Distinct and Common Pathways in the Regulation of Insulin-like Growth Factor-1 Receptor Gene Expression by Angiotensin II and Basic Fibroblast Growth Factor

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Angiotensin II (Ang II) and basic fibroblast growth factor (bFGF) are important modulators of cell growth under physiological and pathophysiological conditions. We and others have previously shown that these growth factors increase insulin-like growth factor-1 receptor (IGF-1R) number and mRNA in vascular smooth muscle cells and that this effect is transcriptionally regulated. To study the mechanisms and the signaling pathways involved, IGF-1R promoter reporter constructs were transiently transfected in CHO-AT1 cells that overexpress angiotensin AT1 receptors. Our findings indicate that Ang II and bFGF significantly increased IGF-1R promoter activity up to 7- and 3-fold, respectively. The effect induced by Ang II was mediated via a tyrosine kinase-dependent mechanism, since tyrphostin A25 largely inhibited the Ang II-induced increase in promoter activity. In addition, co-transfection of dominant negative Ras, Raf, and MEK1 or pretreatment with the MEK inhibitor PD 98059 dose-dependently decreased both the Ang II- and bFGF-induced increase in IGF-1R transcription and protein expression, suggesting that the Ras-Raf-mitogen-activated protein kinase pathway is required for both growth factors. Reactive oxygen species have been shown to act as second messengers in Ang II-induced signaling, and activation of the transcription factor NF-κB is redox-sensitive. While co-transfection of dominant negative IκBα mutant completely inhibited the Ang II-induced increase in transcription, it had no effect on the bFGF signaling. In contrast, co-transfection studies indicated that the transcription factors STAT1, STAT3, and c-Jun and the Janus kinase 2 kinase are required in the signaling pathway of bFGF, whereas only dominant c-Jun inhibited the Ang II-induced effect. In summary, these data demonstrate that Ang II and bFGF increase IGF-1R gene transcription via distinct as well as shared pathways and have important implications for understanding growth-stimulatory effects of these growth factors on vascular cells.

The vascular response to injury requires a coordinated inter-action between hemostatic and inflammatory systems and is regulated by cytokines and growth factors that act locally to regulate cellular proliferation and tissue repair. Among the many growth factors that have been shown to be implicated in the response to vascular injury, angiotensin II (Ang II) is of particular interest. It stimulates a variety of physiological responses related to regulation of blood pressure, salt, and fluid homeostasis (1). However, Ang II has also been shown to function, either directly or indirectly, as a growth factor for vascular smooth muscle cells, cardiac fibroblasts, and cardiac myocytes (2–7). The array of genes induced by Ang II includes proto- oncogenes such as c-fos, c-jun, c-myc, and egr-1 (5, 7–9), genes encoding extracellular matrix proteins such as collagen, fibronectin, and tenasin (10–12), and genes for growth factors like transforming growth factor β, platelet-derived growth factor (PDGF), insulin-like growth factor-1 (IGF-1), and its receptor (5, 7, 12–14). Similarly, basic fibroblast growth factor (bFGF) has been implicated in the vascular injury response. In particular, bFGF increases endothelial cell migration and proliferation and also stimulates angiogenesis in vitro and in vivo (15, 16). The role of bFGF in vessel injury and repair is further supported by evidence that bFGF is released from vessel wall cells after injury (17) and that bFGF mRNA is up-regulated in atherosclerotic lesions (18).

Accumulating evidence has shown that the insulin-like growth factor-1 receptor (IGF-1R) is a convergence point of the control of cell growth. Thus, a functional IGF-1R autocrine loop is required for the mitogenic effects of various growth factors, such as PDGF (19, 20), epidermal growth factor (20–22), thrombin (23), bFGF (24–26), and Ang II (14). Furthermore, we and others have demonstrated that PDGF (24–26), thrombin (27), bFGF (25, 26, 28), and Ang II (25) increase IGF-1R density on vascular cells. Inhibition of this effect by IGF-1R antisense phosphorothioate oligonucleotides inhibits the Ang II-induced cellular growth (29).

