously (10, 14). In brief, 30 μg of total RNA was hybridized to a [^{32}P]UTP-labeled antisense riboprobe generated by T7 polymerase transcription of a linearized plasmid containing the rat IGF I exon 3 and adjacent intron sequences. The plasmid was kindly provided by Dr. P. Rottwein (Washington University School of Medicine, St. Louis, MO). RNA was cohybridized using a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe (33). After RNase digestion, samples were proteinase K treated, phenol extracted, ethanol precipitated, and analyzed on a denaturing polyacrylamide urea (sequencing) gel. Protected IGF I and GAPDH RNA fragments are 182 and 133 bp, respectively. Autoradiograms were exposed for 1–3 d, and protected bands were quantitated by two-dimensional laser densitometry.

IGF I radioimmunoassay. Specific IGF I immunoreactivity of cell conditioned medium was determined as described previously in our laboratory (9). In brief, medium was dialyzed, lyophilized, resuspended in 1 M acetic acid, 0.025 M NaCl, incubated at room temperature for 1 h, and then chromatographed using Bio-Gel P-30 polyacrylamide columns (Bio Rad Laboratories, Hercules, CA). IGF I fractions were assayed using a polyclonal anti–IGF I rabbit antiserum kindly provided by Dr. L. Underwood and Dr. J. J. Van Wyk through the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases. Standard curves were generated using human recombinant IGF I (Ciba-Geigy, Suffern, NY).

Binding assays. To determine the effect of thrombin on IGF I receptor number and binding affinity, quiescent VSMC were exposed to serum-free medium with or without 1 U/ml thrombin for 24 and 48 h. For some experiments cells were exposed to 50 μM SFFLRN for 24 h. Binding assays were performed by incubating cells with 0.1 nM ^{125}I-IGF I and 0–0.1 μM unlabeled IGF I for 90 min at room temperature. Cells were washed in ice-cold binding buffer and solubilized in 2 N NaOH before counting. All assays were performed in duplicate for each experimental point. Data were analyzed using the LIGAND program.

[^{3}H]Thymidine incorporation. Quiescent VSMC were exposed to serum-free medium with or without 1 U/ml thrombin for 24 h. Cells were then incubated with [^{3}H]thymidine (1 μCi/ml) for 24 h in the continued absence or presence of 1 U/ml thrombin. Cells were washed three times with ice-cold PBS, incubated on ice for 15 min with 10% trichloroacetic acid, and after two washes in ice-cold 95% ethanol, radioactivity was extracted with 0.4 N NaOH for assay by liquid scintillation spectrophotometry. To determine the effect of anti–IGF I antiserum on thrombin-induced DNA synthesis, experiments were performed in which cells were exposed with or without agonist and with or without a 1:500 dilution of normal rabbit serum or anti–IGF I antiserum. The anti–IGF I polyclonal antiserum (αIGF I) was raised in rabbits against human recombinant IGF I and showed no cross-reactivity with either insulin or IGF II, as determined by Western immunoblotting and ELISA. This antibody neutralizes up to 100 ng/ml IGF I at a dilution of 1:500. Additional experiments were performed in which thrombin-induced DNA synthesis was determined in the presence of 2 μg/ml monoclonal mouse anti–bovine bFGF antibody (Upstate Biotechnology Incorporated, Lake Placid, NY) or nonimmune IgG. To determine if the effects of IGF I and thrombin on DNA synthesis were additive, quiescent VSMC were incubated in the absence or presence of 1 U/ml thrombin, 50 ng/ml IGF I, or the combination of thrombin and IGF I for 24 h. [^{3}H]Thymidine incorporation was then measured over the subsequent 24 h in the continued presence of agonist. These experiments were also performed in the presence of a 1:500 dilution of αIGF I or a 1:500 dilution of normal rabbit serum.

Growth assay. VSMC were plated at a density of 20,000 cells/ml in 48-well plates. After an overnight incubation in medium containing 10% calf serum, they were exposed to serum-free medium with or without 1 U/ml thrombin and with or without a 1:500 dilution of normal rabbit serum or polyclonal anti–IGF I antiserum (αIGF I). Cells from duplicate wells were counted at 3 d (after harvesting with 25 mM EDTA in PBS).

