Insulin and Insulin-like Growth Factor I Differentially Induce $\alpha_1$-Adrenergic Receptor Subtype Expression in Rat Vascular Smooth Muscle Cells

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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 $\alpha_1$ 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 $\alpha_1$ receptors was detected at $[^3H]$prazosin binding. Both insulin and IGF-I increased $\alpha_1$ receptor number; also, these peptides increased expression of the $\alpha_1D$ receptor gene with no change in expression of the $\alpha_1B$ receptor gene as detected by RNase protection assays. Using Western blotting, we found that these peptides increased expression of the $\alpha_1D$ receptor subtype in these cells. Increased expression of the $\alpha_1D$ 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 $\alpha_1$ 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).

$\alpha_1$ Adrenergic receptors (AR) are a member of family of G protein-coupled membrane receptors. Activation of $\alpha_1$ receptors is a very important modulator of blood pressure, promoting vascular smooth muscle contraction (10, 11). Increasing evidence suggests that activation of $\alpha_1$ adrenergic receptors also regulates cardiovascular growth and hypertrophy, both in the heart and peripheral circulation. For example, overexpression of $\alpha_1$ receptors in the hearts of transgenic mice leads to hypertrophy (12); activation of $\alpha_1$ receptors in myocytes leads to induction of growth-related gene expression which promotes DNA and protein synthesis (13). In addition, activation of $\alpha_1$ 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 $\alpha_1$ 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 target organs, have a good pathophysiological foundation.

In the present study, we asked if insulin and IGF-I modulated expression of $\alpha_1$ adrenergic receptors in cultured smooth muscle cells. Additionally, the possibility that changes in expression of $\alpha_1$ receptors potentiated catecholamine-stimulated mitogenic pathways was examined. The results suggest that insulin and IGF-I increase expression of $\alpha_1$ receptors via a selective activation of the $\alpha_1D$ receptor subtype gene. Also, insulin and IGF-I enhance $\alpha_1$ receptor-mediated mitogenic responses such as activation of mitogen-activated protein (MAP) kinase in rat vascular smooth muscle cells.

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. [35P]dCTP (2,000 Ci/mmol), [35P]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 (6X174 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); [3H]prazosin (19.8 Ci/mmol) from New England Nuclear (Boston, MA); T3 and T7 RNA polymerases, RNase A, single strand synthesis kit and markers (ØX174 DNA/HinfI), RNase A, proteinase K, and acetic anhydride from Promega Corp.(Madison, WI); RNase T1, proteinase K, and acetic anhydride 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, 10% (vol/vol) heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO2/95% air. The cells were harvested for passaging at confluence with trypsin-EDTA and plated in T75 flasks (Nunc). The retained radioactivity was measured using a liquid scintillation counter.

Measurement of α1-receptor number. [3H]Prazosin was used to measure number of α1-receptors as previously described (15, 21). For binding assays and Western blotting, the cultured cells were gently 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 10% (vol/vol) heat-inactivated fetal calf serum at 37°C in a humidified atmosphere of 5% CO2/95% air. The cells were harvested for passaging at confluence with trypsin-EDTA and plated in 100-mm dishes at a density about 5 × 106, with a 80–90% confluence being reached about 7 days later. The medium was replaced every 2 days. Cells were generally used for studies at 8–10 days 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.

Assay of MAP kinase activity. 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 anti-p21ras, anti-actin, and anti-α1-receptor antibodies) 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 nitrocellulose and detected using the ECL Western Blotting Detection System with the α2AR 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 1 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 lystate (400 μg of protein) was immunoprecipitated with anti-p44ERK1 (2 μg/ml protein) and washed four times with lysis buffer and once with kinase buffer. The washed immunocomplexes were resuspended in 40 μl of kinase buffer (25 mM Hepes, pH 7.5, 10 mM MgCl2, 1 mM dithiothreitol, 0.5 mM EGTA, 40 μM ATP, 1 μM of [γ-32P]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 p81 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. 32P 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 Phosphorlmager 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), 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 chloroformisamyl 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 aqeous phase was taken and RNA was precipitated from it with isopropanol (1:1 vol). The resulting RNA pellet was dissolved in TE buffer or autoclaved water to be used in experiments.

