Insulin and Insulin-like Growth Factor I Differentially Induce α_1-Adrenergic Receptor Subtype Expression in Rat Vascular Smooth Muscle Cells

Zhuo-Wei Hu, Xiao-You Shi, and Brian B. Hoffman

Department of Medicine, Stanford University School of Medicine and Geriatric Research, Education and Clinical Center, Veterans Affairs Palo Alto Health Care System, Palo Alto, California 94304

Abstract

Hyperinsulinemia has been implicated as an important risk factor for the development of accelerated cardiovascular disease. We wondered if insulin or IGF-I induced expression of α_1 adrenergic receptors in vascular smooth muscle cells (VSMCs) which could enhance smooth muscle contraction and cell growth activated by catecholamines. Rat aortic VSMCs were incubated with insulin or IGF-I for various times and expression of α_1 receptors was detected using [^{3}H]prazosin binding. Both insulin and IGF-I increased α_1 receptor number; also, these peptides increased expression of the α_{1D} receptor gene with no change in expression of the α_{1B} receptor gene as detected by RNase protection assays. Using Western blotting, we found that these peptides increased expression of the α_{1D} receptor subtype in these cells. Increased expression of the α_{1D} receptor mRNA was inhibited by the receptor tyrosine kinase inhibitor genistein and the PI 3-kinase inhibitor wortmannin but was not inhibited by protein kinase C inhibitor H7 or the L-type calcium channel blocker nifedipine. Preincubation of cells with insulin or IGF-I enhanced subsequent norepinephrine stimulation of mitogen activated kinase activity. These results suggest that insulin/IGF-I regulate expression of α_1 receptors in VSMCs and potentially enhance the effects of catecholamines in settings of hyperinsulinemia. (*J. Clin. Invest.* 1996. 98:1826–1834.) Key words: atherosclerosis • gene expression • hyperinsulinemia • vascular biology

Introduction

There has been growing interest in the hypothesis that insulin resistance and compensatory hyperinsulinemia may contribute to increased blood pressure and accelerated atherosclerosis associated with type II diabetes mellitus or otherwise normal individuals (for reviews, see references 1, 2). Hypertension and other metabolic abnormalities associated with insulin resistance and hyperinsulinemia may function as separate risk factors for the development of accelerated cardiovascular disease (3). However, it is uncertain how hyperinsulinemia might contribute to pathogenesis of hypertension. It has been suggested that hyperinsulinemia may promote sodium reabsorption from the kidney, activate the sympathetic nervous system, and promote increases in $[Ca^{++}]_i$ (4, 5). Another possibility is that hyperinsulinemia may result in increased sensitivity of resistance vessels to vasoconstrictors such as angiotensin II, endothelin, or catecholamines (6–8). Additionally, insulin or insulin-like growth factors stimulate, either directly or indirectly, vascular smooth muscle cell (VSMC)[1] growth (9).

α_1 Adrenergic receptors (AR) are a member of family of G protein coupled membrane receptors. Activation of α_1 receptors is a very important modulator of blood pressure, promoting vascular smooth muscle contraction (10, 11). Increasing evidence suggests that activation of α_1 adrenergic receptors also regulates cardiovascular growth and hypertrophy, both in the heart and peripheral circulation. For example, overexpression of α_1 receptors in the hearts of transgenic mice leads to hypertrophy (12); activation of α_1 receptors in myocytes leads to induction of growth-related gene expression which promotes DNA and protein synthesis (13). In addition, activation of α_1 receptors also stimulates protein synthesis, DNA synthesis, and expression of growth-related proto-oncogenes in vascular smooth muscle cells (14, 15). Tyrosine protein kinase and mitogen-activated protein kinase signaling pathways may be utilized to mediate α_1 receptor stimulation of mitogenesis in myocytes (16, 17) and vascular smooth muscle cells (18).

Interaction between insulin and the sympathetic nervous system (SNS) has been recognized for many years (19). For example, there is evidence suggesting that insulin and the sympathoadrenal system are involved in the pathogenesis of hypertension in obese people (20). Sympathetically mediated stimulation of the heart, vasculature, and kidney contributes to the development of hypertension. Therapeutic strategies aimed at diminishing insulin resistance and lowering insulin concentrations, and diminishing the effects of sympathetic stimulation on target organs, have a good pathophysiological foundation.

In the present study, we asked if insulin and IGF-I modulated expression of α_1 adrenergic receptors in cultured smooth muscle cells. Additionally, the possibility that changes in expression of α_1 receptors potentiated catecholamine-stimulated mitogenic pathways was examined. The results suggest that insulin and IGF-I increase expression of α_1 receptors via a selective activation of the α_{1D} receptor subtype gene. Also, insulin and IGF-I enhance α_1 receptor–mediated mitogenic responses such as activation of mitogen-activated protein (MAP) kinase in rat vascular smooth muscle cells.

Address correspondence to Zhuo-Wei Hu, VA Medical Center, GRECC 182B, 3801 Miranda Avenue, Palo Alto, CA 94304. Phone: 415-858-3933; FAX: 415-855-9437.

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1. *Abbreviations used in this paper:* AR, adrenergic receptor; MAP, mitogen-activated protein kinase; MBP, myelin basic protein; PI3, phosphatidylinositol 3; VSMC, vascular smooth muscle cell.

Methods

Materials. [^{32}P]dCTP (2,000 Ci/mmol), [^{32}P]UTP (3,000 Ci/mmol), Hybond nylon filters, and random primer labeling system were purchased from Amersham Corp.(Arlington, IL); nitrocellulose membranes from Schleicher & Schuell (Keene, NH); DNA molecular markers (ØX174 DNA/HinfI), RNase A, single strand synthesis kit from Promega Corp.(Madison, WI); RNase T1, proteinase K, and actinomycin D from Boehringer Mannheim Biochemicals (Indianapolis, IN); RNase-free DNase I from United States Biochemical (Cleveland, OH); restriction enzymes, T4 DNA ligase from New England Biolabs (Beverly, MA); [^{3}H]prazosin (19.8 Ci/mmol) from New England Nuclear (Boston, MA); T3 and T7 RNA polymerases, RNase protection assay kit from Stratagene, Inc. (La Jolla, CA); cDNAs of rat α_{1B}- and α_{1D}-AR were a gift of Dr. J. Lomasney (Northwestern University); antibodies against the α_{1D}-AR were a gift of Dr. R.J. Lefkowitz (Duke University); RNase T2, cell culture medium, insulin and recombinant human IGF-I, Optim-MEM medium, lipofectamine and newborn bovine serum from Gibco/BRL (Grand Island, NY); collagenase (CLS-IV) from Worthington Biochemical Co. (Freehold, NJ); elastase (EC134) from Elastin (Pacific, MO); soybean trypsin inhibitor (type I, S, T-9003) from Sigma Chemical Co. (St. Louis, MO); bovine serum albumin (fraction V, fatty-acid free) from Miles Scientific (Naperville, IL). Anti-p44^{ERK1}, myelin basic protein (MBP) and protein A/G plus-agarose purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were reagent or molecular biology grade and were obtained from standard commercial sources.