Antisense IGF I receptor ODNs. To determine the effect of IGF I receptor downregulation on thrombin-induced DNA synthesis, VSMC were incubated in serum-free medium for 48 h with or without 5 μM antisense (AS-1) or mismatch (M-1) phosphorothioate ODNs. ODNs were synthesized by the Microchemical Facility, Emory University, and HPLC purified. The AS-1 ODN is 5′-TCCGGAGCCAGACTTCATTC-3′ and is complimentary to a sequence around the AUG site of the rat IGF I receptor mRNA sequence; the M-1 ODN corresponds to AS-1 with 9 of 20 bp differences, 5′-AGCGGTCCCACTCTTGTTTG-3′. Cells were subsequently exposed to 1 U/ml thrombin for 48 h. [^{3}H]Thymidine (1 μCi/ml) was added during the last 24 h of incubation and trichloroacetic acid precipitable counts were determined as described above.

Statistical analysis. Data are expressed as mean±SE. Analysis of repeated measures was performed by ANOVA and comparison between groups was performed using Student's *t* test.

Results

Effects of α-thrombin on IGF I expression. Exposure of VSMC to α-thrombin for 24 h caused a dose-dependent reduction in steady state IGF I mRNA levels (Fig. 1). The reduction was already evident at 0.02 U/ml thrombin and was maximal with 1 U/ml (∼ 27 nM) (76% reduction, compared with control, $P < 0.001$). To determine the time course of this effect, quiescent VSMC were incubated without or with 1 U/ml thrombin for 6–24 h. As shown in Fig. 1 *C* thrombin caused a progressive decrease in IGF I mRNA levels (78% reduction at 24 h, compared with control, $P < 0.0001$). To determine whether the effect was mediated via proteolytic activation of the thrombin receptor, we measured IGF I mRNA levels after exposure of quiescent VSMC to the hexapeptide SFFLRN that acts as the tethered ligand after cleavage of the amino terminus of the thrombin receptor (Fig. 2 *A*). 100 μM SFFLRN markedly reduced IGF I mRNA levels (67±9% decrease, compared with control, mean±SE, $n = 3$), mimicking the effect obtained with the parent agonist thrombin. 1 and 10 μM SFFLRN, were without effect. To further demonstrate that the effect was mediated by a receptor-dependent mechanism we used hirudin to block binding of thrombin to its receptor (Fig. 2 *B*). Hirudin completely inhibited the thrombin-induced reduction in IGF I mRNA. To determine whether changes in IGF I mRNA resulted in changes in IGF I protein we measured immunoreactive IGF I levels in conditioned medium

