BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **242,** 202–208 (1998)
ARTICLE NO. RC977940

Angiotensin II and Endothelin-1 Increase Fibroblast Growth Factor-2 mRNA Expression in Vascular Smooth Muscle Cells

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Received November 26, 1997

The vasoactive hormone angiotensin II (Ang II) can stimulate vascular smooth muscle cell (SMC) hypertrophy and proliferation; thus, it may have an important role in the pathogenesis of hypertension, atherosclerosis and restenosis. Several studies have indicated that Ang II bioactivity on SMC may depend, at least in part, on its ability to induce the expression of polypeptide growth factors that can function in an autocrine manner. Here we report that Ang II treatment of rat aortic SMC increases fibroblast growth factor-2 (FGF-2) but not FGF-1 mRNA levels. Increased FGF-2 mRNA expression is first detectable at 30 min after Ang II addition and maximal levels are present at 8 hr. Ang II induction of FGF-2 mRNA levels is dependent on *de novo* RNA and protein synthesis. The Ang II effect can be blocked by treatment with either the Ang II type 1 receptor-selective antagonist CI-996 or the tyrosine kinase inhibitor genistein. The potent vasoconstrictor and SMC mitogen endothelin-1 can also induce FGF-2 mRNA levels in rat aortic SMC. These results indicate that FGF-2 gene expression is up-regulated by two distinct vasoactive peptides implicated in vascular SMC growth control *in vivo*.

Vascular smooth muscle cell (SMC) accumulation is implicated as a critical event in hypertension, restenosis after vascular injury, and atherosclerosis (reviewed in 1). Numerous studies have demonstrated that the renin-angiotensin system, and in particular the vasoactive octapeptide angiotensin II (Ang II), may play an important role in the regulation of SMC growth, proliferation and migration (reviewed in 2). Ang II-stimulated SMC growth responses may be mediated, at least in part, by endogenously-expressed polypeptides that can function in an autocrine manner. Indeed, Ang II treatment of rat aortic SMC induces platelet-derived growth factor (PDGF) A-chain gene expression (3-6) and PDGF-AA neutralizing antibodies inhibit Ang II-promoted increases in cell size (4) (but not increases in protein (4,5) nor DNA (6) synthesis). Transforming growth factor (TGF)-β1 expression also increases after Ang II addition (6-10). Inhibition of TGF-β1 expression (9) or bioactivity (6,8) potentiates Ang II-stimulated DNA synthesis, indicating that at least under certain conditions, TGF-β1 is an antiproliferative factor. Ang II also up-regulates heparin-binding epidermal growth factor-like growth factor (HB-EGF) mRNA levels (11), insulin-like growth factor (IGF)-1 mRNA and protein expression (12) and fibroblast growth factor (FGF)-2 (basic FGF) protein levels (9,10,13). Treatment of quiescent rat SMC with either anti-IGF-1 antiserum (12) or FGF-2 mRNA-specific antisense oligonucleotides (9) has been shown to inhibit Ang II-stimulated DNA synthesis. Thus, there is evidence that at least four distinct polypeptide growth factors, PDGF-AA, TGF-β1, IGF-1 and FGF-2, may be important in Ang II-mediated vascular SMC growth.

Our laboratory has been studying the regulation of FGF-1 (acidic FGF) and FGF-2 gene expression using vascular SMC cultured *in vitro* (14,15). FGF-1 and FGF-2, two members of a family of structurally related heparin-binding proteins (reviewed in 16), are angiogenic factors (17,18) and potent mitogens for many cell types, including vascular SMC (17,19,20). Two laboratories have reported that Ang II can increase FGF-2 protein levels in rat aortic SMC (9,10,13). In the present study, we have examined this cellular response in detail; additionally, we determined whether Ang II could also regulate FGF-1 expression and whether another SMC mitogenic

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0006-291X/98 $25.00

peptide, endothelin (ET)-1, could alter FGF-1 or FGF-2 mRNA expression in vascular SMC.

