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Insulin-like Growth Factor-I (IGF-I) Regulates IGF-binding Protein-5 Synthesis through Transcriptional Activation of the Gene in Aortic Smooth Muscle Cells*

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Previous studies have shown that porcine aortic smooth muscle cells (SMCs) secrete two insulin-like growth factor-binding proteins (IGFBP), IGFBP-2 and -4, and that these IGFBPs modulate IGF-I-stimulated SMC proliferation and migration. In this study we demonstrate that porcine SMCs express IGFBP-5 mRNA and synthesize and secrete the protein. In this cell type, the biosynthesis of IGFBP-5 is up-regulated by IGF-I. This increase in IGFBP-5 synthesis is accompanied by an increase in the steady-state mRNA levels. The induction of IGFBP-5 mRNA by IGF-I is time- and dose-dependent and requires *de novo* protein synthesis. IGF-II and insulin also increase IGFBP-5 mRNA levels at high doses. An IGF-I analog with normal affinity for the IGF-I receptor but reduced affinity for IGFBPs evokes a similar increase. Another analog that binds to IGFBPs but not to the receptor has no effect, indicating that this effect of IGF-I is mediated through the IGF-I receptor. The IGF-I-induced IGFBP-5 gene expression is cell type-specific because IGF-I had no such effect in other cell types examined. Nuclear run-on assays revealed that IGF-I increased transcription rate of the IGFBP-5 gene, while IGF-I did not change the IGFBP-5 mRNA stability. Furthermore, the IGFBP-5 promoter was 3.5-fold more active in directing expression of the luciferase reporter gene in IGF-I-treated aortic SMCs as compared to control cells, whereas the luciferase activity remained the same in control- and IGF-I-treated fibroblasts. These results suggest that IGF-I up-regulates IGFBP-5 synthesis by transcriptionally activating the IGFBP-5 gene in aortic SMCs.

Many studies have linked the accumulation of aortic smooth muscle cells (SMCs)[1] to the development of atherosclerotic lesions. This accumulation is due to a combination of SMC proliferation, directed migration from the arterial media into the intima (1, 2), and inhibition of apoptosis (3). All of these events are modulated by a number of peptide growth factors including insulin-like growth factors (IGFs). SMCs in culture have been shown to synthesize IGF-I, and this endogenously produced IGF-I stimulates SMC proliferation in an autocrine fashion (4–6). *In vivo*, IGF-I mRNA and immunoreactive IGF-I are detected in intimal lesions that develop after angioplasty (7). IGF-I mRNA and immunoreactive IGF-I levels both increase severalfold after balloon denudation injury, and these increases temporally precede an associated increase in SMC proliferation (8, 9). Likewise, SMCs possess IGF-I receptors and selective inhibition of the receptors by antisense targeting results in marked reduction in SMC proliferation (9, 10). These observations together with the well established fact that IGF-I is a mitogen for SMCs suggest that the local production of IGF-I plays an important role in SMC proliferation (11). In addition to its role in mitogenesis, IGF-I has recently been shown to stimulate SMC migration. Bornfeldt *et al.* (12) showed that IGF-I stimulates SMC directed migration using a Boyden chamber assay. Studies from our laboratory have shown that IGF-I and IGF-II stimulate SMC migration in a monolayer wounding assay, and this response is mediated by the IGF-I receptor (13). Thus, IGF-I is important for SMC proliferation and migration and may therefore play an important role in the development of atherosclerotic lesions.

The bioactivities of IGFs are modulated by a group of high affinity specific binding proteins (IGFBPs). Six distinct IGFBPs, designated as IGFBP-1 to IGFBP-6, have been identified in mammalian systems to date (14, 15). These proteins share relatively high amino acid sequence similarity, but each has distinct structural and biochemical properties that partially determine whether they act to inhibit or potentiate IGF bioactivity. Previous studies from our laboratory have shown that porcine aortic SMCs secrete IGFBP-2 and -4, and that they both modulate IGF-I-stimulated DNA synthesis and cell migration in this cell type (13, 16–18). The availability of IGFBP-2 and -4 in SMCs is regulated by IGFs, PDGF, insulin, and other factors (17, 18). While PDGF or insulin treatment results in moderate increases in IGFBP-2 and -4 synthesis, IGFs accelerate the degradation of the inhibitory IGFBP-4 by activating specific proteases. These findings indicate that the presence of IGFBPs in the area of the vascular lesion may play a role in modifying IGF activity, potentially resulting in modulation of SMC proliferation and migration.

In the present study, we report that porcine aortic SMCs express IGFBP-5 mRNA and synthesize and secrete IGFBP-5. Our data indicate that the synthesis of IGFBP-5 is up-regulated by its own ligand, IGF-I. Of particular interest to us was that IGF-I increased the transcription rate of the IGFBP-5 gene without significantly affecting the mRNA stability and that this response is specific for aortic SMCs.

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[1] The abbreviations used are: SMC, smooth muscle cell; bp, base pair(s); CM, conditioned medium; DRB, 5,6-dichloro-1-β-D-ribofuranosyl-benzimadazole; FBS, fetal bovine serum; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor binding protein; kb, kilobase(s); PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; PDGF, platelet-derived growth factor; Rb, retinoblastoma; PAGE, polyacrylamide gel electrophoresis; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

EXPERIMENTAL PROCEDURES

Materials—Fetal bovine serum (FBS), Dulbecco's minimum essential medium, Eagle's minimum essential medium, and penicillin-streptomycin were purchased from Life Technologies, Inc. Trypsin was obtained from Boehringer Mannheim. Recombinant human IGF-I and rat IGF-II were purchased from Bachem, Inc. (Torrance, CA). Recombinant human FGF and PDGF-BB were purchased from Intergen (Purchase, NY). Two antisera against human IGFBP-5 were prepared as described previously (19). They have no cross-reactivity for IGFBP-2 and -4. Enzymes were purchased from the following commercial suppliers: Promega Corp. (Madison, WI), Boehringer Mannheim, New England Biolabs (Beverly, MA), U. S. Biochemical Corp. Deoxyribonucleotides and radionucleotides were purchased from Boehringer Mannheim, Amersham Corp., and DuPont NEN. TA cloning kit was purchased from Invitrogen (San Diego, CA), and plasmid pGL2-Basic from Promega. Materials for DNA purification were purchased from Qiagen (Chatsworth, CA). Oligonucleotides were synthesized by the Nucleic Acids Core Faculty, University of North Carolina, Chapel Hill.

Cell Culture—Porcine aortic SMCs were isolated from thoracic aortas of 3-week-old piglets. The cells were grown in 10-cm dishes (Falcon Laboratory Division) in Dulbecco's minimum essential medium supplemented with 4.5 g/liter glucose, 4 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml) plus 10% FBS. Human intestinal smooth muscle cells (HISM, American Type Culture Collection, Rockville, MD) were grown in the same medium. Human newborn aortic SMC were a gift from Dr. Stephen Schwartz, University of Washington. These cells were grown in Waymouth's medium supplemented with penicillin (100 units/ml) and streptomycin (100 μg/ml) plus 10% FBS. Human fetal dermal fibroblasts (GM10, Human Mutant Genetic Cell Repository, Camden, NJ) and human glioblastoma tumor cells (T98G, American Type Culture Collection, Rockville, MD) were maintained in Eagle's minimum essential medium supplemented with serine (21 μg/ml), pyruvate (110 μg/ml), asparagine (30 μg/ml), penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FBS. The medium was changed every 4th day until cells became confluent.