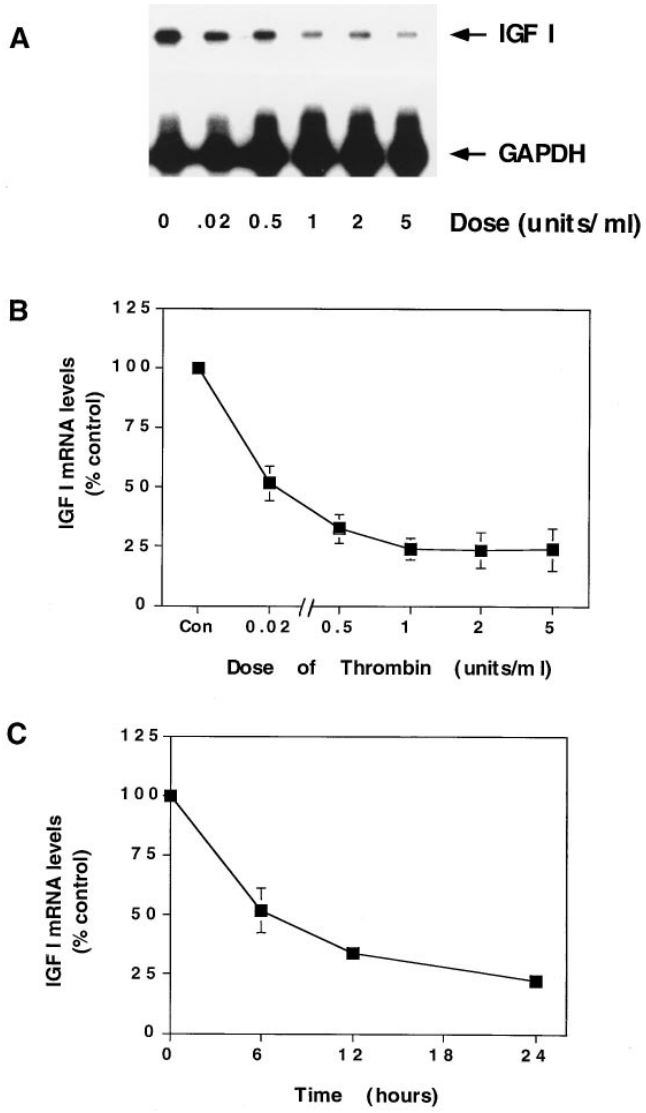

Figure 1. Thrombin downregulates IGF I mRNA levels in VSMC. (*A*) Representative solution hybridization/RNase protection assay. Quiescent VSMC were incubated without or with increasing doses of thrombin for 24 h. Total RNA (30 μg/lane) was cohybridized to ^{32}P-labeled antisense IGF I and GAPDH riboprobes. After RNase digestion, protected bands were analyzed by sequencing gel electrophoresis. (*B*) Densitometric analysis of solution hybridization/RNase protection assays. IGF I mRNA levels (corrected for GAPDH) are expressed as percentage of control. Shown is the mean±SE of determinations from three to four separate experiments for each dose. (*C*) Time course of thrombin-induced downregulation of IGF I mRNA. Quiescent VSMC were exposed to 1 U thrombin/ml for indicated times. IGF I mRNA levels were quantitated by solution hybridization/RNase protection assays, corrected for GAPDH mRNA levels, and are expressed as percentage of control. Shown is the mean±SE of results from two to five separate experiments for each time point.

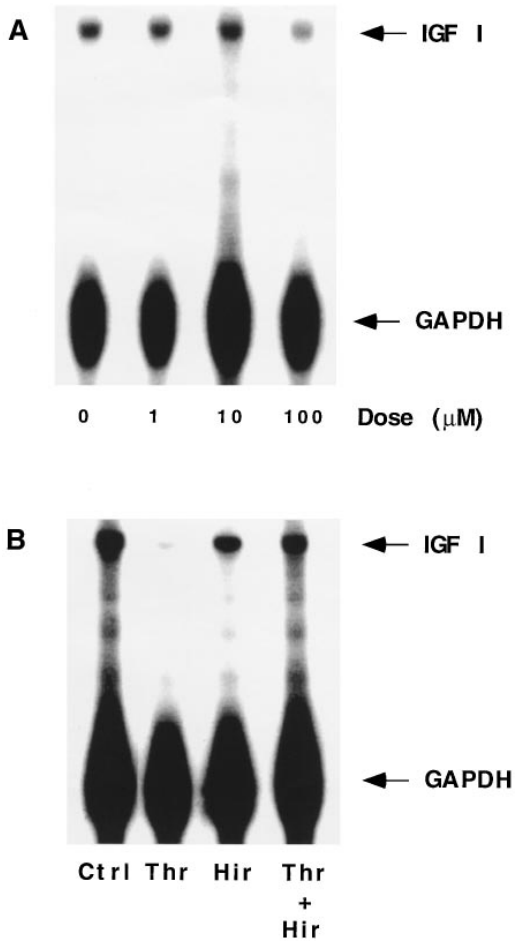

Figure 2. (*A*) Effect of thrombin receptor-specific hexapeptide SFFLRN on IGF I mRNA levels. Quiescent VSMC were exposed to 0–100 μM SFFLRN for 24 h. Total RNA (30 μg/lane) was cohybridized to ^{32}P-labeled IGF I and GAPDH antisense riboprobes. After RNase digestion, fragments were analyzed by sequencing gel electrophoresis. Shown is a representative experiment, which was repeated two additional times. (*B*) Hirudin blocks thrombin-induced downregulation of IGF I mRNA. Quiescent VSMC were incubated without (control, *Ctrl*) or with 1 U/ml thrombin (*Thr*) and/or 1 U/ml hirudin (*Hir*) for 24 h. Total RNA (30 μg/lane) was cohybridized to ^{32}P-labeled IGF I and GAPDH antisense riboprobes. After RNase digestion, products were analyzed by sequencing gel electrophoresis. Shown is a representative experiment, which was repeated once.

from cells exposed to 1 U/ml thrombin for 24 and 48 h. There was no significant change in IGF I levels at 24 h, but there was a 54% reduction at 48 h ($P < 0.001$, Fig. 3 *A*).