MATERIALS AND METHODS

Cell culture. Adult rat (Wistar-Kyoto) thoracic aorta SMC were kindly provided by M. Majesky, Baylor College of Medicine, Houston, TX. The cells were cultured at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Mediatech) supplemented with 5% fetal bovine serum (FBS; Hyclone Laboratories, Inc.), 100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B (JRH Biosciences). Rat SMC cultures were fed every 48 hr and expanded by trypsin-EDTA (JRH Biosciences) treatment and subculturing at a 1:5 split ratio. Cells were incubated for ~72 hr in normal growth medium without FBS but containing 5 μg/ml insulin, 5 μg/ml transferrin and 5 ηg/ml selenious acid (ITS; Collaborative Biomedical Products) to obtain a relatively quiescent SMC population.

RNA isolation and Northern blot hybridization. Serum-starved SMC were treated with the following agents either added alone or in various combinations: Ang II (10^{-7} M unless otherwise noted; Bachem), p-aminophenylalanine[6] Ang II (10^{-7} M; Bachem), ET-1 (10^{-6} M; Peninsula Laboratories), actinomycin D (2 μg/ml; Sigma), cycloheximide (10 μg/ml; Sigma), H7 (50 μM; Seikagaku America), HA1004 (50 μM; Seikagaku America), genistein (5 μg/ml; LC Laboratories), daidzein (5 μg/ml; LC Laboratories), CI-996 (10^{-7} M; Parke-Davis), PD123319 (10^{-7} M; Parke-Davis). Cells were harvested and total RNA was isolated using RNA-Stat 60 (Tel-Test "B" Inc.) according to the manufacturer's instructions. Ten μg of each sample was denatured, loaded onto 1.2% agarose gels containing 2.2 M formaldehyde and subjected to electrophoresis. The gels were stained with ethidium bromide (Sigma) to confirm that the RNA samples were undegraded and that similar amounts were present in each lane. RNA was transferred onto Zetabind nylon membranes (Cuno Inc.) by electrophoresis and then cross-linked to the membrane by UV irradiation. Membrane pre-hybridization, hybridization and washing conditions were as described (15). The cDNA probes used were: (i) rat FGF-1, 517-base pair (bp) EcoRI fragment of pRSV-Neo-HBGF-1; kind gift of W. McKeehan, Albert B. Alkek Institute of Biosciences and Technology, Texas A&M University, Houston, TX; (ii) rat FGF-2, 477-bp XhoI/NcoI fragment of RObFGF.477; kind gift of A. Baird, Scripps Research Institute, La Jolla, CA and (iii) human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 800-bp PstI/XbaI fragment of pHcGAP; American Type Culture Collection, Rockville, MD. The DNA fragments were labeled to high specific activity with [α-^{32}P]dCTP (3000 Ci/mmol; DuPont/NEN) using a random primer labeling kit (Boehringer Mannheim). The blots were air dried and exposed to Kodak X-Omat AR X-ray film. Autoradiographic signals or photographic images were analyzed by densitometry using the BioImage whole band analyzer (Millipore). FGF-1 and FGF-2 mRNA signal intensities were calculated following normalization to either GAPDH mRNA or 18S rRNA levels.

RESULTS

We first determined whether Ang II treatment of serum-starved rat aortic SMC altered FGF-1 or FGF-2 mRNA levels. Cells were either left untreated or treated with Ang II for various time periods, RNA was isolated, and Northern blot hybridization analysis was performed. FGF-1 and FGF-2 transcripts of ~4.4-kilobase (kb) and ~6.0-kb in size, respectively, were co-expressed in rat SMC. Ang II had no detectable effect on FGF-1 mRNA levels (data not shown; also see Fig. 5A) but significantly increased FGF-2 mRNA levels in a time-dependent manner (Fig. 1A). Increased FGF-2 gene expression was first detected at 30 min after Ang II addition; as estimated by densitometry, FGF-2 mRNA levels were elevated ~2-fold. The maximal level of FGF-2 mRNA was apparent after 8 hr of stimulation, representing an ~47-fold induction. FGF-2 mRNA levels were still elevated at 12 hr (~6-fold induction), the latest time point examined.

We next investigated whether Ang II caused a concentration-dependent increase in FGF-2 mRNA levels. Serum-starved SMC were either left untreated or treated for 4 hr with increasing concentrations of Ang II, RNA was isolated, and Northern blot analysis was performed. Ang II increased FGF-2 mRNA levels in a dose-dependent manner (Fig. 1B). Elevated FGF-2 mRNA expression was first apparent when cells were stimulated with 10^{-10} M Ang II (~3-fold induction) and maximal induction (~45-fold) occurred at an Ang II concentration of 10^{-8} M.

