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INSULIN-LIKE GROWTH FACTOR I STIMULATES ELASTIN SYNTHESIS BY BOVINE PULMONARY ARTERIAL SMOOTH MUSCLE CELLS

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Insulin-like growth factor I stimulates mitogenesis in smooth muscle cells, and upregulates elastin synthesis in embryonic aortic tissue. Increased smooth muscle elastin synthesis may play an important role in vascular remodeling in chronic pulmonary hypertension. Therefore, we studied the effect of IGF-I on elastin and total protein synthesis by pulmonary arterial smooth muscle cells in vitro. Tropoelastin synthesis was measured by enzyme immunoassay, and total protein synthesis was measured by [^{3}H]-leucine incorporation. In addition, the steady-state levels of tropoelastin mRNA were determined by slot blot hybridization. Incubation of confluent cultures with various concentrations of IGF-I resulted in a dose-dependent stimulation of elastin synthesis, with a 2.4-fold increase over control levels at 1000 ng/ml of IGF. The increase in elastin synthesis was reflected by a stimulation of the steady-state levels of tropoelastin mRNA. We conclude that IGF-I has potent elastogenic effects on vascular smooth muscle cells, and speculate that it may contribute to vascular wall remodeling in chronic hypertension.

Chronic hypertension in the pulmonary and systemic circulations is associated with remodeling of the vascular wall (1,2). We have shown that chronic hypoxia-induced pulmonary hypertension is associated with extensive changes in the pulmonary arterial wall, including cellular hyperplasia, increased elastin and collagen deposition, and altered reactivity to vasodilators (2,3). The precise mechanisms mediating these changes are unknown and undoubtedly complex, but studies of hypertensive and atherosclerotic systemic arteries suggest that peptide growth factors may play an important role in vascular remodeling (4,5,6,7). These studies implicate PDGF, TGF-*B*, and IL-1 in various aspects of the vascular remodeling process, and additional evidence suggests that insulin-like growth factor I (IGF-I) may also be important in vascular remodeling. For example, IGF-I has mitogenic effects on smooth muscle cells (SMC) (8), immunohistochemical localization of IGF-I within the vascular media varies with vascular load (9), and both fibroblasts and porcine aortic SMC produce IGF-I in vitro (10,11). Furthermore, vascular SMC have receptors for IGF I and II, the expression of which varies during vessel development (12). Finally, a recent study has shown that IGF-I stimulates synthesis of tropoelastin in embryonic chick aortae in organ culture (13).

Abbreviations Used: IGF-I, insulin-like growth factor I; PDGF, platelet-derived growth factor; TGF-*B*, transforming growth factor-beta; FBS, fetal bovine serum; CS, calf serum; DMEM, Dulbecco's Modified Eagles Medium.

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Based on these findings, we examined the effect of IGF-I on elastin synthesis by cultured SMC from the pulmonary arteries of neonatal calves. To determine whether IGF-I regulation of elastin synthesis occurred at a pretranslational level, steady-state tropoelastin mRNA levels were assessed by slot-blot analysis. Our findings suggest that IGF-I may play an important role in the elastogenesis that is associated with pulmonary hypertension.

METHODS

Tissue Culture

Neonatal male dairy calves (age 2-3 weeks) were sacrificed by sodium pentobarbital anesthesia and exsanguination. The heart and lungs were immediately removed, and the pulmonary artery (main and proximal lobar sections) was isolated. The medial layer was aseptically separated from the adventitia, and the luminal side of the medial layer was gently scraped to remove the endothelial lining. In a small amount of DMEM, the medial tissue was minced into small pieces (0.5-1.0mm), which were placed in 75cm^2 flasks with 10% fetal bovine serum (FBS, Hyclone) and DMEM. As the area around an explant became confluent, the explant was removed. SMC obtained in this fashion demonstrated the characteristic "hill and valley" morphology by phase contrast microscopy, and stained in a fibrillar pattern with the muscle-cell specific biotin-conjugated anti-actin antibody HHF35 (14). Cultures were maintained in DMEM supplemented with 10% FBS, 20 mM HEPES, pH 7.4, 10mM MEM non-essential amino-acid solution, 100 units/ml penicillin, 0.1 mg/ml streptomycin, 10 ug/ml gentamycin and 3.7 g/l sodium bicarbonate (all from Sigma). Only early passage (2-5) SMC were used. The cultures were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