Preparation of cultured rat aortic smooth muscle cells. Vascular smooth muscle cells were isolated from the thoracic aortas of 200–250 gram male Sprague-Dawley rats by enzymatic dissociation in Hanks' Balanced Salt solution containing 1 mg/ml collagenase, 0.1 mg/ml elastase, 0.5 mg/ml soybean trypsin inhibitor, 1 mg/ml bovine serum albumin as previously described (15). Cells were grown in DMEM containing 100 U/ml penicillin, 100 mg/ml streptomycin, L-glutamine (2 mM), and 10% (vol/vol) heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO_2-95% air. The cells were harvested for passaging at confluence with trypsin-EDTA and plated in 100-mm dishes at a density about 5×10^5, with a 80–90% confluence being reached about 7 d later. The medium was replaced every 2 d. Cells were generally used for studies at 8–10 d after seeding. Throughout the course of these experiments, cells from third through seventh passage were used. The cells were treated with insulin/IGF-I or vehicle solution (as control) starting from the longest time point and the cells were harvested at the same time.

Measurement of α_1-receptor number. [^{3}H]Prazosin was used to measure number of α_1 receptors as previously described (15, 21). For binding assays and Western blotting, the cultured cells were gently detached with a rubber policeman, pooled ($3–5 \times 10^7$ cells), centrifuged at 200 *g* for 5 min at 4°C, and washed twice in ice-cold PBS. The cells were suspended in 10 mM Tris-HCl (pH 7.5), 0.25 M sucrose and homogenized with a Polytron homogenizer with setting 7 for 2×5 seconds, and setting 10 for 4×5 seconds. The supernatants obtained by centrifugation of homogenates for 5 min at 300 *g* were then centrifuged for 15 min at 45,000 *g*; the pellets (crude membrane extracts) were washed twice with 50 mM Tris-HCl (pH 7.5) and resuspended in the same buffer. Total binding was determined by incubating 100 μl of membrane suspension (100–200 μg protein) with 0.05–8 nM of [^{3}H]prazosin in a 0.5-ml volume of 50 mM Tris-HCl buffer containing 0.1% BSA. Nonspecific binding was determined in the presence of 10 μM phentolamine. After 60-min incubation at 25°C, 2 ml of ice-cold buffer was added into each tube and immediately filtered through a Whatmann GF/B fiberglass filter under vacuum suction and the filters were washed three times with 5 ml of ice-cold buffer. The retained radioactivity was measured using a liquid scintillation counter. Protein concentrations were determined using a protein assay kit (Bio-Rad Laboratories, Richmond, CA). Receptor affinity (K_D) and maximum binding sites of receptor (B_{max}) for [^{3}H]prazosin were calculated from Scatchard plots.

Immunoprecipitation and immunodetection. Cell crude membrane pellets prepared as described above were incubated with lysis buffer (1% NP-40, 25 mm Hepes [pH 7.5], 50 mm NaCl, 50 mM NaF, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml of antipain, aprotinin, and leupeptin) for 30 min on the ice. Insoluble material was removed by centrifugation at 12,100 *g* for 20 min. Membrane extracts were normalized by protein content in each experiment. The membrane extract (2 mg of protein for VSMCs and 3 mg of protein for COS-7 cells) was incubated with an appropriate antibody for 4 h and then further incubated with 25 μl of protein A/G plus-agarose for 2 h. For immunodetection, immunoprecipitates were washed four times with lysis buffer and twice with distilled water, and analyzed by SDS-PAGE. Resolved proteins were transferred to membrane and detected using the ECL Western Blotting Detection System with the α_{1D}-AR antibody and a horseradish peroxidase conjugated secondary antibody.

In vitro assay of MAP kinase activity. Assay of MAP kinase activity was performed following a method described previously (22). To determine MAP kinase activity, cells were incubated in the absence of serum 12 h and then incubated serum-free DME with or without insulin or IGF-I for another 12 h. Medium was changed with serum-free DME for 12 h and the cells stimulated with norepinephrine and other agonists for 10 min. The cells were lysed in 0.4 ml of lysis buffer. After 30-min centrifugation at 4°C, cell lysate (400 μg of protein) was immunoprecipitated with anti-p44^{ERK1} (2 μg/mg protein) and washed four times with lysis buffer and once with kinase buffer. The washed immuocomplexes were resuspended in 40 μl of kinase buffer (25 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM EGTA, 40 μM ATP, 1 μCi of [γ^{32}P]ATP, and myelin basic protein (MBP) (1 mg/ml) as a substrate. The reaction mixture was incubated for 10 min at 30°C. Preliminary experiments suggested that the phosphorylation of MBP is linear for 20–30 min. The reaction was stopped by spotting 10 μl of reaction mixture onto p-81 phosphocellulose paper (Whatman Labsales, Hillsboro, OR) which was then washed in 75 mM phosphoric acid for 1 h and transferred to another washing overnight. The papers washed with acetone for 5 min and dried. ^{32}P was quantitated by scintillation counting. Alternatively, reaction mixtures were loaded on 14% SDS-PAGE and the dried gels were exposed to XAR-5 film (Eastman Kodak Co., Rochester, NY) at −70°C with an intensifying screen for 8–16 h or was visualized after development with a PhosphorImager System (Molecular Dynamics, Sunnyvale, CA).

Total RNA preparation. Isolation of total RNA from the cultured smooth muscle cells was performed as described previously by Hu et al. (15, 21). Briefly, the cultured smooth muscle cells were rinsed with cold calcium-magnesium free phosphate buffered saline and then the cells were homogenized with a Polytron in 10 vol of denaturing buffer containing 4 M guanidinium thiocyanate, 25 mM sodium citrate (pH 7), 0.5% sarcosyl, 0.1 M 2-mercaptoethanol. 1 vol of 2 M sodium acetate (pH 4.0), 10 vol of water-saturated phenol, and 2 vol of chloroformisoamyl alcohol (49:1) were sequentially added to the homogenate with thorough mixing after addition of each reagent. The homogenate were incubated on ice for 20 min and centrifuged at 12,000 *g* for 20 min. The aqueous phase was taken and RNA was precipitated from it with isopropanol (1:1 vol). The resulting RNA pellet was dissolved in the denaturing buffer and again precipitated with isopropanol by cooling and centrifugation. The RNA pellet was washed with 75% ethanol, sedimented, vacuum dried and dissolved in TE buffer or autoclaved water to be used in experiments.