Western Ligand Blot and Immunoblot Analysis—In order to identify the IGFBP-5 secreted by SMC, samples containing 0.5 ml of culture medium were concentrated 20 times by ultrafiltration through a Centricon-10 microconcentrator (Amicon, Berkeley, MA). The proteins were separated by SDS-PAGE using 12.5% polyacrylamide gels under non-reducing conditions as described previously (17). After transfer to filters (Immunobilon P, 0.45-μm pore size, Millipore, Bedford, MA), the filters were probed with ^{125}I-IGF-I and autoradiographs were obtained by exposure to x-ray films (Kodak AR film, Eastman Kodak Co.). Additional filters were immunoblotted using a 1:500 dilution of two human IGFBP-5 antisera. The protein was detected using either an alkaline phosphatase-conjugated goat anti-rabbit or anti-guinea pig second antibodies (Sigma).

Immunoprecipitation—Cells were grown in 6-cm plates (Falcon) and metabolically labeled with [^{35}S]methionine for 6 h. Media were collected, and the plates were rinsed twice with phosphate-buffered saline containing 2 mg/ml bovine serum albumin (Sigma). Cells were lysed with immunoprecipitation (IPT) buffer (25 mM Hepes, 0.1 M NaCl, 1% Triton X-100, 10 mM EDTA) containing 1% bovine serum albumin. ^{35}S-Labeled IGFBP-5 was immunoprecipitated from the medium by the addition of an anti-human IGFBP-5 antibody raised in guinea pig (1:1000 dilution) or normal guinea pig serum. The immune complexes were precipitated by adding protein A-Sepharose (Sigma) and analyzed by 12.5% SDS-PAGE gels, followed by autoradiography.

RNA Isolation and Northern Blot Analysis—RNA was isolated from cell cultures using TriReagent following the manufacturer's instructions (Molecular Research Center, Inc., Cincinnati, OH) and was quantified by measuring UV absorption at $A_{260\ nm}$. RNA samples were size-fractionated on a 1.2% agarose formaldehyde gel, blotted and fixed onto a nylon membrane (ICN Biochemical, Inc., Irvine, CA), and hybridized with the [^{32}P]dCTP-labeled human IGFBP-5 or IGFBP-2 cDNA. A cDNA probe for glyceraldehyde -3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX) was used to assess the specificity. The band densities were quantitated by exposing the filters to phosphor screens, which were scanned on PhosphorImager™ SF followed by image analysis using ImageQuant (Molecular Dynamics).

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR), Cloning, and DNA Sequencing—Based on the published sequences of human, rat and mouse IGFBP-5 (41, 57, 58), a set of PCR primers were designed in the conserved regions to amplify a 393-bp region of the coding sequence of mature IGFBP-5 (sense primer: 5′-GTTTGCCTCAACGAAAAGAGCT-3′; antisense primer: 5′-CTGCTTTCTCTTGTAGAATCCTT-3′). These primers were used in PCR with double-stranded cDNA prepared by reverse transcription from SMC total RNA. Amplification was carried out in a DNA thermal cycler (Perkin-Elmer) using an initial denaturation of 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1 min. A 10-min incubation at 72 °C was performed at the completion of the final cycle. The resulting PCR product was cloned into a pCR™ II vector from a TA cloning kit (Invitrogen). The nucleotide sequence of the insert was sequenced following the dideoxy nucleotide method using Sequenase version 2 (U. S. Biochemical Corp.).

Nuclear Run-on Transcription Analysis—Relative transcription rates of IGFBP-5 and IGFBP-2 genes were measured by nuclear run-on assay. Nuclei from control and IGF-I treated cell cultures were isolated by a previously reported procedure (20). Isolated nuclei were stored in liquid N_2 in glycerol storage buffer (50 mM Tris, pH 8.3, 40% glycerol, 5 mM MgCl, and 0.1 mM EDTA). The run-on assay was performed at 30 °C in 5 mM Tris-Cl (pH 8.0), 2.5 M $MgCl_2$, 150 mM KCl, 100 μCi of [^{32}P]UTP, and 1 mM each of CTP, ATP, and GTP for 30 min. Reactions were quenched with yeast transfer RNA, treated with RNase-free DNase I and proteinase K, and phenol-chloroform-extracted. ^{32}P-Labeled transcript was purified by Quick Spin™ column (Boehringer Mannheim), treated with NaOH, and then ethanol-precipitated. Five μg of plasmid DNA containing IGFBP-5 or IGFBP-2 insert or vector DNA were linearized and NaOH-denatured, slot-blotted (BioDot, Bio-Rad), and hybridized with 1×10^7 cpm ^{32}P-labeled transcript in 10 mM TES (pH 7.4), 10 mM EDTA, 300 mM NaCl, and 0.2% SDS. Equal amounts of radioactivity were used, and the result was quantitated on PhosphorImager™ SF using ImageQuant (Molecular Dynamics).

IGFBP-5 mRNA Stability—To measure the effect of IGF-I on mRNA decay, actinomycin D or 5,6-dichloro-1-β-D-ribofuranosyl-benzimadazole (DRB, Sigma) dissolved in ethanol was added to cell cultures after 18 h of preincubation with or without IGF-I, and total RNA was isolated at 1–24-h intervals. The ethanol diluent was added to control cell cultures. After Northern blotting with IGFBP-5 cDNA probe, the abundance of the mRNA was quantitated on PhosphorImager™ SF (Molecular Dynamics). A cDNA probe for rat 18 S rRNA was used to assess relative amounts of RNA loaded. The $t_{½}$ was defined as the time at which the signal intensity reached 50% of that before inhibitor was added to the cells.

Plasmid Construction and Transfection of Aortic SMC—The construction of IGFBP-5 promoter/luciferase plasmid (pBP-5P/Luc) was described previously (21). The plasmid DNA was purified by a commercial Qiagen kit (Qiagen). Porcine aortic SMC cells were plated at 4×10^4 cells/cm^2 and were maintained in culture medium as described above. After 2 days, the cells were washed with serum-free medium and exposed to 2 μg/well test plasmid DNA and 5 μl of Lipofectamine (Life Technologies, Inc.) for 16 h. After transfection the cells were washed twice and maintained in growth medium. The transfected cells were harvested 3 days later unless otherwise specified. 0.5 μg of pSV-β-galactosidase control vector DNA was cotransfected to determine transfection efficiency. The amount of cellular extract used in the luciferase assay was normalized relative to β-galactosidase activity. Luciferase activities were determined using the Promega luciferase assay system. β-Galactosidase activity was assayed by monitoring the conversion of *o*-nitrophenyl-β-D-galactopyranoside to galactose and *o*-nitrophenyl at $A_{420\ nm}$. Each experiment was repeated three to four times with duplicate samples.

Statistical Analysis—Student's *t* test was used to compare difference between the control and test groups. Values are means ± S.E. $p < 0.05$ was considered significant.

