G-Protein–coupled Receptor Kinase Activity Is Increased in Hypertension

Robert Gros,* Jeffrey L. Benovic,‡ Christopher M. Tan,* and Ross D. Feldman*
**Department of Medicine, Department of Physiology, and Department of Pharmacology and Toxicology, University of Western Ontario, London, Ontario, Canada; and ‡Department of Biochemistry and Molecular Pharmacology, Kimmel Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107-5541*

Abstract

Impaired vascular β-adrenergic responsiveness may play an important role in the development and/or maintenance of hypertension. This defect has been associated with an alteration in receptor/guanine nucleotide regulatory protein (G-protein) interactions. However, the locus of this defect remains unclear. G-Protein–coupled receptor kinases (GRKs) phosphorylate serine/threonine residues on G-protein–linked receptors in an agonist-dependent manner. GRK activation mediates reduced receptor responsiveness and impaired receptor/G-protein coupling. To determine whether the impairment in β-adrenergic response in human hypertension might be associated with altered GRK activity, we studied lymphocytes from younger hypertensive subjects as compared with older and younger normotensive subjects. We assessed GRK activity by rhodopsin phosphorylation and GRK expression by immunoblot. GRK activity was significantly increased in lymphocytes from younger hypertensive subjects and paralleled an increase in GRK-2 (βARK-1) protein expression. In contrast, no alterations in cAMP-dependent kinase (A-kinase) activity or GRK-5/6 expression were noted. GRK activity was not increased in lymphocytes from older normotensive subjects who demonstrated a similar impairment in β-adrenergic–mediated adenylyl cyclase activation.

These studies indicate that GRK activity is selectively increased in lymphocytes from hypertensive subjects. The increase in GRK activity may underlie the reduction in β-adrenergic responsiveness characteristic of the hypertensive state. (*J. Clin. Invest.* 1997. 99:2087–2093.) Key words: adrenoceptors • aging • cyclic AMP–dependent protein kinase • adenylyl cyclase

Introduction

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. We have focused on the hypothesis that an impairment in receptor-mediated vasodilation may contribute.

An impairment in β-adrenergic–mediated vasodilation has been demonstrated both in human hypertension (1) (primarily in younger, Caucasian borderline hypertensive subjects) and in animal models of hypertension (2, 3). Further, the defect has been shown to be selective and reversible (1, 2). In animal models, it has been shown that the impairment in β-adrenergic–mediated vasodilation parallels a decrease in vascular β-adrenergic–stimulated adenylyl cyclase activity (3). Impairment in human β-adrenergic responsiveness as assessed by impaired adenylyl cyclase activity has also been demonstrated in human hypertension (4) (although this finding has not been universal and may depend on other factors including age [5], gender [6], race [7], and sodium intake [1, 2]). These studies have used the human lymphocyte as a model for human vascular β-adrenergic receptor complex. This has been based on the demonstration by ourselves and others that regulation of the lymphocyte β-adrenergic receptor complex parallels the regulation of functional vascular β-adrenergic responsiveness (2, 5, 8).

Activation of the β-adrenergic receptor transmembrane signaling mechanisms requires the interaction of three primary protein components, the receptor, adenylyl cyclase enzyme, and the guanine nucleotide regulatory protein (G-protein)[1] complex linking receptor activation with adenylyl cyclase activation. Studies by ourselves and others have suggested that the impairment in lymphocyte β-adrenergic responsiveness in younger Caucasian male borderline/mild hypertensive subjects is due to an alteration in receptor/G-protein coupling (2, 4, 9). The mechanism underlying this defect remains unclear.

The efficiency with which G-protein–linked receptors (which include β-adrenoceptors) interact with G-proteins is dependent, in part, on the phosphorylation state of the receptor. Increased serine/threonine receptor phosphorylation is associated with reduced β-adrenergic responsiveness (10–12). β-adrenoceptor phosphorylation is mediated by cyclic AMP–dependent protein kinase (A-kinase) activity (10–12) and by members of the G-protein–coupled receptor kinase (GRK) family which include the β-adrenergic receptor kinases (βARK) (10–12).

Alterations in GRK activity linked to impaired receptor/

Address correspondence to Dr. Ross D. Feldman, Room 6L13B, London Health Sciences Center, University Campus, 339 Windermere Rd., P.O. Box 5339, London, Ontario, Canada N6A 5A5. Phone: 519-663-3929; FAX: 519-663-3232; E-mail: ross.feldman@lhsc.on.ca

Received for publication 22 October 1996 and accepted in revised form 13 March 1997.

J. Clin. Invest.

0021-9738/97/05/2087/07 $2.00
Volume 99, Number 9, May 1997, 2087–2093

1. *Abbreviations used in this paper:* A-kinase, cAMP-dependent protein kinase; βARK, β-adrenergic receptor kinase; G-protein, guanine nucleotide regulatory protein; GRK, G-protein–coupled receptor kinase.

G-protein coupling and reduced adenylyl cyclase activation have been described in GRK overexpression systems (13, 14). Additionally, increased GRK expression and activity have been demonstrated in both human and animal models of congestive heart failure and have been linked with the reduced myocardial β-adrenergic responsiveness characteristic of that disease (15, 16). Whether alterations in GRK activity may have a regulatory role in other human diseases is unknown.

Based on these uncertainties, we have assessed GRK activity and expression in human lymphocytes from hypertensive subjects under conditions where we have previously documented reduced lymphocyte β-adrenergic adenylyl cyclase activation. We demonstrate that hypertensive subjects demonstrate a significant enhancement in both GRK activity and GRK-2 (βARK-1) expression.