Effect of α-thrombin on IGF I receptors. To assess the effect of thrombin on IGF I receptors homologous displacement binding experiments were performed. As shown in Fig. 3 *B*, exposure of VSMC to 1 U/ml thrombin for 24 h doubled cell surface IGF I receptor number ($P < 0.05$). This effect was still evident at 48 h (47% increase, $P < 0.01$). Thrombin did not alter IGF I receptor binding affinity: K_d control, 2.6±0.5 nM; K_d thrombin, 2.8±0.4 nM, mean±SE of results from experiments performed at the 24- and 48-h time points ($n = 6$). To determine whether the effect of thrombin was mediated by proteolytic activation of its receptor, we assessed the effect of 50 μM SFFLRN on IGF I receptors. The hexapeptide increased IGF I receptor number by 34% at 24 h: control, 12.4±3.7 fmol/10^5 cells, peptide 16.5±4.6 fmol/10^5 cells, $n = 5$, $P < 0.025$. The peptide did not alter IGF I receptor binding affinity: K_d control, 1.8±0.2 nM; K_d peptide, 2.1±0.3 nM. To determine whether the thrombin-induced increase in IGF I receptor density resulted in increased mitogenic responsiveness to IGF I, we measured [^{3}H]thymidine incorporation in response to IGF

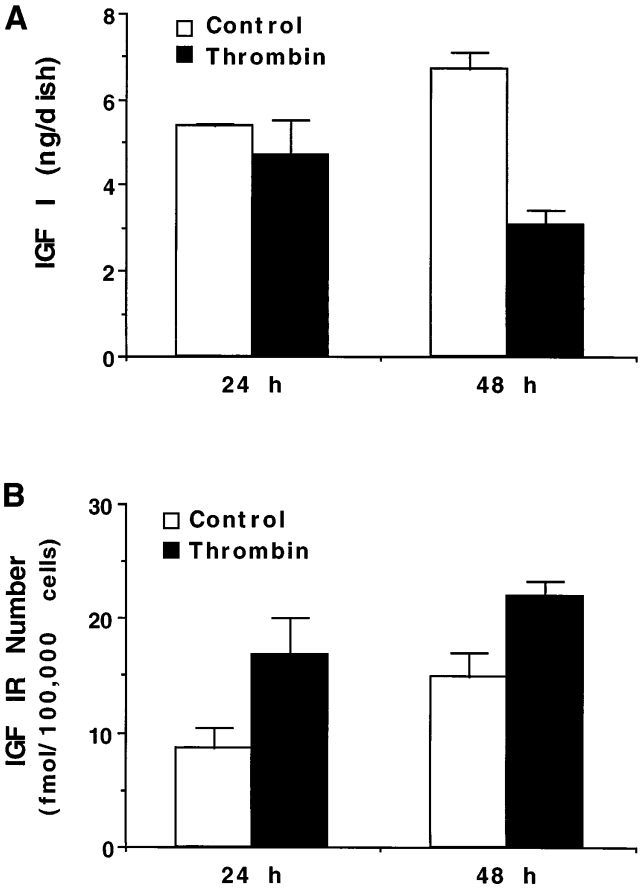

Figure 3. (*A*) Thrombin decreases IGF I secretion from VSMC. Quiescent VSMC were incubated in the absence (control) or in the presence of 1 U/ml thrombin for 24 and 48 h. Conditioned medium was dialyzed, lyophilized, and chromatographed for extraction of IGF I fractions which were assayed by radioimmunoassay. Shown is the mean±SE of triplicate determinations from two separate experiments at 24 h and four separate experiments at 48 h. (*B*) Thrombin increases IGF I receptor density on VSMC. Quiescent VSMC were exposed without (control) or with thrombin 1 U/ml for 24 and 48 h. Radioligand binding studies were performed, and Scatchard analysis using the LIGAND program was performed. Shown is the mean±SE of results from three separate experiments at 24 h and three separate experiments at 48 h.