We also determined whether *de novo* RNA or protein synthesis were required for Ang II induction of FGF-2 mRNA levels. SMC were either left untreated or treated with Ang II in the absence or presence of either actinomycin D, an RNA synthesis inhibitor, or cycloheximide, an inhibitor of translation elongation. Cells were also treated with each of the inhibitors alone. SMC were harvested after 4 hr of treatment, RNA was isolated and Northern blot hybridization analysis performed. Actinomycin D treatment prevented Ang II induction of FGF-2 mRNA levels (Fig. 2); thus, the increase in FGF-2 mRNA expression after Ang II addition is likely to reflect transcriptional activation of the FGF-2 gene. This drug also decreased the basal level of FGF-2 mRNA expression in non-stimulated cells, suggesting that FGF-2 transcripts have a relatively short half-life. Simultaneous treatment with both Ang II and cycloheximide slightly increased FGF-2 mRNA levels; however, the level obtained was similar to that observed when cycloheximide alone was added (Fig. 2). The effect of Ang II treatment alone was significantly greater than the combined effect of Ang II and cycloheximide; thus, protein synthesis is required for maximal induction of FGF-2 mRNA expression.

The various biological activities of Ang II are mediated via binding to specific G protein-coupled cell surface receptors. Two structurally related yet pharmacologically distinct Ang II receptor subtypes have been identified, AT_1 and AT_2 (reviewed in 21). We next investigated whether Ang II regulation of FGF-2 mRNA levels was mediated via the AT_1 and/or AT_2 receptor subtypes. Serum-starved SMC were either left untreated, stimulated with Ang II alone, or pretreated for 30 min with CI-996, an AT_1 receptor-selective antagonist (22), or PD123319, an AT_2 receptor-selective antagonist (23), and then stimulated with Ang II. Cells were also treated with each of the antagonists alone. SMC were

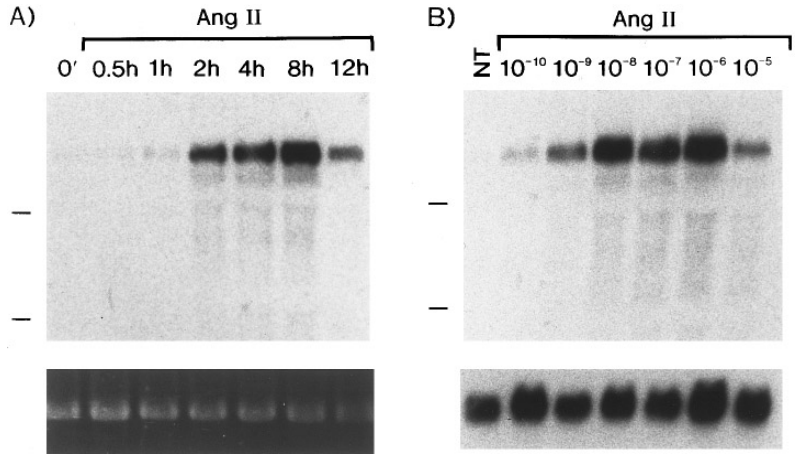

FIG. 1. Effect of Ang II on FGF-2 mRNA levels in SMC: Time-course and dose-response analyses. A, Serum-starved SMC were either left untreated or treated with Ang II for the indicated times. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using an FGF-2 cDNA probe (top). A photograph of the 18S rRNA species visualized by ethidium bromide fluorescence is shown in the bottom panel to demonstrate that similar amounts of RNA were loaded in each gel lane. B, Serum-starved SMC were either left untreated (NT) or treated for 4 hr with the indicated Ang II concentration. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using FGF-2 (top) or GAPDH (bottom) cDNA probes.

harvested 4 hr later, RNA was isolated, and Northern blot hybridization analysis performed. Ang II induction of FGF-2 mRNA expression was inhibited by treatment with CI-996 but not with PD123319 (Fig. 3A). Neither compound alone significantly altered FGF-2 mRNA levels in non-stimulated cells. To confirm that AT_2 receptor binding could not induce FGF-2 mRNA levels, serum-starved SMC were either left untreated or treated for 4 hr with the AT_2 receptor agonist p-aminophenylalanine[6] Ang II (24). RNA was isolated and equivalent amounts were analyzed by Northern blot hybridization. p-aminophenylalanine[6] Ang II did not increase FGF-2 mRNA expression (Fig. 3B). Taken together, these results indicate that Ang II induction of FGF-2 gene expression in rat aortic SMC is mediated by the AT_1 receptor subtype.

