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Genetic Profiling of α_1-Adrenergic Receptor Subtypes by Oligonucleotide Microarrays: Coupling to Interleukin-6 Secretion but Differences in STAT3 Phosphorylation and gp-130

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ABSTRACT

α_1-Adrenoceptor subtypes (α_{1A}-, α_{1B}-, α_{1D}-) are known to couple to similar signaling pathways, although differences among the subtypes do exist. As a more sensitive assay, we used oligonucleotide microarrays to identify gene expression changes in Rat-1 fibroblasts stably expressing each individual subtype. We report the gene expressions that change by at least a factor of 2 or more. Gene expression profiles significantly changed equally among all three subtypes, despite the unequal efficacy of the inositol phosphate response. Gene expressions were clustered into cytokines/growth factors, transcription factors, enzymes, and extracellular matrix proteins. There were also a number of individual subtype-specific changes in gene expression, suggesting a link to independent pathways. In addition, all three α_1-AR subtypes robustly stimulated the transcription of the prohypertrophic cytokine interleukin (IL)-6, but differentially altered members of the IL-6 signaling pathway (gp-130 and STAT3). This was confirmed by measurement of secreted IL-6, activated STAT3, and gp-130 levels. Activation of STAT3 Tyr705 phosphorylation by the α_1-ARs was not through IL-6 activation but was synergistic with IL-6, suggesting direct effects. Interestingly, α_{1B}-AR stimulation caused the dimerization-dependent phosphorylation of Tyr705 on STAT3 but did not activate the transcriptional-dependent phosphorylation of Ser727. The α_{1B}-AR also constitutively down-regulated the protein levels of gp-130. These results suggest that the α_{1B}-AR has differential effects on the phosphorylation status of the STAT3 pathway and may not be as prohypertrophic as the other two subtypes.

α_1-Adrenoceptors (ARs) belong to the superfamily of G-protein-coupled receptors (GPCR) that mediates the functions of catecholamines. Once activated by binding, α_1-ARs initiate the cellular pathways leading to the regulation of physiological effects, including blood pressure maintenance, glucose metabolism, renal sodium reabsorption, and cardiac inotropy (Michelotti et al., 2000; Piascik and Perez, 2001). Three α_1-AR subtypes (α_{1A}-, α_{1B}-, and α_{1D}-AR) have been cloned and characterized pharmacologically (Cotecchia et al., 1988; Lomasney et al., 1991; Perez et al., 1991). All three α_1-AR subtypes display comparable binding affinities to catecholamines and show prevalence for coupling to the G_q family of G proteins, thereby activating IP_3-mediated increases in calcium. Despite having similar binding affinities for the endogenous ligands and predominance toward G_q signaling, all three α_1-AR subtypes are expressed in most tissues, thus raising the question of whether or not α_1-AR subtypes carry out redundant functions.

Discerning the physiological roles of α_1-AR subtypes in vivo has proven difficult, mainly because of the lack of probes with sufficient subtype selectivity (i.e., ligands and low avidity antibodies). This issue has been partly addressed by the use of cell lines transfected with individual α_1-AR subtypes, and transgenic animal models in which α_1-AR subtypes have been either knocked out (Cavalli et al., 1997; Rokosh and Simpson, 2002; Tanoue et al., 2002) or overexpressed (Milano et al., 1994; Zuscik et al., 2000; Lin et al., 2001).

Besides vascular effects, α_1-ARs are known to stimulate hypertrophy of cardiac myocytes by activating established

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ABBREVIATIONS: AR, adrenergic receptor; GPCR, G protein-coupled receptor; IL, interleukin; IP, inositol phosphate; MAPK, mitogen-activated protein kinase; STAT, signal transducer and activator of transcription; ^{125}I-BE-2254, 2-[β-(4-hydroxy-3-[^{125}I]iodophenyl)ethylaminomethyl-]tetralone; DMEM, Dulbecco's modified essential medium; PBS, phosphate-buffered saline; CCF, Cleveland Clinic Foundation; PM, perfectly matched; MM, mismatched; ELISA, enzyme-linked immunosorbent assay; JAK, Janus tyrosine kinase; LIF, leukemia inhibitory factor; VEGF, vascular endothelial growth factor; gp, glycoprotein.

signaling pathways (reviewed in Varma and Deng, 2000). This effect of α_1-ARs may be relevant during the pathogenesis of congestive heart failure, because plasma concentrations of the sympathetic neurotransmitter norepinephrine are elevated during the onset of heart failure. In addition to increased cardiac sympathetic drive, circulating levels of the proinflammatory cytokine IL-6 (and IL-6 family members LIF and cardiotrophin-1) are also elevated in patients with heart failure (Kanda et al., 2000; Eiken et al., 2001; Ng et al., 2002). A role for IL-6 in the development of heart failure has been proposed based upon evidence that mice overexpressing IL-6 or its receptor develop ventricular hypertrophy (Hirota et al., 1995), whereas IL-6 receptor or gp-130 knockout mice develop thin ventricular walls (Taga et al., 1996) or a massive apoptosis before the onset of heart failure (Hirota et al., 1999). Recent evidence indicates that other well-known cardiac stimuli, including angiotensin II (Sano et al., 2000) and activation of β-ARs (Murray et al., 2000), increase expression of IL-6 in the heart, indicating a correlation between neuroendocrine signaling and cytokine release.

α_1-AR subtype-specific signaling has been suggested previously, particularly in studies of α_1-AR stimulation of MAPK pathways (Zhong and Minneman, 1999), which integrate extracellular signals that regulate cell growth, proliferation, and fate. In the present study, we employed oligonucleotide microarrays to provide new insights, and a more sensitive assay of α_1-AR subtype signaling. We compared the profiles of gene expression after short-term (60-min) epinephrine-stimulation of Rat-1 fibroblasts stably transfected with individual α_1-AR subtypes and compare these with stimulated nontransfected cells. Our studies indicate that within the same cellular context, α_1-AR subtypes can stimulate or inhibit identical profiles of expression, despite differences in IP efficacy, as well as profiles that are subtype- and/or pathway-specific. We report that α_1-ARs activate the secretion of IL-6 and that there are synergistic effects on STAT3 activation, suggesting that α_1-AR mediation of STAT3 is through non–IL-6 effects, which was confirmed through neutralizing antibodies. Interestingly, the α_{1B}-AR was not synergistic or as synergistic with IL-6 activation. This could be because the α_{1B}-AR displays constitutive down-regulation of gp-130 protein levels. Our data also suggests that α_{1B}-AR activation can stimulate the dimerization-dependent phosphorylation of Tyr705 on STAT3 but not the transcriptional-dependent phosphorylation of Ser727, the first report of this differential activation of STAT3. These results could be important in regulating pro-hypertrophic pathways and may be differentially regulated by the α_1-AR subtypes.

Materials and Methods

Materials. [^{125}-I]BE-2254, [*myo*-^{3}H]inositol, and [γ-^{32}P]dCTP were obtained from PerkinElmer Life Sciences (Boston, MA). AG 1-X8 resin (formate form), 4 to 15% Ready-Gels, and Bio-Rad protein assay were purchased from Bio-Rad Laboratories (Hercules, CA). Full-length rat p21-cK-Ras and c-fos plasmids were obtained from American Type Culture Collection (Manassas, VA). M-PER lysis reagent and West-Pico enhanced chemiluminescent reagent were from Pierce (Rockford, IL). The gp-130 (M-20) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Both STAT3 and Phospho-STAT3 (Ser727 and Tyr705) antibodies were obtained from Cell Signaling Technology (Beverly, MA). IL-6 and IL-6 neutralizing antibodies were from R & D systems (Minneapolis, MN). Horseradish peroxidase-conjugated anti-rabbit IgG was from Jackson Laboratories (Bar Harbor, ME). (−)-Epinephrine bitartrate, (*S*)-(−)-propranolol hydrochloride, rauwolscine hydrochloride, and prazosin hydrochloride were from Sigma-Aldrich (St. Louis, MO). Phentolamine mesylate was from RBI/Sigma (Natick, MA). Dulbecco's modified Eagle's medium (DMEM), penicillin, streptomycin, trypsin-EDTA, and Ca^{2+}-Mg^{2+}–free phosphate-buffered saline (PBS) were obtained from the Cleveland Clinic Foundation (CCF) house facility (Cleveland, OH). Fetal bovine serum was obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD). G418 was purchased from Calbiochem (La Jolla, CA).

Cell culture. Rat-1 fibroblasts stably transfected with human α_1-AR cDNAs corresponding to either the α_{1A}-, α_{1B}-, or α_{1D}-AR subtype were a gift of GlaxoSmithKline (Uxbridge, UK). These cells were derived from clonal isolates. Cells were propagated in 75-cm^2 flasks in a humidified atmosphere (37°C) in DMEM containing 5% fetal bovine serum, 10 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml of the selection antibiotic G418. Cells were detached by trypsinization (0.05% trypsin, 0.53 mM EDTA) and subcultured at a ratio of 1:5 upon reaching confluence. Nontransfected Rat-1 fibroblasts were maintained under the same conditions but in the absence of G418.

Saturation Binding. Saturation binding experiments were performed using membranes from the Rat-1 cells stably transfected with the α_{1A}-, α_{1B}- or α_{1D}-AR subtypes. Membrane isolations were performed as reported previously (Perez et al., 1991). Binding assays were performed in triplicate in a 0.25-ml assay volume with eight concentrations (0–400 pM) of ^{125}I-BE-2254 and 10 μg of membrane protein per tube. After equilibration for 30 min at 25°C, free and bound radioligand were separated by rapid filtration using a Brandel cell harvester and Whatman GF-C glass-fiber filters. Nonspecific binding was defined as the amount of radioactivity that remained bound to the filters in the presence of 10 μM phentolamine. B_{max} (maximum receptor density) and K_d (affinity) values were obtained using the nonlinear regression function of Prism (GraphPad, San Diego, CA).