Analysis of abundance of α1 receptor subtype mRNAs by RNase protection assays. There are three α1 receptor subtypes expressed in rat aortic smooth muscle cells (15). α1A receptors appear to predominantly mediate contraction of rat smooth muscle (23). To determine if insulin or IGF-1 regulated expression of subtypes of α1-AR in cultured VSMCs, sensitive RNase protection assay was used for detection of α1A, α1B, and α1D subtypes. The antisense 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

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used as an internal control (15). We have confirmed in our previous studies that the level of expression of the $\alpha_{1D}$ receptor mRNA was too low to be quantitated accurately so experiments with this probe were not pursued. The $\alpha_{1B}$ or $\alpha_{1D}$-RNA probes (5 x 10$^6$ cpm) with the $\beta$-actin RNA probe (1-2 x 10$^6$ cpm) as internal control and 50 $\mu$g 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 $\alpha_{1B}$ probe and at 55°C for $\alpha_{1D}$ probe. 250 $\mu$l of ice-cold RNase buffer (50 mM sodium acetate, pH 4.4; 100 mM NaCl; 10 mM EDTA) containing noncoding strand of immobilized to a nylon filter or with immobilized plasmid M13 containing $\alpha_{1D}$-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 x 10$^7$) were 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 x 10$^6$ nuclei/$\mu$l. The prepared nuclei (200 $\mu$l) were added to 200 $\mu$l 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 $\mu$l of [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 $[^3P]$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 x 10$^6$ cpm) was dissolved in 1 ml of hybridization solution (same as described above) and hybridized at 42°C for 36 h with 20 $\mu$g of the M13 plasmid with coding-strand of $\alpha_{1D}$ (329 bp) immobilized to a nylon filter or with immobilized plasmid M13 containing noncoding strand of $\alpha_{1D}$ or $\beta$-actin cDNA as control. The filters were washed with 0.5 x SSPE and 0.1% SDS at 65°C for 30 min and then treated with 2.5 $\mu$g/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 $\alpha_{1D}$-AR cDNA signal to $\beta$-actin cDNA signal.

**Transient transfection of COS cells with $\alpha_{1D}$ receptor subtype expression vectors.** To confirm that the antibody directed against the $\alpha_{1D}$-AR, obtained from Dr. R.J. Lefkowitz’s laboratory, worked in our hands, COS-7 cells were transfected with the rat $\alpha_{1D}$ 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 $\mu$g of lipofectamine and 10 $\mu$g of expression vector containing $\alpha_{1D}$-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 the level of variance of 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 $\alpha_{1D}$ 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-

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**Figure 1.** Effects of insulin and IGF-I on expression of $\alpha_{1D}$-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 [3H]prazosin were performed as described in Methods. There were no significant changes in binding affinity (K$_D$) of $\alpha_{1D}$ receptors for [3H]prazosin in these cells (data not shown). The data are average±SEM of three experiments. *Compared to control, $P < 0.05$; **$P < 0.01$. 

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brane preparations from these cells was isolated and binding assays of [3H]prazosin performed. As illustrated in Fig. 1, A and B, incubation with insulin or IGF-I resulted in increased numbers of α₁-receptors with maximal values detected between 4–24 h. Interestingly, in the later time points, expression of α₁ 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 α₁-receptors in VSMCs.

To evaluate if the increased expression of α₁ 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 α₂ adrenergic receptor antagonist idazoxan (1 μM) for 10 min. Cell lysates (400 mg of protein) were prepared and subjected to immunoprecipitation with an anti-p44/42 MAP 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 α₁D-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 5×10⁵ cpm of riboprobes complementary to α₁D-AR mRNA (A and B) or α₁B-AR mRNA (C) and 2×10⁴ cpm of cRNA probes complementary to β-actin mRNA. The hybridization was performed at 60°C for α₁B or 55°C for α₁D 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 IGF-I (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 α₁ receptors, we determined if the accumulation of α₁B or α₁D-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 α₁B- or α₁D-receptor mRNAs by RNase protection assays as described in Methods. Cultured VSMCs expressed both α₁B and α₁D-AR subtype mRNAs; insulin or IGF-I caused a marked increase in accumulation of α₁D-receptor mRNAs (Fig. 3, A and B). The time course of the induction of α₁D-receptor mRNAs by insulin and IGF-I revealed that an increase in the α₁D-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 α₁D-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 α₁B-receptor subtype mRNAs over 24 h, suggesting that insulin and IGF-I differentially induces expression of α₁D receptor subtype mRNAs in rat aortic smooth muscle cells.

The dose–response relationships for insulin and IGF-I-stimulation of accumulation of α₁D mRNAs are shown in Fig. 4. In-

![Graph](image-url)
creased expression of α₁D-AR mRNAs by insulin could be detected at an insulin concentration as low as 10⁻⁷ M; maximum induction of expression of α₁D-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 α₁D mRNA expression demonstrated effects at a concentration of 10 ng/ml (1.3 nM). The maximum induction of expression of α₁D-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 α₁D mRNA (Fig. 4, lane 8), possibly reflecting desensitization of IGF-I receptors. Fig. 4B demonstrates that IGF-I did not induce α₁B-AR mRNAs at these concentrations of IGF-I.