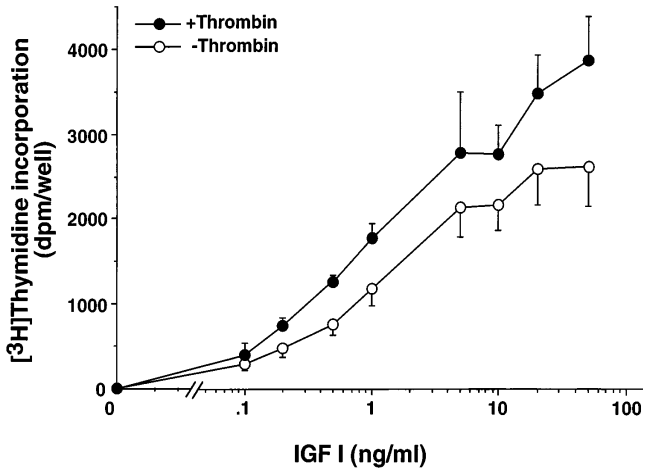

Figure 4. Effect of thrombin on mitogenic response to IGF I. Quiescent VSMC were preincubated in serum-free medium without (−) or with (+) 1 U/ml thrombin for 24 h, and then were exposed to fresh serum-free medium with 0–50 ng/ml IGF I for 24 h. Shown is the IGF I–induced increase in [^{3}H]thymidine incorporation (mean±SE of duplicate determinations from 5 to 11 experiments for each condition).

I in cells preincubated with or without 1 U/ml thrombin for 24 h. Preexposure to thrombin significantly increased DNA synthesis stimulated by IGF I, as indicted by a leftward shift of the dose–response curve ($P < 0.01$, Fig. 4).

Effect of anti–IGF I antiserum on thrombin-induced growth. To determine whether activation of the IGF I receptor was required for thrombin-induced mitogenesis, quiescent VSMC were incubated with 1 U/ml thrombin for 48 h and the effect of polyclonal anti–IGF I antiserum and nonimmune serum on [^{3}H]thymidine incorporation was determined. As shown in Fig. 5 *A*, thrombin induced an approximately twofold increase in DNA synthesis ($P < 0.025$) that was not altered by nonimmune serum but completely blocked by anti–IGF I antibody. It is of note that anti–IGF I antibody decreased basal [^{3}H]thymidine incorporation ($P < 0.01$), as we have shown previously (14). An anti-bFGF antibody decreased thrombin-induced DNA synthesis by 39%, compared with nonimmune IgG ($P = 0.11$). To confirm these results we performed cell counts (Fig. 5 *B*). Thrombin increased cell number by 60% in serum-free medium ($P < 0.025$), whereas in the presence of a 1:500 dilution of anti–IGF I antiserum, thrombin did not significantly increase cell number. Normal rabbit serum (1:500 dilution) increased cell number by 47% ($P = 0.13$) and the addition of thrombin had a further stimulatory effect on cell number (30% increase, $P < 0.05$). To determine if the effects of IGF I and thrombin on DNA synthesis were additive, [^{3}H]thymidine incorporation was measured in cells exposed to 1 U/ml thrombin alone, 50 ng/ml IGF I alone, or their combination. Coincubation of cells with thrombin and IGF I resulted in a 159±51% (mean±SE, $n = 5$) increase in DNA synthesis that was significantly greater ($P < 0.05$) than that induced by thrombin alone (72±15% increase, mean±SE, $n = 5$) or IGF I alone (53±13% increase, mean±SE, $n = 3$). The increase in DNA synthesis induced by the combination of thrombin and IGF I was likewise completely inhibited by a 1:500 dilution of αIGF I and not altered by a 1:500 dilution of normal rabbit serum (not shown).

Effect of antisense IGF I receptor ODNs on thrombin-induced DNA synthesis. We have demonstrated recently that an antisense ODN specific for a sequence at the ATG site of the rat IGF I receptor effectively downregulates IGF I receptors on VSMC, markedly blunting the mitogenic response to serum, angiotensin II, and IGF I. To determine the effect of IGF I receptor downregulation on thrombin-induced DNA synthesis we preincubated VSMC with 5 μM AS-1 or M-1 ODNs for 48 h and measured [^{3}H]thymidine incorporation in response to thrombin. As shown in Fig. 6, 5 μM AS-1 inhibited thrombin-induced DNA synthesis by 91% compared with control ($P < 0.02$), whereas M-1 was without effect.