Methods

Subject protocol. Younger ($n = 16$) and older ($n = 8$) normotensive subjects and younger borderline/mildly hypertensive ($n = 8$) subjects were studied. All subjects studied were male Caucasians. Younger normotensive subjects were between the ages of 19 and 38 yr, healthy, and not taking any medications on a regular basis or any medications for at least 1 mo before the study. Older normotensive subjects were between the ages of 47 and 70 yr, healthy, and not taking any medications on a regular basis or any medications for at least 1 mo before the study. Hypertensive subjects were between the ages of 20 and 36 yr, otherwise healthy, and had neither renal nor cardiovascular complications. Hypertensive subjects had not taken any antihypertensive medications (or any other medications) for at least 1 mo before the study. The criteria for classifying blood pressure status of subjects were as described previously (4). The borderline or mildly hypertensive subjects had pressures > 140/90 mmHg on at least 20% of daytime automatic ambulatory blood pressure readings (model 90207; Spacelabs, Redmond, WA). All subjects were instructed to maintain a high sodium intake for 3 d before study. Compliance with dietary instruction was assessed by urinary sodium determinations based on overnight urine collections. The protocol was approved by the Human Subjects Review Committee, University of Western Ontario.

On the morning of the study subjects were admitted to the Vascular Clinical Investigation Unit, London Health Sciences Center, University of Western Ontario and maintained in a supine position. An intravenous catheter was inserted in an antecubital vein and 20 min later a 120-ml blood sample was obtained.

Sample preparation. Mononuclear leukocytes were separated from EDTA anticoagulated whole blood by the method of Böyum (17) and as described previously (18).

For the preparation of cytosolic fractions, mononuclear leukocytes were resuspended in ice-cold lysis buffer (20 mM Tris-HCl, 2 mM EDTA, pH 7.5, at room temperature, 100 μg/ml PMSF, 10 μg/ml benzamidine, and 5 μg/ml pepstatin A). Cells were subjected to nitrogen cavitation (Parr Bomb, 600 psi, 15 min at 4°C), followed by centrifugation at 45,000 *g* for 30 min at 4°C. The supernatants were collected and frozen in liquid nitrogen. Additionally, whole cell samples were pelleted and frozen at −80°C.

For the preparation of permeable cells for assessment of adenylyl cyclase activity, cells were washed in Hanks' balanced salt solution with 33 mmol/liter Hepes, 0.5 mmol/liter EDTA, and 1 mmol/liter magnesium sulfate (pH 7.4 at 4°C, buffer A). Cells were permeabilized as previously described (4) with the addition of digitonin (10 μg/ml) in buffer A and incubated for 15 min at 4°C. Cells were centrifuged at 400 *g* for 10 min, resuspended in buffer A, washed as above, and resuspended at a concentration of 2×10^7 cells/ml in buffer A for assay of adenylyl cyclase activity.

Assessment of G-protein–coupled receptor kinase activity. GRK enzymatic activity was assessed via determination of the extent of light-dependent phosphorylation of rhodopsin according to our previously published methods (19). Briefly, rod outer segment membranes were prepared from dark-adapted bovine retinas (Rockville Meat Company, Rockville, MD), via stepwise sucrose gradient centrifugation, and then treated with 5 M urea to inactivate endogenous kinase activity. The resulting preparation contains ~95% rhodopsin (19).

To assess the extent of GRK-dependent phosphorylation, 20 μg of lymphocyte cytosolic protein was incubated with 5 μg of the rod outer segment preparation in a buffer containing 20 mM Tris-HCl, pH 7.5 (at room temperature), 2 mM EDTA, 5 mM $MgCl_2$, 130 μM Na_3VO_4, 80 μM ATP with [γ-^{32}P]ATP (20–50 μCi) in a final reaction volume of 55 μl. The reactions were carried out at 30°C for 30 min in the presence or absence of light. The reactions were terminated by the addition of 10 vol of an ice-cold solution of 100 mM sodium phosphate, 5 mM EDTA buffer, pH 7, followed by centrifugation at 13,000 *g* for 3 min (at room temperature). The pellets were solubilized in 80 μl of a solution consisting of 125 mM Tris-HCl, pH 6.8, 20% glycerol, 40 g/liter SDS, 100 g/liter 2-mercaptoethanol, and 0.25 g/liter bromophenol blue and sonicated for 15 min. Proteins were separated using SDS-PAGE. A 4% (wt/vol) polyacrylamide stacking gel with a 12% running gel was used for all studies. Gels were stained with 1 g/liter Coomassie brilliant blue R-250, 50% methanol, 10% acetic acid for 30–50 min, followed by destaining in 50% methanol, 10% acetic acid for 4–5 h. The excised portion of gel containing the rhodopsin band (~ 38 kD) was quantitated via liquid scintillation spectroscopy. The extent of GRK-mediated phosphorylation was determined as the difference between light- and dark-dependent phosphorylation, each determined in triplicate.

Assessment of A-kinase activity. A-kinase activity was determined in cytosolic lymphocyte preparations. A-kinase activity was assessed by ^{32}P-phosphorylation of a synthetic substrate (Kemptide; Sigma Chemical Co., St. Louis, MO) according to our previously published techniques (18). Briefly, 20 μg of cytosolic lymphocyte preparation was incubated for 20 min at 30°C with 0.3 mM Kemptide, 1 mg/ml bovine serum albumin, 0.5 mM ascorbic acid, 6 mM $MgCl_2$, 140 μM ATP, and 1–2 μCi [γ-^{32}P]ATP in the presence or absence of 100 μM cAMP. Phosphorylated substrate was separated by adherence to phosphocellulose paper (Whatman P81 chromatography paper). After five washes in phosphoric acid (75 mM), radioactivity of the phosphocellulose paper was assessed by liquid scintillation spectroscopy. Extent of A-kinase–mediated peptide phosphorylation was determined as the average of duplicate determinations.