To determine the mechanism for increased abundance of the α₁D receptor mRNA, the stability of the α₁D-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. 5A). Insulin/IGF-I treatment did not significantly change the degradation rate of the α₁D-AR mRNAs; the half-life of decay of α₁D-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 α₁D-AR mRNAs. Together, these results suggest that the enhanced accumulation of α₁D-AR mRNAs in the presence of insulin or IGF-I is not due to increased stability of the α₁D-AR mRNAs. We then examined the transcription rate of the α₁D-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 α₁D-AR gene. The transcription rate of the α₁D-AR gene, calculated from the ratio of transcription rate of α₁D-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. 5B).

To evaluate the signaling pathways by which insulin or IGF-I activates expression of α₁D receptor mRNAs, VSMCs were pretreated with genistein, an inhibitor of tyrosine protein kinase. Insulin-induced accumulation of α₁D-AR mRNAs was blocked after pretreatment of cells with genistein (1 μM) for 1 h (Fig. 6), suggesting that insulin-stimulated expression of α₁D 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 α₁D 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 α₁D 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 μM) (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 32P-labeled cRNA probes of α₁D adrenergic receptor or β-actin as outlined under Methods. Data are representative of three experiments. *Compared to control, \( P < 0.05 \); **compared to insulin treatment, \( P < 0.05 \).

**Figure 7.** Immunodetection of α₁ adrenergic receptor subtype expression with anti–α₁D-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 (vector) or rat α₁D-AR expression vectors (α₁D-AR). The membrane extracts (2 mg protein for VSMCs and 3 mg protein for COS-7 cells) were immunoprecipitated with an antibody against α₁D-AR. Immunoprecipitates were subjected to SDS-PAGE, electrotransferred to PVDF membrane, immunodetected with anti–α₁D-AR antibody and visualized by ECL Western Blotting detecting system as indicated under Methods. Positions of α₁D-AR are indicated. Experiments were repeated twice 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 α1D-AR mRNA expression. Pretreatment of cells with wortmannin (10 nM) for 1 h almost completely inhibited insulin-induced expression of α1D-AR mRNA (Fig. 6, lane 5).

In view of the increase in total numbers of α1 receptor expressed in VSMCs and the induction of the α1D receptor gene by IGF-I, we used an antibody against α1D receptors to determine if there was increased expression at the protein level of this receptor subtype (Fig. 7). α1D adrenergic receptors could be detected in COS-7 cells transfected with the α1D receptor expression vector but not in control cells (Fig. 7, lanes 4 and 5). Insulin and IGF-I increased expression of α1D 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 α1 receptor number in cultured smooth muscle cells. These peptide hormones selectively increased expression of the α1D receptor gene with no change in expression of the α1B receptor gene as detected by RNase protection assays. The increase in expression of the α1D 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 α1D receptor subtype at the protein level in these cells. Induced expression of α1D 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 α1 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 α1 receptors to the actions of angiotensin II. Van Klee 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 α1 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 postsynaptic responses of smooth muscle cells to α1 receptor-stimulation (28, 29). We speculated and found that a potentially important response to angiotensin II involved an increase in expression of α1 receptors in vascular smooth muscle (15). Our current results suggest that insulin and IGF-I also induce expression of α1 receptors which could contribute to altered responses to catecholamines in vivo.

Activation of α1 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 α1 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 α1 receptor–mediated growth responses could potentially contribute to adverse effects of catecholamines in hyperinsulinemic subjects. Additionally, insulin and IGF-I enhanced-expression of α1D-receptor gene in VSMCs are associated with an enhanced capacity of α1 receptor agonists to activate mitogen-activated protein kinase. This suggests that the enhanced expression of α1 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 α1 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 α2 receptors in cultured HT29 cells. In addition, Haddock 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 α1B 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 α1D receptor gene suggests that these signalling pathways, known to be activated by these receptors, may play a role in the induction of the α1D receptor. While there is considerable information about the structure of the α1B receptor gene (37–39), little is known about transcriptional regulation of the α1D receptor. However, the differentiation regulation of this receptor gene by insulin/IGF-I suggests that it contains distinct regulatory elements compared to the α1B 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 smooth muscle cells.
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 $\alpha_\text{adrenergic}$ 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 $\alpha_\text{adrenergic}$ 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 $\alpha_\text{adrenergic}$ receptor mRNA is considerably higher. These data suggest that insulin-induced-expression of $\alpha_\text{adrenergic}$ 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 $\alpha_\text{adrenergic}$ 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 $\alpha_\text{adrenergic}$ receptor gene. Insulin and IGF-I increased expression of $\alpha_\text{adrenergic}$ receptors in vascular smooth muscle cells is associated with enhancement of $\alpha_\text{adrenergic}$ 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-$\alpha_\text{adrenergic}$ receptor system.

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References


