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The Impact of Blunted β-Adrenergic Responsiveness on Growth Regulatory Pathways in Hypertension

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ABSTRACT

The effects of vasodilator hormones acting through receptors linked to adenylyl cyclase are impaired in the hypertensive state. This has been ascribed to impaired receptor-G protein coupling. However, these receptors also act via effectors *not* linked to adenylyl cyclase activation. These "alternate" mechanisms may be especially important in growth regulation and might be unaffected (or enhanced) with G protein-coupled receptor-G protein uncoupling. Therefore, we assessed the effects of β-adrenergic activation on 1) regulation of phosphatidylinositol 3-kinase (PI3 kinase) and extracellular signal-regulated kinase (ERK) activation—two tyrosine kinase-dependent enzymes linked to cell growth—and 2) microarray analysis in vascular smooth muscle cells from spontaneously hypertensive rats (SHR). Isoproterenol-stimulated phosphorylation of ERK1/2 was impaired in SHR. The effect of forskolin was unaltered. In contrast, both vasopressin and angiotensin 2-mediated stimulation of ERK activation was enhanced in SHR. In addition, β-adrenergic-mediated inhibition of PI3 kinase activity was attenuated in SHR (whereas the effect of forskolin remained intact). In microarray studies, the effect of isoproterenol to regulate transcription was significantly impaired in SHR (as was the effect of forskolin). Together, these data support the hypothesis that the blunted vasodilator effects of hormones linked to adenylyl cyclase activation are an index of a more generalized impairment in modulating growth regulatory pathways. Furthermore, this study supports the hypothesis that the blunting of β-adrenergic responses relating to increased G protein-coupled receptor kinase 2 expression reflects a "generalized uncoupling" of β-adrenergic-mediated responses and do not support the concept of "enhanced coupling" of "alternate" pathways of β-adrenergic growth regulatory pathways in the hypertensive state.

The increase in vascular resistance characteristic of hypertension is mediated by alterations in both structural (hypertrophy/hyperplasia) and functional determinants (e.g., vascular signaling mechanisms). We (and others) have suggested that the functional defect leading to increased vascular resistance reflects an imbalance between vasoconstrictor and vasodilator mechanisms (Feldman and Gros, 1998). We have focused on the hypothesis that impaired receptor-mediated vasodilation may contribute. In support of this hypothesis, we identified both in human hypertension and in experimental models that the action of G protein-coupled receptors (GPCRs) linked to adenylyl cyclase is attenuated. The prototype GPCR examined in these studies was the β-adrenoceptor. However, this defect is common for a range of GPCRs commonly linked to adenylyl cyclase activation through G protein (G_s) (Feldman and Gros, 1998).

The impairment in GPCR-mediated adenylyl cyclase activation in hypertension has been characterized as an "uncoupling" of the receptor from its G_s (Feldman and Gros, 1998). This has been related to an increased expression of GRK2 (an enzyme that mediates phosphorylation of agonist-occupied GPCRs and consequent uncoupling from its linked G protein). However, it has been appreciated that GPCRs, like the β-adrenoceptor, may have multiple G protein linkages not related to adenylyl cyclase activation (e.g., to G_i) and may also be linked to other effector pathways (Daaka et al., 1997).

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; PI3 kinase, phosphatidylinositol 3-kinase; ERK, extracellular signal-regulated kinase; PKA, protein kinase A; WKY, Wistar Kyoto; SHR, spontaneously hypertensive rat(s); DMEM, Dulbecco's modified Eagle's medium; VSMC, vascular smooth muscle cell; ISO, isoproterenol; FSK, forskolin; PDGF, platelet-derived growth factor; PBS, phosphate-buffered saline; EST, expressed sequence tag; SSC, standard saline citrate; CRE, cAMP response element.

For example, in cardiomyocytes, β-adrenergic antiapoptotic effects have been reported to be mediated via both cAMP-dependent and G_i-dependent coupling to phosphatidyinositol 3-kinase (PI3 kinase) (Chesley et al., 2000; Leblais et al., 2004). Furthermore, a linkage between GPCRs and β-arrestin has been demonstrated, which, in some models, mediates src kinase and ERK activation (Luttrell et al., 1997). Notably several of these linkages (i.e., to G_i and to β-arrestin) are enhanced by increased GRK activity (and/or PKA activity) and consequent GPCR phosphorylation (Shenoy and Lefkowitz, 2003). Thus, whether the uncoupling of GPCRs, like the β-adrenoceptor, represents a global reduction in responses or a "selective" uncoupling of G_s-linked responses (with potentially enhanced non-G_s-mediated responses) is unknown.

The impact of alterations in "coupling" of GPCRs linked to adenylyl cyclase activation has most commonly been studied in the context of short-term vascular re-sponses—primarily by assessing alterations in vasodilatory responses. However, it has also been appreciated that GPCR-mediated adenylyl cyclase activation might have an important role in longer term regulation of vascular growth. Elevations in intracellular cyclic AMP may be either growth inhibitory or growth stimulatory in vascular smooth muscle cells, dependent on the context (Nakaki et al., 1990). The impact of any potential shift in coupling in hypertension from G_s-linked adenylyl cyclase activation to activation of other pathways (e.g., G_i- and β-arrestin-linked pathways) on the "net" growth-modulating effects of β-adrenoceptor activation in the hypertensive state is unknown. Therefore, we have examined the effect of hypertension on 1) β-adrenergic and 2) adenylyl cyclase-mediated activation of growth regulatory pathways (ERK and PI3 kinase) as well as on the regulation of gene expression as assessed by microarray analysis in vascular smooth muscle cells from normotensive and hypertensive rats. Data demonstrate that the effects of β-adrenergic-mediated regulation of both ERK and PI3 kinase are blunted in hypertension. More importantly, there is a blunted effect of β-adrenoceptor activation on early expression of genes important in cell growth. In net, we would conclude that in the hypertensive state there is a global attenuation of the growth regulatory effects of β-adrenoceptor activation that could be an important determinant in the dysregulation of vascular smooth muscle growth characteristic of the hypertensive state. Furthermore, these data do not support an important role of "enhanced β-adrenergic coupling" via "alternate" pathways in the growth regulatory effects of β-adrenoceptor activation.

Materials and Methods

Animal Protocol. Ten- to 12-week-old male normotensive Wistar Kyoto (WKY) rats and spontaneously hypertensive rats (SHR) (Harlan, Indianapolis, IN) were used. The rats were cared for in accordance with Canadian Council on Animal Care guidelines and housed under a 12-h light/dark cycle with free access to standard laboratory chow and drinking water. Indirect tail-cuff measurements of systolic blood pressure were obtained in lightly anesthetized rats as described previously (Gros et al., 1994, 2000). Mean systolic pressures in SHR were significantly higher compared with WKY rats (SHR: 169 ± 4 mm Hg, $n = 10$; WKY: 113 ± 2 mm Hg, $n = 11$; $p < 0.001$).

Vascular Smooth Muscle Cell Primary Cultures. Rat aortic vascular smooth muscle cells primary cultures from SHR and WKY rats were isolated by a modification of the methods of Touyz et al. (1994). In brief, freshly isolated thoracic aortae from both normotensive and hypertensive rats were concurrently digested using collagenase and elastase incubations as described previously (Touyz et al., 1994). After digestion/isolation, vascular smooth muscle cells were resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, gentamicin, and fungizone. For all the experiments, cells were used from the third to the 12th passages.

Experimental Protocol. Cells were serum starved for 24 h (DMEM supplemented with 0.1% bovine serum albumin, gentamicin, and Fungizone) before experimental treatment with drugs.

After serum starvation, VSMCs were incubated in the absence or presence of isoproterenol (ISO; 10 μM), forskolin (FSK; 10 μM), angiotensin II (1 μM), vasopressin (1 μM), and platelet-derived growth factor BB (20 ng/ml) for the times indicated in the figure legends. Vascular smooth muscle cells were then used for various experimental protocols as described below.