RESULTS

Porcine Aortic SMCs Express IGFBP-5 mRNA and Secrete IGFBP-5 Protein—Northern blot analysis of total RNA isolated from porcine aortic SMCs revealed a mRNA band, which hybridized with a human IGFBP-5 cDNA probe under highly stringent conditions (Fig. 1*A*). This transcript was the same size (6 kb) as human IGFBP-5 mRNA found in human fibroblasts but was much less abundant. To make sure that this transcript represents porcine IGFBP-5 mRNA, we performed RT-PCR using a set of primers designed at the conserved regions of mammalian IGFBP-5 and RNA isolated from porcine SMCs as template. A DNA fragment at the predicted size (393 bp) was amplified (Fig. 1*B*). This PCR product was cloned into a plasmid vector and sequenced. The predicted amino acid

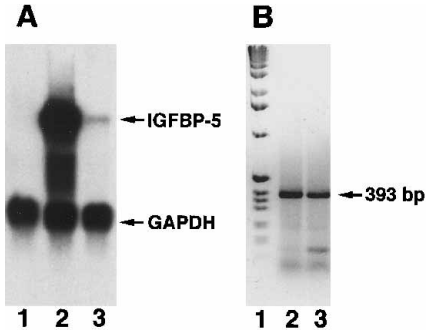

FIG. 1. **Porcine aortic SMCs express IGFBP-5 mRNA.** *A*, Northern blot analyses of IGFBP-5 mRNA levels. Ten μg of total RNA isolated from A673 human rhabdomyosarcoma cells (*lane 1*), GM-10 human fibroblasts (*lane 2*), and porcine aortic SMCs (*lane 3*) was loaded and subjected to Northern blotting using a human IGFBP-5 cDNA probe and a GAPDH cDNA probe. The *arrows* denote the 6-kb IGFBP-5 message and 1.4-kb GAPDH message. *B*, RT-PCR amplification of porcine IGFBP-5 mRNA. One μg of total RNA isolated from GM-10 human fibroblasts (*lane 2*) or porcine aortic SMCs (*lane 3*) was reverse transcribed into cDNA followed by PCR amplification, as described under "Experimental Procedures." *Lane 1* is the 1-kb DNA ladder.

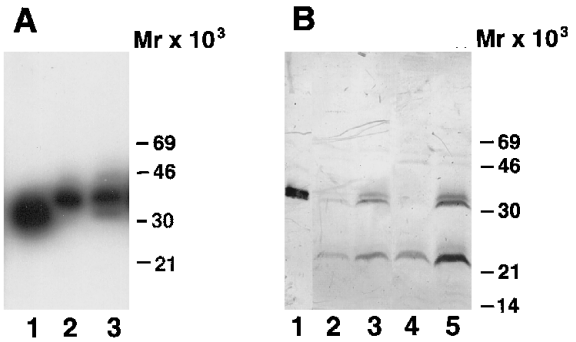

FIG. 2. **Porcine aortic SMCs secrete IGFBP-5.** *A*, ligand blot analysis of porcine SMC conditioned media. The 24-h conditioned medium (0.5 ml) from control (*lane 2*) or IGF-I (100 ng/ml)-treated SMC cultures (*lane 3*) was concentrated 20 times and separated by 12.5% SDS-PAGE gel. Heparin (100 μg/ml) was added 6 h prior to the collection. *Lane 1* contains human IGFBP-5 (100 ng). *B*, immunoblot analysis of porcine SMC conditioned media. The same 24-h conditioned medium samples shown in *panel A* from control (*lanes 2* and *4*) or IGF-I (100 ng/ml)-treated SMCs (*lanes 3* and *5*) were immunoblotted with a human IGFBP-5 antibody prepared in rabbit (*lanes 2* and *3*) or in guinea pig (*lanes 4* and *5*). *Lane 1* contains purified human IGFBP-5 (100 ng).

sequence of this PCR product is as follows: VCLNEKSYRE-QAKIERDSRQHEEPTTSEMAEETYSPKIFRTKHTRISELK-AEAVKKDRRKKLTQSKFVGGAENTAHPVISAPEMRQESE-QGPCRRHMEASLQELKASPRMVPRAVYLPNCDRKGFYKR-KQ (the amino acids that differ from human IGFBP-5 are underlined). This sequence is 96% (126 out of 131 amino acids) identical to the region between Val^{99} and Gln^{229} of human IGFBP-5, indicating that it represents part of porcine IGFBP-5 cDNA. This porcine IGFBP-5 cDNA fragment was ^{32}P-labeled and hybridized to porcine SMC RNA blots. The same 6-kb hybridizing band was observed (data not shown), indicating that this 6-kb transcript is porcine IGFBP-5 mRNA.

To determine if porcine aortic SMCs synthesize and secrete IGFBP-5, conditioned media (CM) from porcine SMC cultures were subjected to Western ligand blot and immunoblot analysis. As reported previously (17), ligand blotting and immunoblotting of CMs from confluent SMCs failed to detect IGFBP-5 (data not shown). This is likely due to the fact that porcine SMC-CM contains abundant proteolytic activity for IGFBP-5 (22, 23). Since IGF-I and heparin have been shown to inhibit IGFBP-5 proteolytic degradation in human fibroblasts (19, 24), IGF-I and/or heparin were added to cell cultures prior to the collection of medium. When heparin and IGF-I were added to subconfluent SMC cultures, an IGFBP at the size of 31 kDa, which comigrated with purified human IGFBP-5, was observed by ligand blotting (Fig. 2*A*). The identity of this 31-kDa IGFBP as IGFBP-5 was confirmed by immunoblotting using two different antibodies to human IGFBP-5 (Fig. 2*B*). The addition of IGF-I increased both intact IGFBP-5 and a 22-kDa IGFBP-5 fragment, suggesting that the IGF-I-induced porcine IGFBP-5 increase may not be simply due to a decrease in degradation.

IGF-I Stimulates IGFBP-5 Synthesis—To determine if the IGF-I-induced increase in accumulated IGFBP-5 is due to an increase in synthesis or decrease in degradation or both, immunoprecipitation of [^{35}S]methionine-labeled newly synthesized IGFBP-5 was performed. As shown in Fig. 3, most newly synthesized IGFBP-5 was degraded in porcine SMCs under basal conditions. Therefore, only a 22-kDa IGFBP-5 fragment was detectable in media from control cultures (Fig. 3*A*, *lane 2*). The addition of heparin decreased the intensity of the IGFBP-5 fragment (50% of the control) and induced the appearance of the intact IGFBP-5 doublet band (Fig. 3*A*, *lane 3*). The addition of IGF-I alone caused a moderate increase in intact IGFBP-5 and a greater increase in the intensity of the IGFBP-5 fragment (435% above control; Fig. 3*A*, *lane 4*, and Fig. 3*B*). The addition of IGF-I together with heparin resulted in a 220% increase in the levels of intact IGFBP-5 compared with heparin alone and a 180% increase in the fragment (Fig. 3, *panel A*, *lane 5*, and *panel B*). When immunoprecipitation was performed using normal guinea pig serum, neither intact nor fragment IGFBP-5 was detected (Fig. 3*A*, *lane 1*). These data indicate that IGF-I increases the IGFBP-5 levels in porcine SMC-CM primarily by stimulating IGFBP-5 synthesis.

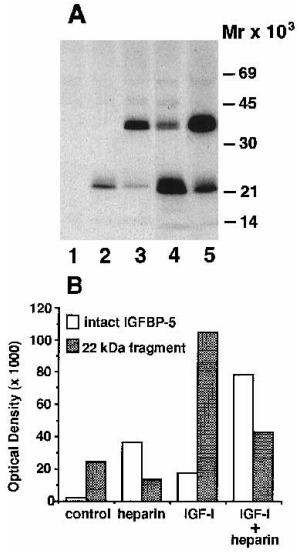

FIG. 3. **IGF-I stimulates IGFBP-5 synthesis in porcine aortic SMCs.** *A*, autoradiogram showing the effect of IGF-I and/or heparin on newly synthesized IGFBP-5. Porcine SMCs were preincubated without (*lanes 1–3*) or with IGF-I (100 ng/ml, *lanes 4* and *5*) for 18 h followed by a 1-h incubation in methionine-free medium before the addition of 50 μCi of [^{35}S]methionine without (*lanes 1*, *2*, and *4*) or with heparin (100 μg/ml, *lanes 3* and *5*). After 6 h, culture media were collected and immunoprecipitated using normal guinea pig serum (*lane 1*) or human IGFBP-5 antiserum prepared in guinea pig (*lanes 2–5*). The pellets were boiled in sample buffer for 10 min and analyzed by SDS-PAGE followed by autoradiography. *B*, phosphorimager analysis. Values are means of two immunoprecipitation experiments as described in *A*.