Analysis of abundance of α_1 receptor subtype mRNAs by RNase protection assays. There are three α_1 receptor subtypes expressed in rat aortic smooth muscle cells (15). α_{1D} receptors appear to predominantly mediate contraction of rat smooth muscle (23). To determine if insulin or IGF-I regulated expression of subtypes of α_1-AR in cultured VSMCs, sensitive RNase protection assay was used for detection of α_{1A}, α_{1B}, and α_{1D} subtypes. The antisence probes for RNase protection assay were made as described previously (15). Antisense probe of rat β-actin was made as described previously by PCR and

used as an internal control (15). We have confirmed in our previous studies that the level of expression of the α_{1A} receptor mRNA was too low to be quantitated accurately so experiments with this probe were not pursued. The α_{1B}- or α_{1D}-RNA probes (5×10^5 cpm) with the β-actin RNA probe ($1–2 \times 10^4$ cpm) as internal control and 50 μg of total RNA, either from control or insulin/IGF-I-treated cells, were coprecipitated in a 1.5-ml microcentrifuge tube and the pellet rinsed with 100% ethanol to facilitate complete supernatant removal. The pellet was thoroughly resuspended in hybridization buffer (80% formamide, 0.4 M NaCl, 50 mM Pipes, and 1 mM EDTA) and hybridized for 36 h at 60°C for α_{1B} probe and at 55°C for α_{1D} probe. 250 μl of ice-cold RNase buffer (50 mM sodium acetate, pH 4.4; 100 mM NaCl; 10 mM EDTA) containing 30 U/ml RNase T2 was added to each assay tube and then incubated for 60 min at 30°C. The reaction was stopped by the addition of an equal volume (280 μl) of 4 M guanidinium thiocyanate solution. After mixing guanidinium thiocyanate solution with the digested mixtures, 3 μl of 10 μg/μl carrier RNA were added. RNase-resistant hybrids were precipitated by adding an equal volume of isopropanol (560 μl) followed by a 15-min centrifugation in a microcentrifuge at top speed (13,000 *g*). Resulted pellets were rinsed with 100% ethanol, suspended directly in 10 μl of loading buffer (80% formamide, 10 mM EDTA, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol), and run on a 6% polyacrylamide, 8 M urea gel. Preliminary experiments demonstrated that under these conditions of hybridization and RNase digestion, the values of α_{1B}, α_{1D}, and β-actin mRNAs on autoradiograms were linear with the amounts of added RNA at least in the range of 5–100 μg. The size of fragments protected was estimated from positions of DNA molecular markers which were 5′-end labeled with [γ^{32}P]ATP and run in the same gel.

Nuclear runoff transcription assays. To determine if insulin or IGF-I changed the transcription rate of the α_{1D}-AR gene, nuclear runoff transcription assays were performed as described previously (15). The cells from two 100-mm dishes were used for isolation of the nuclei. The resulting pelleted nuclei (1×10^7) was resuspended in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 mM EDTA, and 20% glycerol at a concentration of 2×10^5 nuclei/μl. The prepared nuclei (200 μl) were added to 200 μl of reaction buffer composed of 10 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.3 M KCl, 5 mM DTT, 10 mM unlabeled GTP, ATP, CTP, and 10 μl of [α-^{32}P]UTP (3,000 Ci/mmol), incubated for 30 min at 30°C. After RNase-free DNase I and proteinase K treatments, the reaction products were extracted with phenol/chloroform and unincorporated [^{32}P]UTP was removed by trichloroacetic acid precipitation and filtration through a nitrocellulose filter. The radiolabeled RNA was treated again with DNase I and proteinase K, extracted with phenol/chloroform, and precipitated with 75% ethanol. This labeled RNA (3×10^7 cpm) was dissolved in 1 ml of hybridization solution (same as described above) and hybridized at 42°C for 36 h with 20 μg of the M13 plasmid with coding-strand of α_{1D} (329 bp) immobilized to a nylon filter or with immobilized plasmid M13 containing noncoding strand of α_{1D} or β-actin cDNA as control. The filters were washed with 0.5 × SSPE and 0.1% SDS at 65°C for 30 min and then treated with 2.5 μg/ml of RNase A and 5 U/ml of RNase T1 at 37°C for 30 min. The film was scanned with laser densitometry and quantified by calculation of ratio of α_{1D}-AR cDNA signal to β-actin cDNA signal.

Transient transfection of COS cells with α_1 receptor subtype expression vectors. To confirm that the antibody directed against the α_{1D}-AR, obtained from Dr. R.J. Lefkowitz's laboratory, worked in our hands, COS-7 cells were transfected with the rat α_{1D} receptor construct and expression of this subtype was detected by Western blotting. COS-7 cells were cultured in DME with 10% fetal bovine serum at 5% CO_2 and 37°C and transfected at ~ 80% confluence. Transfection was performed in 3.0 ml of Optim-MEM containing 50 μg of lipofectamine and 10 μg of expression vector containing α_{1D}-AR cDNA. 5 h later, 3 ml of DME with 20% FBS was added. 24 hours from the start of transfection, the cells were washed and fresh DME with 10% FBS was replaced; the cells were harvested for immunoblotting on the next day.

Data analysis. Data are presented as mean±SEM, and treatment effects were compared by one-way analysis of variance or Student's paired *t* test (two-tailed). $P < 0.05$ was taken as level of significance.

Results

To test the hypothesis that insulin or IGF-I enhanced expression of α_1 receptors in VSMCs, cells were treated with 100 nM insulin or 100 ng/ml (13 nM) of IGF-I for 1–24 h and mem-