Assessment of Adenylyl Cyclase Activity. Adenylyl cyclase activity in response to isoproterenol (100 μM) or forskolin (10 μM) was determined by the rate of conversion of [α-^{32}P]ATP to [^{32}P]cAMP as reported previously (Gros et al., 1994, 2000). In brief, digitonin-permeabilized vascular smooth muscle cells were resuspended in a solution of Hanks' balanced salt solution with 33 mM HEPES, 0.5 mM EDTA, and 1 mM magnesium sulfate, pH 7.4 at 4°C, added in an aliquot of 40 μl to give a final incubation volume of 100 μl with 1 μCi of [α-^{32}P]ATP, 0.3 mM ATP, 2 mM $MgSO_4$, 0.1 mM cAMP (used in lieu of a phosphodiesterase inhibitor), 5 mM phosphoenol pyruvate, 40 μg/ml pyruvate kinase, and 20 μg/ml myokinase. Incubations were carried out at 37°C for 10 min and terminated by addition of 1 ml of a solution containing 100 μg of ATP, 50 μg of cAMP, and 15,000 cpm of [^{3}H]cAMP. Cells were pelleted by centrifugation at 300*g* for 5 min. cAMP was isolated from the supernatant by sequential Dowex and alumina chromatography and was corrected for recovery with [^{3}H]cAMP as the internal standard. Adenylyl cyclase activity was linear with time and cell number over the ranges used.

Isoproterenol-mediated responses were assessed with the addition of GTP (100 μM). Forskolin-mediated responses were assessed in the presence of $MnCl_2$ (10 mM). As in our previous studies, the extent of isoproterenol-mediated adenylyl cyclase activity was expressed relatively (i.e., as a fraction of forskolin-stimulated activity), thereby minimizing the coefficient of variation seen in the comparison of absolute levels of adenylyl cyclase activity.

Arborization of Vascular Smooth Muscle Cells in Response to Drug Treatment. Short-term β-adrenergic effects on contractile function were determined by assessment of the extent of vascular smooth muscle arborization mechanism (Nabika et al., 1985, 1988). The arborization response mediated by elevations of cAMP has been linked to cytoskeletal changes, including reorganization of actin fibers (Westermark and Portor, 1982; Ben-Ze'ev and Amsterdam, 1987) and assembly of microtubules (Nabika et al., 1985) Vascular smooth muscle cells were cultured onto 35-mm dishes. Plates were placed in a temperature-controlled chamber maintained at 37°C (Bionomic controller; 20/20 Technology, Inc., Wilmington, NC) on an inverted microscope (Axiovert S100; Carl Zeiss, Thornwood, NY). Smooth muscle cell arborization was induced by the addition of isoproterenol (1–100 μM) or forskolin (10 μM). To assess the reversibility of the arborization process, the β-adrenergic antagonist propranolol (1 μM) was added during isoproterenol-induced arborization (i.e., the reversibility of arborization), or vascular smooth muscle cells were pretreated with propranolol for 30 min before isoproterenol stimulation. Progression of arborization was evaluated using time-lapse video microscopy with a digital recording system. Images were obtained every minute and the extent of arborization was assessed by determining the change in image intensity using the threshold setting within the image analysis software (Northern Eclipse 6.0; Empix Imaging, Toronto, ON, Canada). The change in image intensity was expressed as a percentage of basal intensity (before the addition of drug). The change in image intensity was

plotted against time, and slopes were determined from linear regression analysis using Prism 4.0 (GraphPad Software Inc., San Diego, CA).

Vascular Smooth Muscle Cell Proliferation. Vascular smooth muscle cells were cultured in 24-well plates and serum starved for 24 h before experimentation. Vascular smooth muscle cells were incubated in the absence or presence of platelet-derived growth factor-BB (PDGF; 20 ng/ml) or with PDGF in the absence or presence of isoproterenol (100 μM). After 21 h, [^{3}H]thymidine (1 μCi/ml; Valeant Pharmaceuticals, Costa Mesa, CA) was added for an additional 3 h before harvest. After incubation, the medium was aspirated, and the cells washed three times with ice-cold PBS, 10% trichloroacetic acid and then with distilled water and allowed to air dry. Cells were solubilized with 1 ml of 1% SDS, and radioactivity of each sample was determined by liquid scintillation spectrometry.

Immunoblotting. After drug treatment, vascular smooth muscle cells were washed twice with ice-cold PBS and directly lysed in a buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.3% Nonidet P-40, and 1 mM Na_3VO_4 with 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged at 500*g* for 5 min at 4°C. Twenty micrograms of proteins was resolved on 12% SDS-PAGE and blotted electrophoretically onto Immun-Blot polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA). The membranes were blocked with 5% skim milk and incubated either with anti-phospho ERK1/2, anti-ERK1/2, or anti-PI3 kinase p85 antibody (BD Transduction Laboratories, Lexington, KY, or Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and were detected by chemiluminescence as described by the manufacturer's protocol (PerkinElmer Life and Analytical Sciences, Boston, MA).

PI3 Kinase Assay. After drug treatment, vascular smooth muscle cells were washed twice with ice-cold PBS and lysed in a buffer containing 20 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 137 mM NaCl, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM Na_3VO_4. Two hundred to 400 μg of lysates was immunoprecipitated with anti-PY 20 (6 μl) for 2 h at 4°C. Immunoprecipitates were washed once with "lysis" buffer; once with 20 mM Tris, pH 7.4, containing 0.5 mM LiCl; once with water; and once with 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.5 mM $NaVO_3$. Washed immunoprecipitates were resuspended in 50 μl of a buffer containing 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.5 mM EGTA, and 10 μg of phosphatidylinositol. [γ-^{32}P]ATP (10 μCi/assay) and $MgCl_2$ (final concentration 20 mM) and incubated at room temperature for 30 min. The reaction was stopped by addition of 20 μl of 8 M HCl, and 200 μl of chloroform/methanol (50:50) was added to separate the phases. Seventy microliters of the organic phase was taken from each reaction and spotted onto a silica gel 60 plate (Whatman, Maidstone, UK), which was developed in chloroform/methanol/28% ammonium hydroxide/water (60:47:2:11.6) for 2 h. The plate was then air-dried and exposed to radiographic film.

RNA Isolation for Microarray. After treatment, total RNA was extracted from vascular smooth muscle cells using an RNeasy kit (QIAGEN). The concentration and the quality (A_{260}/A_{280} ratio) of the RNA were determined by spectrophotometry. Only RNA samples with A_{260}/A_{280} ratios greater than 1.8 were used. Further assessment of the integrity of the RNA was tested using an Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA) at the London Regional Genomics Centre (Robarts Research Institute, London, ON, Canada; www.lrgc.ca). RNA with sharp and defined 28S and 18S ribosomal peaks validated good RNA integrity. RNA samples with poorly defined or missing peaks indicated degraded RNA and were not used.

Microarray Experiments. All microarray experiments were performed at the London Regional Genomics Centre using the m15K mouse cDNA microarrays from the University Health Network (University of Toronto, Toronto, ON, Canada). These arrays contain 15,296 sequence-verified mouse ESTs obtained from the National Institute on Aging (Bethesda, MD), each spotted in duplicate, and include 392 duplicate clones, 109 triplicate clones, and 30 quadruplicate clones. For a complete gene list, see http://www.microarrays.ca/support/glists.html. For each treatment and each condition, at least two pools of isolated RNA samples were prepared on separate occasions. Each of these "biological replicates" comprised purified RNA from the aortae of at least five animals. Within each biological replicate, three microarray assays were performed (i.e., "technical replicates"), two of which were hybridized with the treated sample labeled with Cy3-dCTP and the untreated control labeled with Cy5-dCTP. This labeling scheme was reversed for the third array (dye flips). Thus, for each condition/animal type studied, six assays were performed, based on RNA isolated from at least 10 animals. The rationale for this strategy reflected several considerations, including 1) use of RNA pools from multiple animals (as opposed to studying RNA sample from individual animals), thereby minimizing biological variability, but also minimizing the opportunity to identify "outliers" of potential interest (Kendziorski et al., 2003; Peng et al., 2003); and 2) the limited impact of the use of larger numbers of replicate chips for each condition to reduce total variability (Han et al., 2004).

Labeling of the cDNA for hybridization to microarrays was carried out using the direct labeling protocol supplied by the University Health Network and done in the dark. In brief, the target was fluorescently labeled by combining 8.0 μl of 5× first strand reaction buffer (SuperScript II; Invitrogen, Carlsbad, CA), 3.75 μM anchored T mRNA primer (5′-TTTTTTTTTTTTTTTTTTTTTVN-3′; V = A,C,G; N = A,C,G,T), 500 μM each of dATP, dGTP, and dTTP, 50 μM dCTP, 0.63 μM Cy3- or Cy5-dCTP (GE Healthcare, Little Chalfont, Buckinghamshire, UK), 10 mM dithiothreitol, and 10 μg of total RNA in a volume of 40 μl. The labeling reaction was incubated in the dark at 65°C for 5 min and then at 42°C for 5 min. Thereafter, 2 μl of reverse transcriptase (SuperScript II; Invitrogen) was added and incubated at 42°C for 2 to 3 h.