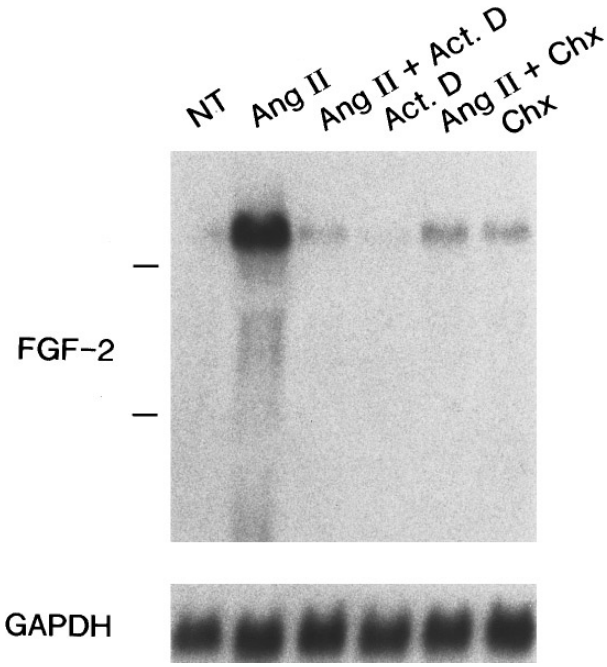

FIG. 2. Effect of actinomycin D or cycloheximide on Ang II-induced FGF-2 mRNA expression in SMC. Serum-starved SMC were either left untreated (NT) or treated for 4 hr with Ang II alone, Ang II and actinomycin D (Act.D), actinomycin D alone, Ang II and cycloheximide (Chx), or cycloheximide alone. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using FGF-2 or GAPDH cDNA probes.

Ang II treatment of rat aortic SMC stimulates several biochemical responses, including protein kinase C (PKC) activation and the tyrosine phosphorylation of numerous cellular proteins (reviewed in 25). We used protein kinase inhibitors to assess the relative importance of Ang II-stimulated PKC and protein tyrosine kinase activity in FGF-2 gene induction. Serum-starved SMC were either left untreated or treated for 4 hr with either Ang II alone or Ang II in combination with H7, HA1004, genistein, or daidzein. H7 and HA1004 inhibit PKC and cyclic nucleotide-dependent protein kinases with H7 being ~7-fold more effective at inhibiting PKC (26). Genistein is an inhibitor of tyrosine-specific protein kinases while the structural analogue daidzein exhibits little, if any, inhibitory activity (27). RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization. H7 or HA1004 treatment had only a small inhibitory effect on Ang II induction of FGF-2 mRNA levels (Fig. 4A); as estimated by densitometry, they decreased induction by ~38 and ~25%, respectively. In comparison, gen-