Ang II exerts its effects through specific G-protein-coupled receptors, predominantly through the AT1 receptor subtype. These receptors induce intracellular calcium mobilization; activation of tyrosine kinases such as p125FAK, p46SHC, and p54Nck; induction of serine/threonine kinases, including protein kinase C and mitogen-activated protein kinases (MAPKs)

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§ The abbreviations used are: Ang II, angiotensin II; IGF-1, insulin-like growth factor-1; IGF-1R, insulin-like growth factor-1 receptor; bFGF, basic fibroblast growth factor; PDGF, platelet-derived growth factor; ETYA, eicosatetrayonic acid; MAPK, mitogen-activated protein kinase; MAPKK, mitogen-activated protein kinase kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; STAT, signal transducer and activator of transcription; UTR, untranslated region; PAG, polyacrylamide gel electrophoresis; SEK, stress-activated extracellular signal-regulated kinase; Jak, Janus kinase; BAPTA/AM, 1,2-bis(aminophenoxy)ethane-N,N,N’,N”-tetraacetic acid tetra(ace-toxyethyl) ester.

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luciferase activities were measured using an EG & G Berthold luminometer (Bad Wildbad, Germany). Firefly luciferase activity was normalized to the internal control Renilla luciferase (Luc/Ren).

Western Blot Analysis—Cultured CHO-AT₁, or CHO-K1 cells were serum-starved for 24 h prior to the addition of Ang II (100 nm) or bFGF (10 ng/ml) for the times indicated. Cells were incubated with the MAPKK inhibitor PD 98059 (10 μM), the tyrosine kinase inhibitor tyrphostin (10 μM), the p38 MAPK inhibitor SB 203580 (10 μM), or ETA (10 μM) 1 h prior to the addition of Ang II or bFGF. Cells were washed in ice-cold phosphate-buffered saline and lysed in lysis buffer containing 150 mM NaCl, 10 mM Tris–Cl, pH 7.4, 5 mM KCl, 1 mM DTT, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM e-aminoacaproic acid, 1 mM sodium orthovanadate, 0.1% sodium deoxycholate, 0.1 μM aprotinin, 10 μg/ml leupeptin, and 10 μg/ml NaF. Lysates were subjected to SDS-PAGE on 7.5% or 12% gels, and separated proteins were transferred to polyvinylidene fluoride membranes. Blots were blocked at 5% dry milk; incubated with polyclonal anti-MAPK, anti-phosphospecific MAPK (p42/p44), or anti-IGF-1Rβ antibodies; and then incubated with peroxidase-conjugated donkey anti-rabbit antibody. Immunopositive bands were visualized by enhanced chemiluminescence. Purified MAPK protein was included as positive control; nonimmune rabbit IgG was used as negative control.

RESULTS

Effect of Ang II on IGF-1R Promoter Activity and Localization of the Ang II-responsive Element—To measure the effect of Ang II on IGF-1R gene expression and to localize the Ang II-responsive region, CHO-AT₁ cells were transiently transfected with IGF-1R promoter constructs containing a luciferase reporter gene under the control of the proximal promoter region of the IGF-1R gene together with the Renilla luciferase thymidine kinase expression vector (pRL-TK) as internal control. Ang II (100 nm) significantly increased IGF-1R promoter activity between 2.3- and 7-fold depending on the promoter constructs (Fig. 1B). No effect was observed when the promoterless pOLUC was used (data not shown). The biggest effect induced by Ang II was seen with the promoter construct containing 476 base pairs of the 5′-flanking region and 640 base pairs of the 5′-untranslated region (UTR) (p = 476/640-Luc)), and a lesser response occurred with the construct containing a shorter 5′-UTR. It appeared that there were some negative regulatory elements between nucleotides 2350 and 476, because the longest reporter construct responded less to Ang II stimulation, compared with (476/640-Luc)). When the sequence between nucleotides −270 and −135 was deleted, the stimulatory effect of Ang II was greatly diminished, although not completely, suggesting that the major Ang II-responsive element may be located between nucleotides −270 and −135 of the 5′-flanking region.