The reaction was stopped by the addition of 4 μl of 50 mM EDTA, pH 8.0, and 2 μl of 10 N NaOH was added and incubated at 65°C for 20 min to hydrolyze the RNA. Then, 4 μl of 5 M acetic acid was added to neutralize the solution. The control and experimental reactions were combined, 100 μl of isopropanol was added, and the DNA was precipitated overnight at −20°C. Labeled cDNA was pelleted by centrifugation at 16,000*g* in the dark at 4°C, the isopropanol was decanted, and the pellet was rinsed with ice-cold 70% ethanol. The samples were then pulse centrifuged, and all remaining alcohol was removed. The pellet was resuspended in 5 μl of RNase- and DNase-free distilled, deionized H_2O (Invitrogen).

Before hybridization, microarrays were prehybridized in 4× SSC, 0.1% SDS, and 0.2% bovine serum albumin for 20 min at 50°C. Prehybridized slides were washed well in filtered, distilled, deionized H_2O and dried in a filtered nitrogen gas stream. Then, 5 μl of yeast tRNA (10 mg/ml; Invitrogen) and 5 μl of calf thymus DNA (10 mg/ml) (Sigma-Aldrich) were added to 100 μl of DIG Easy Hyb solution (Roche Diagnostics, Indianapolis, IN). Eighty microliters of the hybridization solution was then added to each pooled pair of Cy5- and Cy3-labeled cDNA, and the solution was mixed and incubated at 65°C for 2 min and cooled to room temperature in the dark. This solution was applied to the slides and placed into a humidified hybridization chamber incubated at 37°C for 18 h. When the incubation was complete, the slides were washed three times with prewarmed 1× SSC and 0.1% SDS at 50°C for 10 min with constant gentle agitation, quickly rinsed in 1× SSC, and dried in a filtered nitrogen gas stream.

Microarray slides were scanned using the Virtek Chip Reader (Bio-Rad), and the resulting raw scanned images of Cy3 and Cy5 fluorescence intensities were processed using Arrayvision 6.0 (Imaging Research, St. Catharines, ON, Canada). Background-subtracted spot values were then imported into GeneSpring 6.0 (Agilent Technologies) for further analysis.

For all slides, the Cy3 and Cy5 signal intensities were normalized using the intensity dependent Locally weighted linear regression algorithm (LOWESS; Yang et al., 2002) of GeneSpring 6.0, with the smoothing factor set at 20%. Any intensity value of less than 0.01 was set to 0.01. In addition, separate normalization factors were

calculated for each subgrid on the slide to control spatial intensity bias across the slide ("Print-tip group normalization"). Differentially expressed genes were determined using the Student's t test algorithm of GeneSpring 6.0. A gene was said to be differentially expressed if the mean normalized expression ratio from all six arrays was significantly different from 1.0 with a p value of <0.05 set as the minimum level of significance.

Determination of significant differences in the effect of isoproterenol (or forskolin) to mediate early alterations in gene expression in SHR versus WKY vascular smooth muscle cells was determined by a differential effects approach. The rationale for the utilization of this analytical approach reflects 1) appreciation of the common finding of variability in respect to which specific genes are regulated between species/strains and 2) the importance placed in determining whether *quantitatively* the extent of gene regulation mediated by isoproterenol differed between SHR and WKY rats. For each condition and each cell type (WKY versus SHR), the effect of drug on the regulation of gene expression was determined (versus control cells) using the criteria outlined above. The extent of that increase or decrease (each analyzed separately) was compared with the extent of change in the other cell type, and the level of statistical significance determined. Because the genes affected by the same treatment in the two cell types were not uniformly identical, the analysis was repeated but using the extent of change in affected genes in the *opposite* cell type as the index. The conclusion of a significant difference between cell types was made when the extent of change using one cell type as the index was determined to be significantly different from the extent of change using the other cell type as an index. Differences were determined by analysis of variance with $p < 0.05$ as the minimum level of significance. To determine whether a specific gene contained a cyclic AMP response element, we used the searchable CREB Target Gene Database at http://natural.salk.edu/CREB as described recently by Zhang et al. (2005).

Materials. All drugs (unless otherwise specified) were purchased from Sigma-Aldrich. DMEM, Fungizone, and gentamicin were purchased from Invitrogen. Anti-phospho-ERK1/2, anti-ERK1/2, and anti-PI3k p85 were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-PY20 antibody was purchased from BD Transduction Laboratories.

Results

Impaired β-Adrenergic-Mediated Adenylyl Cyclase Activity in SHR Vascular Smooth Muscle Cell Primary Cultures. To determine whether the phenotype of impaired β-adrenergic signaling both at a biochemical and functional level was recapitulated in vascular smooth muscle cell cultures from SHR, we assessed 1) β-adrenergic-mediated adenylyl cyclase activity and 2) β-adrenergic-mediated arborization responses—a measure of short-term functional response.

In freshly isolated cells (including human lymphocytes) a 25 to 50% reduction in isoproterenol-mediated adenylyl cyclase activity has been reported (for review, see Feldman and Gros, 1998). Analogous to those previous findings in freshly isolated tissues, in SHR primary cultures isoproterenol-mediated adenylyl cyclase activity was decreased compared with WKY cultures (WKY: 110 ± 13% of forskolin-stimulated activity; SHR: 77 ± 8% of forskolin-stimulated activity; $n = 4$; $p < 0.05$). In contrast, forskolin-stimulated adenylyl cyclase activity was not significantly decreased in SHR (1997 ± 389 pmol/min/mg protein) compared with WKY cultures (1415 ± 233 pmol/min/mg protein; $n = 6$; $p = 0.17$).

Impaired Arborization of Vascular Smooth Muscle Cells in SHR. In WKY vascular smooth muscle cells, isoproterenol mediates a progressive "arborization" process, characterized by a generalized retraction and an increase in central cellular volume. This arborization of vascular smooth muscle cells was dose-dependent (see below), reversible with time, after isoproterenol washout or with the addition of the β-adrenergic antagonist propranolol (1 μM; Fig. 1A). In addition, isoproterenol (10 μM)-mediated arborization was completely blocked by pretreatment with propranolol (1 μM; Fig. 1B). The effect was mimicked by forskolin, which increases intracellular cAMP by a more direct effect on adenylyl cyclase activation (see below).

Analogous to the impaired vasodilator effects of isoproterenol reported previously in intact vessels from hypertensive animals (and humans; Feldman and Gros, 1998), the β-adrenergic-mediated arborization response was significantly attenuated in vascular smooth muscle cells from SHR compared with WKY vascular smooth muscle cells (Fig. 2). In addition, a comparable impairment in isoproterenol-mediated arborization in SHR smooth muscle cells was observed at both 1 μM (55 ± 10% of WKY) and 100 μM (61 ± 9% of WKY) isoproterenol compared with 10 μM isoproterenol. However, the ED_{50} value for arborization was not significantly different between WKY (589 ± 121 nM; $n = 3$) and SHR (828 ± 226 nM; $n = 3$) vascular smooth muscle cells. In contrast to the difference observed in isoproterenol-mediated arborization, forskolin-stimulated arborization was not significantly different between WKY and SHR vascular smooth muscle cells (Fig. 2). Thus, these studies demonstrate that the impairment in β-adrenergic-mediated responses apparent in other models of hypertension (whether assessed either

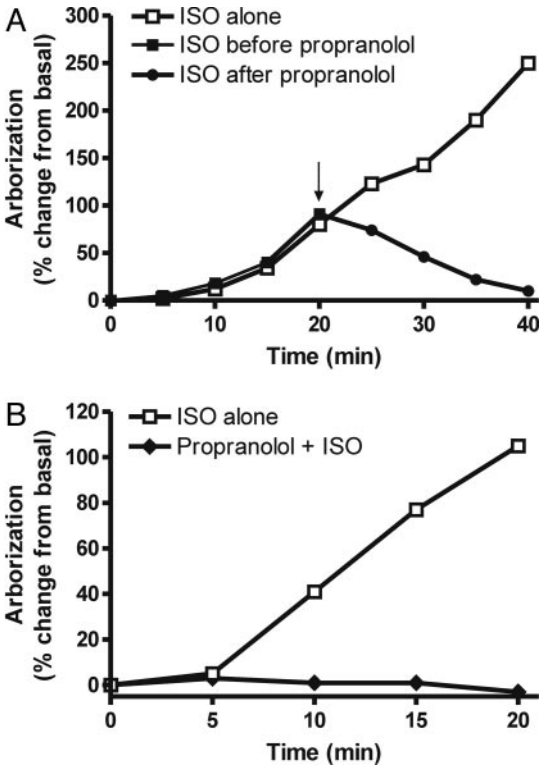

Fig. 1. Effect of propranolol on isoproterenol-mediated smooth muscle cell arborization. A, reversibility of isoproterenol (10 μM)-induced arborization by the addition of the β-adrenergic blocker propranolol (1 μM; point of addition denoted by the arrow). B, pretreatment of vascular smooth muscle cells with propranolol (1 μM; 30 min) completely blocked isoproterenol-mediated arborization.

in vivo or in freshly isolated tissues) is also detectable in cultured cells across the range of passages we used—both at biochemical and functional levels.