Quantitation of Epinephrine-Stimulated Total IP Accumulation. Confluent cells expressing α_1-AR subtypes were grown for 24 h in the presence of 5 μCi of [^{3}H]*myo*-inositol. Propranolol (1 μM) and rauwolscine (0.1 μM) were added to the growth media for 30 min to block putative β- and α_2-ARs, respectively, followed by a 60-min incubation of 10 μM epinephrine in the presence of 10 mM LiCl. After drug incubations, the monolayers were washed with ice-cold PBS and the cells were lysed with 0.4 M perchloric acid. Lysates were scraped, collected, and neutralized by the addition of a 0.5-ml volume of a 0.72 N KOH/0.6 M $KHCO_3$ solution. Soluble [^{3}H]IPs were isolated from the lysates by column chromatography using AG 1X-8 resin-packed columns. The columns were washed with 0.1 M formic acid, and the resin-bound [^{3}H]IPs were displaced by elution with a 0.1 M formic acid solution containing 1.0 M ammonium formate. The eluant was collected in scintillation vials and the radioactivity was detected using a β-counter (Beckman, Irvine, CA). EC_{50} (potency) values and maximal epinephrine responses were compared using Prism (GraphPad).

Preparation of Biotin-Labeled cRNA and Hybridization to Oligonucleotide Arrays. Five pooled flasks (150 mm^2 each) of either nontransfected or α_1-AR subtype-transfected Rat-1 fibroblasts were used for individual hybridization to the oligonucleotide microarray. Two separate hybridizations from two separate RNA preparations were performed per cell group. Briefly, confluent monolayers were first incubated for 30 min with propranolol (1 μM) and rauwolscine (0.1 μM), followed by an additional 60-min incubation with 10 μM epinephrine. After incubations, the media was removed and the monolayers rinsed twice with ice-cold PBS. Poly(A)$^+$ RNA was immediately isolated using the FastTrack 2.0 Kit from Invitrogen (Carlsbad, CA) and stored overnight at −70°C. Double-stranded cDNA was then synthesized from 1.0-μg aliquots of poly(A)$^+$ RNA using the SuperScript Choice double-stranded cDNA synthesis kit

from Invitrogen. From each sample, cDNA was purified by phenol/chloroform extraction and ethanol precipitation, and biotin-labeled cRNA was synthesized via an in vitro transcription reaction using the BioArray high-yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). cRNA transcripts were then purified from the in vitro transcription reaction using the RNeasy Mini kit from QIAGEN (Valencia, CA). The fragmentation of biotin-labeled cRNAs and hybridization of these fragments to the oligonucleotide arrays were both carried out by the Gene Expression Core Service at the CCF. To determine the quality of the mRNA preparations and the subsequent manipulations between test samples, an aliquot of the biotinylated cRNA fragments from each sample were hybridized to an Affymetrix (Santa Clara, CA) "test chip" before the rat genome U34A array was used. This test chip analyzes the percentage of genes that are present in the sample that hybridizes to the genes present in the test chip. The analysis also includes a measure of the amount of full-length cRNA transcribed as determined by the ratios of 3′/5′ regions of both glyceraldehyde-3-phosphate dehydrogenase and β-actin. Aliquots of biotin-labeled cRNA fragments were then hybridized to rat genome U34A Arrays (Affymetrix) containing 7000 known genes and expressed sequence tags from build 74 of the UniGene database. The hybridization signal was amplified by the Antibody Amplification Protocol as described in the Affymetrix GeneChip Expression Analysis Manual.

For data analysis, matrix-based decisions concerning the hybridization of a cRNA to a particular probe set were executed using Affymetrix software. Briefly, each gene probe set was represented on the chip by 25 pairs of perfectly matched (PM) and mismatched (MM) oligonucleotides. Each of the 25 unique PM/MM pairs was different by only a single base-pair change in the MM oligonucleotide, and spanned various places along the gene represented by the probe set to include regions represented by the 5′-untranslated, coding, and 3′-untranslated regions. The MM probes acted as specificity controls that allowed the direct subtraction of both background and cross-hybridization signals. The number of instances in which the PM signal was greater than the MM signal was determined and the average of the logarithm of the PM/MM ratio was calculated. These values were used in a matrix-based algorithm that determined the absence or presence of a cRNA molecule in the experimental sample. To determine the abundance of each RNA, the average of the differences representing PM minus MM for each probe set was calculated after discarding the maximum, the minimum and any outliers beyond three standard deviations. All combinations of pair comparisons were then made between the control (nontransfected) Rat-1 fibroblasts and those transfected with each α_1-AR subtype [i.e., α_{1A}-AR (chip 1) versus control (chip 1); α_{1A}-AR (chip 2) versus control (chip 1), etc.]. Therefore, for each subtype, a four-way comparison was performed. This results in four numerical values for each cell line. Additional comparisons were made between transfected cells (i.e., α_{1A}-AR versus α_{1B}-AR; α_{1A}-AR versus α_{1D}-AR, etc.). For each comparison, changes in the expression of a particular gene had to exhibit an average of 2.0-fold or greater and be present in each of the four-way analyses to be included in the tables. This decision was based upon previous reports that TaqMan verification of the microarray is valid in the 1.7- to 1.8-fold range (Tan et al., 2002). Tables reflect the mean values of the four-way comparisons plus or minus the S.E.M. Expressed sequence tags were excluded from the analysis. The use of two chips reduces the error rate of false positives and false negatives caused by chip-based errors to 1 in 10,000, according to manufacturer's specifications.

Northern Blot Analysis. Two genes that were identified via microarray to either increase or remain unchanged by activation of α_1-AR subtypes (c-fos and p21-cK-Ras) were examined by Northern analysis. Drug incubation protocols were kept identical to those in microarray experiments. Poly(A)$^+$ RNA (10 μg) extracted with the Invitrogen FastTrack 2.0 kit were loaded onto 0.8% agarose-formaldehyde gels for subsequent transfer to nitrocellulose and hybridization. cDNA probes were derived from full-length plasmids obtained from American Type Culture Collection. Probes (c-fos, 5.0-kb *Eco*RI fragment; p21-cK-Ras, 4.4-kb *Eco*RI-*Hin*dIII fragment) were random-primed with [^{32}P]dCTP (6000 Ci/mmol) using the random primed DNA labeling kit from Roche (Indianapolis, IN). Labeled probes (1×10^6 cpm/ml) were hybridized to the membranes overnight at 50°C. Membranes were each washed in 5× then 2× SSC containing 0.1% SDS for 10 min, and exposed to X-ray film for 3 (c-fos) or 48 h (p21-cK-Ras). Qualitative differences in band intensities were compared between α_1-AR subtype transfected and nontransfected Rat-1 cells using the software program NIH Image (http://rsb.info.nih.gov/nih-image/).

Measurement of IL-6 Secretion. Rat-1 fibroblasts stably expressing α_1-AR subtypes were incubated for 24, 48, and 72 h with 10 μM epinephrine in the presence of 1 μM propranolol and 0.1 μM rauwolscine. Additionally, experiments were performed in α_1-AR subtype expressing Rat-1 fibroblasts that were incubated for 48 h with epinephrine in the presence of β-AR and α_2-AR antagonists and the α_1-AR antagonist prazosin (1 μM). Antagonists and epinephrine were replenished in the culture media every 12 h. After incubation, the culture medium was removed and concentrated by vacuum centrifugation. The IL-6 levels in the concentrate were determined by enzyme-linked immunosorbent assay (ELISA) using the rat IL-6 module set from Alexis Biochemicals (San Diego, CA) following the manufacturer's instructions. Sample IL-6 concentrations were extrapolated by spectrophotometric (405 nm) conversion of standard curves ranging in sensitivity from 31 to 2000 pg/ml. To account for IL-6 in serum, background absorbance values (DMEM containing serum and antibiotics) were subtracted from each sample.

Immunoblotting-Immunoprecipitation Studies. To investigate whether changes in transcription for *gp-130* and *STAT3* genes would be reflected in changes at the translational level, antibodies to gp-130 and STAT3 were employed in Western blotting experiments of α_1-AR subtype transfected cells treated with 10 μM epinephrine for 1 or 24 h. Nontransfected Rat-1 fibroblasts were also included in these experiments to exclude any effect of epinephrine incubation on gp-130 and/or STAT3 total protein levels. The effect of α_1-AR subtype stimulation on gp-130 total protein levels was studied after 15, 30, 60, 120, and 180 min of 10 μM epinephrine stimulation. After incubation, the monolayers were washed with ice-cold PBS, and the cells were scraped in M-PER lysis buffer (Pierce) containing protease inhibitors (1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM EDTA, and 0.2 mM aprotinin). Total protein content in the lysates was determined using the Bio-Rad protein assay. Proteins were separated on 4 to 15% gradient gels by electrophoresis. The gels were transferred onto nitrocellulose membranes, blocked for 1 h, and incubated overnight with either STAT3 or gp-130 antibodies. Membranes were incubated with an anti-rabbit HRP-conjugated secondary antibody and visualized by enhanced chemiluminescence.

The epinephrine-induced transcriptional activity of STAT3 was determined in immunoprecipitation-immunoblotting experiments of subtype transfected Rat-1 fibroblasts that had been incubated with 10 μM epinephrine for various times (15, 30, 60, and 120 min). Total STAT3 protein was immunoprecipitated from cellular lysates (100 μg of total protein per time point) using the STAT3 antibody mentioned above. Immunoprecipitated proteins were separated in 4 to 15% gradient gels, which were transferred to nitrocellulose membranes. Membranes were blocked for 1 h and incubated overnight with an antibody designed to recognize the transcriptionally active form of STAT3 (phosphorylated at the Ser727 residue) or the dimerization-dependent phosphorylation of Tyr705. The level of epinephrine-stimulated phosphorylated STAT3 levels at each time point was compared with that of basal levels (time 0, no epinephrine). To compare protein loading, membranes were stripped and reprobed with the same antibody used to precipitate STAT3. To measure IL-6–mediated effects, IL-6 (40 ng/ml; R and D Systems) was added to the medium for 30 min for STAT3 activation or 3 h for gp-130 protein determination. Inhibition of IL-6–mediated biological function was achieved through a neutralizing antibody (R and D Sys-

tems), first given in a dose-response (0.05–5 μg/ml) and then used at its maximal dose (5 μg/ml). Data from immunoblotting-immunoprecipitation studies were analyzed by qualitative comparison of band intensities obtained at each time point versus that of time 0 (basal). Western blots were analyzed and compared in band intensities using the software program NIH Image.

Results

Saturation Binding. The levels of α_1-AR subtype expression in Rat-1 fibroblasts were compared in membrane saturation binding assays using the radioligand ^{125}I-BE-2254. Rat-1 fibroblasts expressed similar densities and affinities of single-site binding curves. The B_{max} values for α_{1A}-, α_{1B}-, and α_{1D}-AR transfected cells were 1.3 ± 0.1, 1.4 ± 0.4, and 2.6 ± 0.5 pmol/mg, respectively. The K_d values for ^{125}I-BE-2254 were also similar among subtypes: α_{1A}-AR, 19.8 ± 1.5 pM; α_{1B}-AR, 30.5 ± 6.5 pM; α_{1D}-AR, 58 ± 6.5 pM. In all binding assays, nonspecific binding at the K_d ranged from 9.2 to 15% of the total radioligand bound, and no more than 10% of total radioactivity added was bound.