IGF-I Increases the Steady-state Levels of IGFBP-5 mRNA—To examine if IGF-I-induced increase in IGFBP-5 synthesis was regulated at the level of mRNA abundance, total RNA was isolated from SMCs treated with or without IGF-I

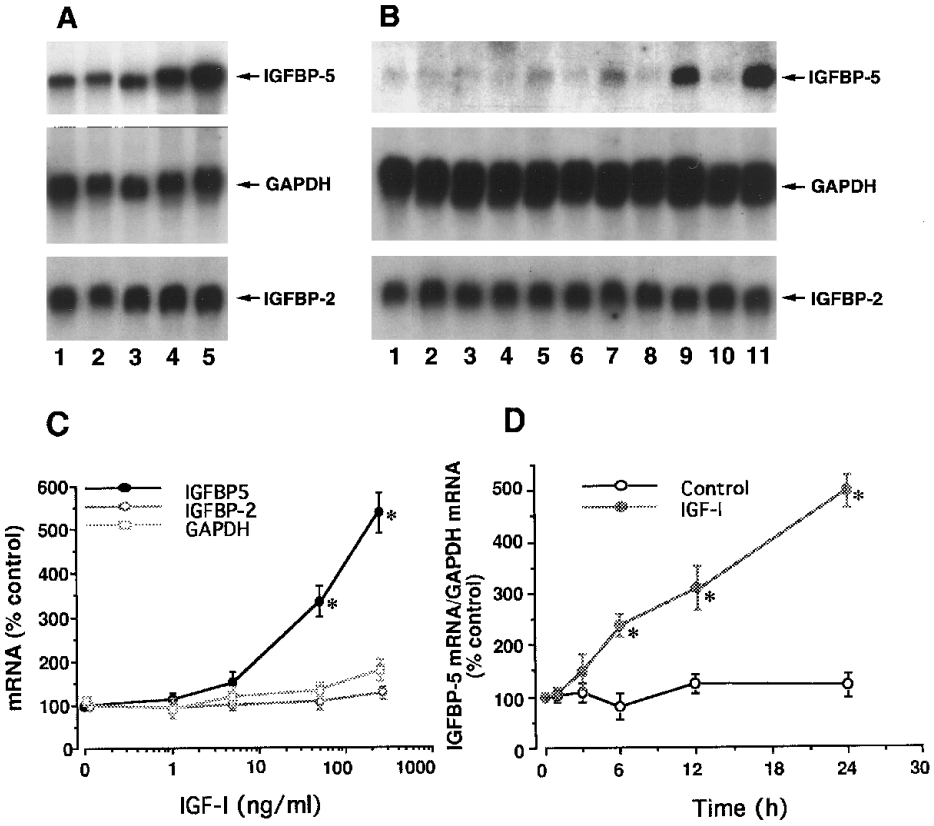

FIG. 4. **IGF-I increases the steady-state levels of IGFBP-5 mRNA in porcine aortic SMCs.** *A*, autoradiogram showing dose-dependent effect of IGF-I. Porcine SMC cultures were treated without (*lane 1*) or with 1 (*lane 2*), 5 (*lane 3*), 50 (*lane 4*), and 250 ng/ml IGF-I (*lane 5*) for 24 h. Total RNA was isolated from SMC cultures and subjected to Northern blot with cDNA probes for IGFBP-5, IGFBP-2, and GAPDH. *B*, autoradiogram showing the time-course effect of IGF-I. Porcine SMC cultures were preincubated in serum-free medium for 24 h and then treated without (*lanes 1*, *2*, *4*, *6*, *8*, and *10*) or with 100 ng/ml IGF-I (*lanes 3*, *5*, *7*, *9*, and *11*) for 0 (*lane 1*), 1 (*lanes 2* and *3*), 3 (*lanes 4* and *5*), 6 (*lanes 6* and *7*), 12 (*lanes 8* and *9*), and 24 h (*lanes 10* and *11*). *C* and *D*, phosphorimager analyses of the concentration dependence and time-course experiments, respectively. Values are means ± S.E. of four (*C*) or three (*D*) separate experiments. They are expressed as a percentage of mRNA levels in the control, untreated samples. *, significantly different from the controls ($p < 0.05$).

and subjected to Northern blot analysis. IGF-I treatment caused significant increases in the steady-state levels of IGFBP-5 mRNA in a dose-dependent manner (Fig. 4, *A* and *C*). When incubated for 24 h, IGF-I treatment significantly increased IGFBP-5 mRNA levels using concentrations from 50 to 250 ng/ml ($p < 0.05$). IGF-I (250 ng/ml) caused a 448% increase, while 228% and 57% increases were observed using concentrations of 50 and 5 ng/ml, respectively. In contrast to IGFBP-5, IGF-I treatment had no effect in on IGFBP-2 mRNA levels. IGF-I at the highest concentration (250 ng/ml) appeared to slightly increase GAPDH mRNA levels (63%, Fig. 4, *A* and *C*). This increase, however, was much less than the increase in IGFBP-5 mRNA and could reflect an increase in total protein synthesis that occurs with IGF-I treatment of SMCs (18). IGF-I induced IGFBP-5 mRNA levels in a time-related fashion (Fig. 4, *B* and *D*). IGF-I (100 ng/ml) caused significant 147% and 217% increases in IGFBP-5 mRNA levels after 6 and 12 h of incubation, respectively. A greater response (497% of the control values) was seen after 24 h. The induction of IGFBP-5 mRNA levels appeared to be specific for IGF-I. Addition of FGF, either alone or in combination with IGF-I, did not further increase IGFBP-5 mRNA levels (Fig. 5, *A* and *B*). PDGF, on the other hand, caused a moderate decrease in the presence (40%) or absence of IGF-I (21%). These results indicated that the stimulation of IGFBP-5 synthesis by IGF-I is regulated at the level of mRNA abundance in porcine SMCs and that this effect is IGF-I-specific.

The IGF-I-induced IGFBP-5 mRNA Expression Is Mediated through the IGF-I Receptors—The effect of IGF-I was compared with two IGF-I-related peptides, IGF-II and insulin, which bind to the IGF-I receptors with lower affinity. As shown in Fig. 6 (*A* and *C*), IGF-II and insulin were effective at high concentrations, but less potent. IGF-II at the concentration of 50 ng/ml caused a 110% increase in comparison to a 243% increase by IGF-I at the same concentration. Insulin had little effect at this dose. A very high dose of insulin (1 μg/ml) evoked a moderate