Effect of "Vasoconstrictor" Hormones on ERK Phosphorylation. To examine the effects of hormones acting through GPCRs linked to G_q on ERK phosphorylation, we examined the short-term (5-min) effects of vasopressin and angiotensin II. The effects of these hormones on ERK activation were compared with that of PDGF (i.e., a hormone acting through a tyrosine kinase receptor pathway, as opposed to a GPCR pathway). In WKY cells, vasopressin, angiotensin II, and PDGF all significantly increased ERK1/2 phosphorylation (Fig. 3). In SHR, whereas the effects of both angiotensin II and vasopressin were significantly enhanced (versus their effects in WKY cells), the effects of PDGF were not significantly different between WKY and SHR cells (Fig. 3).

Effect of β-Adrenoceptor Activation on Phosphorylation of ERK. In initial studies, we determined that short-term treatment (5 min) with either forskolin or isoproterenol had no significant effects on phosphorylation levels of ERK1/2 in either SHR or WKY vascular smooth muscle cells (WKY, ISO: 98 ± 5% of basal; FSK: 126 ± 12% of basal; and SHR, ISO: 98 ± 17% of basal; FSK: 122 ± 24% of basal; n = 4–5; p = N.S.). We therefore examined whether longer durations (90 min) of treatment with either isoproterenol or forskolin could alter the ERK phosphorylation state. With 90 min of incubation, both isoproterenol and forskolin treatment significantly enhanced ERK1/2 phosphorylation in isolated vascular smooth muscle cells obtained from WKY rats (Fig. 4). Phospho-ERK1/2 content was not significantly different between WKY and SHR vascular smooth muscle cells under basal conditions (SHR: 83 ± 11% of WKY; n = 5; p = N.S.). In contrast, isoproterenol-mediated effects on ERK1/2 phosphorylation were significantly impaired in SHR aortic smooth muscle cells compared with the effect observed in WKY cells (Fig. 4). However, the forskolin-mediated enhancement of ERK phosphorylation remained intact (versus the effect observed in WKY vascular smooth muscle cells; Fig. 4). Total ERK protein content was not significantly altered by isoproterenol or forskolin treatment in either WKY- or SHR-treated cells (Fig. 4, inset).

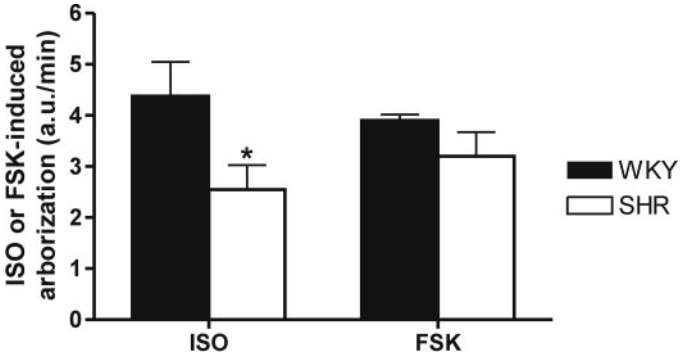

Fig. 2. Effect of isoproterenol and forskolin on vascular smooth muscle cell arborization. Vascular smooth muscle cells from either WKY rats or SHR were treated with either isoproterenol (10 μM) or forskolin (10 μM). The extent of arborization was determined by the change of image intensity, which was plotted against time, and slopes were determined with linear regression analysis. Isoproterenol-induced arborization was significantly reduced in smooth muscle cells from SHR. Data represent the mean ± S.E.M. from three to 10 independent experiments performed under identical conditions. *, $p < 0.05$ versus WKY by paired t test.

Effect of Isoproterenol and Forskolin on PI3 Kinase Activity. In vascular smooth muscle cells obtained from WKY rats, 90 min of treatment with either isoproterenol or forskolin resulted in a comparable decrease in PI3 kinase activity (Fig. 5). Protein expression of regulatory p85 subunit of PI3 kinase did not change with treatments (Fig. 5, inset).

Under basal conditions, PI3 kinase activity was significantly greater in SHR versus WKY vascular smooth muscle cells (148 ± 15% of WKY activity; n = 8; $p < 0.05$), consistent with the findings seen in other models of hypertension (Loberg et al., 2003). It is noteworthy that this was not associated with any increase in p85 immunoreactivity (98 ± 6% of WKY expression; n = 3; p = N.S.). In SHR vascular smooth muscle cells, the ability of isoproterenol to inhibit PI3 kinase activity was significantly attenuated (Fig. 5). However, the inhibitory effect of forskolin remained intact (Fig. 5) and was not different from the extent of inhibition seen by forskolin in WKY vascular smooth muscle cells.

Effect of Isoproterenol and Forskolin Treatment on Gene Expression. Using a microarray approach, we examined the effect of isoproterenol and forskolin on the regulation of gene expression in vascular smooth muscle cells obtained from WKY rats and SHR. The overall coefficient of variation between the two pools of RNA for WKY rats and SHR (including isoproterenol and forskolin treatment groups versus control) was 10.2%.

Considering all 15,296 genes represented on the microarray slide (including ESTs) in WKY cells, isoproterenol treatment significantly changed 2.7% of the genes (Table 1). In contrast, forskolin significantly changed 5.2% of the genes. However, the overall extent of change did not differ between isoproterenol and forskolin treatments in WKY vascular smooth muscle cells (Table 1). Forty genes were commonly significantly affected by isoproterenol and forskolin, of which five were ESTs and two were duplicated. It is noteworthy that of those 31 unique genes for which data were available in the CREB Target Gene Database (see *Materials and Methods*) in 18 (58%), a cyclic AMP response element could be identified.

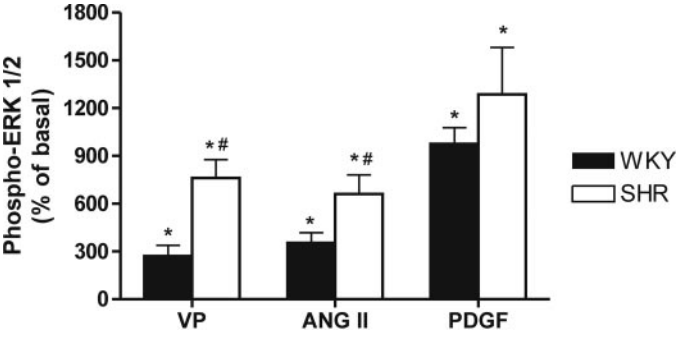

Fig. 3. Short-term effect of vasopressin, angiotensin II, and PDGF on mitogen-activated protein kinase activity as assessed by phospho-ERK. Vascular smooth muscle cells from either WKY rats or SHR were incubated in the absence or presence of vasopressin (1 μM), angiotensin II (1 μM), or PDGF (20 ng/ml) for 5 min. Densitometric analysis of phospho-ERK1/2 content expressed as a percentage of basal (untreated) vascular smooth muscle cells. All treatments significantly increased phospho-ERK1/2. However, enhanced ERK1/2 phosphorylation was observed in response to vasopressin and angiotensin II in vascular smooth muscle cells obtained from SHR. Data represent the mean ± S.E.M. from four to five independent experiments performed under identical conditions. *, $p < 0.05$ versus basal (untreated cells). #, $p < 0.05$ versus WKY-treated vascular smooth muscle cells. (VP, vasopressin; Ang II, angiotensin II).

In SHR cells, isoproterenol treatment altered expression of a comparable number of genes, but to a lesser extent than that seen with WKY cells. In SHR cells, isoproterenol treatment resulted in significant changes in 2.9% of the gene array (Table 1) of which 36 were common to those altered in WKY cells. In SHR, forskolin mediated changes in a marginally greater number of genes (versus isoproterenol-treated cells, 4.1% of the genes; Table 1) (70 common to those regulated in WKY cells). However, in SHR, the overall extent of change did not differ between isoproterenol and forskolin treatments, except for marginal reduction in forskolin- versus isoproterenol-mediated responses (Table 1).