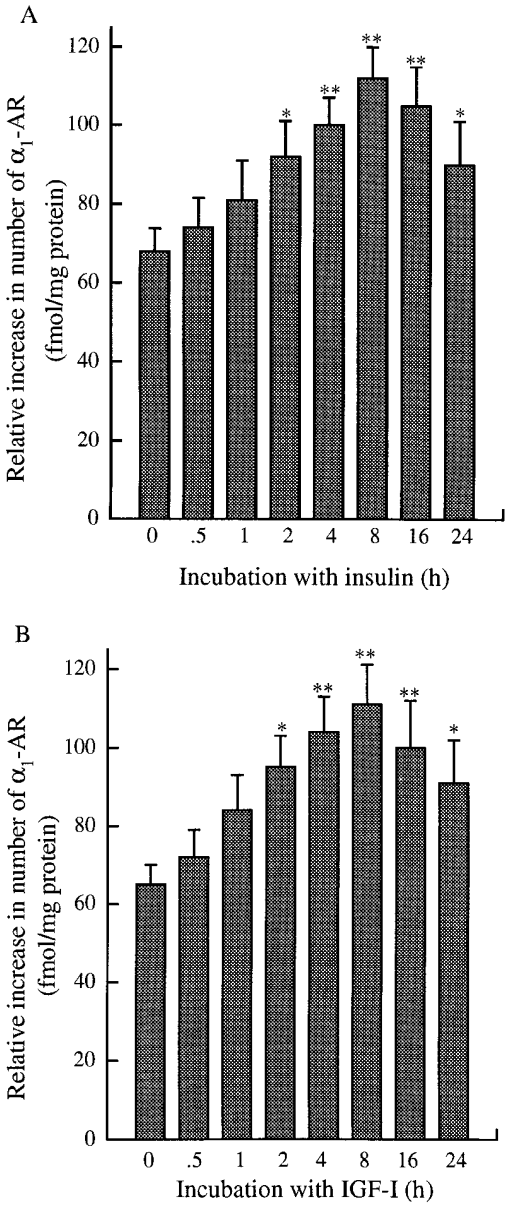

Figure 1. Effects of insulin and IGF-I on expression of α_1-adrenergic receptors. Rat vascular smooth muscle cells were grown to near confluence in a series of 100-cm^2 dishes. The cells were incubated with insulin (100 nM) (*A*) or IGF-I (80 ng/ml) (10 nM) (*B*) for the times indicated. Cell membranes were prepared and the binding assays using [^{3}H]prazosin were performed as described in Methods. There were no significant changes in binding affinity (K_D) of α_1 receptors for [^{3}H]prazosin in these cells (data not shown). The data are average±SEM of three experiments. *Compared to control, $P < 0.05$; **$P < 0.01$.

brane preparations from these cells was isolated and binding assays of [^{3}H]prazosin performed. As illustrated in Fig. 1, *A* and *B*, incubation with insulin or IGF-I resulted in increased numbers of α_1-receptors with maximal values detected between 4–24 h. Interestingly, in the later time points, expression of α_1 receptors gradually returned to basal values. This could relate to desensitization of IGF-I receptors after prolonged exposure of cells to these growth factors. Degradation of IGF-I in the culture dish is much less likely since the response to IGF-1 actually decreased at a high concentration. However, these experiments demonstrated that insulin and IGF-I significantly enhanced expression of α_1-receptors in VSMCs.

To evaluate if the increased expression of α_1 receptors induced by insulin and IGF-I potentiates catecholamine stimulated-mitogenic effects in rat vascular smooth muscle cells, norepinephrine stimulation of MAP kinase activity was per-

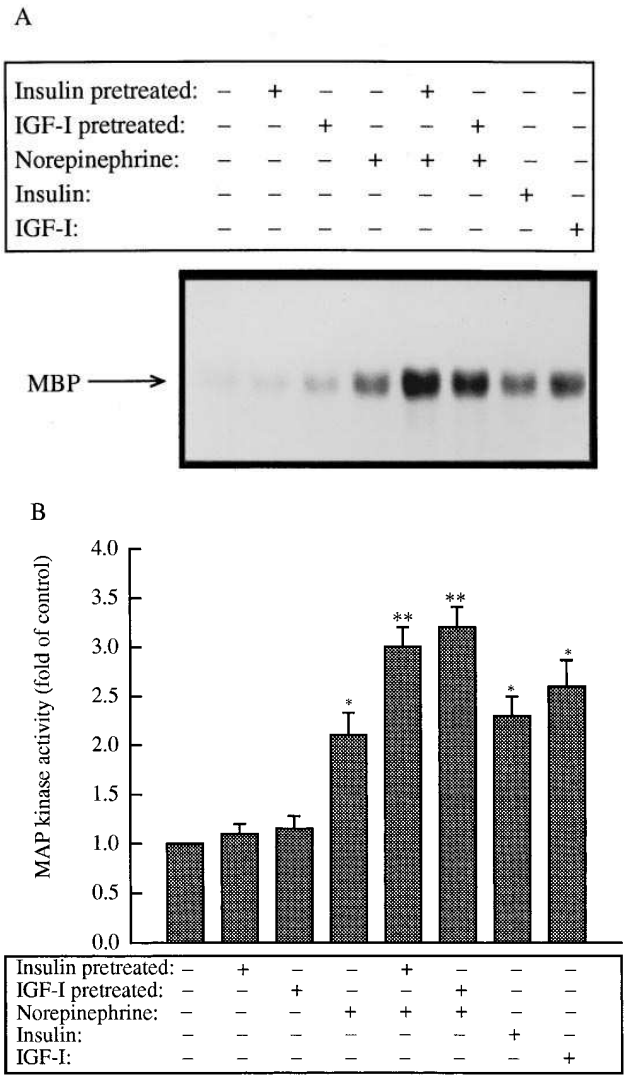

Figure 2. Preincubation of vascular smooth muscle cells with insulin or IGF-I enhances norepinephrine-stimulated MAP kinase activity. Smooth muscle cells were grown to near confluence and incubated with serum-free DME for 12 h. Cells were then incubated with either insulin (100 nM), IGF-I (80 ng/ml) (10 nM), or vehicle for 12 h in the serum-free DME. Medium was changed to serum-free DME for additional 12 h and treated with or without norepinephrine (10 μM), insulin (100 nM), or IGF-I (80 ng/ml) (10 nM) in the presence of a β-adrenergic receptor antagonist timolol (1 μM) and an α_2 adrenergic receptor antagonist idazoxan (1 μM) for 10 min. Cell lysates (400 mg of protein) were prepared and subjective to immunoprecipitation with an anti-p44^{ERK1} antibody. After intensively washing, in vitro MAP kinase activity in the immunocomplexes was performed as described under Methods. (*A*) Representative of three experiments. (*B*) Average±SEM of three experiments. * Compared to control, $P < 0.05$; ** compared to norepinephrine stimulation, $P < 0.05$.