Discussion

VSMC proliferation and migration are important features of a variety of pathophysiological conditions, including the development of traditional and posttransplantation atherosclerosis, hypertensive vascular remodeling, postangioplasty restenosis,

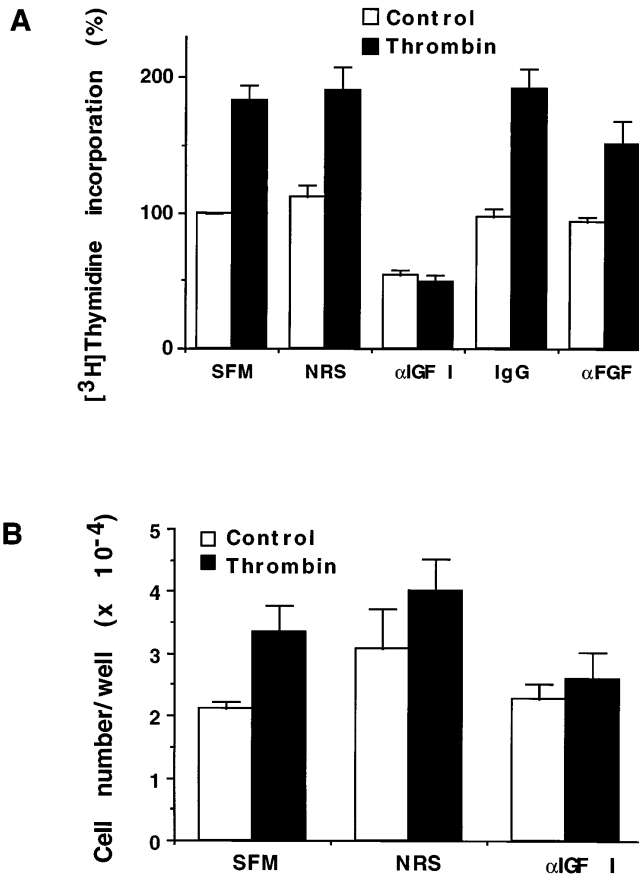

Figure 5. Effect of anti–IGF I antiserum on thrombin-induced DNA synthesis and cell proliferation. (*A*) Quiescent VSMC were exposed without (control) or with 1 U/ml thrombin for 48 h. Experiments were performed in serum-free medium (*SFM*) alone or in the presence of 1:500 dilution of normal rabbit serum (*NRS*), 1:500 dilution of αIGF I, 2 μg/ml nonimmune IgG (*Ig G*), or 2 μg/ml anti-FGF antibody (*α*FGF). [^{3}H]Thymidine incorporation was assessed and is expressed as percent control in SFM. Shown is the mean±SE of duplicate measurements from 6 to 15 separate experiments for each condition. (*B*) Quiescent VSMC were incubated without (control) or with 1 U/ml thrombin in serum-free medium alone (*SFM*) or in the presence of a 1:500 dilution of normal rabbit serum (*NRS*) or polyclonal anti–IGF I antiserum (*αIGF I*). Cells were counted at 3 d. Shown is the mean±SE of duplicate determinations from four separate experiments.

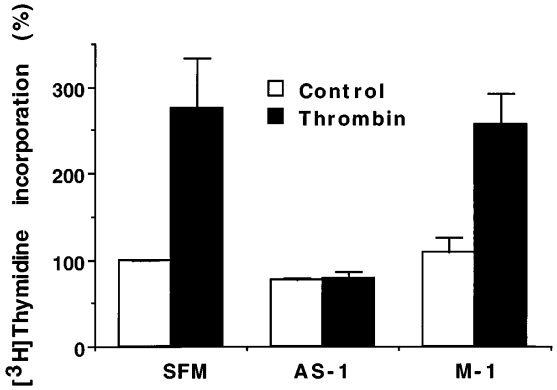

Figure 6. Antisense IGF I receptor ODNs block thrombin-induced DNA synthesis. VSMC were incubated in serum-free medium alone for 48 h (*SFM*) or in the presence of 5 μM antisense (*AS-1*) or mismatch (*M-1*) phosphorothioate ODNs directed against the AUG site of the IGF I receptor mRNA. Cells were then incubated without (control) or with 1 U/ml thrombin for 48 h and [^{3}H]thymidine incorporation was measured. Shown is the mean±SE of duplicate determinations from three separate experiments, expressed as percent control in SFM.

and graft occlusive disease (1–3). Identification of cytokines and growth factors central to this process is thus of enormous clinical importance.