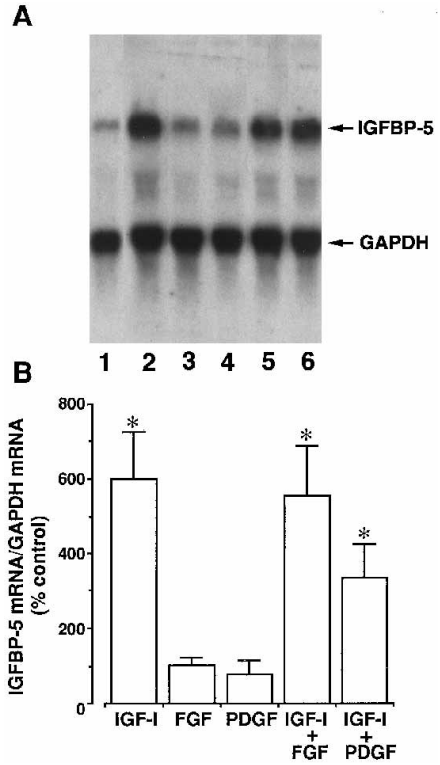

FIG. 5. *A*, autoradiogram showing the effects of PDGF and FGF on IGF-I-induced IGFBP-5 mRNA expression. Porcine aortic SMCs were incubated in serum-free medium without (*lane 1*) or with IGF-I (100 ng/ml, *lane 2*), PDGF (5 ng/ml, *lane 3*), FGF (50 ng/ml, *lane 4*), IGF-I plus PDGF (*lane 5*), or IGF-I plus FGF (*lane 6*) for 24 h. Total RNA was isolated from SMC cultures and subjected to Northern blotting with cDNA probes for IGFBP-5 and GAPDH. *B*, phosphorimager analysis of three experiments as described in *A*. Values are means ± S.E. expressed as a percentage of mRNA levels in the control, untreated samples. *, significantly different from the controls ($p < 0.05$).

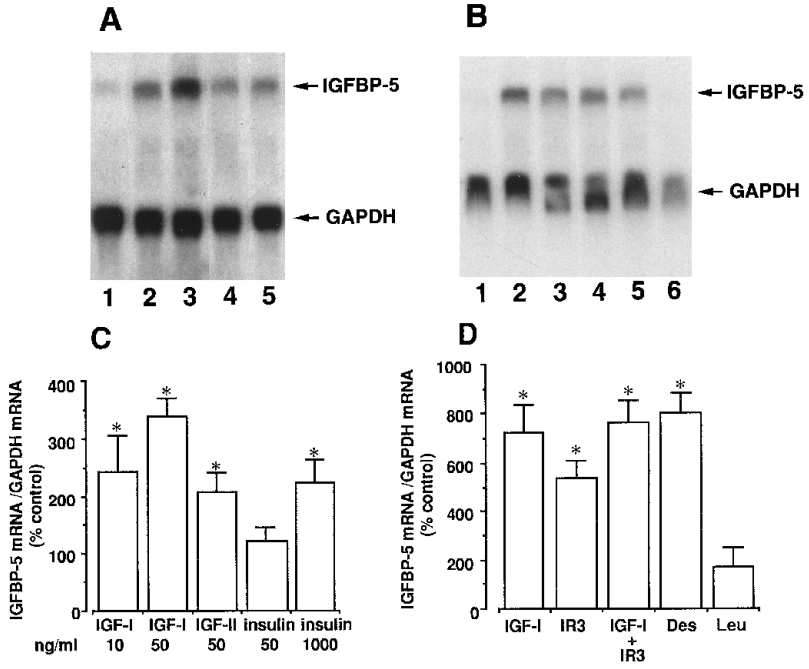

FIG. 6. **The effect of IGF-I on IGFBP-5 gene expression is mediated through the IGF-I receptor.** *A*, autoradiogram showing the effects of IGF-I, IGF-II, and insulin. Porcine aortic SMCs were incubated with serum-free medium (*lane 1*), or serum-free medium plus IGF-I (10 ng/ml, *lane 2*; 50 ng/ml, *lane 3*), IGF-II (50 ng/ml, *lane 4*), or insulin (1 μg/ml, *lane 5*) for 24 h. *B*, autoradiogram showing the effects of IGF analogs and the IGF-I receptor blocking antibody αIR3. Porcine SMCs were incubated with serum-free medium (*lane 1*), or serum-free medium with IGF-I (100 ng/ml, *lane 2*), αIR3 (10 μg/ml, *lane 3*), IGF-I (100 ng/ml) plus αIR3 (10 μg/ml, *lane 4*), Des(1–3)-IGF-I (100 ng/ml, *lane 5*), or [Leu^{24}]IGF-I$_{(1-60)}$ (100 ng/ml, *lane 6*) for 24 h. *C* and *D*, phosphorimager analyses of three experiments as described in *A* and *B*, respectively. Values are means ± S.E. expressed as a percentage of mRNA levels in the control, untreated samples. *, significantly different from the controls ($p < 0.05$).

increase (140%). These data suggested that the IGF-I receptor mediated this effect. We attempted to determine if this effect of IGF-I was mediated by the IGF-I receptor by selectively blocking the receptor using the monoclonal blocking antibody, αIR3. Surprisingly, the antibody did not inhibit the IGF-I-induced increase (Fig. 6*C*, *lane 4*) and caused a 436% increase in IGFBP-5 mRNA levels in porcine SMCs (Fig. 6*C*, *lane 3*). This is unlikely to be due to the use of this anti-human IGF-I receptor antibody in a heterologous system, since αIR3 induced a similar increase of IGFBP-5 mRNA expression in human aortic SMCs (data not shown). Thus, the induction of IGFBP-5 by IGF-I could not be assessed under conditions of selective receptor blockage. We next examined the effects of IGF analogs, Des(1–3)-IGF-I and [Leu^{24}]IGF-I$_{(1-60)}$. Des(1–3)-IGF-I is an IGF-I analog with normal affinity for IGF-I receptors but with remarkably reduced affinity for IGFBPs. This peptide evoked a similar 705% increase in IGFBP-5 mRNA levels in porcine SMCs (Fig. 6, *panel C*, *lane 5*, and *panel D*). On the other hand, [Leu^{24}]IGF-I$_{(1-60)}$, which binds to IGFBPs normally but does not bind to the IGF receptors, had no effect (Fig. 6, *panel C*, *lane 6*, and *panel D*). Therefore, the stimulation of IGFBP-5 expression by IGF-I appears to be mediated through the IGF-I receptor.

IGF-I Induces IGFBP-5 Expression through Transcriptional Activation of the IGFBP-5 Gene—The elevation in IGFBP-5 mRNA levels and protein synthesis observed after IGF-I treatment could reflect transcriptional activation of the gene and/or posttranscriptional events. To determine if IGF-I induces IGFBP-5 mRNA levels by transcriptional mechanisms, nuclear run-on assays were performed on nuclei isolated from control and SMCs that had been treated with IGF-I. As shown in Fig. 7, IGF-I treatment increased the level of IGFBP-5 transcription rate an average of 289% above base line in two separate experiments, whereas no difference was seen with IGFBP-2 transcripts, suggesting that the increase in the steady-state levels of IGFBP-5 mRNA induced by IGF-I is due at least in part to an increase in the rate of transcription.