Quantitation of Epinephrine-Stimulated Total [^{3}H]IP Accumulation. As a measure of α_1-AR subtype-G_q signaling, concentration responses to epinephrine stimulation of [^{3}H]IP accumulation were performed in α_1-AR subtype transfected Rat-1 fibroblasts (Fig. 1). No significant differences in potency ($-\log EC_{50}$, molar) were noted for epinephrine stimulation of total [^{3}H]IP accumulation among α_1-AR subtypes: α_{1A}-AR, 7.16 ± 0.15; α_{1B}-AR, 7.86 ± 0.24; α_{1D}-AR, 7.72 ± 0.15. However, the maximal responses to epinephrine varied among subtypes, and were significantly higher in α_{1A}-AR cells than those of α_{1B}-AR (by 4.1-fold) and α_{1D}-AR (by 2.8-fold) cells. The maximal responses to epinephrine in α_{1D}-AR cells were modestly higher than those in α_{1B}-AR cells (by 1.2-fold). There were no differences in basal or agonist-independent [^{3}H]IP counts among α_1-AR subtype transfected fibroblasts (data not shown).

Comparison of mRNA Quality between Samples. The results of the "test chip" analysis between the samples used in the microarray study revealed that both hybridizations from separate RNA preparations had reproducible parameters of quality. All three parameters that determine the amount (percentage present) and quality of mRNA (3′/5′ ratios) for both glyceraldehyde-3-phosphate dehydrogenase and β-actin were comparable between samples and are considered excellent.

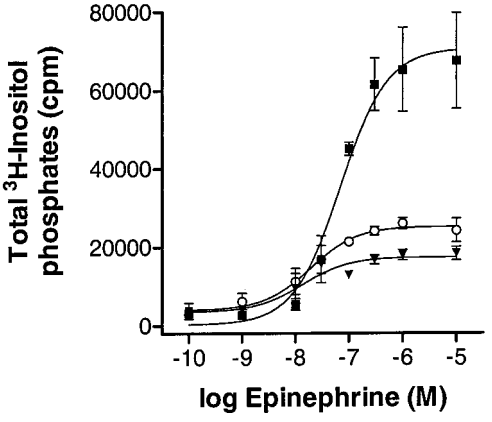

Fig. 1. α_1-AR subtype-mediated total [^{3}H]IP accumulation in Rat-1 fibroblasts expressing α_{1A}-AR (■), α_{1B}-AR (▼), or α_{1D}-AR (○) subtypes. Cells expressing α_1-AR subtypes were incubated with increasing concentrations of epinephrine (10^{-10}–10^{-5} M) for 60 min in the presence of β- and α_2-AR blockers, and the soluble [^{3}H]IPs were eluted by column chromatography and quantified by scintillation counting. Each point represents the mean ± S.E.M. of three independent experiments measured in counts per minute performed in triplicate. Despite equal receptor density as determined by ligand binding studies, the α_{1A}-AR subtype displays greater efficacy in the IP response. EC_{50} values are not significant from one another, $p > 0.23$.

Genes commonly modified by α_1-AR subtypes. The gene expression profiles of Rat-1 fibroblasts stably expressing α_1-AR subtypes is shown in Table 1. Common to all three subtypes, gene expression changed for 29 genes by at least two-fold relative to nontransfected Rat-1 fibroblasts. These common gene expressions have been divided into four clusters, which include genes that code for: 1) secreted endogenous peptide ligands (cytokines and growth factors); 2) DNA binding proteins (transcription factors and immediate early genes); 3) signaling and/or catabolic enzymes; and 4) extracellular matrix proteins. In each cluster, genes have been arranged in decreasing order of magnitude of expression changes, starting with the highest fold-values of α_{1A}-AR fibroblasts as reference. Thus, the strongest stimulations for each cluster were for the gp-130 related cytokine, IL-6, the immediate-early gene c-fos, the tyrosine phosphatase, CL100, and fibronectin, respectively. Figure 1 shows that even though the epinephrine-stimulated accumulation of total IPs was most efficacious in α_{1A}-AR cells, all three subtypes changed the gene expressions of most of the genes in Table 1 by similar folds. In addition, some genes (GD-VEGF, CELF, rNFIL-6, Arc, RNR-1) were more strongly changed in their expression in α_{1B}-AR and/or α_{1D}-AR cells than α_{1A}-AR cells.

Table 1 also shows that within each cluster there were genes belonging to particular families: VEGF3 and GD-VEGF (heparin binding FGF); IL-6 and LIF (gp-130 binding cytokines) in cluster 1; c-fos and c-jun (activator protein-1); Egr-1 and Egr-2 (Zinc-finger DNA binding) in cluster 2; and Tyrosine phosphatases CL100 and BAD2 (dual purpose, threonine/tyrosine phosphatase) in cluster 3.

Analysis of the microarray data also revealed that α_1-AR subtypes commonly inhibited the gene expression of 9 genes by at least 2-fold versus nontransfected cells (Table 2). The fold-decreases in the gene expressions for each of the 9 "commonly-inhibited" genes were of similar magnitude among α_1-AR subtypes.

α_1-AR subtype-unique changes in gene expression. Table 3 shows that compared with nontransfected Rat-1 fibroblasts, multiple changes in gene expression that were specific to each α_1-AR subtype existed. These included, for each subtype, both positive and negative changes, which varied in magnitude and number of genes modified. The α_{1B}-AR modified the greatest number of genes (17), followed by the α_{1D}-AR (12) and α_{1A}-AR (6). We noted that in α_{1B}-AR cells, a relatively large number of gene expressions modified have been associated with neurodegeneration and apoptosis, including tau and synuclein (Golbe, 2002), transforming growth factor β3 (Dunker et al., 2001), and caspase-6 (MacLachlan and El-Deiry, 2002).

Differential regulation of IL-6 signaling genes by α_1-AR subtypes. In addition to changes in gene expression that were common to all subtypes, and to subtype-specific changes, the expression of certain genes changed in a different way: stimulated by two, but not all three subtypes. Table

4 shows that this was true of genes that code for members of the IL-6 signaling pathway (gp-130, STAT3 and Ras). Table 4 shows that although all subtypes increased the gene expression of the cytokine, IL-6, only α_{1A}-AR and α_{1D}-AR subtypes were linked to increases in gene expression of gp-130 (the IL-6 high affinity receptor and signal transducer), and STAT3 (prototype IL-6–stimulated transcription factor). p21-Ras has been included in this pathway becauseit has been shown to relay IL-6/gp-130 signals in some cells (Taga and Kishimoto, 1997).

Northern blots. To validate some of the data obtained with the microarray technique, Northern blotting analyses were carried out for two different genes (c-fos and p21-cK-Ras) present in the microarray. Drug incubation protocols for obtaining northern and microarray data were kept identical. Figure 2 shows that in nontransfected cells epinephrine had no effect in the transcription of c-fos (NT lane). On the other hand, epinephrine robustly stimulated the transcription of c-fos in α_1-AR subtype transfected cells. The increase in c-fos transcription by northerns followed a similar pattern to those

TABLE 1

Increased gene expression changes in Rat-1 fibroblasts, common to all three α_1-AR subtypes

Data indicate the fold increase ± S.E.M. in gene expression with the indicated accession number. For each gene cluster, the genes are listed in decreasing order of magnitude, taking into account the α_{1A}-AR fold values as reference point. Data are from two separate hybridizations from two separate RNA preparations and compared in a four-way analysis as described under *Materials and Methods*. Fold changes are similar among the subtypes.

		Subtype		
	Accession	α_{1A}-AR	α_{1B}-AR	α_{1D}-AR
Cytokines/growth factors				
IL-6	M26744	57.5 ± 4.5	62 ± 12	21.2 ± 2.9
VEGF3	L20913	8.6 ± 3.6	16 ± 9.1	6.6 ± 2.8
BDNF	D10938	6.1 ± 2.3	7.3 ± 2.9	5.5 ± 1.8
Glioma-derived VEGF	M32167	4.4 ± 0.7	11.3 ± 3.2	3.6 ± 0.1
KGF	X56551	4.1 ± 0.1	2.1 ± 0.2	2.7 ± 0.1
LIF	AB010275	2.9 ± 0.3	2.1 ± 0.1	2.4 ± 0.15
Transcription factors				
c-fos	X06769	63.8 ± 23.4	43.5 ± 12.5	61 ± 13
c-jun	X17163	19.1 ± 0.1	23.6 ± 0.1	23 ± 1.0
CREM	S66024	16.3 ± 0.3	10.8 ± 0.5	17 ± 0.7
CELF	M65149	9.1 ± 0.5	17.4 ± 0.3	16.9 ± 1.5
NGFI-B	U17254	6.4 ± 0.1	2.7 ± 0.1	6.2 ± 0.1
rNFIL-6	S77528	6.6 ± 0.1	5.3 ± 0.4	12.4 ± 0.3
Arc	U19866	6.6 ± 0.4	4.3 ± 0.1	10.7 ± 0.7
NF-1-X1	AB012235	4.5 ± 1.7	4.8 ± 1.8	7.9 ± 3.3
MSX-2	U12514	3.9 ± 0.3	2.3 ± 0.2	2.0 ± 0.2
rHox	S82911	2.6 ± 0.1	2.3 ± 0.2	3.4 ± 0.1
RNR-1	L08595	2.3 ± 0.2	4.1 ± 0.7	11.0 ± 1.2
NGFI-A (Egr-1, Krox-24)	M18416	2.1 ± 0.1	3.1 ± 0.2	2.7 ± 0.1
Krox-20 (Egr-2)	U78102	2.0 ± 0.1	2.2 ± 0.1	2.8 ± 0.3
Krox-24 3′-UTR (Egr-1, NGFI-A)	U75397	2.0 ± 0.1	2.7 ± 0.2	2.5 ± 0.1
Enzymes				
Tyrosine phosphatase-CL100	S74351	12.9 ± 1.5	13.4 ± 1.4	14.4 ± 1.7
Tyrosine phosphatase-BAD2	U02553	8.5 ± 0.2	6.7 ± 0.5	7.6 ± 0.0
Stearoyl-CoA-desaturase 2	AF036761	6.9 ± 0.5	9.4 ± 0.1	10.7 ± 0.6
Collagenase	M60616	5.9 ± 0.2	8.4 ± 0.6	3.6 ± 0.4
Hexokinase	D26393	4.9 ± 1.7	4.4 ± 1.0	7.1 ± 1.5
proCathepsin L	S85184	2.1 ± 0.1	2.7 ± 0.3	4.1 ± 0.2
Calcineurin A α	D90035	2.0 ± 0.0	2.2 ± 0.1	2.6 ± 0.1
Extracellular matrix proteins				
Fibronectin	M28259	4.4 ± 1.5	3.7 ± 1.1	3.8 ± 1.3
Collagen III	M21354	2.4 ± 0.2	2.1 ± 0.1	4.6 ± 0.2

BDNF, brain-derived neurotrophic factor; KGF, keratinocyte growth factor; CREM, cAMP response element modulator protein; CELF, C/EBP-like factor; NGFI-B, nerve growth factor-induced B factor; rNFIL-6, rat nuclear factor induced by IL-6; MSX, muscle-segment homeobox; RNR, ribonucleotide reductase; NGFI-A, nerve growth factor-induced protein A; UTR, untranslated region.