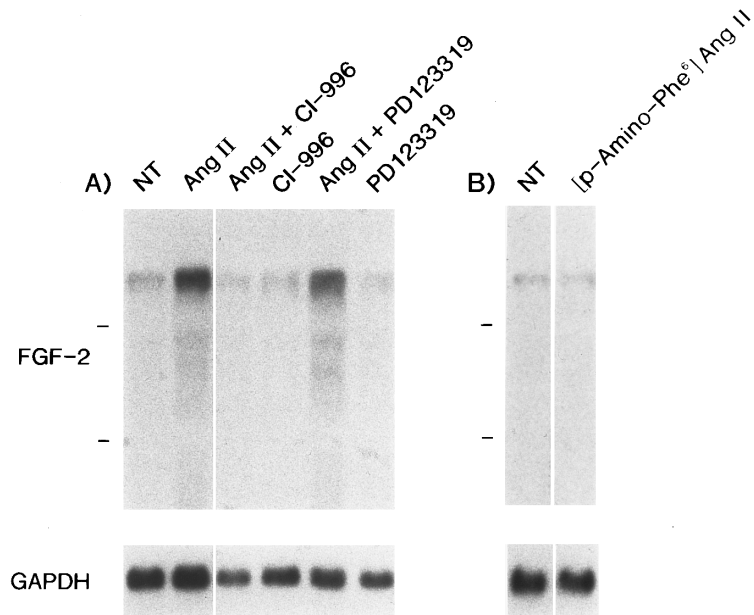

FIG. 3. Effect of AT_1 receptor- or AT_2 receptor-specific antagonists on Ang II-induced FGF-2 mRNA expression and the effect of an AT_2 receptor agonist on FGF-2 mRNA levels in SMC. A, Serum-starved SMC were either left untreated (NT), treated with Ang II, CI-996 or PD123319 alone, or pretreated for 30 min with either CI-996 or PD123319 and then stimulated with Ang II. Cells were collected 4 hr later, RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using FGF-2 or GAPDH cDNA probes. B, Serum-starved SMC were either left untreated (NT) or treated for 4 hr with p-Aminophenylalanine[6] Ang II. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using FGF-2 or GAPDH cDNA probes.

istein almost completely blocked (~92% inhibition) Ang II induction of FGF-2 mRNA expression while daidzein had no detectable effect. When the compounds were each added alone to serum-starved SMC only H7 had an effect on basal levels of FGF-2 mRNA expression (Fig. 4B). These results indicate that tyro-

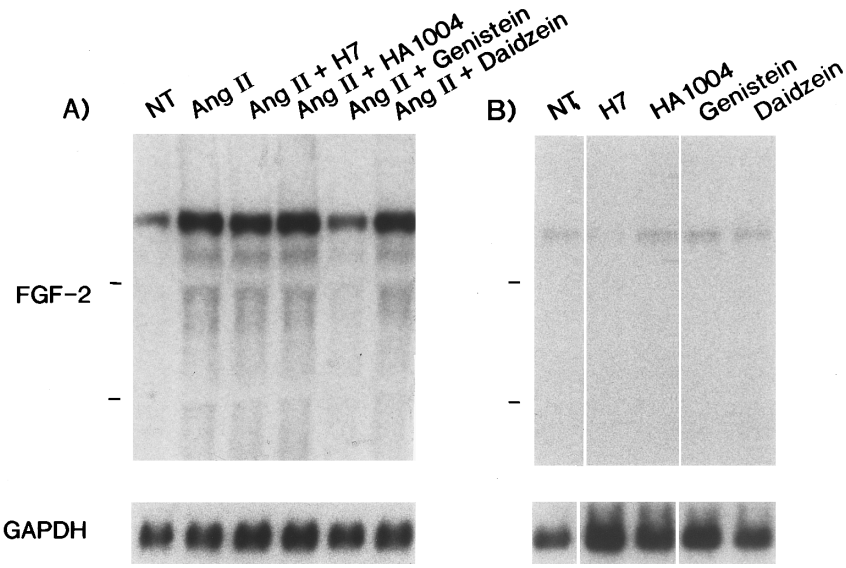

FIG. 4. Effect of protein kinase C or protein tyrosine kinase inhibitors on Ang II-induced FGF-2 mRNA expression in SMC. A, Serum-starved SMC were either left untreated (NT) or treated for 4 hr with either Ang II alone or Ang II in combination with H7, HA1004, genistein or daidzein. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using FGF-2 or GAPDH cDNA probes. B, Serum-starved SMC were either left untreated (NT) or treated for 4 hr with H7, HA1004, genistein or daidzein. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using FGF-2 or GAPDH cDNA probes.