Assessment of GRK and arrestin expression. Assessment of GRK and arrestin protein expression was determined by immunoblotting. GRK-2 protein expression was determined using a 1:100 dilution of a mouse monoclonal antibody 3A10, raised against purified recombinant bovine GRK-2. The GRK-2 antibody recognizes a domain in the COOH-terminal region of bovine GRK-2 that is identical in sequence to human GRK-2 (Marr, R.S., and J.L. Benovic, unpublished observations). GRK-5 and GRK-6 protein expression was determined using a rabbit polyclonal antibody which was generated against the COOH-terminal 102 amino acids of GRK-5, and recognizes both GRK-5 and GRK-6 (20). The GRK-5/6 antibody was used at a dilution of 1:2,000. Protein expression for arrestins was determined using the arrestin mouse monoclonal antibody F4C1 (21), generously provided by Dr. Larry Donosco (Wills Eye Hospital, Philadelphia, PA). This antibody recognizes an epitope found in all arrestins and was used to determine β-arrestin and arrestin-3 protein expression. The antiarrestin antibody was used at a dilution of 1:2,000.

Samples containing 20 μg of protein were mixed with sample buffer (as above) and boiled for 5 min before application to 4% stacking/10% running polyacrylamide gels. Electrophoresis (minigel system; Bio-Rad Laboratories, Richmond, CA) was performed at 100 V for 90–100 min. After electrophoresis, gels were soaked for 20 min in transfer buffer (48 mM Tris-HCl, 39 mM glycine, 0.375 g/liter SDS, 20% methanol). Proteins were transferred to presoaked polyvinylidenedifluoride membranes (Immobilon-P transfer membrane; Millipore Corp., Bedford, MA) at 15 V for 20 min (trans-blot semi-

dry transfer cell; Bio-Rad Laboratories). Membranes were soaked overnight in blocking buffer solution of 1% bovine serum albumin in Tris-buffered saline which contained 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, and 0.1% Tween 20. Membranes were incubated with antibodies (anti–GRK-2 or anti–GRK-5/6 or antiarrestin) for 3 h at room temperature. Membranes were washed once with blocking buffer, followed by three washes with Tris-buffered saline and then incubated for 1 h at room temperature with either peroxidase-conjugated goat anti–mouse IgG (Sigma Chemical Co.) at a 1:1,000 dilution (for both anti–GRK-2 and antiarrestin antibodies) or peroxidase-conjugated goat anti–rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1:7,500 (for anti–GRK-5/6 antibody). Immunoreactivity was detected with enhanced chemiluminescence detection system (ECL; Amersham Corp., Arlington Heights, IL). Autoradiographs were assessed densitometrically using NIH Image 1.6 software.

Assays of adenylyl cyclase activity in permeabilized lymphocytes. Assays of adenylyl cyclase activity are performed on permeabilized cell preparations according to our previously published methods (4). Permeabilized cells resuspended in buffer A were added in an aliquot of 40 μl to give a final incubation volume of 100 μl with 1 μCi [α-^{32}P]ATP, 0.3 mmol/liter ATP, 2 mmol/liter $MgSO_4$, 0.1 mmol/liter cAMP (used in lieu of a phosphodiesterase inhibitor), 5 mM phosphoenolpyruvate, 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 ATP, 50 μg cAMP, and 15,000 cpm [^{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.

β-adrenoceptor stimulation of adenylyl cyclase activity was assessed by isoproterenol (100 μmol/liter) in the presence of GTP (100 μmol/liter). Maximal catalytic activity was assessed with forskolin (10 μmol/liter). Isoproterenol and forskolin-stimulated activities were expressed relative to GTP-stimulated activity. This proportional method of expression was selected prospectively and is consistent with that used in our previous studies comparing stimulated levels of adenylyl cyclase activity in subject groups (4, 8).

Data analysis. Analysis was by ANOVA followed by group *t* test comparisons where appropriate. A value of $P < 0.05$ on a two-tailed test was used as a minimum level of significance.

Results

Both systolic and diastolic blood pressures were significantly higher in the borderline/mildly hypertensive subjects compared with the younger and older normotensive subjects. No other differences were apparent in body mass index or sodium excretion between younger and older normotensive and younger hypertensive subjects (Table I).

Table I. Clinical Data for Subjects

	Younger normotensive	Younger hypertensive	Older normotensive
	(n = 16)	*(n = 8)*	*(n = 8)*
Age (yr)	26±1	30±2	53±3*
Sodium excretion (meq/24 h)	138±12	153±17	138±24
Body mass index (kg/m^2)	23.3±0.6	24.4±0.7	24.0±1.4
Daytime systolic pressure (mmHg)	119±1	133±2*	119±2
Daytime diastolic pressure (mmHg)	73±1	87±3*	75±1

Results are mean±SEM. *$P < 0.05$ vs. younger normotensive controls.

GRK activity in human lymphocytes. Incubation of rod outer segments with lymphocyte cytosolic fractions resulted in the phosphorylation of a 38-kD band, consistent with the labeling of rhodopsin (19). Light exposure resulted in a greater than threefold increase in rhodopsin phosphorylation. The light-dependent phosphorylation of rhodopsin was completely inhibited by the addition of 10 μg/ml of heparin (Fig. 1, *inset A*). The addition of protein kinase A inhibitor (PKI 10 μg/ml) or cAMP (1 mM) did not significantly alter cytosolic GRK-mediated phosphorylation of rhodopsin (data not shown). GRK activity was restricted predominantly to the cytosolic fraction (81±4% [$n = 4$] of total cellular activity).

In cytosolic fractions from younger hypertensive subjects GRK activity was significantly increased (45±9%) as compared with activity in samples from young normotensive subjects or compared with GRK activity in samples from older normotensive subjects (Fig. 1, *inset B*).