We also sought to determine if IGF-I treatment affects IGFBP-5 mRNA stability. Porcine aortic SMCs were incubated with or without IGF-I for 18 h, and then treated with actinomycin D. Although there was no difference in the calculated $t_{1/2}$

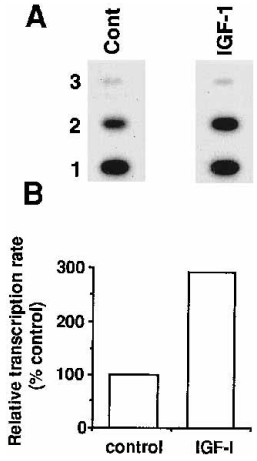

FIG. 7. **IGF-I stimulates the transcription rate of the IGFBP-5 gene in porcine aortic SMCs.** *A*, autoradiogram showing the effect of IGF-I. Nuclei from control and IGF-I-treated cultures were isolated and nuclear run-on assays performed in the presence of [^{32}P]UTP for 30 min. The nascent ^{32}P-labeled transcripts were hybridized to slots of filter-bound IGFBP-2 (*lane 1*), IGFBP-5 (*lane 2*), and pBluescript DNA (*lane 3*). *B*, phosphorImager analysis of two separate experiments. Values are means expressed as a percentage of mRNA levels in the control, untreated samples.

of IGFBP-5 mRNA in the control and IGF-I-treated groups, treatment of actinomycin D caused a transient rise in IGFBP-5 mRNA levels (data not shown). This actinomycin D-associated increase in IGFBP-5 mRNA levels has previously been observed in human breast carcinoma cells (25) and complicated the interpretation of these data. We next performed similar experiments using the RNA polymerase II inhibitor DRB. Addition of DRB to porcine SMCs led to a progressive decline in IGFBP-5 abundance (Fig. 8*A*) with a calculated $t_{1/2}$ for both control and IGF-I-treated groups of approximately 18 h (Fig. 8*B*). Thus, IGF-I treatment does not cause an alternation in IGFBP-5 mRNA stability.

These results indicate that the increase in the levels of IGFBP-5 mRNA induced by IGF-I is primarily due to the activation of the IGFBP-5 gene. We wondered whether IGF-I modulates IGFBP-5 transcripts by direct interaction with the 5′- or

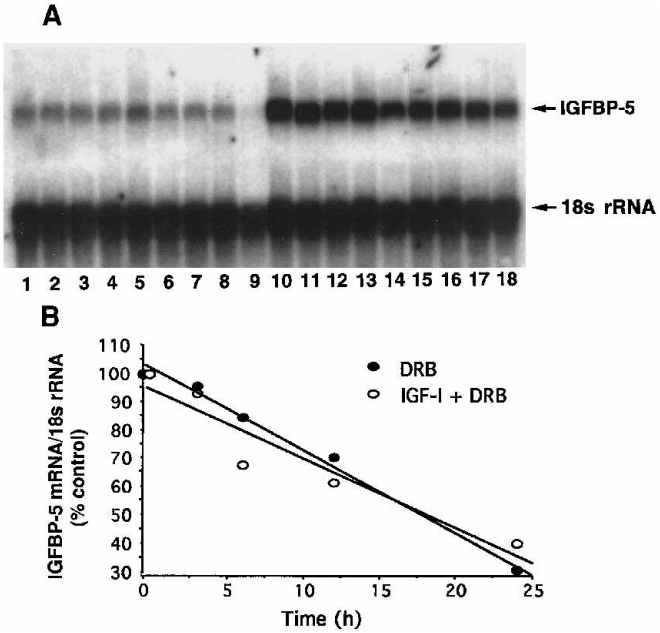

FIG. 8. **IGF-I does not cause alteration in the stability of IGFBP-5 mRNA in porcine aortic SMCs.** *A*, autoradiogram showing a representative Northern blot. Porcine aortic SMCs were incubated without (*lanes 1–9*) or with IGF-I (*lanes 10–18*) for 18 h, followed by the addition of vehicle (*lanes 2–5* and *11–14*) or DRB at 75 μM concentration (*lanes 6–9* and *15–18*). The cells were harvested at 0 (*lanes 1* and *10*), 3 (*lanes 2*, *6*, *11*, and *15*), 6 (*lanes 3*, *7*, *12*, and *16*), 12 (*lanes 4*, *8*, *13*, and *17*), and 24 h (*lanes 5*, *9*, *14*, and *18*) after the addition of DRB or vehicle. Total RNA was isolated and subjected to Northern blotting with cDNA probes for IGFBP-5 and 18 S rRNA. *B*, effect of IGF-I on IGFBP-5 mRNA decay in transcriptionally blocked porcine SMCs. Values are means of two separate experiments expressed as a percentage of levels in the control, untreated samples.

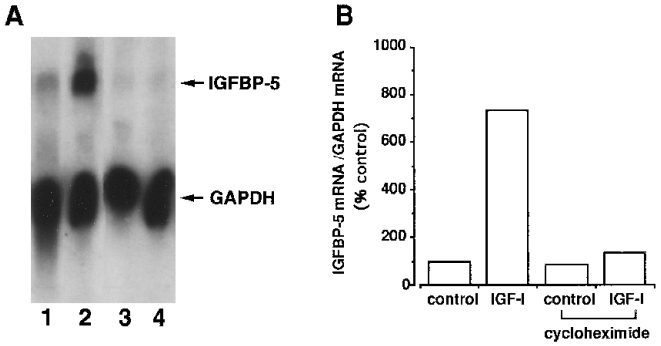

FIG. 9. **The protein synthesis inhibitor cycloheximide abrogates the IGF-I-induced IGFBP-5 expression.** *A*, autoradiogram showing the inhibitory effect of cycloheximide. Porcine aortic SMC cultures were incubated without (*lanes 1* and *3*) or with IGF-I (100 ng/ml, *lanes 2* and *4*) in the presence (*lanes 1* and *2*) or absence (*lanes 3* and *4*) of cycloheximide (10 μg/ml) for 24 h. Total RNA was isolated from SMC cultures and subjected to Northern blotting with cDNA probes for IGFBP-5 and GAPDH. *B*, phosphorimager analysis. Values are means of two separate experiments expressed as a percentage of levels in the control, untreated samples.

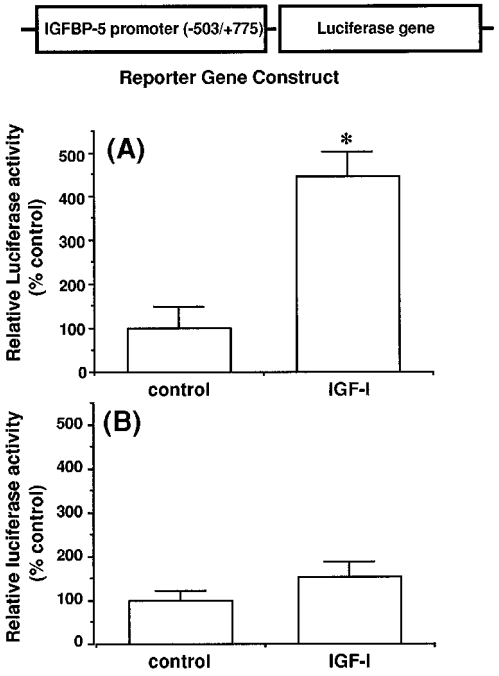

FIG. 10. **Effect of IGF-I on human IGFBP-5 promoter activity in aortic SMCs (*A*) and fibroblasts (*B*).** A 1278-bp DNA fragment of the human IGFBP-5 gene 5′-flanking region was fused to a luciferase reporter gene (pGL2-Basic) and transiently transfected into porcine SMCs and GM-10 fibroblasts. After growing in complete medium for 72 h, cells were incubated in serum-free medium with or without IGF-I (100 ng) for another 6 h. The cellular extracts were prepared and the luciferase activity was measured as described under "Experimental Procedures." The relative luciferase activities represent the relative value normalized by galactosidase activity. Values are means ± S.E. expressed as a percentage of the levels in the controls. * Significantly different from the controls ($p < 0.05$).

3′-regulatory sequences or, alternatively, by inducing the synthesis of an intermediate regulatory factor(s). Accordingly, porcine SMCs were treated with IGF-I in the presence and absence of cycloheximide. As shown in Fig. 9, while IGF-I alone induced a 656% increase, co-incubation with cycloheximide completely blocked the IGF-I-induced IGFBP-5 gene expression (151% of the controls), suggesting this effect of IGF-I requires *de novo* protein synthesis.