TABLE 2

Decreased gene expression changes in Rat-1 fibroblasts, common to all three α_1-AR subtypes

Data indicate the fold decrease ± S.E.M. in the gene expression with the indicated accession number. The genes are listed in decreasing order of magnitude, taking into account the α_{1A}-AR fold values as reference point. Data are from two separate hybridizations from two separate RNA preparations and compared in a four-way analysis as described under *Materials and Methods*. Fold changes are similar among the subtypes.

Gene	Accession	α_{1A}-AR	α_{1B}-AR	α_{1D}-AR
Cutaneous fatty acid binding protein.	S69874	−8.3 ± 0.7	−8.3 ± 0.7	−9.4 ± 0.1
Lamina associated peptide 2	U18314	−4.1 ± 1.0	−3.2 ± 1.3	−4.0 ± 1.2
C-CAM4	U23056	−5.9 ± 2.0	−6.6 ± 2.4	−6.0 ± 1.4
Protein kinase MUK2	U49953	−4.9 ± 1.0	−7.1 ± 1.3	−6.2 ± 0.0
Mint-3	AF029109	−2.6 ± 0.3	−3.2 ± 0.4	−2.3 ± 0.2
Lysyl oxidase	S77494	−2.5 ± 0.1	−4.2 ± 0.3	−2.0 ± 0.0
Smooth muscle cell LIM protein	U44948	−2.3 ± 0.1	−5.4 ± 0.3	−3.3 ± 0.1
Dimethylargininase	D86041	−2.3 ± 0.1	−4.4 ± 0.3	−4.1 ± 0.1
Malic enzyme	M26594	−2.1 ± 0.1	−5.8 ± 0.5	−2.8 ± 0.3

C-CAM, cell-cell adhesion molecule; MUK, MAPK-upstream kinase.

obtained by microarrays: α_{1A}-AR (by 63.8 ± 23.4-fold), α_{1B}-AR (43.5 ± 12.5-fold), and α_{1D}-AR (61 ± 13-fold) cells relative to nontransfected controls. However, these changes were significantly higher in the microarray analysis, which is consistent with previous reports that the array is a more sensitive assay for detecting changes in mRNA levels (Yun et al., 2003).

Northern blots also showed increased transcript levels of p21-cK-Ras in α_{1A}-AR and α_{1D}-AR fibroblasts, whereas these levels remained unchanged in α_{1B}-AR cells, relative to nontransfected controls. Qualitatively, these transcriptional changes also mimic those obtained by microarrays: α_{1A}-AR (increased by 5.3 ± 0.4 fold), α_{1B}-AR (no change), and α_{1D}-AR (8.4 ± 0.6) compared with nontransfected cells.

Epinephrine-Stimulated Secretion of IL-6 by α_1-AR Subtypes. Because IL-6 has been associated with cardiac hypertrophy and heart failure and was one of the most changed in gene expression, we decided to explore the IL-6 pathway in more detail. The concentration of secreted IL-6 in culture medium was measured in ELISA experiments. The first set of experiments showed that in Rat-1 fibroblasts, α_1-AR subtypes stimulated an increase in the concentration of IL-6 in culture medium after epinephrine incubation. The effect was time-dependent. No IL-6 could be detected in culture medium during the first 24 h of epinephrine incubation; however, longer incubation times (48 and 72 h) resulted in increased IL-6 levels (α_{1A}-AR, 7.3- and 24-fold; α_{1B}-AR, 5.5- and 12.5-fold; α_{1D}-AR, 8.5 and 12.6-fold, respectively). This is probably a result of the sensitivity of the ELISA assay and is similar to previous reports that examined IL-6 secretion (Norris and Benveniste, 1993). Separate experiments summarized in Fig. 3B showed that at 48 h of epinephrine incu-

TABLE 3

α_1-AR subtype-unique changes in gene expression

Data indicate the fold increase (positive values) or decrease (negative values) ± S.E.M. in gene expression with the indicated accession number. For each subtype-specific cluster, the genes are listed in decreasing order of magnitude. Data are from two separate hybridizations from two separate RNA preparations and compared in a four-way analysis as described under *Materials and Methods*.

Gene	Accession	α_{1A}-AR	α_{1B}-AR	α_{1D}-AR
Lactate dehydrogenase B	U07181	6.0 ± 0.9	N.C.	N.C.
Myogenic regulatory factor	M84176	3.0 ± 0.5	N.C.	N.C.
EC-Superoxide dismutase	Z24721	−6.8 ± 0.7	N.C.	N.C.
Heme oxygenase-2	J05405	−5.5 ± 0.9	N.C.	N.C.
Kinesin light chain C	M75148	−4.3 ± 1.3	N.C.	N.C.
GTP-binding protein (Gαi-1)	M17527	−3.5 ± 0.3	N.C.	N.C.
Neuritin	U88958	N.C.	25.3 ± 4.3	N.C.
Lumican	X84039	N.C.	14.4 ± 0.7	N.C.
Nucleolin	M55017	N.C.	8.5 ± 0.8	N.C.
Tau protein kinase	X73653	N.C.	7.4 ± 0.4	N.C.
Cartilage homeoprotein (Cart-1)	L14018	N.C.	7.1 ± 0.1	N.C.
Mast cell protease 10	U67913	N.C.	4.6 ± 0.1	N.C.
Versican V3-isoform precursor	AF072892	N.C.	4.4 ± 0.6	N.C.
Tricarboxylate transporter	L12016	N.C.	3.7 ± 0.2	N.C.
COMT	M60753	N.C.	3.6 ± 0.7	N.C.
Sensory neuron synuclein	X86789	N.C.	−33.9 ± 0.6	N.C.
L-Glutamine amidohydrolase	J05499	N.C.	−9.9 ± 0.7	N.C.
α-7-integrin-α-chain	X65036	N.C.	−8.9 ± 0.1	N.C.
Vascular α-actin	X06801	N.C.	−7.1 ± 0.1	N.C.
Transforming growth factor β3	U03491	N.C.	−5.5 ± 0.1	N.C.
Thy-1 glycoprotein	X02002	N.C.	−5.1 ± 1.3	N.C.
Caspase-6	AF025670	N.C.	−4.7 ± 0.4	N.C.
Caveolin	Z46614	N.C.	−2.9 ± 0.3	N.C.
Adipsin	M92059	N.C.	N.C.	20.4 ± 2.2
SHPS-1	D85183	N.C.	N.C.	7.5 ± 0.4
Presenilin-2	X99267	N.C.	N.C.	7.5 ± 0.3
α-Crystallin-B chain	M55534	N.C.	N.C.	6.5 ± 2.1
Chemokine CX3C	AF030358	N.C.	N.C.	5.8 ± 0.8
Sugar transporter GLUT5	D28562	N.C.	N.C.	4.7 ± 0.1
Flavin monooxygenase 1	M84719	N.C.	N.C.	4.7 ± 0.4
c-erb-A-thyroid hormone receptor	X12744	N.C.	N.C.	4.7 ± 0.5
PKC binding protein and substrate	U41453	N.C.	N.C.	4.5 ± 0.4
c-erb-A-α-2 related protein	M31174	N.C.	N.C.	4.2 ± 0.5
RYB-a	D28557	N.C.	N.C.	−4.1 ± 0.2
90-kDa Heat shock protein	S45392	N.C.	N.C.	−3.0 ± 0.1

N.C., no change; EC, extracellular; SHPS-1, Src homology protein substrate-1; CX3C, cysteine-3 intervening residues-cysteine; GLUT, glutamate transporter; PKC , protein kinase C.

TABLE 4

α_1-AR subtype specific gene expression changes in IL-6 signaling

Data indicate the fold increase ± S.E.M. in gene expression with the indicated accession number. Data are from two separate hybridizations from two separate RNA preparations and compared in a four-way analysis as described under *Materials and Methods*. Although both the α_{1A}- and α_{1D}-AR caused the gene expression to change in the downstream signals of this pathway, the α_{1B}-AR did not, despite the stimulation of IL-6.

Gene	Accession	α_{1A}-AR	α_{1B}-AR	α_{1D}-AR
IL-6	M26744	57.5 ± 4.5	62 ± 12	21.2 ± 2.9
gp-130	M92340	4.4 ± 0.05	N.C.	5.2 ± 0.2
STAT3	X91810	4.0 ± 0.8	N.C.	10.1 ± 0.5
p21-Ras	U09793	5.3 ± 0.4	N.C.	8.4 ± 0.6

N.C., no change.

bation, the accumulated IL-6 concentration in medium from α_1-AR subtype expressing cells was completely abolished by prazosin (1 μM, 48 h) in α_{1A}- and α_{1B}-AR expressing cells and mostly blocked in α_{1D}-AR cells. No IL-6 protein accumulation was detected in nontransfected Rat-1 fibroblasts treated with identical epinephrine conditions (data not shown).