GRK-2 expression assessed by immunodetection. Quantitative Western blotting was performed to determine whether the increase in GRK-mediated phosphorylation of rod outer segments in lymphocytes from hypertensive subjects represented an increase in immunodetectable GRK-2. On Western blots of cytosolic lymphocyte preparations, the anti–GRK-2-specific 3A10 antibody recognized a peptide with a molecular mass of 80 kD, which migrated identically with recombinant GRK-2

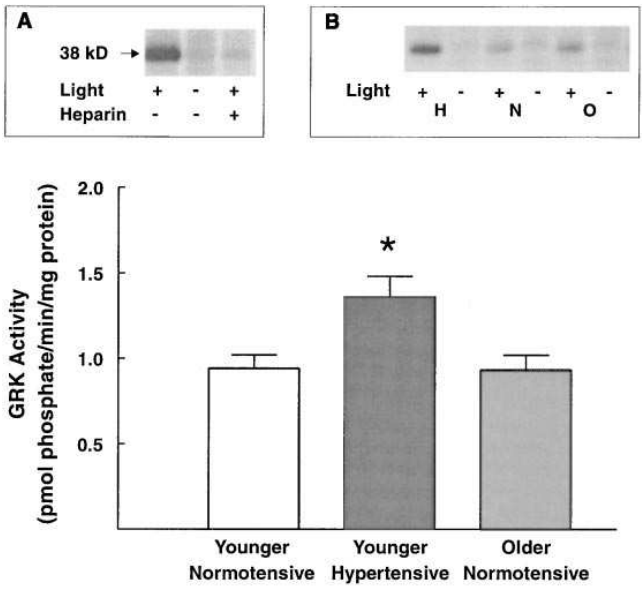

Figure 1. Assessment of GRK activity in lymphocyte cytosolic fractions from younger and older normotensive subjects and younger hypertensive subjects. The data represent mean±SEM. *$P < 0.01$ vs. younger normotensive control subjects. (*Inset A*) Assessment of GRK activity in lymphocyte cytosolic fraction. Autoradiograph depicting light-dependent phosphorylation of rhodopsin (~ 38 kD). Lane *1* represents activity in the presence of light, lane *2* in the absence of light. Lane *3* depicts the effect of heparin on light-dependent rhodopsin phosphorylation. (*Inset B*) Assessment of GRK activity in lymphocytes from younger hypertensive (*H*) and from younger (*N*) and older (*O*) normotensive subjects; representative autoradiograph.

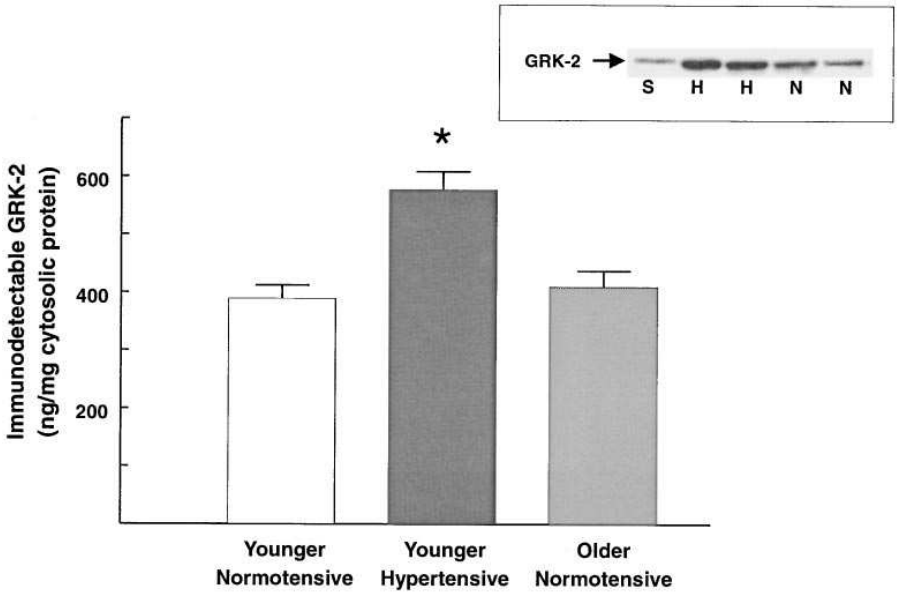

Figure 2. Assessment of GRK-2 expression in cytosolic fractions from younger hypertensive and normotensive subjects. The data represent the mean±SEM. $*P < 0.01$ vs. younger normotensive control subjects. (*Inset*) A representative autoradiograph of a Western blot depicting labeling of 5 ng recombinant GRK-2 standard (*S*), and immunodetectable GRK-2 in cytosolic fractions from younger hypertensive (*H*) subjects and younger normotensive (*N*) subjects.

(Fig. 2, *inset*). Immunodetectable GRK-2 was increased by 40±6% in cytosolic fractions from hypertensive subjects as compared with normotensive subjects ($P < 0.01$) (Fig. 2). Also, a comparable increase (30±7%) in GRK-2 expression in hypertensive subjects was detected when whole cell pellets were assessed (data not shown). In contrast, immunodetectable GRK-2 protein concentrations were not altered in samples from older normotensive subjects (Fig. 2).

To determine the relationship between alterations in GRK-2 expression and β-adrenergic–mediated response, we assessed isoproterenol-stimulated adenylyl cyclase activity in permeabilized lymphocytes in a subset of the younger normotensive ($n = 8$) and younger hypertensive ($n = 4$) subjects studied under identical conditions as those described above. Isoproterenol-stimulated adenylyl cyclase activity was significantly reduced in lymphocytes from hypertensive subjects as compared with normotensive controls (normotensives = 86±4% above GTP-stimulated levels; hypertensives = 64±7%, $P < 0.02$). Isoproterenol-stimulated adenylyl cyclase activity was significantly inversely correlated with GRK-2 protein expression (Fig. 3); i.e., those subjects with higher levels of GRK-2 protein expression demonstrated a lesser extent of β-adrenergic–mediated adenylyl cyclase activation. In contrast, forskolin-stimulated adenylyl cyclase activity and GRK-2 expression were not correlated (data not shown).