Effect of bFGF on IGF-1R Gene Transcription—The stimulatory effect induced by bFGF in CHO-AT₁ cells ranged between 1.7- and 3.2-fold (Fig. 1C), and the same was found in the parental cell line CHO-K1 (data not shown). Previous studies have suggested that the bFGF-response element may be located between nucleotides −476 and −476, because the longest reporter construct responded well to Ang II stimulation, compared with (−476/640-Luc)). When the sequence between nucleotides −270 and −135 was deleted, the stimulatory effect of Ang II was greatly diminished, although not completely, suggesting that the major Ang II-responsive element may be located between nucleotides −270 and −135 of the 5′-flanking region.

Effect of Ang II and bFGF on IGF-1R Protein Levels—To assess whether Ang II or bFGF increased IGF-1R protein levels, cell lysates of cells treated with or without the corresponding growth factor were assayed for IGF-1R protein levels by Western immunoblot. Fifty micrograms were fractionated on a 7.5% reducing SDS-PAGE. Ang II and bFGF increased IGF-1R

EXPERIMENTAL PROCEDURES

Materials—Cell culture media and LipofectAMINE were purchased from Life Technologies (Baltimore, MD) and Ham’s F-12 medium was purchased from New England Biolabs (Beverly, MA). The MAPK pathway (42–44), activation of the Jak/STAT cascade (45, 46), anti-MAPK and phosphospecific anti-MAPK (p42/p44) antibodies, and the horseradish peroxidase-conjugated anti-rabbit immunoglobulin were purchased from New England Biolabs (Beverly, MA). The full length promoter of the IGF-1R was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell culture—CHO-AT₁ cells (kindly provided by Dr. E. Clauser, INSERM, Paris) stably overexpressing the Ang II AT₁ receptor (49) were grown in Ham’s F-12 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.75 μg/ml G418 and incubated at 37 °C in a humidified 5% CO₂ atmosphere. The parental cell line CHO-K1 (ATCC, Rockville, MD) was cultured under the same conditions but without the addition of G418.

Plasmids and Transfections—The full length promoter of the IGF-1R (−2350/+640-Luc) and a shorter promoter construct (−476/+640-Luc) were generously gift from Dr. H. Werner (National Institutes of Health, Bethesda, MD). Deletion fragments were made from the full-length promoter construct and subcloned upstream of the firefly luciferase cDNA, resulting in fragments extending from nucleotides −476 to +21, −416 to +21, −330 to +21, −270 to +21, and −135 to +21. The constructs have previously been described: dominant negative mutant p21(WAF1) (N17) (50), dominant negative Raf (301) (51), dominant negative MAPKK I (MEK1) mutant A221 (52), dominant negative Jun kinase (stress-activated extracellular signal-regulated kinase (SEK1)) (53), dominant negative c-Jun (Tam67) (54), dominant negative STAT1 Tyr701 (55), dominant negative STAT3 Tyr705 (56), kinase-deficient Jak2 kinase (57), and dominant negative IκBα K21/22R (58). To control for transfection efficiency and interwell variation, cells were co-transfected with the internal control vector pRL-TK according to the manufacturer (Promega Corporation, Madison, WI), and the MAPK pathway (42–44), the Jak/STAT cascade (45, 46), and activation of the MAPK pathway (42–44), the Jak/STAT cascade (45, 46), and activation of the Jak/STAT cascade (45, 46). Similarly to Ang II, bFGF has been shown to induce the expression of the early response gene c-fos (47). However, the signaling pathways by which Ang II or bFGF increase transcriptional activity of the IGF-1R gene are unknown, with the exception that the bFGF, but not the Ang II effect, is protein kinase C-dependent (48).

The purpose of the present studies was to localize the Ang II- and the bFGF-responsive elements in the IGF-1R promoter and define the signaling cascades whereby these two growth factors increase IGF-1R gene transcription or protein expression. We show that Ang II and bFGF positively regulate transcriptional activity of the IGF-1R gene and that they increase IGF-1R gene expression via common as well as distinct signaling pathways.
protein expression, with a maximum seen after 24 h of incubation (Fig. 2). Incubation with Ang II or bFGF for 48 h did not further increase IGF-1R protein levels (not shown).