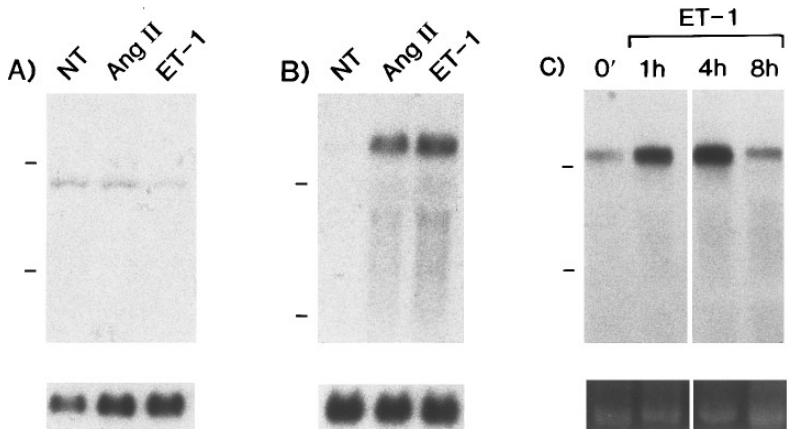

FIG. 5. Effect of ET-1 on FGF-1 and FGF-2 mRNA levels in SMC. A, Serum-starved SMC were either left untreated (NT) or treated for 4 hr with Ang II or ET-1. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using FGF-1 (top) or GAPDH (bottom) cDNA probes. B, The same three RNA samples were also analyzed by Northern blot hybridization using FGF-2 (top) or GAPDH (bottom) cDNA probes. C, Serum-starved SMC were either left untreated or treated with ET-1 for the indicated times. RNA was isolated and equivalent amounts of each sample were analyzed by Northern blot hybridization using an FGF-2 cDNA probe (top). A photograph of 18S rRNA is also shown to demonstrate similar amounts of RNA were loaded in each gel lane (bottom).

sine kinase activity, and to a lesser extent PKC activity, is required for Ang II-mediated FGF-2 mRNA induction.

Finally, we determined whether ET-1, a 21-amino acid vasoactive hormone that is also implicated in vascular SMC growth control (reviewed in 28,29), could alter FGF-1 or FGF-2 mRNA levels in rat SMC. Serum-starved SMC were either left untreated or treated with equivalent concentrations of Ang II (as a control) or ET-1 for 4 hr, RNA was isolated and Northern blot hybridization analysis was performed. ET-1 had no significant effect on FGF-1 mRNA levels (Fig. 5A) but increased FGF-2 mRNA levels to a similar degree as Ang II (Fig. 5B). To investigate the kinetics of ET-1-induced FGF-2 mRNA expression, RNA was isolated from untreated SMC or SMC treated with ET-1 for 1, 4 or 8 hr and FGF-2 mRNA levels were assayed by Northern blot hybridization. Increased FGF-2 mRNA expression was first detected at 1 hr after ET-1 addition and maximal FGF-2 mRNA levels were apparent after 4 hr of stimulation (Fig. 5C). Thus, ET-1 induces FGF-2 mRNA levels in a time-dependent manner with more rapid kinetics than observed after Ang II addition.

DISCUSSION

We report here that Ang II treatment of serum-starved rat aortic SMC cultures promotes the rapid and transient induction of FGF-2 mRNA levels. The kinetics of FGF-2 mRNA accumulation are similar to those reported for Ang II up-regulation of PDGF A-chain (3,4,7) and IGF-1 (12) mRNA levels. In contrast, Ang II induces a more rapid and transient increase in HB-EGF mRNA levels (11) and a more delayed elevation in TGF-β1 mRNA levels (7). The Ang II effect on FGF-2 mRNA levels was dose-dependent with a threshold concentration of 10^{-10} to 10^{-9} M, in agreement with previous studies examining Ang II-induced TGF-β1 (8) and HB-EGF (11) mRNA expression in rat aortic SMC.

Ang II does not induce FGF-2 mRNA levels to the maximal extent if RNA synthesis is inhibited using the drug actinomycin D. This finding is consistent with a transcriptional control mechanism for Ang II-mediated FGF-2 mRNA induction. The Ang II effect on IGF-1 mRNA levels in rat SMC has also been shown to be due to transcriptional activation (12). Ang II induction of FGF-2 mRNA levels also did not occur in the presence of cycloheximide. This indicates that newly synthesized proteins, presumably transcription factors encoded by Ang II-inducible immediate-early response genes, are necessary for increased FGF-2 mRNA expression. Ang II up-regulation of PDGF A-chain (3,4), TGF-β1 (8) and IGF-1 (12) mRNA levels is also dependent on *de novo* protein synthesis.