A-kinase activity. To determine whether the increase in GRK-mediated activity and GRK-2 expression in cytosolic preparations from hypertensive subjects was related to a generalized increase in cytosolic kinase activity, we examined the activity of A-kinase. Basal A-kinase activity (1,322±37 pmol phosphate/min/mg cytosolic protein) was significantly increased by cAMP (to 2,873±104 pmol phosphate/min/mg cytosolic protein). In contrast to the increased GRK activity seen in lymphocyte cytosolic fractions from hypertensive subjects, neither basal nor cAMP-stimulated A-kinase activities were altered (Fig. 4). Notably, basal A-kinase activity was marginally but significantly reduced in samples from older subjects. However, cAMP-stimulated A-kinase activity was not significantly different between older and younger subjects (Fig. 4).

Assessment of GRK-5/6 expression by immunodetection. To determine whether the increase in GRK-2 in hypertensive subjects might be associated with a generalized increase in the expression of other GRKs, we assessed immunodetectable GRK-5/6 in lymphocytes. The anti–GRK-5/6 antisera predominantly labeled a 68-kD lymphocyte peptide which comigrated with recombinant GRK-5, consistent with labeling of lymphocyte GRK-5 (Fig. 5, *inset*). (It should be noted that GRK-6 migrates essentially identically to GRK-5 on SDS-PAGE [20]. Therefore, in the absence of a more specific antibody, a contribution of GRK-6 to the immunodetectable labeling with the antisera used cannot be ruled out.) In contrast to the increase in GRK-2 expression in samples from hypertensive subjects, immunodetectable GRK-5/6 levels were not significantly al-

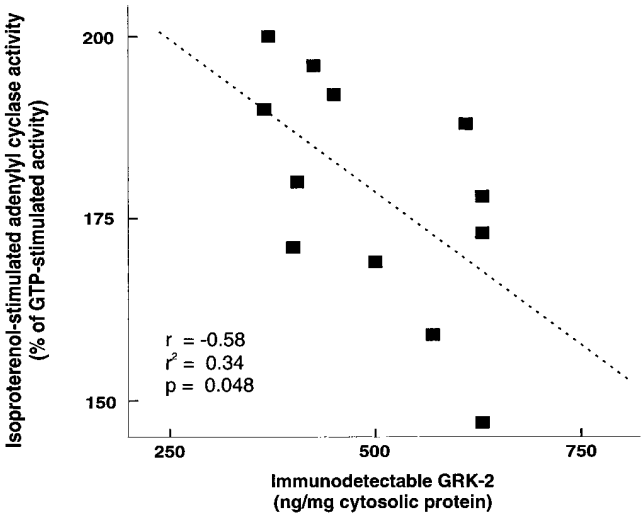

Figure 3. The correlation between GRK-2 expression and isoproterenol-stimulated adenylyl cyclase activity in younger normotensive and hypertensive subjects. Basal adenylyl cyclase activity = 1.23±0.13 pmol/min/10^6 cells, GTP-stimulated activity = 1.76±0.17.

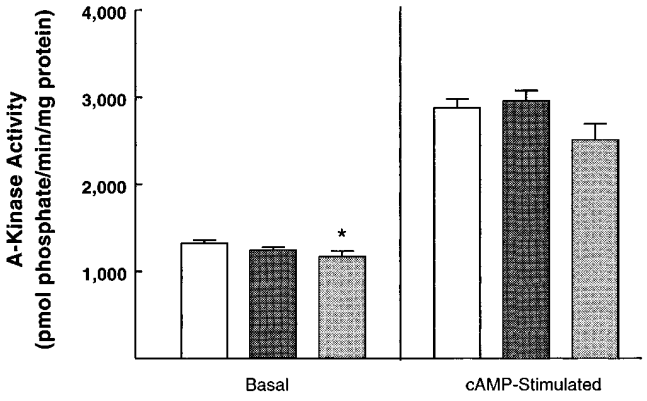

Figure 4. Assessment of A-kinase activity in lymphocyte cytosolic fractions from younger normotensive (*white bars*), older normotensive (*light shaded bars*), and younger hypertensive (*dark shaded bars*) subjects. A-kinase activity is expressed as picomoles of phosphate per minute per milligram of cytosolic protein. The data represent mean activity±SEM. *$P < 0.05$ vs. younger normotensive subjects.

tered in either whole cell pellets (Fig. 5) or in cytosolic fractions (101±9% of the expression in younger normotensive subjects). In whole cell pellets, GRK-5/6 expression was not significantly altered in older subjects. However, cytosolic fractions from older subjects demonstrated a marginal but statistically significant reduction in GRK-5/6 expression (78±7% of the expression in younger normotensive subjects, $P < 0.05$).

Assessment of β-arrestin expression by immunodetection. We next determined whether the increase in immunodetectable GRK-2 in samples from hypertensive subjects was associated with increased immunodetectable β-arrestin levels. The antiarrestin antibody recognized two proteins migrating at ~ 55 and ~ 52 kD, consistent with labeling of β-arrestin and arrestin-3, respectively (Fig. 6). Immunodetectable levels of the arrestins were not significantly different in samples from either younger hypertensive or older normotensive subjects (hypertensive subjects: 98±6% of expression in younger normotensive subjects; older subjects: 91±5% of expression in younger normotensive subjects).