**Common and Distinct Signaling Pathways of Ang II and bFGF on the Stimulation of IGF-1R Gene Expression and on Protein Level**—The Ang II AT₁ receptor belongs to the family of G-protein-coupled seven-transmembrane domain receptors (60), whereas the bFGF receptor is characterized by a single transmembrane domain that has intrinsic tyrosine kinase activity (37–39). To define signal transduction pathways by which Ang II and bFGF stimulate transcription of the IGF-1R, cells were transfected with the indicated promoter reporter constructs and either pretreated with various inhibitors or co-transfected with increasing doses of dominant negative expression constructs prior to the addition of Ang II or bFGF. The protein-tyrosine kinase inhibitor tyrphostin A25 (10 \( \mu \)M) de-

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**Fig. 1. Regulation of IGF-1R promoter activity by Ang II and bFGF.** A, IGF-1R promoter constructs used. CHO-AT₁ cells were transiently transfected with 1 \( \mu \)g of the various reporter plasmids and 5 ng of pRL-TK and 20 h later incubated with or without Ang II (100 nM) (B) or bFGF (10 ng/ml) (C) for 24 h. The luciferase values are normalized to Renilla luciferase activity and presented as mean ± S.E. from five separate experiments. Hatched bars, control cells; filled bars, cells stimulated with Ang II or bFGF, respectively.

**Fig. 2. Western blot analysis of the effect of Ang II and bFGF on IGF-1R levels in CHO-AT₁ cells.** After 24 h of serum starvation, cells were treated with 100 nM Ang II or 10 ng/ml bFGF for the times indicated. Total proteins from cell lysates were subjected to SDS-PAGE under reducing conditions on 7.5% gels and transferred to polyvinylidene fluoride membranes. Membranes were then probed with an antibody recognizing the \( \beta \)-subunit of the IGF-1R. Lanes 1 and 5, control cells; lanes 2–4 and 6–8, cells were incubated with Ang II or with bFGF for 4, 8, and 24 h, respectively. Fifty \( \mu \)g of protein were loaded.
CHO-AT1 cells were transfected with the p(-270/+21-Luc) and the p(-476/+640-Luc) constructs and the pRl-TK plasmid to correct for interwell variation. Twenty hours after transfection, cells were incubated with or without tyrphostin A25 (10 μM) prior to the addition of Ang II (100 nM) for 1 h prior to the addition of Ang II (100 nM). Twenty four hours later, cells were lysed, and luciferases were measured. Data are presented as Luc/Ren and are mean ± S.E. of three separate experiments. Tyrphostin A25 alone had no effect on basal luciferase activity. Tyrphostin A25 had the same inhibitory effect on all other IGF-1R promoter constructs (data not shown). B, cells were treated with or without genistein (60 nM) or ETYA (10 μM) prior to the addition of Ang II for 24 h. Ten micrograms of total protein were subjected to SDS-PAGE on a 12% reducing gel and probed with anti-phosphospecific p44/p42 antibody.

Fig. 3. Effect of protein tyrosine kinase inhibition and lipoxygenase inhibition on the Ang II-induced increase in IGF-1R transcription and on AngII-induced MAPK phosphorylation. A, CHO-AT, cells were transfected with the p(-476/+640-Luc) and the p(-270/+21-Luc) construct and the pRl-TK plasmid to correct for interwell variation. Twenty hours after transfection, cells were incubated with or without tyrphostin A25 (10 μM) for 1 h prior to the addition of Ang II (100 nM). Twenty four hours later, cells were lysed, and luciferases were measured. Data are presented as Luc/Ren and are mean ± S.E. of three separate experiments. Tyrphostin A25 alone had no effect on basal luciferase activity. Tyrphostin A25 had the same inhibitory effect on all other IGF-1R promoter constructs (data not shown). B, cells were treated with or without genistein (60 nM) or ETYA (10 μM) prior to the addition of Ang II for 24 h. Ten micrograms of total protein were subjected to SDS-PAGE on a 12% reducing gel and probed with anti-phosphospecific p44/p42 antibody.