Measurement of Elastin Synthesis

Following trypsinization, SMC were passed at equal density into each well of a 24-well tissue culture plate. The cells were grown to confluence in DMEM with 10% FBS, then the cells were incubated for 24 hr in DMEM with 0.1% calf serum (CS). Medium was removed, and 1 ml of DMEM with 0.1% CS containing various concentrations of IGF-I was added with the elastin cross-linking inhibitors beta-aminoproprionitrile (BAPN) (Sigma) at 100 ug/ml and penicillamine (Sigma) at 50 ug/ml. IGF-I was obtained from Bachem (Torrence, CA), and was produced by solid-phase synthesis (15). It is bioequivalent to the native peptide, and bovine and human IGF-I have identical amino acid sequences (16). A dose range of 0 ug to 1000 ng/ml of IGF-I was studied, using standard logarithmic dosing increments. The cells were incubated under these conditions for 48 hr at 37°C. Medium was removed and assayed for tropoelastin. Cells from each well were trypsinized and counted manually in a hemocytometer.

Samples were assayed for tropoelastin utilizing a direct enzyme immunosorbent assay (EIA) developed by Prosser et al. (17). Bovine tropoelastin for standards and rabbit polyclonal antiserum against bovine tropoelastin were kindly provided by Dr. Robert Mecham (Washington University Medical Center). Goat anti-rabbit IgG antibody conjugated to horse radish peroxidase (Kirkegaard & Perry Laboratories, Gaitherburg, MD) and substrates 2.2^1-azino-di-[3-ethyl-benzthiazoline sulfonate] (ABTS) and hydrogen peroxide (Kirkegaard & Perry) were used for detection. A tropoelastin standard curve was run with each assay, and all samples were assayed in duplicate. Optical density was read at 414 nm on an automatic plate reader (Titertek Multiskan MC, Flow Laboratories). Elastin synthesis was normalized to cell number.

Measurement of Total Protein Synthesis

SMC were grown to confluence in 24-well tissue culture plates as described above. At confluence, cells were washed for 24 hr in DMEM with 0.1% CS, and DMEM with 0.1% CS and various concentrations of IGF-I were then applied for 48 hr. Medium was then removed and replaced with leucine-free MEM (Gibco Laboratories, Grand Island, NY) for 1.5 hr. Cells were then pulsed overnight with leucine-l-[3,4,5-^{3}H(N)](NEN) at 10 uCi/ml. Medium was then removed, and a 100 ul aliquot was added to 900 ul of cold 10% trichloracetic acid (TCA) (Sigma). The precipitate was collected on Whatman GF/B filter paper circles, rinsed with 2 ml of 95% ethanol, air dried, and placed in a scintillation vial with 10 ml of ScintiVerse E (Fisher Scientific) scintillation fluid, and counted using a Packard Tri-Carb 1500 Liquid Scintillation Analyzer (Packard, Laguna Hills, CA). Cell layers were solubilized in 0.1N NaOH, neutralized with 0.1N HCl, precipitated with cold 10% TCA, filtered, and incorporated [^{3}H] leucine was determined as before. Medium and cell-associated counts were summated to approximate total protein synthesis.

RNA Isolation and Slot-Blot Analysis

Cells were harvested, washed twice with PBS and extracted with 4M guanidine isothiocyante, 25 mM Na-citrate (pH 7), 100 mM *B*-mercaptoethanol, and extracts were passed five times through a 25 guage syringe to shear DNA. RNA was isolated by isopycnic centrifugation through cesium chloride as described (18). For slot-blots, total RNA was denatured in 6% formaldehyde, diluted serially 1:2 and applied to nitrocellulose filters as described (19). For tropoelastin mRNA analysis, slots contained 0, 5.6, 11.25, 22.5 and 45 ng of adsorbed RNA. As an internal control of RNA loading, samples were further diluted, and 0, 0.18, 0.35, 0.7 and 1.4 ng of total RNA were adsorbed to nitrocellulose and probed for 28 S rRNA. Blots were dried at 80°C and hybridized under conditions described (19). Tropoelastin mRNA was detected with probe [^{32}P]T66, an exon-specific cDNA of the bovine elastin gene (20); 28 S rRNA was hybridized to a DNA probe provided by Dr. David Schlessinger, Washington University School of Medicine. DNAs were excised from plasmid, gel purified, and nick translated to high specific activity with [^{32}P] dCTP (NEN) as described (19). Relative RNA levels were determined by densitometric scanning of autoradiographs. Data were expressed in arbitrary units, and the slope of the line formed by plotting the densitometry value against nanograms RNA blotted was determined. Data are presented as the quotient of the elastin mRNA slope divided by the 28 S rRNA slope determined from the same set of dilutions.