However, comparing across animal types, the *extent* of change seen with either isoproterenol *or* forskolin treatment was significantly less in SHR versus WKY rats. With isoproterenol treatment, both up-regulation and down-regulation of gene expression was significantly blunted (Table 2). With forskolin treatment, up-regulation of gene expression was significantly blunted in SHR, whereas the effect of forskolin

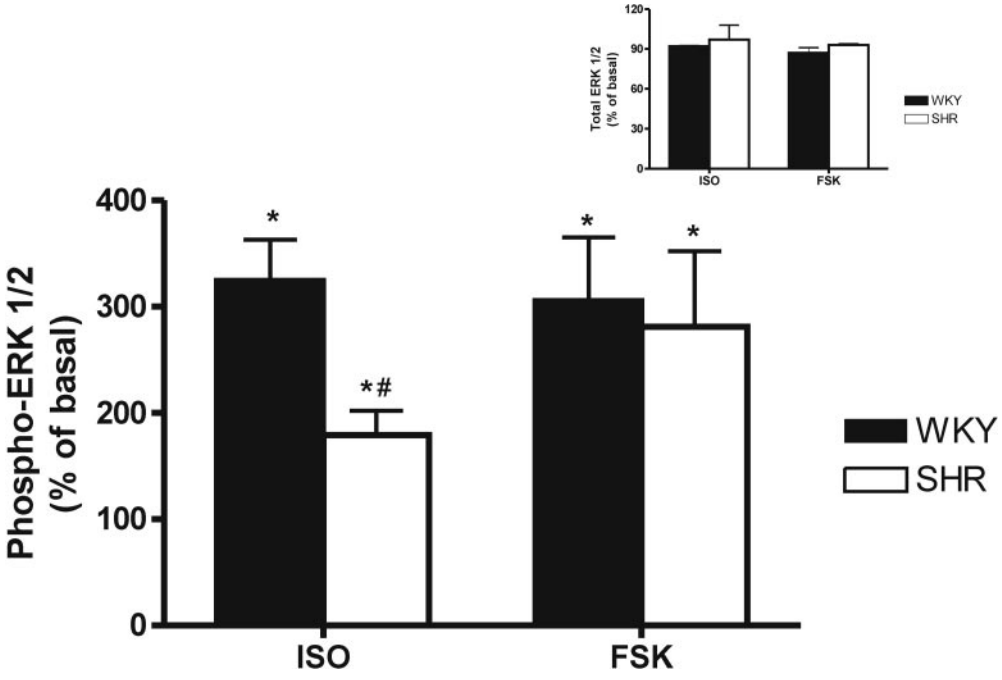

Fig. 4. Effect of isoproterenol and forskolin on ERK1/2 phosphorylation. Vascular smooth muscle cells from either WKY rats or SHR were incubated in the absence or presence of isoproterenol (10 μM) or forskolin (10 μM) for 90 min. Densitometric analysis of phospho-ERK1/2 content expressed as a percentage of basal (untreated) vascular smooth muscle cells. Impaired isoproterenol-mediated enhancement of ERK1/2 phosphorylation in vascular smooth muscle cells obtained from SHR. Inset, densitometric analysis of total ERK1/2 in cell lysates from untreated and treated cells. Data represent the mean ± S.E.M. from three to seven independent experiments performed under identical conditions. *, $p < 0.05$ versus basal (untreated vascular smooth muscle cells). #, $p < 0.05$ versus WKY-treated vascular smooth muscle cells.

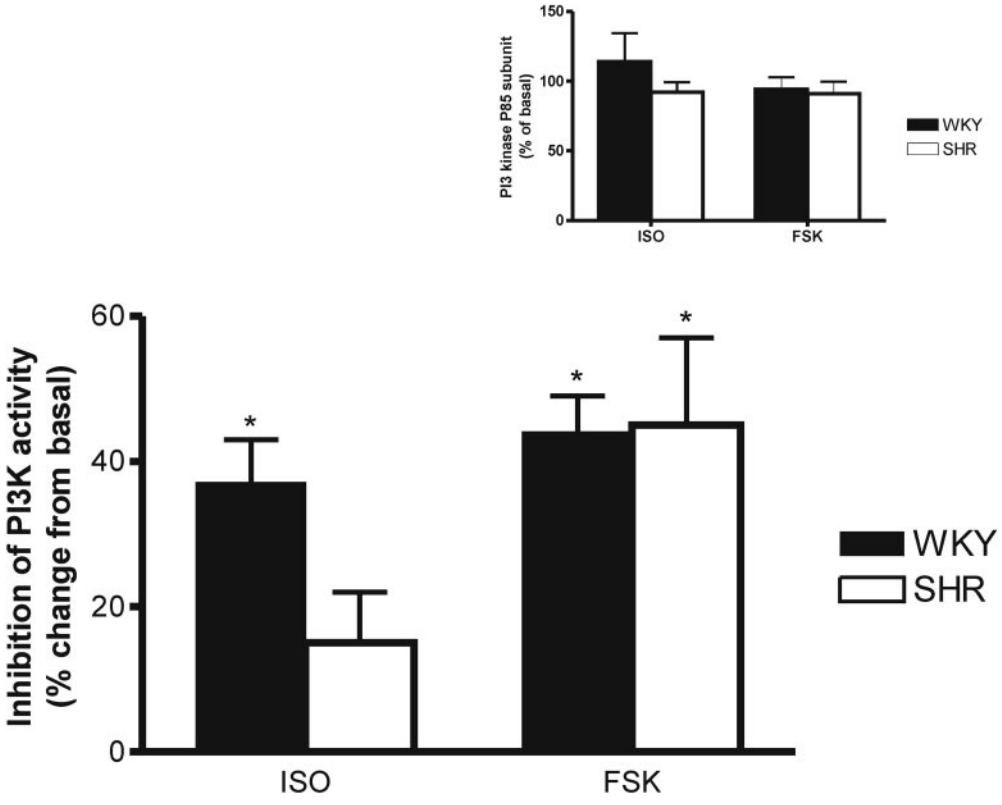

Fig. 5. Effect of isoproterenol and forskolin on PI3 kinase activity in vascular smooth muscle cells. PI3 kinase activity was assessed as described under *Materials and Methods*. Inhibition of PI3 kinase by isoproterenol (10 μM) was significantly attenuated in SHR vascular smooth muscle cells. Inset, assessment of the expression of p85 subunit from PI3 kinase. Data represent the mean ± S.E.M. from three to eight independent experiments performed under identical conditions. *, $p < 0.05$ versus untreated vascular smooth muscle cells.

on down-regulating gene expression was more comparable (Table 2).

Interestingly, only 20 genes (19 up-regulated and one down-regulated) were commonly regulated both by isoproterenol and forskolin in both WKY and SHR vascular smooth muscle cells (Table 3). These included the regulator of G protein signaling, which has recently been found to play a key role in regulating vascular function (Le and Coffman, 2003), and two Krüppel-like factor genes, for which an emerging role in vascular biology is being established (Feinberg et al., 2004) (Table 3)—all of which were up-regulated. Of the 20 genes in common, two were ESTs and one gene was duplicated (as separate clones). Of these 17 unique genes for which data were available in the CREB Target Gene Database (see *Materials and Methods*) in 12 (71%) contained an identifiable cyclic AMP response element.

To determine the relationship between the alterations in gene expression and the blunted effects of isoproterenol on growth regulatory enzyme (PI3 kinase and ERK), we examined those 553 genes identified as linked to either cell growth and maintenance and signal transduction ontologies (as determined by GeneSpring software). Common to the pattern seen in the entire gene array, in WKY rats, isoproterenol changed gene expression significantly in 28 genes (20 increased, eight decreased) versus 28 in SHR (17 increased and 11 decreased) (Table 4). Although the extent of down-regulation mediated by isoproterenol was comparable in SHR versus WKY, the ability of isoproterenol to up-regulate gene expression was significantly impaired in cells from SHR (Table 5). It is noteworthy that the proportion of unique genes identifiable as having a CRE (i.e., which would be expected to be primarily regulated by isoproterenol-mediated increases in cAMP) was comparable in SHR (43%) and WKY rats (46%).

For the genes identified as linked to cell growth and maintenance/signal transduction ontologies, forskolin significantly changed 49 genes (38 increased and 11 decreased) in WKY vascular smooth muscle cells versus 33 in SHR (22 increased and 11 decreased) (Tables 6 and 7). The proportion of forskolin-regulated genes identifiable as having a CRE was comparable with the proportion seen with isoproterenol treatment (WKY, 46%; SHR, 56%). Similar to that observed with isoproterenol treatment, the extent of down-regulation by forskolin was comparable between WKY rats and SHR (Table 5). However, the ability of forskolin to up-regulate genes from these selected ontologies was significantly impaired in SHR vascular smooth muscle cells (Table 5). In total, these data support the conclusion that isoproterenol-mediated gene alterations in SHR are blunted, an effect that parallels blunted forskolin-mediated effects.