Increased the Ang II response by 60–70%, suggesting that protein tyrosine phosphorylation is involved in the pathway of Ang II (Fig. 3A). Similar results were obtained when genistein, another tyrosine kinase inhibitor was used (54 ± 1.1 to 82 ± 5.3% inhibition of the Ang II response, depending on the promoter construct used; mean ± S.E. of three experiments).

We have previously shown that Ang II signals through a lipoxygenase-dependent pathway to increase macrophage-mediated oxidative modification of low density lipoprotein (61). Therefore, we were interested to see if Ang II would also signal through that pathway to increase IGF-1R transcription. Indeed, ETYA almost completely inhibited the stimulatory effect of Ang II on IGF-1R transcription (representative experiments as follows: for −476/+640, control, 9.5; Ang II, 36.8; and ETYA/Ang II, 17.9 Luc/Ren, respectively; or for −416/+21, control, 5.4; Ang II, 16.5; and ETYA/Ang II, 7.2 Luc/Ren), whereas it had no effect on the increase induced by bFGF (−2350/+640: control, 10.1 ± 3.6; bFGF, 16.8 ± 1.4; ETYA, 14.7 ± 4.8; and ETYA/bFGF, 19.8 ± 5.9, Luc/Ren respectively (mean ± S.D. of two experiments). ETYA alone had no effect on basal luciferase activity (data not shown). In agreement with the above mentioned experiments using genistein or ETYA to block the Ang II stimulation of IGF-1R gene transcription, both blockers also inhibited Ang II-induced MAPK phosphorylation, suggesting that a tyrosine kinase- and a lipoxygenase-dependent step are upstream of MAPK activation (Fig. 3B).

It is documented that Ang II activates the MAPK pathway in vascular smooth muscle cells (62, 63) and that this activation is partially dependent on protein kinase C (62) and apparently requires prior activation of a Ca²⁺-dependent tyrosine kinase (64). Co-transfection experiments with dominant negative Ras, Raf, and MEK1 suggested that the Ras-Raf-MAPKK pathway is involved in the transcriptional activation of the IGF-1R by Ang II because all inhibited the luciferase response induced by Ang II (Fig. 4), whereas the empty vectors had no effect (data not shown). Similarly, the specific MAPKK inhibitor PD 98059 (100 μM) blocked the Ang II response to control levels in all promoter constructs without having any effect on basal luciferase activity (−2350/+640: control, 3.3 ± 0.8; Ang II, 13.9 ± 2.7; PD 98059/Ang II, 5.1 ± 1.2 Luc/Ren, respectively; −135/+21: control, 6.8 ± 1.1; Ang II, 20.7 ± 2.2; and PD 98059/Ang II, 8.6 ± 1.9 Luc/Ren, respectively (mean ± S.E. of five independent experiments)). In contrast, while the response induced by Ang II required the p44/p42 MAPK activation, the p38 MAPK inhibitor SB 203580 had no effect on the Ang II response, suggesting that p38 MAPK was not involved (−476/+21: control, 6.5 ± 0.6; SB 203580, 5.7 ± 0.6; and SB 203580/Ang II, 24.2 ± 1.7 Luc/Ren, respectively (mean ± S.E. of four experiments)). To confirm the specificity of these findings, cells were treated with or without Ang II for various times, and total proteins were immunoblotted with phosphospecific antibodies against p44 and p42 (extracellular signal-regulated kinases 1 and 2). Ang II induced a rapid phosphorylation of p44/p42 already after 2 min, with a maximum at 5 min. PD 98059 completely inhibited the Ang II-induced phosphorylation of p44/p42 (data not shown). It is known that MAPKs in turn phosphorylate numerous cellular proteins, including c-Jun among many others (65). When dominant negative c-Jun (Tam67) was co-transfected with the p(−476/+21-Luc), it completely reduced the stimulatory effect of Ang II to control values, whereas the empty vector had no effect (Fig. 5).