Discussion

An impairment in β-adrenergic–stimulated adenylyl cyclase activity has been demonstrated in lymphocytes from young borderline (primarily Caucasian) hypertensive subjects (4). These studies demonstrate that this defect in signal transduction is associated with increased expression of GRK-2 (βARK-1). No alterations in other kinases were demonstrated. Specifically, cytosolic A-kinase activity was not significantly increased nor was the expression of GRK-5/6.

Impairment in receptor/G-protein interactions has been described previously in hypertensive subjects (4). To date, studies have linked impaired β-adrenergic–stimulated adenylyl cyclase activity with alterations in receptor affinity for agonists (2, 8, 9). This has been conventionally believed to represent an alteration in efficiency of interaction between the receptor and G-protein complex (9). However, neither receptor density (22) nor G-protein expression (4) has been demonstrated to be reduced. An alteration in receptor phosphorylation leading to impaired receptor G-protein coupling would be a potential explanation for the reduction in receptor/G-protein coupling in lymphocytes from hypertensive subjects. However, in the absence of the ability to assess the extent of receptor serine-threonine phosphorylation in vivo, this hypothesis remained unproven. Studies to date have not demonstrated increased A-kinase activity in hypertension (23) (which could then lead to increased receptor phosphorylation and impaired receptor/G-protein coupling).

An increase in GRK function and expression leading to increased receptor phosphorylation would provide an alternative mechanism for receptor/G-protein uncoupling in hypertension. The correlation between increased GRK activity and impaired receptor G-protein coupling has been demonstrated in vitro and in vivo. Increased GRK activity associated with βARK overexpression systems has been demonstrated to parallel an impairment of β-adrenergic stimulation of adenylyl cy-

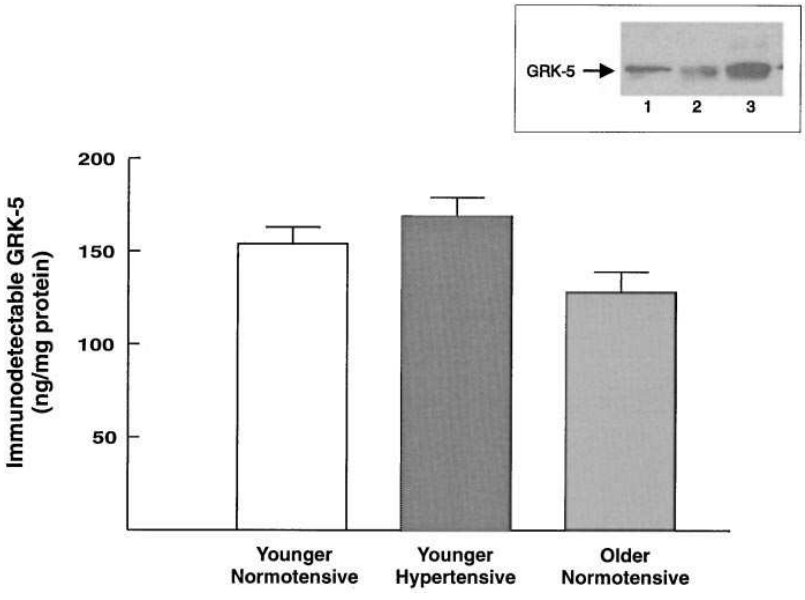

Figure 5. Immunodetectable GRK-5/6 in whole cell pellets from older and younger normotensive and younger hypertensive subjects. The estimates of immunodetectable protein concentrations were based on recombinant GRK-5 standards. The data represent the mean±SEM. (*Inset*) A representative autoradiograph of a Western blot. Lane *1* represents labeling of recombinant GRK-5 (5 ng). Lane *2* represents labeling of lymphocyte GRK-5/6. Lane *3* represents labeling of a 50:50 mix of recombinant GRK-5 and lymphocyte GRK-5/6.

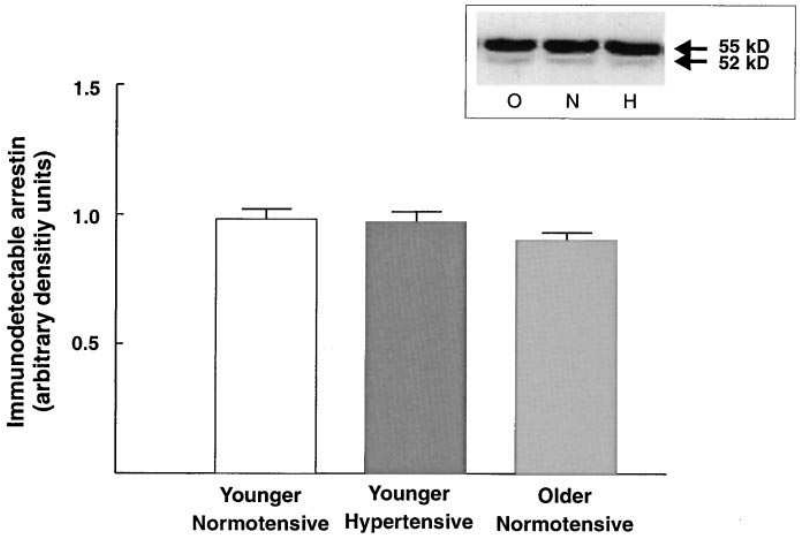

Figure 6. Immunodetectable arrestins in lymphocyte cytosolic fractions from older and younger normotensive subjects and younger hypertensive subjects. The data represent the mean±SEM. (*Inset*) Immunodetectable β-arrestin/arrestin-3 in older (*O*) and younger normotensive (*N*) and younger hypertensive (*H*) subjects. A representative autoradiograph of a Western blot is depicted.

clase (14), although a reduction in basal adenylyl cyclase activity with βARK overexpression has also been reported (13). Further, βARK inhibitor–expressing mutants have been associated with increased β-adrenergic–mediated responses (13) and attenuation of β-adrenergic receptor desensitization (24). As discussed above, pathologically, an increase in GRK activity has been directly linked to the impairment in β_1-adrenergic–stimulated myocardial effects in congestive heart failure in humans (15). Therefore, these studies would generally support the hypothesis that increased GRK activity could mediate an increase in receptor phosphorylation and hence the uncoupling between the receptor and G-protein characteristic of the human hypertensive state.