Impaired β-Adrenergic-Mediated Enhancement of PDGF-Stimulated Vascular Smooth Muscle Cell Proliferation. Finally, to assess the net effect of blunted β-adrenergic-mediated growth regulatory responses on cell growth, we assessed isoproterenol-mediated stimulation of vascular smooth muscle cell proliferation after PDGF exposure. PDGF alone significantly increased [^{3}H]thymidine incorporation in vascular smooth muscle cells obtained from WKY rats (523 ± 91% of basal; $n = 5$; $p < 0.05$). The effect of PDGF on vascular smooth muscle cells proliferation was significantly greater in SHR (177 ± 53%; $n = 5$; $p < 0.05$) compared with WKY vascular smooth muscle cells, consistent with previous studies (Hadrava et al., 1989).

In WKY, isoproterenol mediated a further significant enhancement of PDGF-mediated proliferation (153 ± 12% of PDGF alone; $n = 6$; $p < 0.05$). In contrast, in SHR vascular smooth muscle cells, isoproterenol did not significantly enhance PDGF-mediated proliferation (96 ± 7% of PDGF alone; $n = 6$; p = N.S.).

Discussion

Previous studies in both human and animal models have confirmed that with regard to short-term effects on vascular reactivity, the hypertensive state is characterized by impairment in vasodilator mechanisms (acting through GPCRs

TABLE 1

Extent of change of significantly altered genes with isoproterenol or forskolin treatment

p value represents ISO vs. FSK comparisons.

	ISO-Based	FSK-Based	*p*
WKY			
Decreasers	−0.21 ± 0.02 (n = 190)	−0.20 ± 0.02 (n = 362)	N.S.
Increasers	0.20 ± 0.03 (n = 223)	0.23 ± 0.01 (n = 448)	N.S.
SHR			
Decreasers	−0.11 ± 0.01 (n = 234)	−0.12 ± 0.01 (n = 365)	N.S.
Increasers	0.15 ± 0.01 (n = 213)	0.09 ± 0.01 (n = 262)	<0.01

ISO-based, (ISO/CONTROL) − (FSK/CONTROL); FSK-based, (FSK/CONTROL) − (ISO/CONTROL).

N.S., not significant.

TABLE 2

Extent of change of significantly altered genes in WKY and SHR

p value represents WKY vs. SHR comparisons.

	WKY-Based	SHR-Based	*p*
Isoproterenol			
Decreasers	−0.19 ± 0.01 (n = 190)	−0.14 ± 0.02 (n = 234)	<0.001
Increasers	0.24 ± 0.03 (n = 223)	0.13 ± 0.04 (n = 213)	<0.05
Forskolin			
Decreasers	−0.14 ± 0.005 (n = 362)	−0.11 ± 0.01 (n = 365)	<0.001
Increasers	0.19 ± 0.02 (n = 448)	0.04 ± 0.03 (n = 262)	<0.001

WKY-based, $(WKY_{ISO}/WKY_{CONTROL}) - (SHR_{ISO}/SHR_{CONTROL})$ or $(WKY_{FSK}/WKY_{CONTROL}) - (SHR_{FSK}/SHR_{CONTROL})$; SHR-based, $(SHR_{ISO}/SHR_{CONTROL}) - (WKY_{ISO}/WKY_{CONTROL})$ or $(SHR_{FSK}/SHR_{CONTROL}) - (WKY_{FSK}/WKY_{CONTROL})$.

TABLE 3

Common genes significantly changed with both isoproterenol and forskolin treatment in both WKY and SHR vascular smooth muscle cells

NIA Clone ID	Gene Description
Increased	
H3053A12	$RGS2_1$
H3066F01	$RGS2_2$
H3041H12	Leukemia inhibitory factor (Lif)
H3057B07	Gly96
H3015B01	Gut-enriched Kruppel-like factor (GKLF)
H3051F10	GRO1 oncogene (Gro1)
H3075C08	Kruppel-like factor 4 (gut) (Klf4)
H3054C02	Growth arrest and DNA-damage-inducible, γ
H3085G10	AXIN1 up-regulated 1 (AXUD1)
H3154E06	Inhibitor of DNA binding 3 (Idb3)
H3080B10	cDNA FLJ13448 fis, clone PLACE1002993
H3088G03	N.A.
H3008A09	RhoB
H3062E09	Protein tyrosine phosphatase 4a1 (Ptp4a1)
H3115D06	cDNA-FLJ21375 fis, clone COL03223
H3108G06	N.A.
H3046C10	Isopentenyl-diphosphate δ isomerase (IDI1)
H3116A06	Proline-rich protein with nuclear targeting signal (B4–2)
H3026A08	IκBα
Decreased	
H3092E09	Early growth response 1 (Egr1)

N.A., not applicable; NIA, National Institute on Aging.

linked to G_s/adenylyl cyclase activation) (Feldman and Gros, 1998). The current findings demonstrate that the growth regulatory effects of β-adrenoceptor activation (the prototype GPCR linked to G_s/adenylyl cyclase) are comparably impaired with regard to β-adrenergic-mediated effects on proliferation, ERK activation, inhibition of PI3 kinase activity as well as the regulation of gene expression as determined by microarray analysis.

β-Adrenergic-mediated vasodilation and β-adrenergic-stimulated adenylyl cyclase activation are impaired in hypertension. These effects have been demonstrated in both human and experimental models of hypertension and both in vivo and in vitro (Feldman and Gros, 1998). The current studies confirm that this functional impairment in β-adrenergic-mediated responses is still apparent in cultured single cell vascular systems in spontaneously hypertensive rats, a commonly used genetic model of hypertension. It is noteworthy that analogous "durability" of altered regulation of adenylyl cyclase in hypertension has been reported in "immortalized" lymphoblasts from hypertensive patients (Siffert et al., 1995). The persistence of this "phenotype" of impaired β-adrenergic responses in cultured cell systems would suggest that these defects are not due to short-term in vivo regulatory effects in hypertension, which would be expected to be diminished and ultimately be undetectable when assessed in vitro in cultured cells with ongoing passaging.

The short-term defect in β-adrenergic-mediated arborization responses paralleled impairment in the effect of β-adrenergic receptor activation on PDGF-stimulated vascular smooth muscle cell proliferation. Previous studies have reported vascular smooth muscle hyper-responsiveness in hypertension both under basal conditions as well as after treatment with a range of growth factors (Hamet et al., 1988; Battle et al., 1994; Begum et al., 1998; Tanner et al., 2003). However, these differences in growth rates have been shown to be specific for individual growth factors. The present studies indicate that for β-adrenergic-mediated responses, growth stimulatory effects are not enhanced in SHR but instead are blunted.

The growth regulatory enzymes examined in this study (i.e., β-adrenergic regulation of PI3 kinase activity and ERK phosphorylation) demonstrated patterns of regulation in SHR paralleling the blunted responses to β-adrenergic stimulation seen in both arborization and proliferative responses. With regard to ERK phosphorylation, although transient exposure to either isoproterenol or forskolin had no effect on phospho-ERK expression, prolonged exposures enhanced phospho-ERK expression. In previous studies, the effect of β-adrenoceptor activation on ERK activity has been generally found to be stimulatory (Luttrell et al., 1997; Yeh et al., 2005). Both cAMP-dependent and cAMP-independent (via G_i and β-arrestin) mechanisms have been suggested to be important in mediating this effect. In vascular smooth muscle cells, however, the stimulatory effect of β-adrenoceptor activation on ERK activity parallels that of direct adenylyl cyclase activation by forskolin. The effect of β-adrenoceptor activation on ERK activity was impaired in cells from SHR, although forskolin-mediated response was preserved. These data are consistent with a defect in β-adrenoceptor signaling occurring "upstream" from adenylyl cyclase activation that

TABLE 4
Significantly changed genes from cell growth, maintenance, and signal transduction ontologies following isoproterenol treatment in WKY and SHR vascular smooth muscle cells