This inhibitory effect of dominant negative c-Jun could also be observed with the smaller IGF-1R promoter constructs p(−270/+21-Luc) and p(−135/+21-Luc) (data not shown).

We have seen that the Ang II-induced IGF-1R gene expression is calcium-dependent and is mediated via a redox-sensitive pathway. Indeed, intracellular Ca²⁺ chelation using BAPTA/AM (10 μM) decreased the stimulatory effect of Ang II to control levels without having any effect on basal luciferase activity, suggesting that intracellular Ca²⁺ is required (−476/+640: control, 10.8 ± 1.3; BAPTA/AM, 13.4 ± 1.9; Ang II, 31.5 ± 2.5; and BAPTA/AM/Ang II, 8.7 ± 7.0 Luc/Ren, respectively (mean ± S.D. from two experiments)). Also, reactive oxygen species have been shown to have act as second messengers in Ang II-induced signaling (66) and activation of the transcription factor NF-κB is redox-sensitive (67). Co-transfection of the IGF-1R promoter construct p(−476/+640-Luc) with K212/22R, the IκBα mutant that shows a defect in degradation and in ubiquitin conjugation and therefore inhibits translocation of NF-κB to the nucleus (58), completely inhibited the Ang II-induced increase in IGF-1R transcription (Fig. 6A). This was also true when the shorter construct p(−270/+21-Luc) was used (data not shown). Interestingly, the IκBα mutant had no effect on the Ang II response when the short IGF-1R promoter construct p(−135/+21-Luc) was used, suggesting that a puta-
Quite in contrast to Ang II, however, co-transfection with IGF-1R activation of IGF-1R promoter activity. Cells were transfected with the p(-476/+640-Luc) IGF-1R promoter construct and increasing doses of dominant negative Raf, Ras, and MEK1. After transfection, cells were then stimulated with or without Ang II (100 nM) for 24 h. The experiments were performed twice with essentially identical results.

The Raf-Ras-MAPK kinase pathway is required in the transcriptional stimulation of the IGF-1R promoter and IGF-1R protein by Ang II. CHO-AT1 cells were transfected with the p(-476/+640-Luc) IGF-1R promoter construct and increasing doses of dominant negative Tyrphostin A25, 6.5

Cells were transfected with the p(-476/+21-Luc) construct and increasing doses of Tam67. Twenty hours later, cells were stimulated with or without Ang II. Data are expressed as mean ± S.E. of three experiments.

While both Ang II and bFGF stimulated IGF-1R gene transcription (Fig. 1), the signal pathway by which these two growth factors mediate the increase in IGF-1R expression showed common but also distinct features. Thus, tyrphostin A25 reduced the stimulatory effect of bFGF on IGF-1R transcription by 44.4 ± 3.8% (−476/+21: control, 3.8 ± 0.10; tyrphostin A25, 3.1 ± 0.08; bFGF, 11.8 ± 0.46; and tyrphostin A25/bFGF, 6.5 ± 0.29, respectively (mean ± S.E. of four experiments)). The Ras-Raf-MAPK pathway seemed also to be required in the transcriptional activation of the IGF-1R by bFGF, as we found with Ang II. Dominant negative Ras, Raf, and MEK1 inhibited the bFGF-induced increase in luciferase activity to a similar degree as seen with Ang II (Fig. 7). Accordingly, the MAPKK inhibitor PD 98059 (100 μM) completely abrogated the bFGF-induced stimulation of IGF-1R transcription and inhibited the bFGF-induced phosphorylation of p44/p42 MAPK (data not shown). Furthermore, BAPTA/AM inhibited the bFGF-induced stimulation of IGF-1R expression to control values (representative experiment −476/+640: control, 9.9; bFGF, 33.3; and BAPTA/bFGF, 13.7 Luc/Ren, respectively); however, the p38 MAPK inhibitor SB 203580 did not inhibit the bFGF response similarly to Ang II-stimulated IGF-1R expression (data not shown). The c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) has been shown to phosphorylate and regulate the activity of several transcription factors including c-Jun, ELK-1, and ATF-2 (68–72). The JNK/SAPK is phosphorylated, resulting in its activation by JNK kinase (JNK kinase/SEK1) (53, 73–75). Increasing doses of dominant negative SEK1 expression construct produced a dose-dependent decrease in the IGF-1R transcriptional activity induced by bFGF when the p(-476/+21-Luc) reporter construct was used, suggesting that the SEK1/JNK/SAPK pathway was involved (Fig. 8A). It is of interest that the same dominant negative SEK1 did not have any effect on IGF-1R transcriptional stimulation by Ang II (data not shown), despite the inhibitory effect of dominant negative c-Jun in the response to Ang II (Fig. 5). It is of note that dominant negative c-Jun also dose-dependently reduced the stimulatory effect seen with bFGF (Fig. SB).