Data Analysis

Data are presented as arithmetic means $\pm$ standard error of the mean. The Student's t-test was used for group comparison (two-tailed). Data were considered significantly different if $p \leq 0.05$.

RESULTS

IGF-I stimulated secretion of tropoelastin by pulmonary arterial SMC in a dose-dependent manner (Fig 1). Incubation of SMC with DMEM with 0.1% FBS alone (no IGF-I added) resulted in a basal production of 51.2 $\pm$ 3.4 ng tropoelastin per 10^5 cells. At 1 and 10 ng/ml IGF-I, no significant increase in elastin secretion was detected. However, at 100 ng/ml IGF-I, a significant increase in elastin secretion was noted (92.9 $\pm$ 4.6 ng/10^5 cells). A dose of 1000 ng/ml IGF-I resulted in a further increase of about 2.4-fold over basal to 120.3 ng/10^5 cells which was remarkably similar to the level (122.8 $\pm$ 8.2 ng/10^5 cells) secreted by cells incubated with DMEM containing 5% calf serum (Fig 1).

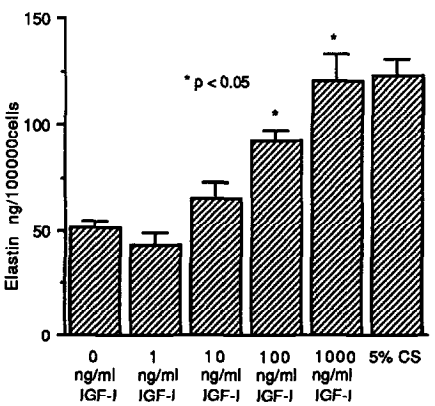

Fig. 1. IGF-I Stimulates Elastin Secretion by Neonatal PA SMC. IGF-I induced a dose-dependent increase in tropoelastin secretion by neonatal bovine PA SMC. Confluent cells were incubated for 48hr. with various concentrations of IGF-I in DMEM and 0.1% CS. At the maximal dose of IGF-I tested (1000ng/ml) elastin secretion was roughly equivalent to that induced by 5% CS. Secreted tropoelastin was measured by enzyme immunoassay.

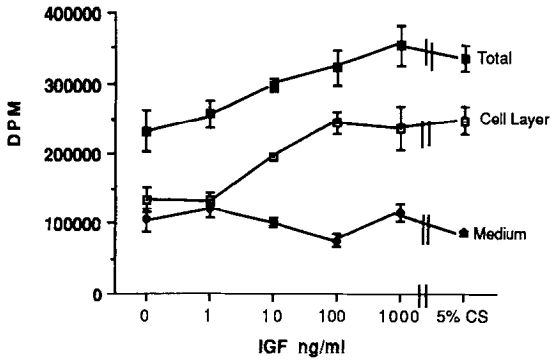

Fig 2. IGF-I Stimulates Total Protein Synthesis by PA SMC. IGF-I induced a dose-dependent stimulation of total protein synthesis by PA SMC. Cells exposed to IGF-I for 48 hr. were pulsed overnight with 3H-leucine, and counts measured in TCA-precipitated protein from the medium and cell layer extract. These were summated to give an approximation of total protein synthesis. Total protein synthesis at the highest dose of IGF-I (1000 ng/ml) was roughly equivalent to that induced by 5% CS.

Total protein synthesis, determined by incorporation of [^{3}H]-leucine, was also stimulated by IGF-I. Maximal stimulation of total protein synthesis occurred at a dose of 1000 ng/ml IGF-I, with a 52% increase over basal levels (Fig 2).

To assess the mechanism by which IGF-I regulated elastin synthesis by SMC, relative expression of tropoelastin mRNA was measured by slot blot analysis. IGF-I at doses of 1 and 10 ng/ml resulted in a 2-fold increase in steady-state tropoelastin mRNA levels relative to 28s mRNA, whereas doses of 100 and 1000 ng/ml stimulated tropoelastin mRNA 3- and 4-fold, respectively; and 5% CS resulted in a 3-fold increase in relative elastin mRNA expression (Fig 3).