Epinephrine-Stimulated Changes in STAT3 Protein and Ser727-Phosphorylation by α_1-AR Subtypes. Fig. 4A shows the effect of epinephrine incubation on STAT3 total protein levels in Rat-1 fibroblasts transfected with α_1-AR subtypes. After 1 h of incubation with 10 μM epinephrine, we observed no differences in STAT3 total protein levels between epinephrine-stimulated and nonstimulated subtype-transfected cells. Longer epinephrine exposure (24 h) resulted in substantially higher levels of STAT3 total protein, particularly for α_{1D}-AR cells and, to a lesser extent, for α_{1A}-AR expressing cells. We observed no differences in total STAT3 protein levels between epinephrine-stimulated and nonstimulated α_{1B}-AR fibroblasts at either incubation time.

Fig. 4B shows the effect of epinephrine on stimulation of the Ser727 phosphorylated form of STAT3 in Rat-1 fibroblasts expressing individual α_1-AR subtypes. We observed that activation of both the α_{1A}- and α_{1D}-AR subtypes increased the phosphorylation of Ser727 STAT3 in a time-dependent manner. On the other hand, epinephrine incubation of α_{1B}-AR transfected cells consistently reduced the phosphorylation status of Ser727 STAT3 below basal levels (time 0, no agonist). In addition, Fig. 4B shows that epinephrine had no effect on total STAT3 protein levels of α_1-AR subtype transfected Rat-1 fibroblasts at any time point.

α_1-AR Subtype Regulation of gp-130 Protein Levels in Rat-1 Fibroblasts. The effect of α_1-AR subtype activation on total gp-130 protein levels was studied by Western blotting experiments with α_1-AR subtype-transfected and non-transfected fibroblasts that were incubated with epinephrine (10 μM) for 1 and 24 h (Fig. 6A). At 1 h incubation, we observed no differences in gp-130 protein levels between epinephrine-stimulated and nonstimulated fibroblasts (applies to both nontransfected and α_1-AR subtype-transfected fibroblasts). However, we observed that the α_{1B}-AR reduced the levels of gp-130 total protein in an agonist-independent manner compared with nontransfected and α_{1A}- and α_{1D}- cells. This agonist-independent reduction in total gp-130 protein in α_{1B}-AR cells was also apparent at 24 h of epinephrine incubation. Although epinephrine had little or no effect in α_{1A}- and α_{1D}-AR cells after 1 h of treatment, incubation for 24 h almost completely abolished the levels of gp-130 total protein relative to those of nonstimulated cells.

The reduction in levels of total gp-130 protein in epinephrine-stimulated α_{1A}- and α_{1D}-AR cells was characterized in a time-course experiment (Fig. 5B). By 2 and 3 h of epinephrine incubation, a reduction in total gp-130 protein levels was apparent in α_{1A}- and α_{1D}-AR cells, whereas no changes were observed in α_{1B}-AR cells (even when using higher protein loading to compensate for the loss of gp-130 expression by α_{1B}-AR transfection alone). Figure 5C indicates that at 3 h of epinephrine incubation, the observed reduction in total gp-130 protein content in α_{1A}-AR and α_{1D}-AR cells can be blocked by the α_1-AR antagonist prazosin (1 μM).

IL-6 Regulation of STAT3 Activation and gp-130 Levels. To ensure that a correct dose of the neutralizing antibody was used, the IL-6–mediated phosphorylation of Tyr705 was

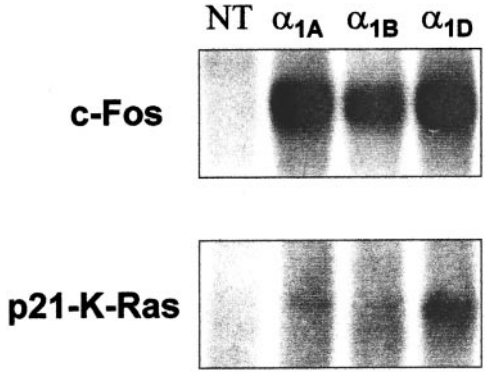

Fig. 2. Effect of α_1-AR subtype-stimulation on steady-state c-fos and p21-cK-Ras mRNA levels in nontransfected Rat-1 fibroblasts (NT) or Rat-1 fibroblasts transfected with α_{1A}-AR, α_{1B}-AR or α_{1D}-AR subtypes. Cells were exposed to 10 μM epinephrine for 60 min, in the presence of β- and α_2-AR blockers, then mRNA was prepared. Northern blot hybridizations were performed using 10 μg of poly(A)$^+$ RNA per lane with c-fos and p21-cK-Ras cDNA probes. Hybridizations and washing conditions were performed as indicated in the text. These results are representative of two individual Northern blot experiments. c-fos mRNA seems to be equally stimulated by all three subtypes, with somewhat lower levels with the α_{1B}-AR, consistent with the microarray results (Table 1). Fold-intensity differences (NIH Image) compared with control are 7.83, 6.13, and 7.58 for the α_{1A}-, α_{1B}-, and α_{1D}-AR, respectively). With p21-cK-Ras mRNA, the α_{1B}-AR does not stimulate, whereas both the α_{1A}- and the α_{1D}-AR do so, with a higher efficacy from the stimulation of the α_{1D}-AR subtype, again consistent with the microarray results (Table 4). Fold-intensity differences (NIH Image) compared with control are 1.9, 1.1, and 2.20 for the α_{1A}-, α_{1B}-, and α_{1D}-AR, respectively).

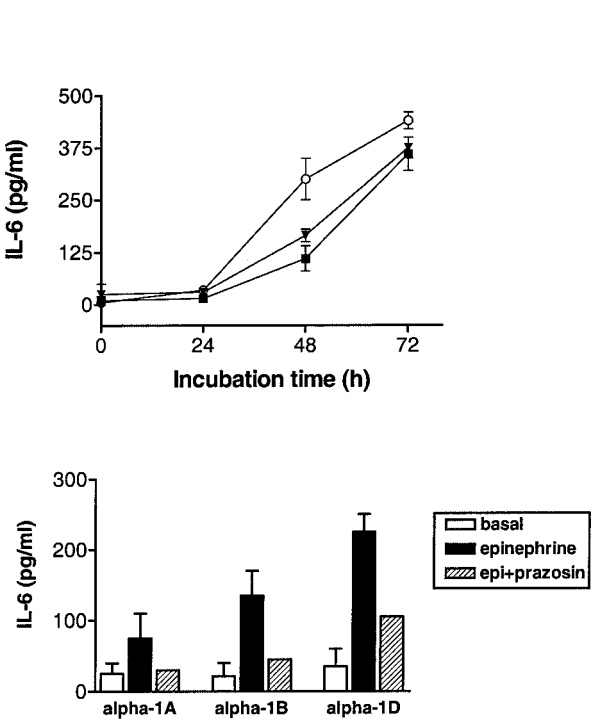

Fig. 3. α_1-AR stimulation of IL-6 secretion in Rat-1 fibroblasts. A, in time-course studies, IL-6 was secreted into the culture medium of α_{1A}-AR (■), α_{1B}-AR (▼), and α_{1D}-AR (○) cells in a time-dependent fashion using a maximum concentration of epinephrine (10 μM). B, selecting the 48-h time point, IL-6 secretion can be stimulated by all three subtypes. IL-6 secretion can be blocked with the α_1-AR antagonist prazosin (1 μM) incubated in the culture medium before and during the addition of epinephrine, demonstrating that the secretion of IL-6 is mediated through stimulation of the α_1-AR receptor subtypes. IL-6 secretion was determined on the parental cells but was negligible.

first used in a dose-response to increasing concentrations of the neutralizing antibody (Fig. 6). Using a range of concentrations from 0.05 μg/ml to 5 μg/ml, only the 5 μg/ml dose provided sufficient blockage of the IL-6 response.

Using an antibody that recognizes the dimerization-dependent Tyr705 phosphorylation state of STAT3, the exogenous addition of IL-6 to the culture medium resulted in increased levels of the phospho-protein in all three α_1-AR subtype cell lines (Fig. 7). This activation could be blocked with the addition of a neutralizing antibody to IL-6 at 5 μg/ml. Interestingly, all three α_1-AR subtypes also caused increases in Tyr705 phosphorylation, including the α_{1B}-AR, which previously did not activate Ser727 phosphorylation. Epinephrine-mediated Tyr705 phosphorylation could not be blocked by the IL-6 neutralizing antibody, suggesting direct effects. This is also supported by the fact that epinephrine and IL-6 together caused a synergistic phosphorylation of Tyr705 but was weak for the α_{1B}-AR.

To determine the effects of IL-6 on the protein levels of gp-130, IL-6 was added to the culture medium for 3 h, a condition in which α_1-ARs cause a down-regulation of the protein (Fig. 8). Whereas α_{1A}- and α_{1D}-AR activation cause the down-regulation of gp-130, the addition of IL-6 caused an increase in gp-130 levels. The α_{1B}-AR cell line, as before, shows no changes in gp-130 levels and is persistently down-regulated because five times the protein needed to be loaded