IGF I is a 70–amino acid peptide whose pleiotropic effects are mediated by a membrane receptor with intrinsic tyrosine kinase activity (34). These include stimulation of cellular growth and differentiation, metabolic effects, stimulation of hematapoiesis, and vasodilatory effects, among others (35). The primary role of IGF I in developmental growth is demonstrated by the lethality that results from targeted mutation of the IGF I receptor, and by the significant growth retardation and mortality produced by targeted mutation of the IGF I gene (36, 37). IGF I circulates at high levels in plasma, and is also produced in multiple tissues, where it has an autocrine and paracrine function (35). Thus VSMC in vitro and in vivo express IGF I and its receptor (4–13).

The potential role of IGF I in vascular growth responses in vivo is supported by a variety of studies including the demonstration that VSMC IGF I expression is increased after balloon injury (5, 11), increases in vascular load (33), and induction of hypertension (12). An important question that remains unsolved is the relative importance of IGF I, compared with other growth factors, in the control of vascular proliferation. In a broader sense the control of the cell cycle by various growth factors is only partially understood. In mouse fibroblasts IGF I acts as a progression factor, allowing entry of cells into S phase (38). In smooth muscle cells IGF I is important for the growth response to PDGF and to angiotensin II, since neutralizing anti–IGF I antiserum blocks growth responses to these agonists (6, 14). Thrombin is a serine protease that activates a G-protein coupled receptor and has a well-established role in thrombus formation (15). More recently thrombin has been shown to have proinflammatory effects (16–18) and to be a potent mitogen for VSMC (19–21). It has thus been suggested that thrombin may have an important role in vascular proliferative responses. Cellular mechanisms involved in thrombin's mitogenic effects are poorly understood, but the delayed onset of thrombin-induced DNA synthesis has suggested the participation of autocrine factors (21). The purpose of this study was to study potential interactions between thrombin and the VSMC IGF I–IGF I receptor axis and to determine the role of this autocrine pathway in thrombin-induced growth of VSMC.

Our findings unequivocally demonstrate that thrombin progressively reduced IGF I secretion from VSMC. This effect is mediated by a receptor-dependent mechanism. It is blocked by the thrombin inhibitor hirudin and is reproduced by exposure to the peptide SFFLRN, which can function as a tethered ligand after cleavage of the amino terminus of the receptor. In contrast, however, thrombin markedly increased IGF I receptor density, and this increase preceded the reduction in ligand. The increase in IGF I receptors induced by thrombin resulted in a potentiation of the mitogenic response to IGF I. To determine whether endogenously produced IGF I was required for thrombin-induced mitogenic signaling we tested the effect of neutralizing anti–IGF I antiserum on thrombin-induced DNA

synthesis and proliferation. This antibody markedly reduced thrombin-induced growth responses, indicating an absolute requirement for IGF I in the thrombin-stimulated mitogenic signaling pathway. Furthermore, coincubation experiments indicated that although the effects of thrombin and IGF I on DNA synthesis were additive, an anti–IGF I antiserum completely suppressed DNA synthesis induced by the combination of thrombin and IGF I. It is of note that we cultured our cells in a defined serum-free medium that contains insulin and that has been shown to maintain VSMC in an anabolic, quiescent state (39). Although insulin may bind with low affinity to the IGF I receptor, our findings cannot be related to an antiinsulin effect since the antibody does not cross-react with insulin. To confirm these results we preincubated VSMC with 5 μM AS-1 ODNs for 48 h, which we have previously reported to decrease IGF I receptor binding sites by ~ 50%, without altering binding affinity (31). This preincubation markedly inhibited subsequent thrombin-induced DNA synthesis. The M-1 ODN, which we have previously shown to have no effect on IGF I receptor number or binding affinity, did not alter thrombin-induced DNA synthesis.