DISCUSSION

The findings presented here demonstrate that IGF-I mediated a dose-dependent stimulation of tropoelastin synthesis and mRNA levels in neonatal pulmonary artery SMC. At the highest dose tested, 1000 ng/ml, tropoelastin synthesis increased 2.4-fold over basal levels. This stimulation of

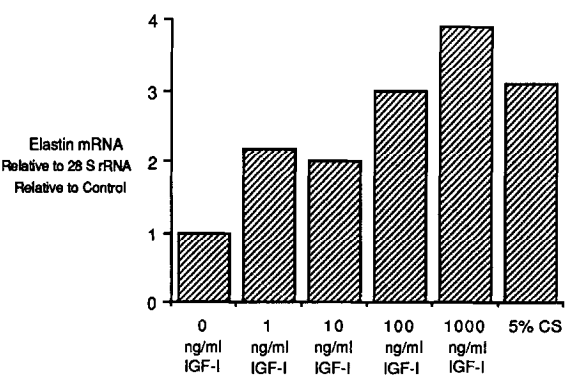

Fig. 3. IGF-I Stimulates Elastin mRNA Expression by PA SMC. Incubation of SMC with various concentraions of IGF-I for 48 hr. resulted in a dose-dependent stimulation in relative tropoelastin mRNA levels. At the highest dose of IGF-I tested (1000 ng/ml) this exceeded the stimulation caused by 5% CS. Data are normalized to 28S r RNA.

elastin synthesis was similar to that reported by Foster et al. (13) in chick embryonic aortae organ cultures and indicates that IGF-I is a potent stimulus for elastin synthesis by mammalian vascular SMC.

IGF-I-mediated upregulation of tropoelastin mRNA levels (Fig 3) was similar to the pattern seen for tropoelastin secretion (Fig 1). However, increased mRNA was detected at 1 and 10 ng/ml IGF-I, doses at which no stimulation of tropoelastin protein was seen, and the magnitude of the increase in mRNA levels was greater than that for protein at the higher doses used. These data suggest that IGF-I stimulation of elastin may be complex, involving both pre- and post-translational controls. For example, the data for all doses of IGF-I tested suggest that tropoelastin gene transcription was stimulated, but secreted protein levels increased to a lesser degree and at higher doses of IGF-I. These findings may indicate that the message was not translated efficiently or that the secretion of translated tropoelastin peptides was somehow hindered. Consistent with this hypothesis, IGF-I regulates the synthesis of both collagen and glycosaminoglycan at pre-and post-translation levels (13,21,22).

IGF-I has been shown to have numerous effects on various cells, including stimulation of amino acid transport, synthesis of DNA, RNA and protein, and incorporation of sulfate and proline into proteoglycan and collagen, respectively (23-25). The current study indicates that IGF-I stimulates both elastin and total protein synthesis. Foster et al. (13) found that 53% of total protein synthesis by IGF-I-stimulated embryonic chick aortae in organ culture was devoted to tropoelastin. Liu and Davidson (26) recently reported that TGF-*B* stimulated elastogenesis by porcine aortic SMC, and although total protein synthesis was not measured in that study, the authors speculate that this effect on matrix production was not purely selective for elastin expression. Similarly, the effect of IGF-I on elastin synthesis by pulmonary artery SMC may not be selective.

There is additional evidence to support a role of IGF-I in matrix remodeling associated with hypertensive arteries. Exposure to increased intraluminal pressure leads to medial hypertrophy in arteries, and increased deposition of matrix proteins by SMC is believed to be largely responsible for adaptation to vascular load (1). In support of a role for IGF-I in medial remodeling in hypertension, Hansson et al. (9) has shown that the localization of IGF-I by immunostaining is markedly increased in the arterial media after only 24 hr. of exposure to increased vascular load. Immunostaining of the media was further increased at 48 hr. This suggests that IGF-I is either locally produced within, or translocated to, the vascular media in response to hemodynamic stress.