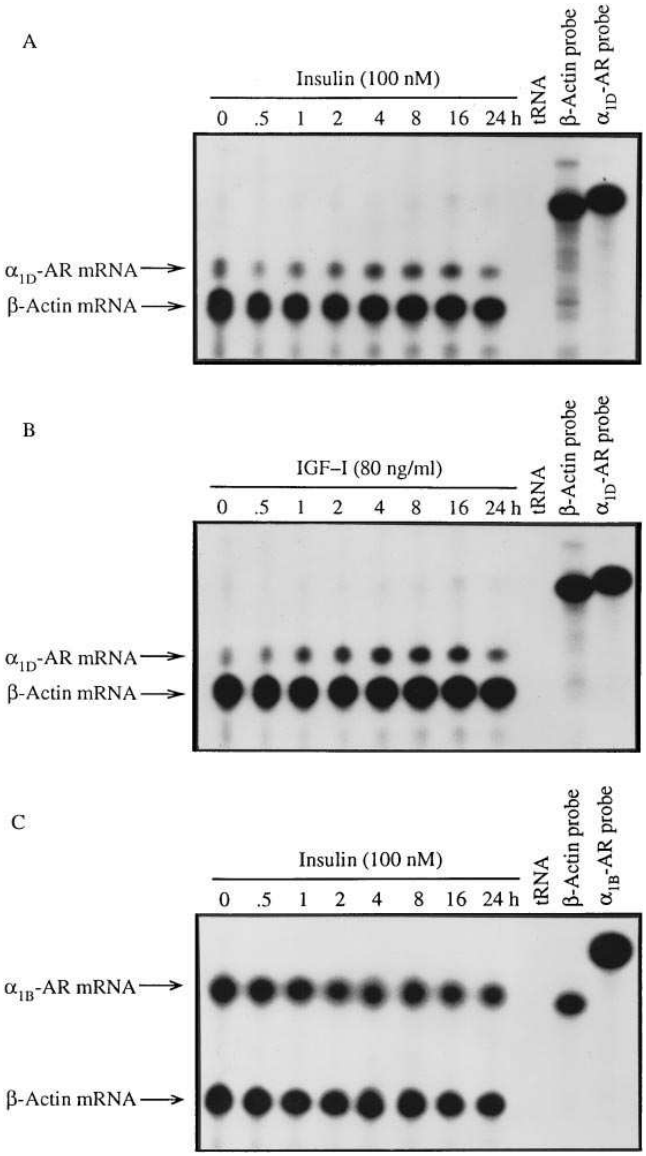

Figure 3. Insulin or IGF-I-induced a time-dependent increases in accumulation of α_{1D}-adrenergic receptor mRNAs. Total RNAs were isolated from the VSMCs that had been incubated either in the absence or in the presence of 100 nM insulin or 80 ng/ml (10 nM) of IGF-I for the indicated times. 50 μg RNA was coprecipitated and hybridized to 5×10^5 cpm of riboprobes complementary to α_{1D}-AR mRNA (*A* and *B*) or α_{1B}-AR mRNA (*C*) and 2×10^4 cpm of cRNA probes complementary to β-actin mRNA. The hybridization was performed at 60°C for α_{1B} or 55°C for α_{1D} for 36 h. RNase digestion and recovery of RNase-resistant hybrids were conducted as outlined in the section of Methods. β-Actin mRNA was used as an internal control. The exposure times of the autoradiograms in *A* and *B* were 16 h and *C* was 12 h. Experiments were repeated at least three times with similar results.

formed in cells pretreated with or without insulin or IGF-I. VSMCs were pretreated with insulin (100 nM) or IGFI (80 ng/ml) (10 nM) for 12 h and then medium was replaced by a fresh medium for 12 h. Cells were then stimulated with norepinephrine (1 μM) for 10 min which led to a 2.3±0.1-fold increase in MAP kinase activity in VSMCs. Pretreatment of cells with insulin or IGF-I significantly enhanced norepinephrine stimulation of MAP kinase activity (3.1±0.2-fold for insulin pretreatment and 3.0±0.2-fold for IGF-I pretreatment, respectively), indicating that insulin and IGF-I potentiates catecholamine-induced mitogenic responses (Fig. 2, *A* and *B*).

To investigate the mechanisms for enhanced expression of α_1 receptors, we determined if the accumulation of α_{1B} or α_{1D}-receptor mRNAs was changed by insulin or IGF-I. Total RNAs were isolated from controls or cells incubated with insulin (100 nM) or IGF-I (80 ng/ml) (10 nM) for the indicated times to examine expression of α_{1B}- or α_{1D}-receptor mRNAs by RNase protection assays as described in Methods. Cultured VSMCs expressed both α_{1B} and α_{1D}-AR subtype mRNAs; insulin or IGF-I caused a marked increase in accumulation of α_{1D} receptor mRNAs (Fig. 3, *A* and *B*). The time course of the induction of α_{1D}-receptor mRNAs by insulin and IGF-I revealed that an increase in the α_{1D}-receptor mRNAs could be detected as early as 0.5 h (1.3±0.2-fold of control, $P < 0.05$) and the maximum expression occurred by 2 h (3.2±0.3-fold of control, $P < 0.01$). At 24 h of insulin or IGF-I treatment, the values of α_{1D}-receptor mRNAs remained higher than the controls (Fig. 3, *A* and *B*). However, neither insulin (Fig. 3 *C*) nor IGF-I (data not shown) changed expression of α_{1B}-AR subtype mRNAs over 24 h, suggesting that insulin and IGF-I differentially induces expression of α_{1D} receptor subtype mRNAs in rat aortic smooth muscle cells.

The dose–response relationships for insulin and IGF-I-stimulation of accumulation of α_{1D} mRNAs are shown in Fig. 4. In-

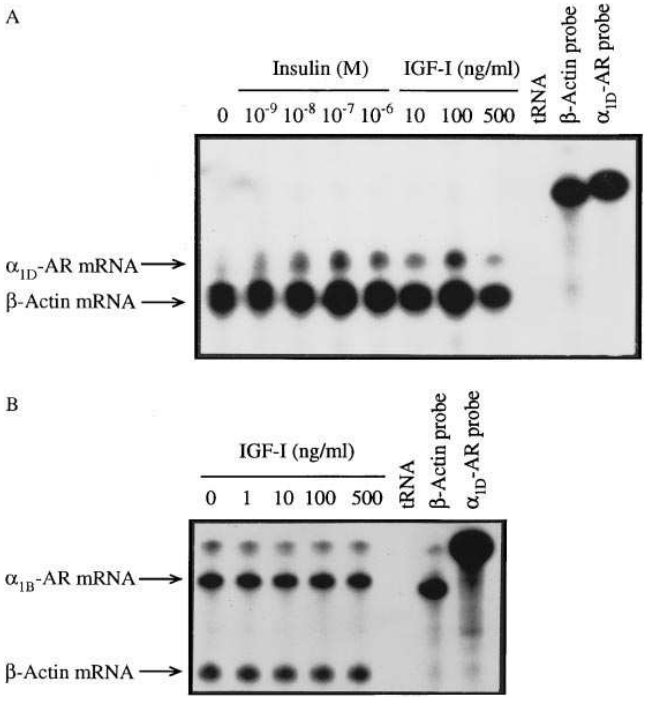

Figure 4. Dose-response relation of insulin or IGF-I–induced increases in the α_{1D} receptor mRNAs. Quiescent vascular smooth muscle cells were treated with indicated concentrations of insulin or IGF-I for 3 h. Total RNA was isolated and 50 μg of total RNAs from either control or insulin/IGF-I–treated cells was coprecipitated and hybridized to 5×10^5 cpm of riboprobes complementary to α_{1D}-AR mRNA (*A*) or α_{1B}-AR mRNA (*B*) and 2×10^4 cpm of cRNA probes complementary to β-actin mRNA. RNase protection analysis was performed as described under Methods. The data are representative of three experiments.