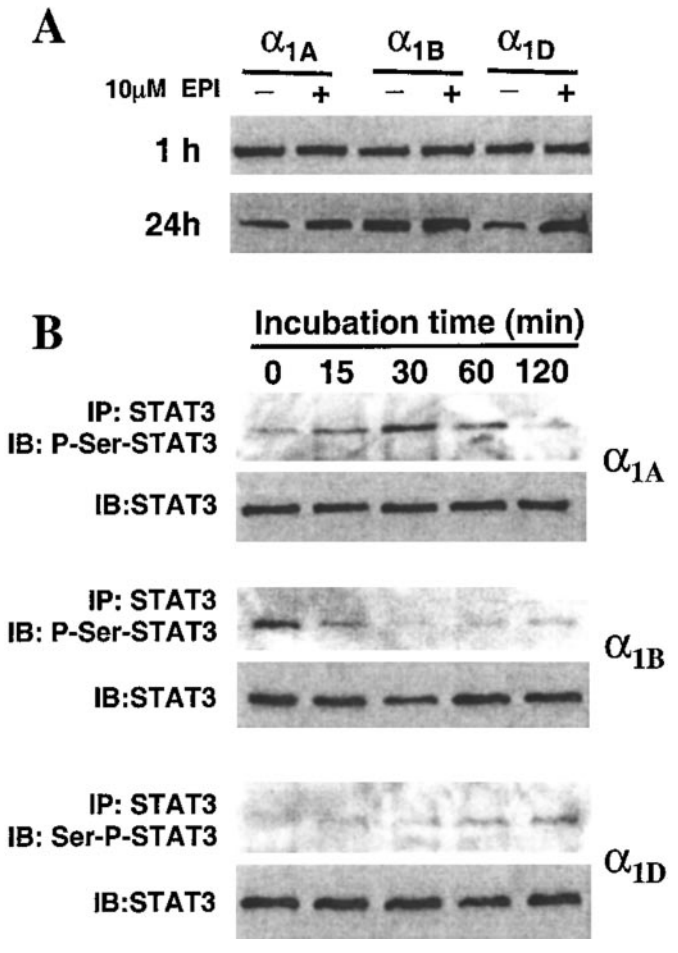

Fig. 4. Time-course studies of α_1-AR subtype stimulation on total STAT3 protein (A) and transcriptional function of STAT3 (Ser727-phosphorylation) (B) in Rat-1 fibroblasts. Total STAT3 protein levels were compared between subtype-stimulated (+) and nonstimulated (−) cells in Western blotting experiments with an antibody to STAT3. Total STAT3 levels increased upon 24-h incubation with the α_{1A}- (1.70×) and α_{1D}-AR subtypes (2.70×) but were unchanged for the α_{1B}-AR (1.11×). Fold-changes in intensities are determined by NIH Image software using nonstimulated as the control. Active STAT3 was detected using an antibody that only recognizes the Ser727-phosphorylated form of STAT3 afterSTAT3 immunoprecipitation (IP). Both the α_{1A}- (fold time-course intensities: 1, 1.65, 5.84, 3.93, and 1.42) and α_{1D}-AR (fold time-course intensities: 1, 0.61, 1.25, 2.74, and 3.72) increase the transcriptionally active form of STAT3 upon stimulation. However, the α_{1B}-AR (fold time-course intensities: 1, 0.47, 0.41, 0.33, and 0.28) decreases its formation.

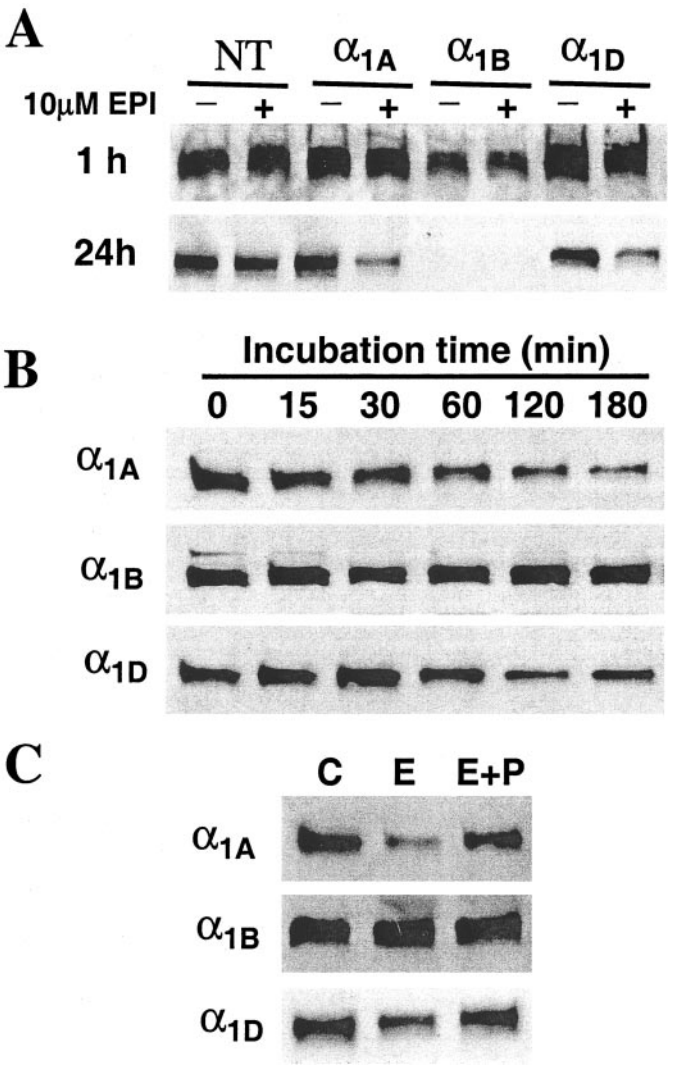

Fig. 5. α_1-AR subtype regulation of gp-130 protein levels in Rat-1 fibroblasts. A, individual α_1-AR subtypes were stimulated in the presence or absence of 10 μM epinephrine (with β- and α_2-AR blockers) for either 1 or 24 h. Although no changes were observed at 1 h, both the α_{1A}- (fold-intensity change, 0.36) and the α_{1D}-AR (fold-intensity change, 0.47) decreased gp-130 levels at 24 h, whereas the α_{1B}-AR displayed neither basal nor stimulated levels of the protein. Fold changes in intensities are determined by NIH Image software using nonstimulated as the control. B, time course studies of epinephrine-stimulation revealed that although the α_{1B}-AR stimulation of gp-130 levels (time-course intensity changes: 1, 0.91, 1.03, 1.02, 1, and 0.99) was not changed, the α_{1A}- (time-course intensity changes: 1, 0.85, 0.67, 0.55, 0.36, and 0.30) and the α_{1D}-AR subtype (time-course intensity changes: 1, 1.15, 1.26, 0.87, 0.44, and 0.54) displayed time-dependent decreases, consistent with A. The α_{1B}-AR lane is overloaded by 5-fold for visualization of the protein. C, the decrease in gp-130 protein levels by α_{1A}- and α_{1D}-AR stimulation can be reversed with the α_1-AR antagonist prazosin (1 μM), suggesting mediation through stimulation of the α_1-AR.

to observe a band. Interestingly, the IL-6–mediated effects on gp-130 were abolished in the α_{1B}-AR cell line. With α_{1A}- or α_{1D}-AR activation, the addition of both epinephrine and IL-6 down-regulated gp-130 levels, but neither effect could be reversed by the addition of the IL-6 neutralizing antibody.

The combination of epinephrine and IL-6 together was also probed with the anti-phosphoSer727 antibody (Fig. 9). Although both α_{1A}- and α_{1D}-AR activation increased the phosphorylation of Ser727, α_{1B}-AR activation did not, as shown in Fig. 4. The addition of IL-6 by itself also increased Ser727 phosphorylation as expected, but the combination of the two systems did not produce the synergism seen with Tyr705 phosphorylation.

Discussion

Although much effort has focused on the regulation of α_1-AR subtype structure-function relationships and activation of common signaling pathways, no study has directly explored the comprehensive regulation of gene expression downstream of each subtype in the same cellular environment. This study represents the first microarray analysis of a GPCR in a transfected cell system. A common question raised in the GPCR field is whether each subtype can couple to different functions, or are they merely degenerate receptors? With the use of microarrays, we obtained the gene expression profiles generated by the α_1-AR subtypes (α_{1A}-, α_{1B}-, and α_{1D}-) (epinephrine-treated) stably expressed in Rat-1 fibroblasts, and compared them with those of control cells (epinephrine-treated, nontransfected Rat-1 fibroblasts). We found that of 7000 genes in the microarray, epinephrine modified the expression of a total of 47, 55, and 53 genes by at least 2-fold relative to nontransfected controls for the α_{1A}-, α_{1B}-, and α_{1D}-ARs, respectively. Of these, a profile was established that was highly similar to the three α_1-AR subtypes (38 genes; Tables 1 and 2). However, minor profiles that were unique to each α_1-AR subtype (Table 3) and a profile (IL-6 signaling genes) activated by two but not all three subtypes (Table 4) were also produced. The changes in gene expression, which were invoked by a 1-h activation protocol, are likely to be different from profiles generated from sustained activation. We chose the 1-h stimulation time point because many second-messenger studies use this same condition.

However, a limitation of our system is the use of a separate parental cell line that lacks the α_1-AR as the control instead of using each stably-transfected cell line as its own internal control of stimulated versus nonstimulated transcription. We did this for practical reasons. Therefore, with our experimental system, we cannot rule out gene expression changes that are caused by differences in cell line propagation. However, these should be rare events. Verification of the protein changes, however, used stimulated versus nonstimulated conditions within the same cell line. Along these same lines, we cannot rule out effects that are caused by subtype-specific

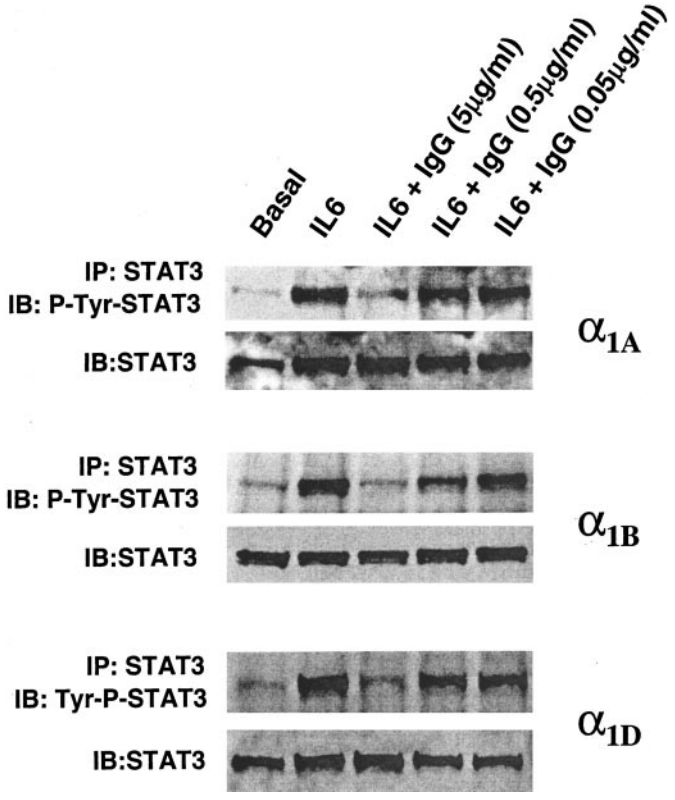

Fig. 6. Dose-dependent antagonism of IL-6–mediated tyrosine phosphorylation of STAT3 with a neutralizing antibody against IL-6. IL-6 (40 ng/ml) was added to the culture medium of each α_1-AR subtype cell line for 30 min in the presence or absence of an IL-6–neutralizing antibody at the indicated dose. Cell lysates were probed for STAT3 activation using an antibody that recognizes the Tyr705 phosphorylated form of STAT3. The blots were stripped and reprobed for total STAT3 protein levels. A level of 5 μg/ml of the neutralizing antibody was found to block IL-6 function.