In additon to the possibility that IGF-I is produced by vascular wall cells, peptide growth factors present in serum may have increased access to the vascular media under conditions leading to increased endothelial permeability (5,6). Since IGF-I is present in significant concentration in serum (up to 1 ug/ml), it may be able to diffuse through an altered endothelium in quantities sufficient to stimulate SMC proliferation and matrix protein production (e.g., elastin). Furthermore, IGF-I has a synergistic effect with PDGF, another potent growth factor present in serum, on SMC thymidine incorporation (27). The possibility that these growth factors may also have a synergistic effect on elastin synthesis has not been examined.

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REFERENCES

1. Folkow, B. (1982) Physiol Rev 62,347-504.
2. Stenmark, K.R., Fasules, J., Voelkel, N.F., Henson, J., Tucker, A., Wilson, H., and Reeves, J.T. (1987) J. Appl. Physiol. 62,821-830.
3. Orton, E.C., and Stenmark, K.R. (1988) J. Appl. Physiol. (In Press)
4. Libby, P., Warner, S.J.C., Solomon, R.N., and Birinyi, L.K. (1988) N. Engl. J. Med. 318,1493-1498.
5. Ross, R., Raines, E.W., Bowen-Pope, D.F. (1986) Cell 40,155-169.
6. Ross, R. (1985) J. Cardiovascular Pharmacology 7(Suppl. 3),5186-5190.
7. Liu, J. and Davidson, J.M. (1988) Biochem. Biophys. Res. Comm. 154,895-901.
8. Pfeifle, B., Ditschuneit H.H., Ditschuneit H. (1982) Horm. Metab. Res. 14,409-414.
9. Hansson, H.A., Jennische, E., Skottner, A. (1987) Acta Physiol. Scand. 129,165-169.
10. Clemmons, D.R. (1985) Circ. Res. 56,418-426.
11. Atkinson, P.R., Weidman, E.R., Bhanmick, B., and Bala, R.M. (1980) Endocrinology 106,2006-2012.
12. Lee, P.D.K., Hintz, R.L., Rosenfeld, R.G., Benitz, W. E. (1987) In Vitro Cell. Dev. Biol. 24,921-926.
13. Foster, J., Rich, C.B., and Florini, J.R. (1987) Collagen Rel. Res. 7,161-169.
14. Tsukada, T., Tippens, D., Gordon, D., Ross, R., and Gown, A. (1987) Am. J. Pathol. 126,51-60
15. Rosenfeld, R.G., Conover, C.A., Hodges, D., Lee, P.D.K., Mistra, P., Hintz, R.L., Li, Ch.H. (1987) Biochem. Biophys. Res. Comm. 143,199-205.
16. Hogenner, A., and Hambel, R.E. (1986) J. Biol. Chem. 261,569-575.
17. Prosser IW, Whitehouse LA, Mecham RP. Direct measurement of tropoelastin in cell culture medium by EIA: improvements using a highly specific polyclonal antibody preparation. Annalytical Biochem (Submitted).
18. Wrenn, D.S., Parks, W.C., Whitehouse, L.A., Crouch, E.C., Kucich, U., Rosenbloom, J., Mecham, R.P. (1987) J. Biol. Chem. 262,2244-2249.
19. Parks, W.C., Secrist, H., Wu, L.C., Mecham, R.P. (1988) J. Biol. Chem. 263,4416-4423.
20. Yeh, H., Ornstein-Goldstein, N., Indik, Z., Sheppard, P., Anderson, N., Rosenbloom, J.C., Cicila, G., Yoon, K., Rosenbloom, J. (1987) Collagen Rel. Res. 7,235-247.
21. Kemp, S.F., Hintz, R.L. (1980) Endocrinology 106,744-749.
22. Willis, D.H., Liberti, J.P. (1985) Biochem. Biophys. Acta 844,72-80.
23. Phillips, L.S., Vassilopoulon-Sellin, R. (1980) N. Engl. J. Med. 302,371-377.
24. Froesch, E.R., Schmid, C.R., Schwander, J., and Zapf, J. (1985) Ann. Rev. Physiol. 47,443-467.
25. Guenther, H.L., Guenther, H.E., Froesch, E.R., and Fleish, H. (1982) Experentia 38,979-980.
26. Liu, J., and Davidson, J.M. (1988) Biochem. Biophys. Res. Comm. 154,895-901.
27. Clemmons, D.R. (1985) Endocrinology 117,77-83.