In order to gain insight into the promoter region(s) of the IGFBP-5 gene responsive to IGF-I, we transfected SMCs with a 1278-bp segment of human IGFBP-5 promoter fused to the reporter luciferase gene. This segment of the IGFBP-5 promoter contains identical sequences of a number of well defined regulatory elements, including a TATA box, a CAAT box, and several AP-2 elements, which previously have been shown to be responsible for the cAMP-induced activation of this gene (21). As shown in Fig. 10*A*, relative luciferase activity in IGF-I-treated SMCs was 345% higher than those of the control SMCs ($p < 0.05$). This indicates that this 1278-base pair promoter region contains a *cis*-acting element(s) that is responsible for the IGFBP-5 gene response to IGF-I.

The Regulation of IGFBP-5 Gene Expression by IGF-I Is Cell Type-specific—Since previous studies showed that IGF-I treatment does not result in a significant change in IGFBP-5 mRNA levels in human fibroblasts, human osteosarcoma, and breast carcinoma cells (19, 26, 27, 37, 38), we wondered if the stimulation of IGFBP-5 gene expression by IGF-I seen in porcine SMCs reflects a cell type-specific regulation or is simply due to different species used. To determine this, we examined the effect of IGF-I on IGFBP-5 expression in a number of human cell lines derived from different tissues. Similar to porcine aortic SMC, IGF-I treatment (100 ng/ml, 24 h) resulted in a significant increase (320 ± 53%) in the steady-state levels of IGFBP-5 mRNA in human newborn aortic SMCs (Fig. 11*A*). In contrast to aortic SMCs, human fetal skin fibroblasts (GM-10), glioblastoma cell (T98G), and human intestinal SMCs had abundant IGFBP-5 mRNA levels under basal conditions (Fig. 11, *B–D*). IGF-I treatment did not change the IGFBP-5 mRNA levels in these cells. In addition, in human fibroblasts transfected with the IGFBP-5 promoter/luciferase chimerical constructs, relative luciferase activity did not change when IGF-I was added (Fig. 10*B*). These results indicate that the up-regu-

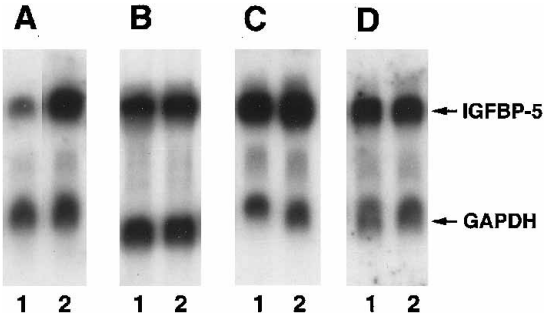

FIG. 11. **IGF-I stimulates IGFBP-5 gene expression in human aortic SMCs (*A*) but not in human fibroblasts (*B*), glioblastoma (*C*), and human intestinal SMCs (*D*).** Confluent cells were incubated without (*lane 1*) or with IGF-I (100 ng/ml, *lane 2*) for 24 h. Total RNA was isolated, and 15-μg RNA aliquots were loaded and subjected to Northern blotting with cDNA probes for IGFBP-5 and GAPDH.

lation of IGFBP-5 gene expression by IGF-I occurs in human and porcine aortic SMCs and this regulation is cell type-specific.

DISCUSSION

The present study demonstrates that porcine as well as human aortic SMCs express IGFBP-5 mRNA and secrete the protein. In this cell type, the biosynthesis of IGFBP-5 is stimulated by IGF-I. IGF-I regulates IGFBP-5 synthesis at the mRNA level. This effect of IGF-I appears to be mediated by the IGF-I receptor and requires *de novo* protein synthesis. The increase in IGFBP-5 mRNA levels that is induced by IGF-I is primarily due to an elevation in the transcription rate of the IGFBP-5 gene rather than an alteration in the stability of the transcript, suggesting that IGF-I regulates IGFBP-5 expression primarily by transcriptional activation of the gene in aortic SMCs.

The expression of the IGFBP-5 gene is cell type-specific. High levels of IGFBP-5 mRNA have been found in fibroblasts, glioblastoma cells, skeletal muscle cells, osteoblasts, chondrocytes, granulosa cells, and thyroid cells but not in hepatoma or rhabdomyosarcoma cells (19, 21, 26, 28–30, 55, 56). Although the aortic SMC has been extensively used as a model to study the IGF system, there was no previous report regarding the expression of IGFBP-5 in this cell type. Several experimental difficulties seem to be partly responsible. First, aortic SMC-CM contains abundant proteolytic activity for IGFBP-5. SMC-CM has been shown to rapidly degrade exogenously added human IGFBP-5 (22, 23). As shown in this study, the endogenously secreted IGFBP-5 was completely degraded yielding small fragments under basal conditions unless IGF-I and/or heparin was added to the culture medium (Figs. 2 and 3). Second, endogenously produced IGFBP-5 (31 kDa) was difficult to distinguish from 32-kDa IGFBP-2, which is the predominant form of IGFBP secreted by porcine SMCs that is detected by Western ligand blotting. Third, the IGFBP-5 production by SMCs is inversely correlated to cell density. IGFBP-5 mRNA levels were 4–5-fold lower in postconfluent cultures as compared with subconfluent SMC cultures.[2] In this study, we used subconfluent cultures, whereas in previous studies confluent cultures were used (17, 31).

The fact that aortic SMCs synthesize IGFBP-5 and that its synthesis is under the regulation of IGF-I implies that this binding protein may play an important role in modulating IGF-induced SMC proliferation and migration. IGFBP-5 has been shown to have the unique property of adhering to extracellular matrix (32). When associated with the extracellular matrix, it has been shown to potentiate the effect of IGFs on fibroblast growth. In addition, IGFBP-5 may also be involved in muscle cell differentiation. The expression of IGFBP-5 is greatly increased during terminal differentiation of the mouse myoblast cell lines (33–35). This process is also stimulated by IGFs. The exact physiological function(s) of IGFBP-5 in aortic SMC proliferation and migration is currently under investigation.

The abundance of IGFBP-5 is influenced by a number of factors. Of the substances studied, IGF-I was the most potent regulator in aortic SMCs. IGF-I increased the IGFBP-5 protein as well as IGFBP-5 mRNA levels. The effect of IGF-I in inducing IGFBP-5 expression is specific. IGF-I had no effect on IGFBP-2 mRNA level. There was a small increase in GAPDH mRNA, but this change was negligible in comparison to a severalfold increase in IGFBP-5. Two other SMC mitogenic factors, FGF and PDGF, had no stimulatory effect. In fact, PDGF acts as a inhibitor of IGFBP-5 expression either added alone or in combination with IGF-I. This inhibitory effect of PDGF was previously observed in rat osteoblasts (36). An increase in IGFBP-5 abundance in CM induced by IGF-I was previously reported in human fibroblasts and other cells (19, 26–29, 37, 55, 56). However, inconsistent and confusing results have been documented regarding the mechanisms accounting for this increase. In human fibroblasts, U2 osteosarcoma cells and breast carcinoma cells, IGF-I treatment significantly increases the IGFBP-5 protein concentrations without affecting the IGFBP-5 mRNA abundance (19, 26, 27, 37, 38). This effect of IGF-I may be attributed to the inhibition of IGFBP-5 proteolysis rather than an alteration of the biosynthesis in these cells. In rat FRTL-5 thyroid cells and osteoblasts, on the other hand, IGF-I stimulated IGFBP-5 concentrations with a concomitant increase in IGFBP-5 mRNA levels, suggesting that IGF-I may regulate the IGFBP-5 abundance by simulating its synthesis (28, 29).