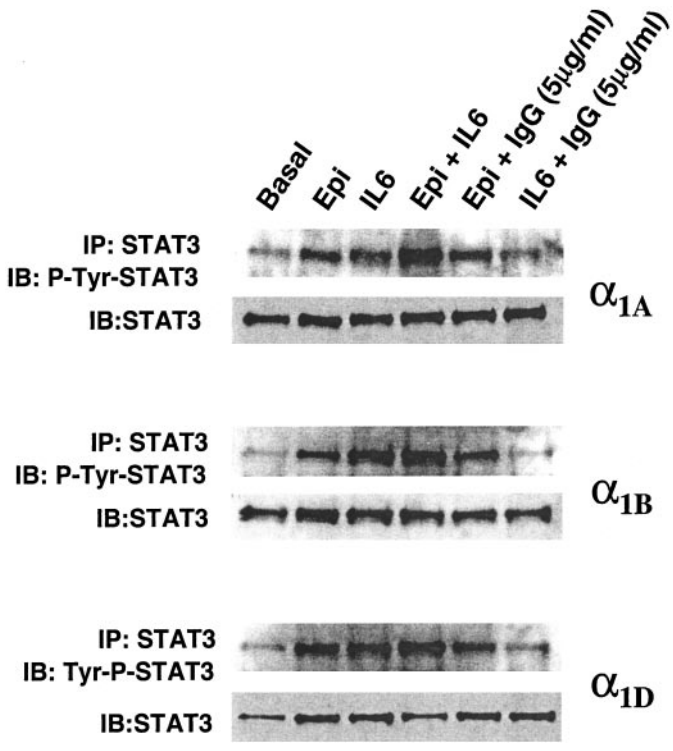

Fig. 7. α_1-AR- and IL-6–mediated activation of STAT3 tyrosine-phosphorylation. Epinephrine (10 μM) and/or IL-6 (40 ng/ml) were added to the culture medium of each of the three α_1-AR subtype cell lines for 30 min in the presence or absence of the IL-6 neutralizing antibody (5 μg/ml). Cell lysates were probed for total STAT3 and Tyr705 phosphorylation status. All three α_1-AR subtypes and IL-6 activated Tyr705 phosphorylation of STAT3. The neutralizing antibody only blocked the IL-6 activation. The combination of epinephrine and IL-6 was synergistic for only the α_{1A}- and α_{1D}-AR subtypes.

but promiscuous couplings, because expression of the receptors is above physiological levels.

In these signaling studies, we used the endogenous α_1-AR ligand epinephrine and Rat-1 fibroblasts, a cell model commonly used in α_1-AR signaling studies (Garcia-Sainz et al., 1998; Chen et al., 1999), which we found to express similar densities of α_1-AR subtypes. Besides gene expression, another measure of α_1-AR function in Rat-1 fibroblasts included the activation of total IP accumulation by epinephrine. As expected, epinephrine stimulated the accumulation of IPs with higher efficacy in α_{1A}-AR fibroblasts than the other two subtypes, consistent with previous studies of α_1-AR subtype-stimulated second messenger activation (reviewed in Zhong and Minneman, 1999). Interestingly, the gene expression changes in common between the three α_1-AR subtypes had similar fold changes, suggesting that the efficacy of the IP response does not influence the degree of expression changes. This could be because the IP effectors were saturated for all three subtypes, and the excess IP response may not be physiologically relevant. On the other hand, it may also suggest that a non-IP signaling molecule(s) may be responsible for the gene expression changes.

The largest profile generated by the α_1-AR subtypes was characterized by gene expressions commonly activated or repressed by all three α_1-AR subtypes (Tables 1 and 2), suggesting that a certain level of redundancy among α_1-AR subtypes exists. Several of these genes have been previously associated with α_1-AR activation in primary cell lines and tissues. For example, increases in mRNA levels for c-*fos*, an immediate-early gene that was highly up-regulated in the present study, have been reported after α_1-AR activation of cardiomyocytes (Deng et al., 1998), vascular smooth muscle cells (Okazaki et al., 1994), cerebral cortex (Shen and Gundlach, 2000), aortic rings (Carcillo and Hough, 1995), and hepatocytes (Im et al., 1998). α_1-AR activation in cardiomyocytes also results in activation of c-jun and egr-1 (Iwaki et al., 1990; Jin et al., 2000), both up-regulated in the present study. α_1-AR–mediated signaling in hepatocytes, renal tubular cells, and cardiomyocytes invoke protein tyrosine phosphatases and calcineurin (Aperia et al., 1992; Nguyen and Gao, 1999; Sugden, 2001). Furthermore, NF-1-X, a transcription factor up-regulated in this study, has been shown to bind to the α_{1B}-AR promoter and regulate the expression of the α_{1B}-AR gene (Gao and Kunos, 1998). Overall, evidence from the literature provides a certain measure of confirmation of the observed changes in the microarray and suggests that α_1-AR signals in Rat-1 fibroblasts can extend to other cell types and tissues. However, as an independent test of confirmation, we measured by Northern blotting the expression levels of two genes that showed significant changes in expression in the microarray. We found that the expression profiles of both c-fos and p21-cK-Ras were similar to actual mRNA levels (Fig. 2), but the increased sensitivity of the microarray analysis does lead to higher fold-changes.

A few additional genes that were commonly changed in expression by α_1-AR subtypes have never been associated with α_1-AR signaling (Table 1). These code for proteins that

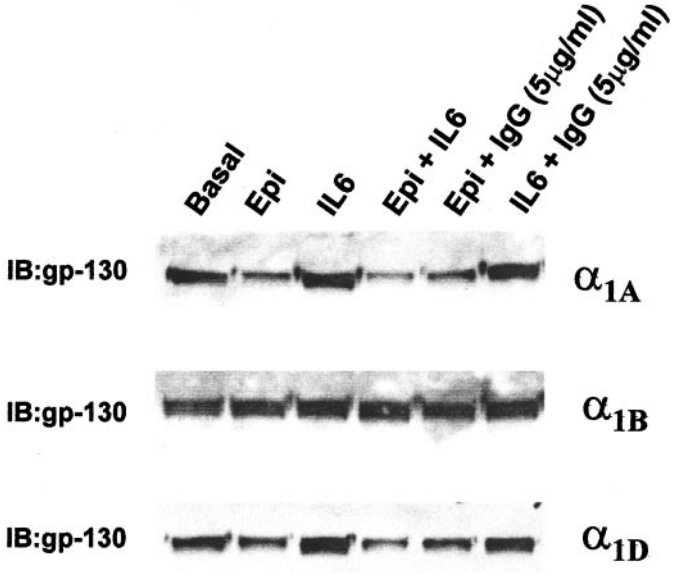

Fig. 8. α_1-AR- and IL-6–mediated effects on gp-130 protein levels. Epinephrine (10 μM) and/or IL-6 (40 ng/ml) were added to the culture medium of each of the three α_1-AR subtype cell lines for 3 h in the presence or absence of the IL-6 neutralizing antibody (5 μg/ml). Cell lysates were probed for gp-130 protein levels. The α_{1B}-AR lane is overloaded by 5-fold for visualization of the protein. At 3 h of incubation, epinephrine caused the down-regulation of gp-130 protein levels for the α_{1A}- and α_{1D}-ARs but was constitutively down-regulated by the α_{1B}-AR, as shown in Fig. 5. The IL-6 neutralizing antibody could not block this effect. However, the addition of IL-6 caused the up-regulation of gp-130, which was not blocked by the neutralizing antibody. The combination of epinephrine and IL-6 caused a synergistic effect on gp-130 by both the α_{1A}- and α_{1D}-AR subtypes but not the α_{1B}-AR. The α_{1B}-AR cell line was unresponsive to any treatment.

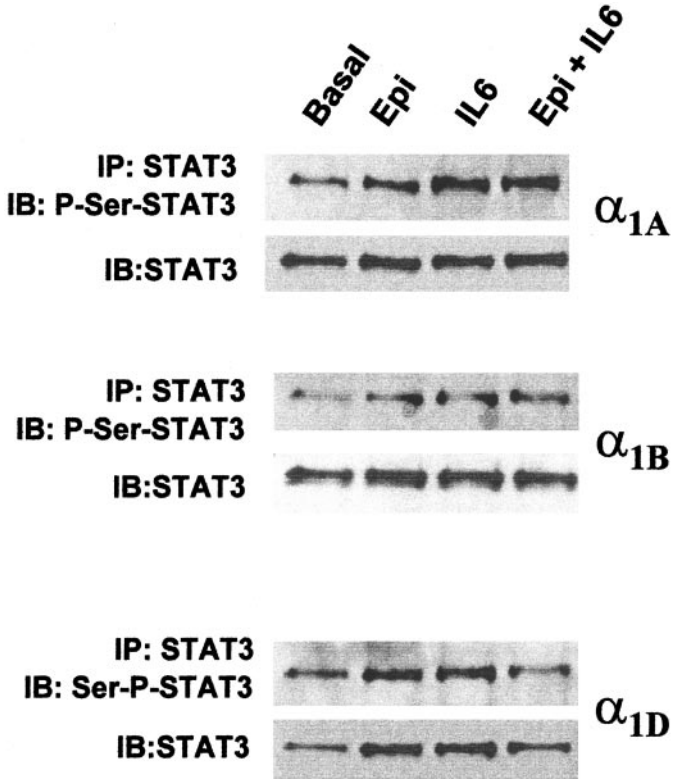

Fig. 9. α_1-AR- and IL-6–mediated effects on STAT3 Ser727 phosphorylation levels. Epinephrine (10 μM) and/or IL-6 (40 ng/ml) were added to the culture medium of each of the three α_1-AR subtype cell lines for 30 min. Cell lysates were probed for total STAT3 and Ser727 phosphorylation status. As before, the α_{1A}- and α_{1D}-AR activation increased Ser727 phosphorylation status, whereas α_{1B}-AR activation did not. The addition of IL-6 also increased Ser727 phosphorylation but was not synergistic with the α_1-AR.

are primary regulators in cAMP-mediated responses (cAMP response element modulator protein and C/EBP-like factor), angiogenesis (VEGF and glioma-derived VEGF), fatty acid metabolism (stearoyl-CoA-desaturase 2), and formation of extracellular matrix (fibronectin, collagen III, and collagenase).