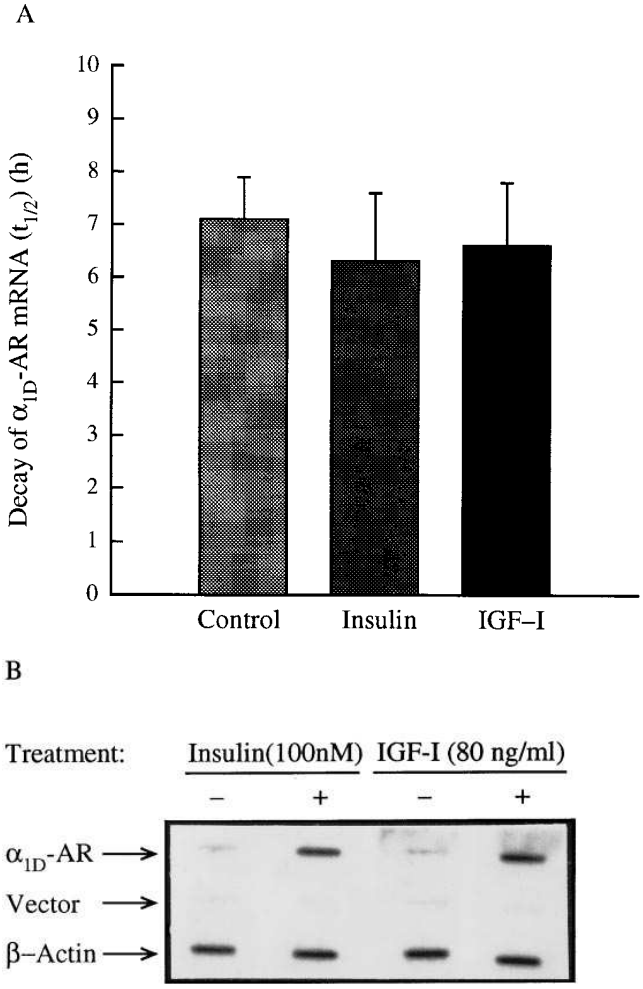

Figure 5. Insulin and IGF-I induction of α_{1D}-adrenergic receptor gene expression by increase in transcription rate of the gene. (*A*) Insulin or IGF-I treatment does not change stability of the α_{1D} receptor mRNAs. Cells were preincubated with 5 μg/ml actinomycin D for 3 h to block transcription and then the cells were treated with vehicle (control) or 100 nM of insulin or 80 ng/ml (10 nM) of IGF-I for different times as indicated. The total RNAs were isolated and RNase assays were performed as described under Methods. The data are the average±SEM of three experiments. (*B*) VSMCs grown in DME were exposed to insulin (100 nM) or IGF-I (80 ng/ml) (10 nM) for 2 h. The cells were harvested and nuclei prepared as outlined under Methods. Transcript elongation was allowed to continue in the presence of [^{32}P]UTP and unlabeled nucleotide. After elongation, equal amounts of radiolabeled RNA were hybridized either to plasmids (20 μg/slot) harboring coding-strand of rat α_{1D}-adrenergic receptor cDNA, β-actin cDNA, or to vector plasmids as control. All of the plasmids were linearized before immobilization to the nylon membrane. The exposure time of film is 24 h with an intensive screen. Data are a representative of three experiments.

creased expression of α_{1D}-AR mRNAs by insulin could be detected at an insulin concentration as low as 10^{-9} M; maximum induction of expression of α_{1D}-AR mRNAs occurred with an insulin concentration of 10^{-7} M (3.0±0.2-fold, $P < 0.01$). The dose-response effect of IGF-I on α_{1D} mRNA expression demonstrated effects at a concentration of 10 ng/ml (1.3 nM). The maximum induction of expression of α_{1D}-AR mRNAs occurred with IGF-I concentration of 100 ng/ml (13 nM); further increases in concentration of IGF-I resulted in a decreased expression of α_{1D} mRNA (Fig. 4, lane *8*), possibly reflecting desensitization of IGF-I receptors. Fig. 4 *B* demonstrates that IGF-I did not induce α_{1B}-AR mRNAs at these concentrations of IGF-I.

To determine the mechanism for increased abundance of the α_{1D} receptor mRNA, the stability of the α_{1D}-receptor mRNAs in the presence of transcriptional inhibitor actinomycin D (5 μg/ml) was compared in control and in insulin/IGF-I–treated cells (Fig. 5 *A*). Insulin/IGFI treatment did not significantly change the degradation rate of the α_{1D}-AR mRNAs; the half-life of decay of α_{1D}-AR mRNAs was ~ 7.1±0.8 h in control cells, 6.3±1.3 h in insulin treated-, or 6.6±1.2 in IGF-I–treated VSMCs. After incubation of cells with actinomycin D for 2 h, insulin or IGF-I did not increase accumulation of α_{1D}-AR mRNAs. Together, these results suggest that the enhanced accumulation of α_{1D}-AR mRNAs in the presence of insulin or IGFI is not due to increased stability of the α_{1D}-AR mRNAs. We then examined the transcription rate of the α_{1D}-AR gene in control and insulin or IGF-I-treated VSMCs using nuclear runoff assays. These experiments demonstrated that insulin (100 nM) or IGF-I (80 ng/ml) (10 nM) led to a marked elevation in transcription rate of the α_{1D}-AR gene. The transcription rate of the α_{1D}-AR gene, calculated from the ratio of transcription rate of α_{1D}-AR gene to that of β-actin gene, was increased by 3.7±0.7-fold ($P < 0.01$) and 3.4±0.5-fold ($P < 0.05$) of control in nuclei from cells treated with insulin or IGF-I, respectively (Fig. 5 *B*).