More recently, a novel nuclear signaling pathway has been described that regulates a large family of transcription factors called STATs (76). This pathway, initially described for the interferon receptors, has subsequently been shown to be involved in hormone and growth factor signaling, such as growth hormone (77), Ang II (36), or bFGF (45, 46). We have previously shown that Ang II directly stimulates the Jak/STAT pathway in rat aortic smooth muscle cells by phosphorylation of the intracellular Jak2 kinase and its substrates STAT1 and STAT2 (36). We therefore investigated whether kinase-deficient Jak2 or dominant negative STAT1 Tyr701 and STAT3 Tyr705 mutants could inhibit the increase in IGF-1R transcriptional activity induced by Ang II or bFGF. While all mutants had no effect on the Ang II response (data not shown), they greatly reduced the effect seen with bFGF (Fig. 9), suggesting that the Jak/STAT pathway is involved in the response to bFGF but not to Ang II in this cell model.

Similarly to the transcriptional assays, the MAPKK inhibitor PD 98059 and the protein tyrosine kinase inhibitor tyrphostin A25 decreased the stimulatory effect of Ang II or bFGF on IGF-1R protein expression, whereas the lipoxygenase inhibitor ETYA blocked only the Ang II response. Furthermore, the p38 MAPK inhibitor SB 203580 did not inhibit the Ang II or bFGF effect (Fig. 10), confirming the results observed in the reporter assays.

**DISCUSSION**

It has previously been demonstrated that growth factors such as PDGF, thrombin, Ang II, and bFGF increase IGF-1R on vascular smooth muscle cells and that this effect is transcriptionally regulated (23, 25, 28). Furthermore, the ability of Ang II to up-regulate IGF-1R is a critical determinant of its mitogenic activity on vascular cells, since the Ang II-induced increase in DNA synthesis was inhibited by IGF-1R-specific antisense oligonucleotides (29). The present studies show by which mechanisms and signaling pathways Ang II and bFGF increase IGF-1R gene transcription. By deletional analysis of...
the IGF-1R promoter region, we determined that the Ang II-responsive region is located in the proximal promoter, between nucleotides −270 and −135 upstream of the transcription start site, as is the bFGF-responsive element. In addition, stimulation of IGF-1R gene promoter activity by Ang II or bFGF in transient transfection experiments correlates well with its effect on endogenous IGF-1R protein levels. Both increased IGF-1R protein expression after 8–24 h. This is in good agreement with the previous reports of Du et al. (48) and Ververis et al. (25), which showed that Ang II and bFGF caused a significant increase in IGF-1R mRNA peaking at 3 h and 6–9 h, respectively. Of note, Hernandez-Sanchez et al. (59) reported that the bFGF-responsive element was located between nucleotides −476 and −188. These findings are somewhat different from ours; however, our studies were performed using different cells, and our deletion constructs contained less 5′-UTR sequence. Our data indicate loss of basal activity between nucleotides −270 and −135 but conservation of a bFGF-responsive element.