The observed increase in GRK activity appears to be selectively associated with an increase in βARK-1 (GRK-2) expression. No alterations in GRK-5/6 expression were demonstrated. Further, no alterations in A-kinase activity were apparent in cytosolic fractions in hypertensive versus normotensive controls. The lack of alterations in A-kinase activity has several implications. First, A-kinase activation is the other dominant mechanism mediating receptor serine-threonine phosphorylation leading to impaired activity (10–12). Further, the lack of any difference in either basal or stimulated A-kinase activity in cytosolic fractions from younger normotensive and hypertensive subjects would rule out a nonspecific alteration in kinase activity or a blood pressure–related difference in composition of the cytosolic fractions between hypertensive and normotensive subjects as the explanation for the alteration in GRK activity.

It is notable that the increase in GRK activity and expression was unique to the hypertensive subjects, and was not common to older normotensive subjects. Initial studies by us and others had suggested a common impairment of β-adrenergic–mediated adenylyl cyclase activation in lymphocytes from older and hypertensive subjects (5, 8). More recently, we have suggested that the regulation of the cholera- and pertussis toxin–mediated labeling of G-proteins differs between older and hypertensive subjects (4). The current studies extend these findings to further differentiate the altered pattern of regulation of β-adrenergic responsiveness in aging versus hypertension.

If generalized, the increase in GRK activity (paralleling the impairment in β-adrenergic–stimulated adenylyl cyclase activity) could be the explanation for the impairment in vascular β_2-adrenergic responsiveness characteristic of borderline hypertensive subjects. As discussed above, previous studies by us and others have demonstrated that regulation of lymphocyte β-adrenergic–stimulated adenylyl cyclase activity parallels the regulation of vascular β-adrenergic responsiveness in hypertension. However, hypertension-related, lymphocyte-specific explanations for the altered GRK activity should be considered, including alterations in lymphocyte activation profiles and in lymphocyte subset distribution.

Lymphocyte activation increases GRK activity (25). However, three lines of evidence would suggest that a selective increase in lymphocyte activation in hypertensive subjects is not the explanation for the increase in GRK function. First, other studies have indicated that basal and proliferative responses are not increased (and may be suppressed) in lymphocytes of hypertensive subjects (26). Second, in contrast to the decrease in basal levels of adenylyl cyclase activity seen with lymphocyte activation (27), our previous studies of hypertensive subjects (studied under identical conditions) have not detected any decrease in basal or forskolin-stimulated levels of adenylyl cyclase activity (4). Third, lymphocyte activation increases A-kinase activity (28). Thus, if the increase in GRK activity in hypertensives was related to lymphocyte activation, one would anticipate a parallel increase in both basal adenylyl cyclase activity and A-kinase activity. The divergence between increased GRK activity and unaltered basal levels of adenylyl cyclase and A-kinase activity in hypertensives would argue against a lymphocyte-specific explanation for these findings.

An alteration in the distribution of lymphocyte subpopulations in hypertensive subjects (if those subsets differed in GRK-2 expression) might also explain our findings. However, no alterations in lymphocyte subpopulations have been detected in human hypertensive subjects (29).

It should be pointed out that increased GRK activity may not be the unique defect leading to the impairment in β-adrenergic receptor activation characteristic of the hypertensive state. Recent studies from our laboratory have demonstrated functional G-protein alterations in hypertension which could

not be explained by increased GRK activity (4). It is notable that in congestive heart failure a similar pattern of alterations in receptor/G-protein coupling related to increased GRK activity and altered G-protein function has been described (15, 30).

The mechanism of GRK-2 regulation in hypertensive subjects is unclear. In congestive heart failure, increased GRK-2 expression could be linked to the increase in sympathoadrenal activity (31–33). However, that linkage in hypertension would be somewhat more tenuous. First, increased sympathetic activity has not been universally seen in hypertensive populations (34). Second, our previous studies have demonstrated that the defect in β-adrenergic responsiveness in hypertensive subjects is corrected after dietary salt restriction, a maneuver which is associated with increased sympathetic activity (8). Thus, the pattern of dynamic regulation of β-adrenergic responsiveness in hypertension would not be consistent with the classical models of agonist-induced desensitization. Hence, although the mechanism of GRK-2 regulation in hypertension remains unknown (and is the focus of ongoing study in our laboratory), a linkage with increased sympathetic activity is less likely.

In summary, the present studies have demonstrated an increase in GRK activity and function in lymphocytes from young borderline Caucasian hypertensive subjects. The generalizability of these findings, both to other populations of hypertensive subjects, and to the vasculature of hypertensive humans and animals, has yet to be demonstrated. However, these findings suggest that an alteration in GRK function could underlie the defect in β_2-adrenergic responsiveness, contributing to the impairment in vascular function characteristic of the hypertensive state.

Acknowledgments

These studies were supported by grants-in-aid from the Medical Research Council of Canada and from the National Institutes of Health (GM44944). Dr. Feldman is supported by a Career Investigator Award from the Heart and Stroke Foundation of Ontario. Dr. Benovic is an Established Investigator of the American Heart Association.

References

1. Feldman, R.D. 1990. Defective venous β-adrenergic response in borderline hypertensive subjects is corrected by a low sodium diet. *J. Clin. Invest.* 85: 647–652.