The existence of gene expression changes that were unique to each α_1-AR subtype suggests that nonredundant functions may exist (Table 3). One of the gene expressions that is the most robustly repressed in our study was synuclein (unique to the α_{1B}-AR), which we have recently shown to be abnormally modified in transgenic mouse brains overexpressing this subtype (Papay et al., 2002). In addition, the modification of proapoptotic genes and those associated with neurodegeneration, including tau, synuclein, transforming growth factor β3, and caspase-6 by the α_{1B}-AR subtype, is also supported by a neurodegenerative and apoptotic phenotype in the same transgenic mouse model (Zuscik et al., 2000), as well as a microarray analysis in transgenic brains (J. Yun, R. J. Gaivin, A. Boongird, Z. Ying, P. J. Gonzalez-Cabrera, R. S. Papay, I. Najm, and D. M. Perez, submitted), which contain caspase-3–mediated apoptosis, or in the heart, which contained terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive myocytes (Yun et al., 2003).

Gene expressions can be grouped into a third profile in Rat-1 fibroblasts through the activation of two but not three subtypes (Table 4) of the prototypic members of the IL-6 signaling pathway (STAT3 and gp-130). IL-6 is an endocrine cytokine that is secreted from fibroblasts, myocytes, and other cell types after inflammatory and stressful stimuli; its receptor mechanism includes the signal-transducing receptor gp-130. IL-6 signals through the JAK/STAT pathway induced by gp-130 (Taga and Kishimoto, 1997). Our results indicate that protein levels for gp-130 and STAT3 were differentially regulated by the three α_1-AR subtypes. Recent evidence suggests that increased protein levels of STAT1 by itself, which is not phosphorylated, is sufficient to drive the transcription of a subset of genes (Chatterjee-Kishore et al., 2000a,b), suggesting that α_1-AR–mediated increases in STAT3 protein may be physiologically relevant. Although the microarray studies successfully predicted changes in the protein levels for IL-6, gp130, and STAT3, the activational status for these proteins was more complicated. Our data showed that although all three subtypes stimulated the synthesis and secretion of IL-6 in Rat-1 fibroblasts (Fig. 3), only α_{1A}- and α_{1D}-AR subtypes increased both the protein level and the Ser727 phosphorylated form of STAT3 (Fig. 4), whereas α_{1B}-AR did not. Figure 6 confirms that α_1-AR activation of STAT3 could be direct; at the very least, however, it occurs through non–IL-6 effects because a neutralizing antibody to IL-6 could not block it. Direct effects are also suggested by the synergistic stimulation of Tyr705 phosphorylation by both epinephrine and IL-6. However, this synergistic effect with IL-6 was weak or nonexistent with α_{1B}-AR activation. The synergistic activation of STAT3 may have implications in heart failure, where there are elevations in both circulating catecholamines and IL-6. However, the most interesting effect was the α_{1B}-AR activation of Tyr705 phosphorylation (Fig. 7) but not of Ser727 phosphorylation (Figs. 4 and 9). There is precedent for the differential phosphorylation of STATs. IL-1 has been shown to induce the phosphorylation of STAT1 on Ser727 but not Tyr 701 (H. Nguyen, M. Chatterjee-Kishore, Z. Jiang, Y. Qing, C. V. Ramana, J. Bayes, M. Commane, X. Li, and G. R. Stark, submitted).

STAT3 is activated by tyrosine phosphorylation at Tyr705, which induces dimerization, nuclear translocation, and DNA binding (Darnell et al., 1994). Transcriptional activation seems to be stimulated by serine phosphorylation at Ser727, apparently through MAPK or mammalian target of rapamycin (mTOR) pathways (Wen et al., 1995; Yokogami et al., 2000). Both phosphorylation events are not coupled but are independent of one another, although both are thought to be involved in transcriptional processes to achieve maximal effects (Wen et al., 1995). In the same Rat-1 fibroblasts that we used here, it has been shown previously that the α_1-AR subtypes can activate the MAPK pathways differentially with α_{1B}-AR activation, resulting in increased p38 activity but not JNK activity, whereas the α_{1D}-AR activated JNK but not p38 (Waldrop et al., 2002). In addition, α_{1B}-AR–mediated inhibition of STAT3 in hepatocytes was found to be determined by p42/44 MAPK (Nguyen and Gao, 1999). These differences in MAPK activation could be the determining factor why the α_{1B}-AR does not phosphorylate Ser727. Because the combination of epinephrine and IL-6 is not synergistic with Ser727 phosphorylation (Fig. 9), this also suggests that the two pathways converge at Ser727 phosphorylation, unlike Tyr705 phosphorylation. Nevertheless, the ability of the α_{1B}-AR to suppress Ser727 phosphorylation is likely to produce differences in the transcriptional activity and eventual biological activity of STAT3.

Interestingly, whereas gp-130 protein levels were inhibited by long-term α_{1A}- or α_{1D}-AR stimulation (Fig. 5), they were constitutively down-regulated by the α_{1B}-AR. Stimulation with IL-6 produced the opposite effect by increasing gp-130 protein levels (Fig. 8). The IL-6 neutralizing antibody could inhibit neither the epinephrine response nor the IL-6 response, suggesting that the IL-6–mediated changes in gp-130 protein levels are not a result of its biological function or signaling. However, the α_1-AR–mediated effects on gp-130 could be blocked with prazosin (Fig. 5C). The combination of both epinephrine and IL-6 were again synergistic, displaying even greater changes in gp-130 protein levels than either alone, suggesting independent events. The nonsignaling mechanism of the IL-6–mediated changes in gp-130 protein levels and the lack of α_1-AR–mediated IL-6 coupling, suggests that α_1-AR- and IL-6–mediated effects on gp-130 protein are different. α_1-AR–mediated changes are probably caused primarily by direct transcriptional events, as supported by the microarrays, and are not caused by activational paradigms with IL-6. However, the IL-6–mediated changes in gp-130 are probably caused by protein stability through complex formation (i.e., with IL-6/IL-6R/gp-130 trimer), which does not require IL-6 activation.

IL-6 is reported to increase in astrocytes and hepatocytes upon α_1-AR stimulation (Norris and Benveniste, 1993; Jung et al., 2000), confirming that our microarray data can be translated to endogenous systems. In addition, the α_{1B}-AR transgenic mouse model also constitutively down-regulated gp-130 protein levels in the heart (Yun et al., 2003), again confirming the fibroblast results. Confirmatory for the α_{1A}-AR–mediated increases in STAT3, Ser727 phosphorylation is also seen in the normal rat myocyte (P. J. Gonzalez-Cabrera, J. Yun, B. R. Rorabaugh, D. F. McCune, and D. M. Perez, in preparation), which is composed of both the α_{1A}- and α_{1B}-AR

subtypes, but heart function is thought to be mainly driven by the α_{1A}-AR (Lin et al., 2001). α_1-ARs as well as the Angiotension AT1 receptor have been previously linked to JAK/STAT activation (McWhinney et al., 1997; Zhong et al., 2000). Although there is controversy about whether this is caused by a direct coupling of the receptors to JAK/STAT, our data suggest at least a non–IL-6–mediated mechanism, because the IL-6–neutralizing antibody could not block STAT3 activation.

One of the most striking features of gene expression by α_1-AR subtypes in the present study was the appearance of numerous genes with known roles in the processes relevant to cellular growth or hypertrophy. A major effect of α_1-AR stimulation in the heart is the activation of growth-promoting pathways that lead to cardiac hypertrophy or vascular cell proliferation (Varma and Deng, 2000). Activation of all three α_1-AR subtypes increased the transcription of three potent inducers of myocyte hypertrophy (IL-6, LIF, and calcineurin), whereas the α_{1B}-AR seems to inhibit STAT3 Ser727 phosphorylation and possibly gp-130–mediated pathways. The inhibition of both STAT3 and gp-130 protein levels has been implicated in wall thickening, heart failure, and apoptosis (Hirota et al., 1999). This suggests that the α_{1B}-AR may not be as potent an inducer of hypertrophy as the other two α_1-AR subtypes. This was also suggested in Yun et al. (2003), in which many genes/proteins associated with hypertrophy were down-regulated in the heart by the α_{1B}-AR in a transgenic mouse model. Although pharmacological studies have implicated the α_{1A}-AR subtype in mediating the growth of rat myocytes (Rokosh et al., 1996), α_{1A}-AR transgenic mice models have discarded this subtype as the sole mediator of hypertrophy (Lin et al., 2001; Rokosh and Simpson, 2002). Other studies have shown that overexpressed α_{1B}-AR constitutively active mutants manifest a mild hypertrophic phenotype (Milano et al., 1994; Zuscik et al., 2001), whereas no evidence exists for the α_{1D}-AR subtype in mediating any heart function (Tanoue et al., 2002). One study has even suggested that both the α_{1A}- and α_{1B}-AR subtypes need to be coactivated to achieve the previously reported robust hypertrophy phenotype seen in cultured cell models (McWhinney et al., 2000). Thus, although the subtypes may be differentially coupled to blood pressure homeostasis and induction of cardiac hypertrophy, it still seems that more than one α_1-AR subtype might be needed to mediate the same function.

Recent evidence indicates that vascular fibroblasts that make up the adventitial layer in blood vessels express functional α_1-AR subtypes (Faber et al., 2001; Zhang et al., 2002). These fibroblasts respond to catecholamines by activating growth and migratory pathways (vascular remodeling) as seen in vascular injury models. Here, too, gene expression modified by α_1-ARs in Rat-1 fibroblasts may be part of a coordinated regulation of groups of genes (i.e., IL-6, collagenase, VEGF, and fibronectin) whose products act in the remodeling (apoptosis, migration, extracellular matrix changes) process of vessels. Another gene cluster modified by the α_1-AR subtypes not reported here includes cell-cycle regulatory genes (i.e., cyclins, cyclin-dependent kinases, cyclin-dependent kinase inhibitors, etc.) that may be involved in differential α_1-AR subtype coupling to proliferative responses. In this cluster, we observe that the α_{1B}-AR subtype signals differently from α_{1A}- and α_{1D}-AR cells, which display identical profiles with respect to this cluster (P. J. Gonzalez-Cabrera, J. Yun, B. R. Rorabaugh, D. F. McCune, and D. M. Perez, in preparation).

In conclusion, we show an in-depth comprehensive comparison of the gene expression changes caused by the α_1-AR subtypes. Although most of the gene expression changes were similar among the subtypes, some subtype-specific profiles were generated. This was verified at the protein and/or activational level for IL-6, gp-130, and STAT3 and suggests α_1-AR subtype-dependent differential coupling to pathways that can mediate hypertrophy and vascular remodeling.

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