There has been significant interest generated by the observation that growth factors and cytokines, which possess structurally different receptors, with or without intrinsic tyrosine kinase activity, may signal through a common pathway to the nucleus. In order to define the mechanisms and the signaling cascade involved in the Ang II or bFGF regulation of IGF-1R gene expression, we transiently transfected various IGF-1R promoter constructs into CHO-AT1 or CHO-K1 cells and used different approaches to block the signaling pathways at different levels. Our findings clearly show that Ang II and bFGF share common but also quite distinct pathways. Thus, both Ang II and bFGF increase IGF-1R transcriptional activity via the Ras-Raf-MAPKK-MAPK pathway, since transfection of dominant negative expression constructs for Ras, Raf, or MEK1 dose-dependently reduced the stimulatory effects of these growth factors on IGF-1R promoter activity, whereas they had no effect on IGF-1R promoter activation in the absence of Ang II or bFGF. Further evidence for the involvement of this signaling pathway in the activation of the IGF-1R promoter by Ang II or bFGF was provided by experiments using PD 98059. This compound, which is a specific inhibitor of MAPKK phosphorylation and activation (78, 79), completely reversed the stimulatory effect on luciferase activity induced by Ang II or bFGF. Furthermore, analysis at the protein level clearly demonstrated that both Ang II and bFGF induced a rapid phosphorylation of MAPK, which was inhibited by upstream blockade of MAPKK by PD 98059, and inhibition of the MAPKK reduced the stimulatory effect of Ang II and bFGF on IGF-1R protein levels. Thus, the Ras-Raf-MAPK pathway is clearly required for Ang II and bFGF induction of IGF-1R gene and protein expression.

Although the Ang II AT1 receptor does not possess intrinsic tyrosine kinase activity, its activation leads to intracellular second messenger protein tyrosine phosphorylation by cytosolic tyrosine kinases (80). Thus, our finding that the protein-tyrosine kinase inhibitors genistein and tyrphostin A25 inhibited the Ang II-induced stimulation of IGF-1R gene expression and phosphorylation of MAPK demonstrates a requirement for protein-tyrosine kinase(s) in Ang II-stimulated IGF-1R expres-
We have previously shown that lipoxygenases may be involved in the signaling pathway of Ang II (61). Our present study demonstrates that Ang II-induced activation of the IGF-1R promoter requires lipoxygenase activity, since this stimulation was blocked by ETYA, a lipoxygenase inhibitor (81). ETYA and genistein not only reduced the stimulatory effect of Ang II on IGF-1R promoter transcriptional activity to basal levels but also inhibited the Ang II-induced phosphorylation of MAPK, suggesting that protein tyrosine phosphorylation and lipoxygenase activation is upstream of MAPK activation. Consistent with the results observed in the transcriptional assays, tyrosine kinase and lipoxygenase inhibition abolished the increase in IGF-1R protein level induced by Ang II.

The regulation of IGF-1R transcription is not well understood. The IGF-1R gene promoter lacks TATA or CAAT motifs;
nylidene fluoride membranes, and then blotted with anti-IGF-1R
induced increase in IGF-1R protein expression.

were transfected with expression vectors encoding dominant
region of the IGF-1R gene responsive to Ang II or bFGF, cells
as well as a PDGF-responsive element (85) and potential AP-2
contains putative consensus sequences for a number of well
_9_ 5
of the IGF-1R promoter extending from nucleotide
fation on the proximal promoter region upstream of the transcrip-
IGF-1R gene expression by Ang II and bFGF and the main
pathways by which these growth factors increase
IGF-1R transcriptional activity and IGF-1R protein expression.
Both growth factors increase IGF-1R promoter activity by act-
ing on the proximal promoter region upstream of the transcrip-
t start site. Although Ang II and bFGF possess structurally
different receptors, they transduce signaling through common
pathways, notably the Ras-Raf-MAPKK-MAPK and c-Jun
pathways. However, they also use unique signaling pathways,
such as lipoygenase-mediated MAPK activation or the in-
volvement of the transcription factor NF-κB in the case of Ang
II or the JNK/SAPK cascade and the Jun kinase and Jak/STAT
pathway in the bFGF-induced regulation of IGF-1R gene
expression. These studies have important implications for un-
derstanding growth-stimulatory effects of these growth factors.

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