In this study, we have directly demonstrated that IGF-I induces an increase in IGFBP-5 synthesis in aortic SMCs by using metabolic labeling of porcine SMCs coupled with immunoprecipitation. Although porcine SMC conditioned medium contains IGFBP-5 protease activity, IGF-I does not appear to greatly affect proteolysis. IGF-I treatment resulted in an increase in the newly synthesized intact IGFBP-5 as well as the proteolytically degraded fragment, while heparin only increased the intact protein levels by inhibiting the degradation (Fig. 3). Therefore IGF-I regulates the IGFBP-5 abundance in porcine SMCs primarily by increasing the biosynthesis. These results are consistent with the previous data obtained in rat thyroid cells and osteoblasts, but different from those of human fibroblasts, human osteosarcoma, and breast carcinoma cells. Since the cell models used in the above studies are derived from different tissues and species, this discrepancy may reflect cell-specific regulation or species differences. A recent study using human and bovine fibroblasts suggested a possible species difference may exist (27). We addressed this question by examining IGF-I-induced IGFBP-5 gene expression in a number of human cell types. Our data with human aortic SMCs, human skin fibroblasts, human glioblastoma cells, and intestinal SMCs (Fig. 11) indicate that the stimulation of IGFBP-5 gene transcription by IGF-I is a cell type-specific event. This conclusion is in agreement with the fact that the structure of the IGFBP-5 promoter is highly conserved among mammalian species. The proximal 200 bp of the IGFBP-5 gene promoter, which has been shown to contain the primary promoter activity (39), is more than 90% identical among human, mouse, and rat (40–42). Therefore, it is unlikely species difference is the sole factor accounting for the different regulation of IGFBP-5

[2] C. Duan and D. R. Clemmons, unpublished observation.

synthesis.

Although previous studies have demonstrated IGF-I-induced IGFBP-5 mRNA steady-state levels in other cell types (27–29), the pathways and mechanisms responsible for the rise in IGFBP-5 mRNA levels have not been established. In this study, we attempted to delineate whether IGF-I operates through the IGF-I receptor to induce IGFBP-5 gene expression. Because the anti-IGF-I receptor antibody (αIR3) behaved as a partial agonist in stimulating IGFBP-5 expression, definite proof that type I IGF-I receptor stimulation accounts for IGFBP-5 induction could not be obtained. The partial agonist activity seen here with αIR3 has been reported previously with IGF-I-induced c-*myc* induction in human SMCs (43). Our experiments using IGF-II, insulin, and IGF analogs, however, strongly suggest that the stimulation of IGFBP-5 expression by IGF-I is mediated through the IGF-I receptor.

The data presented in this study demonstrate that IGF-I treatment transcriptionally activates the IGFBP-5 gene without altering the transcript stability. The IGFBP-5 gene expression is activated within 6 h of exposure to IGF-I, as indicated by Northern blotting and transient gene transfer studies. Coupled with the relatively long half-life of IGFBP-5 mRNA in porcine aortic SMCs (18 h), the progressive acceleration of the transcription rate is sufficient to result in a substantial increase in mRNA expression, protein synthesis, and secretion into the culture medium. The transcriptional activation of the IGFBP-5 gene by IGF-I in aortic SMCs was further ascertained by the results of gene transfer studies in SMCs. A fusion plasmid containing 1278 bp of 5′-flanking region of human IGFBP-5 gene showed an IGF-I-induced rise in directing reporter gene expression (Fig. 10). These observations indicate that IGF-I is able to activate the IGFBP-5 gene through *cis*-acting element(s) residing on this 1278-bp region. IGF-I has been shown to regulate transcription of a number of genes, *e.g.* c-*myc* (43), growth hormone (44), thyroglobulin (45), δ1-crystallin (46), elastin (47), P-450 cholesterol side chain cleavage gene (48), and others (49). However, little is known regarding the *cis*-DNA sequences responsive to IGF-I. Recently, IGF-I was shown to regulate chicken δ1-crystallin gene expression through a GC-rich sequence that binds to a Sp-1-like protein (46). In the rat elastin promoter, a similar but not identical GC-rich sequence is capable of binding to IGF-I-regulated proteins and responsible for the IGF-I-responsiveness of this gene (47). One of the proteins has been shown to be Sp-1 (50). The IGFBP-5 promoter contains several GC-rich regions superficially resembling the Sp-1 element. In particular, the DNA sequence 5′-CCCCACCCCCACCC-3′ at position −147 to −134 has this potential. Although this highly conserved sequence contains two overlapping AP-2 elements (5′-CCCCACCC-3′) and is capable of binding to AP-2 *in vitro*, it does not appear to mediate the AP-2 regulation under basal condition *in vivo* (21). This sequence contains sequences identical to the retinoblastoma (Rb) control element 5′-CCACCC-3′. The Rb control element motif has been identified as a Sp-1-binding sequence responsible for Rb-induced *trans*-activation (51). A recent study by Jensen *et al.* (50) suggested that IGF-I may disrupt Sp-1 binding to the GC-rich domain of the elastin gene by affecting the phosphorylation state of Rb in rat SMCs. Studies using transient transfection assays are currently under way to determine if this sequence and/or other sequence(s) is required for IGFBP-5 gene to be activated in response to IGF-I.

Our results have shown that IGF-I up-regulates IGFBP-5 gene expression in a dose-dependent fashion and in a time frame consistent with that of an intermediate effect. The finding that cycloheximide abrogates IGF-I-induced IGFBP-5 transcription suggests a requirement for the synthesis of an intermediate protein(s). It has been reported that IGF-I stimulates the expression the immediate-early gene c-*fos* in several cell types (52, 59). The encoded proteins Fos can dimerize with Jun and form the AP-1 complex, a transcriptional activator that regulates many genes (53). An elevation in AP-1 transcriptional activity induced by IGF-I has recently been observed in IEC-6 intestinal epithelial cells (60). Although a consensus AP-1 element is present in the rat IGFBP-5 promoter region (−314 to −320 bp; Ref. 42), there is no evidence that this AP-1 element is functional in rat. Moreover, this sequence is not conserved even among mammalian species; a single residue alteration from T to C leads to the ablation of the potential AP-1 element in the human IGFBP-5 promoter (40). One of the other genes that responds rapidly to IGF-I is the prereplicative (G_1) phase-specific cyclin D1. This gene is activated by IGF-I treatment within 1 h in MG63 human osteosarcoma cells (54). D-type cyclins are known to be able to form complexes with Rb and affect Rb phosphorylation status (61, 62). The phosphorylation states of Rb are reported to be affected by IGF-I in aortic SMCs, and this change appears to be related to the IGF-I-induced disruption of Sp-1 binding of the rat elastin gene (50). Further studies are needed to determine which mechanism(s) IGF-I uses to activate IGFBP-5 gene transcription in aortic SMCs.

In summary, aortic SMCs synthesize IGFBP-5 in addition to previously identified IGFBP-2 and -4. The abundance of this important modulator of IGF activity is regulated by its own ligand, IGF-I. IGF-I regulates IGFBP-5 synthesis by activating transcription of the IGFBP-5 gene rather than altering the stability of the transcript. This effect of IGF-I appears to be mediated by the IGF-I receptor and is aortic SMC-specific. Since IGF-I is important for aortic SMC proliferation and migration, analysis of the regulation of IGFBP-5 gene expression and action in SMCs should provide insight into the role of the IGF system in the development of atherosclerotic lesions.

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