To evaluate the signaling pathways by which insulin or IGF-I activates expression of α_{1D} receptor mRNAs, VSMCs were pretreated with genistein, an inhibitor of tyrosine protein kinase. Insulin-induced accumulation of α_{1D}-AR mRNAs was blocked after pretreatment of cells with genistein (1 μM) for 1 h (Fig. 6), suggesting that insulin-stimulated expression of α_{1D} receptor gene requires a tyrosine kinase-induced phosphorylation. Activation of protein kinase C and raising intracellular calcium may also mediate biologic responses induced by insulin or IGF-I; a protein kinase C (PKC) inhibitor, H7 (1 μM), and L-type calcium blocker nifedipine (1 μM) did not inhibit insulin-induced increase in expression of α_{1D} mRNA (Fig. 6, lanes *4* and *6*). Recent studies suggest that phosphatidylinositol 3 (PI 3)-kinase plays an important role in signalling

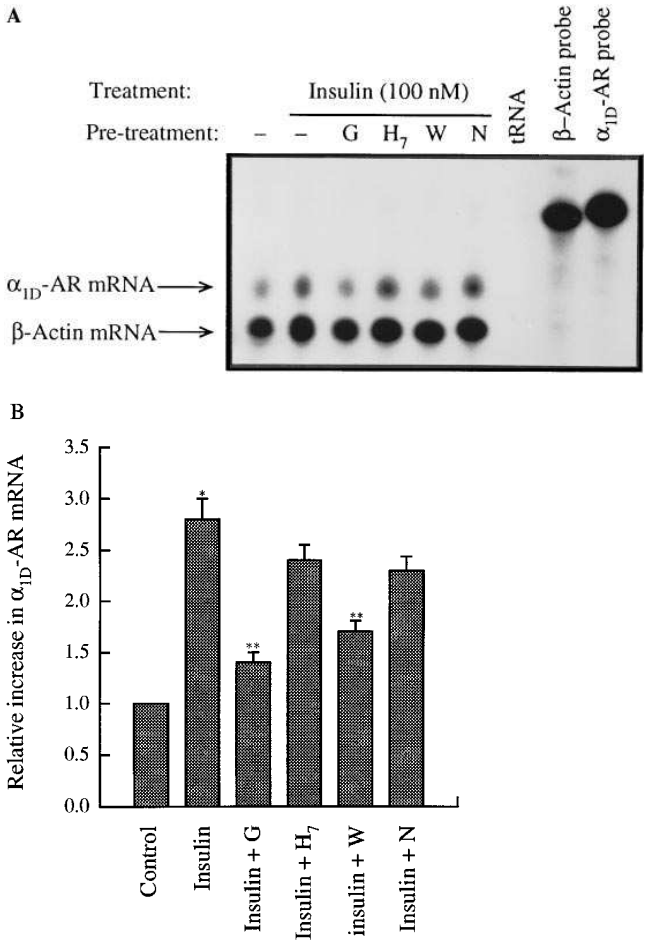

Figure 6. Induction of α_{1D} receptor gene expression by insulin can be inhibited by antagonists of tyrosine protein kinase. (*A*) Total RNAs were prepared from vascular smooth muscle cells incubated under control conditions (lane *1*) or with insulin (100 nM) (lane *2*), insulin with pretreatment of genistein (10 μM) (lane *G*), or insulin plus H7 (10 μM) (lane *H7*), insulin plus wortmannin (10 nM) (lane *W*), and insulin plus nifedipine (10 μM) (lane *N*) for 3 h. Inhibitors were added before 2 h of treatment. RNase protection analysis of these RNAs was carried out using ^{32}P-labeled cRNA probes of α_{1D} adrenergic receptor or β-actin as outlined under Methods. Data are representative of three experiments. (*B*) is average±SEM of three experiments. *Compared to control, $P < 0.05$; **compared to insulin treatment, $P < 0.05$.

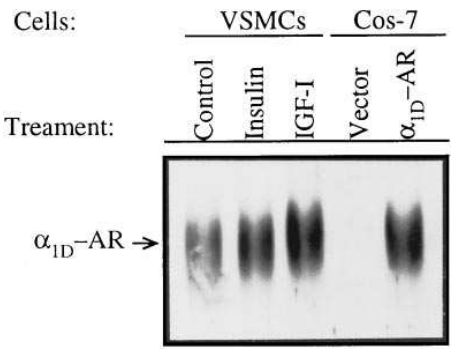

Figure 7. Immunodetection of α_1 adrenergic receptor subtype expression with anti–α_{1D}-AR peptide antibodies. Near confluent rat VSMCs were incubated in serum-free DME for 24 h and then treated with or without insulin (100 nM) or IGF-I (80 ng/ml) (10 nM) for 12 h. Cell membrane extracts were prepared from control, insulin/IGF-I-treated VSMCs or from COS-7 cells transiently transfected with control vectors (*vectors*) or rat α_{1D}-AR expression vectors (α_{1D}-*AR*). The membrane extracts (2 mg protein for VSMCs and 3 mg protein for COS-7 cells) were immunoprecipitated by an antibody against α_{1D}-AR. Immunoprecipitates were subjected to SDS-PAGE, electrotransferred to PDV membrane, immunodetected with anti–α_{1D}-AR antibody and visualized by ECL Western Blotting detecting system as indicated under Methods. Positions of α_{1D}-AR are indicated. Experiments were repeated trice with similar results.

pathways of insulin (24). The PI 3-kinase inhibitor wortmannin was used to determine possible effect of PI 3 -kinase in regulation of α_{1D}-AR mRNA expression. Pretreatment of cells with wortmannin (10 nM) for 1 h almost completely inhibited insulin-induced expression of α_{1D}-AR mRNA (Fig. 6, lane *5*).

In view of the increase in total numbers of α_1 receptors expressed in VSMCs and the induction of the α_{1D} receptor gene by IGF-I, we used an antibody against α_{1D} receptors to determine if there was increased expression at the protein level of this receptor subtype (Fig. 7). α_{1D} adrenergic receptors could be detected in COS-7 cells transfected with the α_{1D} receptor expression vector but not in control cells (Fig. 7, lanes *4* and *5*). Insulin and IGF-I increased expression of α_{1D} receptor subtype in cultured vascular smooth muscle cells (Fig. 7, lanes *1–3*).

Discussion

The results of the current studies demonstrate that insulin and IGF-I increase total α_1 receptor number in cultured smooth muscle cells. These peptide hormones selectively increased expression of the α_{1D} receptor gene with no change in expression of the α_{1B} receptor gene as detected by RNase protection assays. The increase in expression of the α_{1D} receptor gene occurred via increased transcription of this gene rather than to changes in mRNA stability. Using Western blotting, we found that there was increased expression of the α_{1D} receptor subtype at the protein level in these cells. Induced expression of α_{1D} receptor mRNA was inhibited by the receptor tyrosine kinase inhibitor genistein and the PI 3-kinase inhibitor wortmannin, suggesting that these pathways may be involved in the activation of the expression of this gene.