Ang II induction of FGF-2 mRNA expression was inhibited by the AT_1 receptor-selective antagonist CI-996 but not by the AT_2 receptor-selective antagonist PD123319. Furthermore, p-aminophenylalanine[6] Ang II treatment of serum-starved SMC did not alter FGF-2 mRNA levels. These results indicate that Ang II regulation of FGF-2 gene expression is mediated by the AT_1 receptor subtype. This result is in agreement with numerous reports indicating that Ang II-induced responses in rat aortic SMC (e.g., DNA synthesis (6,30), hypertrophy (31), gene induction (30,31)) require binding to AT_1 cell surface receptors.

As an initial approach to investigate the signal transduction mechanisms involved in Ang II regulation of FGF-2 mRNA expression we determined whether this effect required PKC and/or tyrosine kinase activity.

PKC inhibition with H7 had only a minimal effect on FGF-2 mRNA induction by Ang II and this effect was similar to that noted after HA1004 treatment. This result is in agreement with a report by Ali et al. (13) indicating that the PKC inhibitors calphostin C and staurosporine do not significantly inhibit Ang II-induced FGF-2 protein expression. However, genistein, a general non-specific tyrosine kinase inhibitor, almost completely blocked Ang II induction of FGF-2 mRNA levels. It has been reported that genistein does not interfere with Ang II binding to AT_1 receptors and effectively inhibits Ang II-stimulated tyrosine phosphorylation in rat aortic SMC (32). Therefore, it appears likely that the Ang II-stimulated increase in FGF-2 mRNA expression is mediated primarily via a pathway that involves tyrosine phosphorylation.

ET-1, like Ang II, is a potent vasoconstrictor that can, at least under certain conditions, stimulate SMC proliferation *in vitro* (reviewed in 33). Furthermore, administration of exogenous ET-1 following balloon injury of the rat carotid artery promotes neointimal SMC accumulation (34) and ET-1 receptor antagonists can inhibit balloon injury-induced vascular lesion formation (34,35). We found that ET-1 treatment of serum-starved SMC increased FGF-2 but not FGF-1 mRNA levels. The kinetics of ET-1-induced FGF-2 mRNA accumulation were more rapid and transient than the kinetics observed after Ang II stimulation. Previous studies have shown that ET-1 can also induce PDGF A-chain and TGF-β1 mRNA levels in rat aortic SMC (36).

In summary, the present study demonstrates that Ang II and ET-1 can induce FGF-2 mRNA levels in rat aortic SMC. If FGF-2 gene expression is also regulated by these peptides *in vivo,* then FGF-2 may play a role in Ang II- and ET-1-stimulated cellular responses. For example, the ability of Ang II to promote angiogenesis *in vivo* (37,38) may be mediated, at least in part, by FGF-2 production at the peptide administration site. The importance of FGF-2 in Ang II-induced vascular SMC hypertrophy or SMC accumulation following vascular injury is presently unknown but several studies have examined the role of FGF-2 in Ang II-stimulated hypertrophy or DNA synthesis *in vitro.* Ali et al. (13) reported that Ang II-induced FGF-2 expression can occur without concomitant SMC hypertrophic growth. Thus, enhanced FGF-2 production may be important but is not sufficient to promote this cellular response. Itoh et al. (9) reported that antisense oligonucleotides targeted to FGF-2 mRNA suppress Ang II-stimulated DNA synthesis. However, Weber et al. (6) found that FGF-2 neutralizing antibodies had no effect on Ang II-stimulated DNA synthesis, suggesting that if endogenous FGF-2 is indeed important, it may be functioning in an intracrine manner. Finally, recent studies using rat SMC indicate that FGF-2 can (i) autoinduce its own expression (14), (ii) induce ACE gene expression (39) and (iii) decrease AT_1 receptor gene expression (40). Taken together with the results reported here, it is clear that there are multiple interactions between FGF-2 and several components of the angiotensin ligand:receptor system. These interactions may contribute to the pathogenesis of vascular diseases such as hypertension, atherosclerosis and restenosis.

ACKNOWLEDGMENTS

We thank Dr. Majesky, Dr. McKeehan and Dr. Baird for generously providing reagents. We are also grateful to K. Wawzinski and D. Weber for excellent secretarial assistance. This work was supported in part by National Institutes of Health grant HL-39727 (J.A.W.). Portions of this work were performed by Ms. Peifley in partial fulfillment of the requirements for the Masters degree in Biomedical Sciences at Hood College, Frederick, MD.

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