Our findings not only establish that IGF I is an essential requirement for thrombin-induced mitogenesis, but they also suggest that upregulation of IGF I receptors (and thus receptor availability) may play a key role in maintaining an active IGF I–IGF I receptor signaling pathway. Thus, both PDGF and angiotensin II upregulate IGF I receptors on VSMC (40, 41) and their mitogenic effects are blocked by anti–IGF I antiserum (6, 14). The concept that upregulation of IGF I receptors may serve a key function in mitogenic responses to agonists is supported by our recent demonstration that VSMC mitogenic responses to angiotensin II are almost completely inhibited by downregulation of IGF I receptors (31). Furthermore, the effect of IGF I receptor downregulation cannot be reversed by high exogenous doses of IGF I. Similar findings have been reported in SV-40 T antigen–transformed BALB/c3T3 cells. These cells manifest a marked increase in IGF I secretion and antisense oligonucleotide mediated downregulation of the IGF I receptor inhibits their growth (42). We have demonstrated recently that inducible and constitutive transcription of an antisense IGF I receptor cDNA construct in VSMC reduces IGF I receptor number by ~ 50% and inhibits the growth response to 10% serum by 60% (13). This finding establishes that IGF I receptor availability plays a key role in VSMC proliferative responses.

It is important to note that tyrosine kinase activity has been shown previously to be essential for thrombin-induced mitogenesis of VSMC (29). Weiss et al. (30) have proposed that this activity is contributed by the bFGF receptor, since an anti-bFGF antibody at high concentrations (30 μg/ml) reduced thrombin-induced DNA synthesis by 60%. Our data indicate that 2 μg/ml anti-bFGF antibody reduced thrombin-stimulated DNA synthesis by 39%, which was not statistically significant ($P = 0.11$), although this reduction may be biologically relevant. Increasing the concentration to 5 μg/ml had no additional effect (not shown). The difference in our results may relate to a difference in cells (Weiss and co-workers used newborn rat cells) or culture conditions. Importantly, however, these authors observed that the tyrosine kinase inhibitor herbimycin A diminished thrombin's mitogenic effect by > 90%, while anti-bFGF antibody was partially effective. It is of note that bFGF upregulates IGF I receptors on VSMC (41) and thus one may speculate that the inhibitory effect of anti-bFGF antibody described by Weiss et al. (30) may be related to a reduction in IGF I binding sites.

An unresolved issue is why an intact IGF I–IGF I receptor signaling axis is required for thrombin-induced mitogenesis. Possibly, substrates of the IGF I receptor tyrosine kinase or kinases that are activated more distally are essential cofactors for thrombin-induced mitogenic signaling. Alternatively it is possible that upregulation of IGF I receptors by thrombin is sufficient to induce signaling through the IGF I receptor pathway (in spite of reduced total ligand availability), thereby stimulating mitogenesis. In this respect it is important to note that although thrombin reduces total IGF I secretion, available free extracellular IGF I may potentially be increased, because thrombin markedly reduces levels of the inhibitory IGF I binding protein, IGFBP-4, in VSMC-conditioned medium.[2] Furthermore, we have shown recently that thrombin increases phosphorylation of insulin receptor substrate-1, an important early intermediate in the IGF I receptor signaling pathway.[3] This is consistent with an interaction between the thrombin receptor and IGF I receptor signaling pathways.

We thus conclude that thrombin has opposing effects on the VSMC IGF I–IGF I receptor axis, inducing a progressive decrease in IGF I synthesis and a marked upregulation of IGF I receptors. The upregulation of IGF I receptors likely overrides the effect of reduced total ligand availability. Thus neutralization of extracellular IGF I inhibits thrombin-induced DNA synthesis, as does downregulation of IGF I receptors. These data provide compelling evidence that the IGF I–IGF I receptor axis is essential for thrombin-induced mitogenesis, consistent with a novel interaction between G-protein coupled and tyrosine kinase receptor signaling pathways. Our findings have important implications for understanding mechanisms whereby thrombin induces VSMC growth in vivo.

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