-Fold Change	Isoproterenol WKY Gene Description	-Fold Change	Isoproterenol SHR Gene Description
7.99	*(RGS2)1*	6.04	*(RGS2)1*
2.79	*(RGS2)2*	2.43	*(RGS2)2*
2.12	α-4 Integrin subunit gene	1.98	*Growth arrest and DNA-damage-inducible, γ*
1.79	*Growth arrest and DNA-damage-inducible, γ*	1.60	Transforming growth factor β1 (Tgfb1i4)1
1.68	Protein tyrosine phosphatase, nonreceptor type 21 (Ptpn21)	1.54	Transforming growth factor β1 (Tgfb1i4)2
1.54	Hypothetical protein 2-cell	1.43	*Amino acid transporter system A (ATA2)*
1.41	*Amino acid transporter system A (ATA2)*	1.42	Calcium-binding protein D9k
1.39	B-cell translocation gene 2 (Btg2)	1.37	*Protein tyrosine phosphatase 4a1 (Ptp4a1)1*
1.37	*Protein tyrosine phosphatase 4a1 (Ptp4a1)1*	1.36	Heat shock protein 65 (HSP65)
1.35	VEGF164	1.24	Solute carrier family 16, member 1 (Slc16a1)
1.33	Serine/threonine kinase receptor-associated protein (Strap)	1.20	Transforming growth factor β1 (Tgfb1i4)3
1.26	*Protein tyrosine phosphatase 4a1 (Ptp4a1)2*	1.18	*Protein tyrosine phosphatase 4a1 (Ptp4a1)2*
1.23	Serine/threonine kinase 10 (Stk10)	1.17	Growth response protein (CL-6)
1.22	Mouse c-fos gene	1.13	*IκBα* gene
1.13	Growth factor receptor-bound protein 7 (Grb7)	1.11	Solute carrier family 39 (iron-regulated transporter), member 1 (Slc39a1)
1.12	Protein tyrosine phosphatase, nonreceptor type 2 (Ptpn2)	1.10	*Fibroblast growth factor inducible 14 (Fin14)*
1.10	*IκBα*	1.08	Heat shock protein 90 (HSP90)
1.09	*Fibroblast growth factor inducible 14 (Fin14)*		
1.07	β-Transducin repeat-containing protein (Btrc)		
1.07	Programmed cell death 8 (Pdcd8)		
−1.08	Thiazide-sensitive Na-Cl cotransporter	−1.07	Oxidative-stress responsive 1 (OSR1)
−1.11	Fas-activated serine/threonine FAST kinase	−1.08	Cell division cycle 2-like 5 (CDC2L5)
−1.14	Choline transporter (CHOT1)	−1.09	Cystathionine β-synthase (CBS)
−1.16	Tumor necrosis factor, α-induced protein 2 (Tnfaip2)	−1.09	Folylpolyglutamate synthetase precursor (Fpgs)
−1.17	Bone morphogenetic protein (Bmp-1)	−1.10	Protein tyrosine phosphatase, receptor type, G (Ptprg)
−1.18	Moloney leukemia virus10 (Mov10)	−1.15	Golgi transport complex protein (90 kDa) (GTC90)
−1.27	K-Cl cotransporter KCC1 (Slc12a4)	−1.16	Heat shock protein 40 (HSPF1)
−2.71	*Early growth response 1 (Egr1)*	−1.16	Gas5 growth arrest-specific gene
		−1.28	Calcium-binding protein D9k
		−1.33	Transforming growth factor, β receptor II (Tgfbr2)
		−1.81	*Early growth response 1 (Egr1)*

parallels the defect in short-term regulation of vascular reactivity. As noted above, in several animal models of hypertension (and in human hypertension) GRK2 activity/protein content is increased and parallels the receptor-G protein uncoupling that characterizes the defect in adenylyl cyclase activation in the hypertensive state. This might have been predicted to result in an enhancement of β-adrenergic ERK activation via other pathways (e.g., via β-arrestin-mediated pathways). This was not the case. It is notable that a similar impairment in β-adrenergic-mediated ERK activation also occurs with vascular overexpression of GRK2 (Eckhart et al., 2002), supporting the concept that enhanced β-adrenoceptor phosphorylation by GRK2 impairs ERK regulation, which would not be expected for a β-adrenoceptor/β-arrestin or β-adrenoceptor/G_i pathway of ERK activation; or alternatively that in hypertension these "other pathways" are comparably uncoupled by a concurrent (but as yet unidentified) mechanism.

In contrast to the impairment in β-adrenergic-mediated ERK activation in hypertension, vasopressin- and angiotensin II-mediated responses (presumably acting via a G_q-dependent pathway) were enhanced. This is consistent with an enhancement of ERK signaling due to enhanced receptor/G protein coupling (or enhanced phospholipase C activation). This effect seems to be GPCR-selective, because PDGF-mediated responses were not altered. It is noteworthy that these

TABLE 5

Extent of change of significantly altered genes from cell growth, maintenance, and signal transduction ontologies

p value represents WKY vs. SHR comparisons.

	WKY-Based	SHR-Based	*p*
Isoproterenol			
Decreasers	−0.09 ± 0.02 (*n* = 8)	−0.10 ± 0.02 (*n* = 11)	N.S.
Increasers	0.28 ± 0.11 (*n* = 20)	0.04 ± 0.14 (*n* = 17)	<0.05
Forskolin			
Decreasers	−0.10 ± 0.02 (*n* = 11)	−0.14 ± 0.05 (*n* = 11)	N.S.
Increasers	0.32 ± 0.16 (*n* = 38)	−0.28 ± 0.26 (*n* = 22)	<0.05

WKY-based, $(WKY_{ISO}/WKY_{CONTROL}) - (SHR_{ISO}/SHR_{CONTROL})$ or $(WKY_{FSK}/WKY_{CONTROL}) - (SHR_{FSK}/SHR_{CONTROL})$. SHR-based, $(SHR_{ISO}/SHR_{CONTROL}) - (WKY_{ISO}/WKY_{CONTROL})$ or $(SHR_{FSK}/SHR_{CONTROL}) - (WKY_{FSK}/WKY_{CONTROL})$.
N.S., not significant.

TABLE 6

Significantly *increased* genes from cell growth, maintenance, and signal transduction ontologies following forskolin treatment in WKY and SHR vascular smooth muscle cells

-Fold Change	Forskolin WKY Gene Description	-Fold Change	Forskolin SHR Gene Description
10.92	*(RGS2)1*	5.12	*(RGS2)1*
2.88	Carnitine palmitoyltransferase I (CPT I)	2.37	*Growth arrest and DNA-damage-inducible, γ*
2.60	*(RGS2)2*	2.13	*(RGS2)2*
2.27	*Growth arrest and DNA-damage-inducible, γ*	1.37	Transforming growth factor β1 (Tgfb1i4)1
1.84	Acid transporter system A (ATA2)	1.29	*Protein tyrosine phosphatase 4a1 (Ptp4a1)1*
1.75	*B-cell translocation gene 2 (Btg2)*	1.28	*B-cell translocation gene 2 (Btg2)*
1.62	Transforming growth factor β1 (Tgfb1i4)1	1.25	Growth response protein (CL-6)
1.47	Heat shock protein 105 (HSP105)	1.24	*Transforming growth factor β1 (Tgfb1i4)2*
1.47	JAK1 protein tyrosine kinase	1.21	Heat shock 70-kDa protein 5 (glucose-regulated protein, 78 kDa) (Hspa5)
1.34	*Protein tyrosine phosphatase 4a1 (Ptp4a1)1*	1.20	*IκBα gene*
1.33	*Transforming growth factor β1 (Tgfb1i4)2*	1.18	Voltage-dependent anion channel 2 (Vdac2)
1.31	Heat shock 70 protein (HSP70)	1.18	Nucleolar GTPase (HUMAUANTIG)
1.28	Voltage-dependent anion channel 1 (Vdac1)	1.17	Transforming growth factor, β receptor II (Tgfbr2)
1.28	*IκBα gene*	1.14	IQ motif-containing GTPase activating protein 1 (Iqgap1)
1.27	BCL2/adenovirus E1B 19-kDa-interacting protein 1, NIP3 (Bnip3)	1.12	Rab3 GTPase-activating protein, noncatalytic subunit (150 kDa) (RAB3-GAP150)
1.24	Protein tyrosine phosphatase 4a1 (Ptp4a1)2	1.12	Potassium voltage-gated channel, shaker-related subfamily, β member 3 (Kcnab3)
1.23	Solute carrier family 31 (copper transporters), member 2 (SLC31A2)	1.12	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)
1.21	Growth response protein (CL-6)	1.11	Lactate dehydrogenase 2, B chain (Ldh2)
1.20	Heat shock protein 030 (HSP030)	1.11	Pat-12 product (Pat-12)
1.19	Stathmin gene	1.11	Heat shock protein 65 (HSP65)
1.18	MO25 gene	1.07	Janus kinase 2 (Jak2)
1.18	Protein tyrosine phosphatase 4a2 (Ptp4a2)	1.05	Tax1 (human T-cell leukemia virus type I) binding protein 1 (TAX1BP1)
1.18	Heat shock protein, 74 kDa, A (HSPa9a)		
1.18	Transforming growth factor β1 (Tgfb1i4)		
1.17	Heat shock protein 90 (HSP90)		
1.16	Heat shock 70 protein (HSP70)		
1.16	Transmembrane-trafficking protein (TMP21)		
1.16	Transport-secretion protein 2.2 (TTS-2.2 gene)		
1.15	Serine threonine-tyrosine kinase (STY)		
1.15	COP9 (constitutive photomorphogenic), subunit 7a (Cops7a)		
1.14	Tumor necrosis factor-α-converting enzyme (TACE/ADAM17)		
1.14	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV)		
1.14	ATPase, H + transporting, lysosomal (vacuolar proton pump) (ATP6H)		
1.14	*Homo sapiens* X28 region		
1.13	Proliferation-associated protein 1 (Plfap)		
1.11	Wingless-related MMTV integration site 4 (Wnt4)		
1.08	Solute carrier family 39 (iron-regulated transporter), member 1 (Slc39a1)		
1.08	Cell division cycle 37 homolog (Cdc37)		