Elucidation of metabolic cardiovascular risk factors in the development of coronary and peripheral atherosclerosis is highly clinically relevant. There is growing recognition that insulin resistance is an important cardiovascular risk factor. Resistance to insulin-stimulated glucose uptake is widespread in the population (25). Most of these people do not have diabetes but rather require higher than normal concentrations of insulin to maintain normal concentrations of plasma glucose ('insulin resistance' in tissues such as skeletal muscle and fat). Insulin resistance has been found to be associated with cardiovascular risk factors such as dyslipidemia, hyperuricemia, increased concentrations of plasminogen activator inhibitor 1, and hypertension (26, 27). Systemic insulin administration has an excitatory effect on sympathetic activity even when normal plasma glucose concentrations are maintained by glucose infusions. Consequently, high normal or pharmacological concentrations of insulin lead to activation of firing rate of sympathetic nerves, enhanced norepinephrine release, and increases in heart rate and blood pressure (27). These types of sympathetic alterations may be the earliest precursor of adult hypertension and these types of abnormalities in hypertensives are associated with insulin resistance.

We wondered if insulin or IGF-I might enhance expression of α_1 receptors in vascular smooth muscle which would potentially magnify the capacity of catecholamines to elevate blood pressure in hyperinsulinemic subjects. This hypothesis was motivated in part by previous findings relating α_1 receptors to the actions of angiotensin II. Van Kleef et al. (28) found that enhanced DNA synthesis in rat arteries due to a continuous infusion of angiotensin II was blocked by prazosin independently of changes in blood pressure demonstrating that α_1 receptors are involved in the angiotensin II–induced increase of medial smooth muscle cell DNA synthesis. It is known that angiotensin II can activate sympathetic nervous system activity either through effects in the central nervous system, facilitation of neurotransmission, or enhancement of postjunctional responses of smooth muscle cells to α_1 receptor-stimulation (28, 29). We speculated and found that a potentially important response to angiotensin II involved an increase in expression of α_1 receptors in vascular smooth muscle (15). Our current results suggest that insulin and IGF-I also induce expression of α_1 receptors which could contribute to altered responses to catecholamines in vivo.

Activation of α_1 receptors stimulates the proliferation of smooth muscle cells in culture (30, 31). Nakaki et al. (32) reported that norepinephrine stimulates DNA synthesis in quiescent cloned rat aortic smooth muscle cells. In rat aorta, activation of α_1 receptor markedly induces c-*fos* gene expression and induces other growth-stimulating genes including PDGF-A (33). In an in vitro preparation of aorta, c-*fos* expression is induced exclusively in the medial smooth muscle cell layer as determined by in situ hybridization (14). Consequently, enhanced α_1 receptor–mediated growth responses could potentially contribute to adverse effects of catecholamines in hyperinsulinemic subjects. Additionally, insulin and IGF-I enhanced-expression of α_{1D}-receptor gene in VSMCs are associated with an enhanced capacity of α_1 receptor agonists to activate mitogen-activated protein kinase. This suggests that the enhanced expression of α_1 receptors may enhance the effects of insulin and IGF-I on growth control of vascular smooth muscle cells. These findings suggest the hypothesis that the use of α_1 receptor antagonists in the treatment of hypertension in type II diabetics may have special benefit in inhibiting blood vessel hypertrophy in this setting.

Very little is known about the capacity of insulin to regulate expression of adrenergic receptors. Devedjian et al. (34) demonstrated that insulin inhibited expression of α_2 receptors in cultured HT29 cells. In addition, Haddcock et al. (35) demonstrated that insulin inhibited effects mediated by β adrenergic receptors, possibly associated with tyrosine phosphorylation of these receptors. We have found previously that activation of protein kinase C leads to increased transcription of the α_{1B} receptor gene (21). While insulin or IGF-I may activate protein kinase C (36), we found in the current studies that transcription of the α_{1B} receptor gene was not increased. Indeed, we have found in the current studies that the protein kinase C antagonist H7 did not inhibit activation of expression of the α_{1D} receptor gene. The capacity of the tyrosine protein kinase inhibitor genestein and PI 3-kinase inhibitor wortmannin to inhibit insulin/IGF-I induction of the α_{1D} receptor gene suggests that these signalling pathways, known to be activated by these receptors, may play a role in the induction of the α_{1D} receptor. While there is considerable information about the structure of the α_{1B} receptor gene (37–39), little is known about transcriptional regulation of the α_{1D} receptor. However, the differentiation regulation of this receptor gene by insulin/IGF-I suggests that it contains distinct regulatory elements compared to the α_{1B} receptor gene.

Although there is evidence suggesting that cultured vascular smooth muscle cells have functional responses to insulin which may be mediated by both insulin receptors and IGF-I receptors (40), a number of other studies (41–43) demonstrate that only specific receptors for IGF-I are abundant in cultured

rat and human aortic smooth muscle cells (41, 44). IGF-I binding to IGF-I receptors has a K_d of $\sim$ 1.5 nM. The biologic actions of insulin, in the presence of high concentrations (often needing 100-fold of IGF-I), is likely mediated by IGF-I receptors in vascular smooth muscle cells. In our study, IGF-I at a concentration of 1.3 nM stimulated an increase in expression of α_{1D} adrenergic receptor mRNA with a maximal response at about 10 nM in these cells. Indeed, further increase in concentration of IGF-I led to a blunted expression of α_{1D} receptor mRNA, suggesting that desensitization of IGF-I receptors was occurring at this higher concentration. However, the concentration of insulin needed to produce a maximal induction of expression of α_{1D} adrenergic receptor mRNA is considerably higher. These data suggest that insulin induced-expression of α_{1D} adrenergic receptors in vascular smooth muscle cells is likely mediated by the IGF-I receptor signaling pathway.

We have found that both insulin and IGF-I induce expression of the α_{1D} receptor gene. Moreover, the effects of insulin and IG-I are due at least in part to an increase in the rate of transcription initiation of the gene although other mechanisms such as increased translational efficiency may also contribute the increase in expression of the α_{1D} receptor gene. Insulin and IGF-I increased expression of α_{1D} adrenergic receptors in vascular smooth muscle cells is associated with enhancement of α_1 adrenergic agonist activation of MAP kinase, suggesting that hyperinsulinemia may contribute to the growth of smooth muscle cells via a novel mechanism of cross-talk with components of the sympathetic-α_1 adrenergic receptor system.

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