GPCR/G_q-dependent pathways might have been expected to be similarly affected by increased GRK2 expression (and GPCR/G protein uncoupling) in the hypertensive state (i.e., leading to blunted ERK activation). However, in SHR, G_q-dependent ERK activation seems to be resistant to the impact of increased GRK2 activity. Interestingly a similar "resistance" to the effect of increased expression of GRK2 on G_q-mediated pathways (versus G_s-mediated pathways) has been described previously in in vitro models of GRK2 overexpression (Iacovelli et al., 1999).

β-Adrenergic regulation of PI3 kinase activity and its alteration in SHR cells follows an analogous pattern as that seen for ERK activation. β-Adrenoceptor activation and elevation of intracellular cAMP levels have been reported to have both inhibitory and stimulatory effects on PI3 kinase activity (Colombo et al., 2003; Smith et al., 2005). The stimulatory effects of β-adrenoceptor activation on PI3 kinase activity have been suggested to be G_i-dependent (and divergent from the effects of cAMP-elevating agents) and generally occur in models where β-adrenoceptor activation stimulates cell growth (Chesley et al., 2000). However, in vascular smooth muscle cells, where β-adrenoceptor activation inhibits its proliferation, isoproterenol inhibited PI3 kinase activity, an effect that paralleled that of direct adenylyl cyclase stimulation with forskolin. In SHR, the isoproterenol-mediated inhibition of PI3 kinase was blunted, although forskolin-mediated responses remained intact (analogous to the effects of isoproterenol and forskolin on ERK activation in SHR). In aggregate, these data are consistent with either 1) a model where for β-adrenergic-mediated responses, adenylyl cyclase activation is the predominant mechanism mediating PI3 kinase regulation or 2) a model where β-adrenergic-mediated responses through alternate pathways are comparably impaired in SHR. Regardless of the model, these data do not support a scenario where the growth regulatory effects of β-adrenoceptor activation are enhanced in the hypertensive state.

Short-term β-adrenergic activation has been associated with regulation of a range of genes (Brand et al., 1993; Shen and Gundlach, 2000). The current study demonstrates that isoproterenol mediates up-regulation and (to a lesser extent) down-regulation of a number of genes, including those previously shown to be very sensitive to increases in intracellular cAMP (i.e., regulator of G protein signaling proteins). Of those regulated genes linked to cell signaling/growth ontogenies, almost half had identifiable CRE elements. This proportion is higher than that reported in other surveys of the effect of PKA activation on gene regulation [e.g., Zambon et al. (2005) found only 9% of genes regulated by PKA activation in S49 cells]. This speaks to the multiplicity of mechanisms of cAMP-mediated genetic regulation beyond regulation of CRE as well as the variability in the genetic targets of cAMP-mediated effects between tissues/species (as has been reported for the genetic regulatory effects of a range of hormones/agents linked to elevations in cAMP/PKA activation) (McLean et al., 2002; Tierney et al., 2003; Meyer-Kirchrath et al., 2004).

Forskolin tended to have a greater effect on regulation of gene expression, paralleling its greater effects on cAMP activation (versus isoproterenol). However, it is notable that the pattern of effects of forskolin versus isoproterenol on gene expression differed (i.e., there was incomplete concordance between these two agents regarding specific genes regulated). Although this could represent differential mechanisms of effects of isoproterenol versus forskolin (including some of the purported non-cAMP-mediated effects of forskolin), this could also be a reflection of the intrinsic variability in profiling patterns of biological responses based on microarray methodologies. Furthermore, the proportion of regulated genes expressing CRE were comparable with isoproterenol versus forskolin treatment, suggesting that the mechanism(s) of regulation might be similar (although the subsets affected differed, perhaps relating to differences in extent/duration of adenylyl cyclase activation).

In the current study, we report impairment in the effects of β-adrenoceptor activation on vascular gene expression in SHR vascular smooth muscle cells. This finding parallels the pattern of impaired responses seen with regard to ERK and PI3 kinase activation. However, whether this defect in early gene expression is β-adrenoceptor/G protein-specific is unlikely, because the impairment in isoproterenol-mediated responses paralleled impairment in forskolin-mediated responses. In addition, it should be noted that given the relatively small numbers of animals studied and the use of a single age of study, our findings with regard to the impact of hypertension on regulation of any individual gene are lim-

TABLE 7

Significantly *decreased* genes from cell growth, maintenance, and signal transduction ontologies following forskolin treatment in WKY and SHR vascular smooth muscle cells

-Fold Change	Forskolin WKY Gene Description	-Fold Change	Forskolin SHR Gene Description
−1.07	Mas proto-oncogene and Igf2r gene for insulin-like growth factor type 2	−1.05	Voltage-dependent anion channel 3 (Vdac3)
−1.10	Protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF)	−1.07	Folylpolyglutamate synthetase precursor (Fpgs)
−1.11	Pregnancy-specific glycoprotein 19 (Psg19)	−1.07	Tumor necrosis factor-α-converting enzyme (TACE)
−1.11	Sodium channel, voltage-gated, type II, β polypeptide (SCN2B)	−1.08	Heat shock protein 65 (HSP65)
−1.14	Cell division cycle 42 homolog (Cdc42)	−1.11	Coxsackie virus and adenovirus receptor (CXADR)
−1.14	Protein tyrosine phosphatase 4a3 (Ptp4a3)	−1.14	Mas proto-oncogene and Igf2r gene for insulin-like growth factor type 2
−1.20	Tumor necrosis factor-α-converting enzyme (TACE)	−1.14	Signal transducer and activator of transcription 2 (Stat2)
−1.21	FUSED serine/threonine kinase	−1.16	Zinc finger transcription factor (GATA-6)
−1.22	Receptor for MCF virus 1 (Rmc1)	−1.22	Gas5 growth arrest-specific gene
−1.57	UNC51.1 serine/threonine kinase (Unc51.1)	−1.61	RGS11
−1.60	*Early growth response 1 (Egr1), mRNA*	−1.67	*Early growth response 1 (Egr1)*

ited. Specifically, given our calculated coefficient of variation between sample pools (10%), the risk of a type 2 error for findings related to regulation of any specific gene (versus the overall pattern of effects) would be expected to be high. However, notwithstanding these limitations, these data demonstrating "down-regulation" of isoproterenol-mediated effects *do not* support our preliminary hypothesis that the uncoupling of β-adrenoceptor/G_s protein interactions demonstrated previously in hypertension leads to enhanced coupling via other pathways suggested to be potentially important in β-adrenoceptor regulation of growth/proliferative pathways.

In total, these data suggest that in the hypertensive state, the defect in β-adrenoceptor coupling to adenylyl cyclase activation is indicative of a generalized impairment in signaling via both short-term vasoregulatory pathways as well as pathways mediating longer term effects on vascular smooth muscle cell growth and development. In addition, these data suggest the primacy of adenylyl cyclase-dependent pathways in mediating these developmental effects of β-adrenoceptor activation in vascular smooth muscle cells both in the normotensive and hypertensive state. Furthermore, these data support the hypothesis that the blunted vasodilator effects of hormones linked to adenylyl cyclase activation may be an index of their blunted effects in modulating growth regulatory pathways. Most importantly perhaps, these data *do not* support the concept that the GRK2-mediated uncoupling of receptor-G_s interactions in hypertension leads to enhanced coupling via alternate pathways important in β-adrenoceptor-mediated growth regulation. This would infer that in hypertension, these alternate pathways are also uncoupled and/or that quantitatively they contribute little to the growth regulatory effects of β-adrenoceptor activation.

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