2. Naslund, T., D.J. Silberstein, W.J. Merrell, J.H. Nadeau, and A.J.J. Wood. 1990. Low sodium intake corrects abnormality in β-receptor-mediated arterial vasodilation in patients with hypertension: correlation with β-receptor function in vitro. *Clin. Pharmacol. Ther.* 48:87–95.

3. Feldman, R.D. 1987. Beta-adrenergic receptor alterations in hypertension: physiological and molecular correlates. *Can. J. Physiol. Pharmacol.* 65: 1666–1672.

4. Feldman, R.D., C.M. Tan, and J. Chorazyczewski. 1995. G-Protein alterations in hypertension and aging. *Hypertension (Dallas).* 26:725–732.

5. Feldman, R.D. 1992. A low sodium diet corrects the defect in β-adrenergic response in older subjects. *Circulation.* 85:612–618.

6. Mills, P.J., M.G. Ziegler, R.A. Nelesen, and B.P. Kennedy. 1996. The effects of the menstrual cycle, race, and gender on adrenergic receptors and agonists. *Clin. Pharmacol. Ther.* 60:99–104.

7. Venter, C.P., S. Daya, P.H. Joubert, and W.J. Strydom. 1985. Ethnic differences in human lymphocytic cyclic AMP production after isoprenaline stimulation and propranolol blockade. *Br. J. Clin. Pharmacol.* 19:187–190.

8. Feldman, R.D., W.J. Lawton, and W.L. McArdle. 1987. Low sodium diet corrects the defect in lymphocyte β-adrenergic responsiveness in hypertensive subjects. *J. Clin. Invest.* 79:290–294.

9. Feldman, R., L.E. Limbird, J. Nadeau, D. Robertson, and A.J.J. Wood. 1984. Leukocyte beta receptor alterations in hypertensive subjects. *J. Clin. Invest.* 73:648–653.

10. Hausdorff, W.P., M.G. Caron, and R.J. Lefkowitz. 1990. Turning off the signal: desensitization of β-adrenergic receptor function. *FASEB (Fed. Am. Soc. Exp. Biol.) J.* 4:2881–2889.

11. Lefkowitz, R.J., W.P. Hausdorff, and M.G. Caron. 1990. Role of phosphorylation in desensitization of the β-adrenoceptor. *TIPS (Trends Pharmacol. Sci.).* 11:190–194.

12. Barnes, P.J. 1995. State of the art. Beta-adrenergic receptors and their regulation. *Am. J. Resp. Crit. Care Med.* 152:838–860.

13. Koch, W.J., H.A. Rockman, P. Sarmama, R. Hamilton, R.A. Bond, C.A. Milano, and R.J. Lefkowitz. 1995. Cardiac function in mice overexpressing the β-adrenergic receptor kinase or a βARK inhibitor. *Science (Wash. DC).* 268:1350–1353.

14. Pippig, S., S. Andexingert, K. Daniel, M. Puzicha, M.G. Caron, R.J. Lefkowitz, and M.J. Lohse. 1993. Overexpression of β-arrestin and β-adrenergic receptor kinase augment desensitization of β_2-adrenergic receptors. *J. Biol. Chem.* 268:3201–3208.

15. Ungerer, M., M. Bohm, J.S. Elce, E. Erdmann, and M.J. Lohse. 1993. Expression of β-arrestins and β-adrenergic receptor kinases in the failing human heart. *Circulation.* 87:454–463.

16. Ping, P., A. Li, C. Bentley, and H.K. Hammond. 1995. Increased G-protein receptor kinase activity and expression during development of heart failure. *Circulation.* 92:57*a*. (Abstr.)

17. Böyum, A. 1968. Isolation of mononuclear cells and granulocytes from human blood. *Scan. J. Clin. Lab. Invest.* 21(Suppl. 97):77–89.

18. Feldman, R.D. 1993. Insulin-mediated sensitization of adenylylcyclase activation. *Br. J. Pharmacol.* 110:1640–1644.

19. Benovic, J.L., F. Mayor, Jr., C. Staniszewsi, R.J. Lefkowitz, and M.G. Caron. 1987. Purification and characterization of the β-adrenergic receptor kinase. *J. Biol. Chem.* 262:9026–9032.

20. Loudon, R.P., B. Perussia, and J.L. Benovic. 1996. Differentially regulated expression of the G protein-coupled receptor kinases, βARK and GRK6, during myelomonocytic cell development *in vitro. Blood.* 88:4547–4557.

21. Donosa, L.A., D.S. Gregerson, L. Smith, S. Robertson, V. Knospe, T. Vrabec, and C.M. Kalsow. 1990. S-antigen: preparation and characterization of site-specific monoclonal antibodies. *Curr. Eye Res.* 9:343–355.

22. Brodde, O.-E., A.E. Daul, N. O'Hara, and A.M. Khalifa. 1985. Properties of α- and β-adrenoceptors in circulating blood cells of patients with essential hypertension. *J. Cardiovasc. Pharmacol.* 7:S162–S167.

23. Silver, P.J., R.J. Michalak, and S.M. Kocmund. 1985. Role of cyclic AMP protein kinase in decreased arterial cyclic AMP responsiveness in hypertension. *J. Pharmacol. Exp. Ther.* 232:595–601.

24. Kong, G., R. Penn, and J.L. Benovic. 1994. A β-adrenergic receptor kinase dominant negative mutant attenuates desensitization of the β_2-adrenergic receptor. *J. Biol. Chem.* 269:13084–13087.

25. De Blasi, A., G. Parruti, and M. Sallese. 1995. Regulation of G protein–coupled receptor kinase subtypes in activated T lymphocytes. *J. Clin. Invest.* 95: 203–210.

26. Shasha, S.M., U. Shasha, B. Kristal, M. Barzilai, O. Steinberger, and T. Shkolnik. 1991. Proliferative response of T lymphocytes to mitogenic lectins in essential hypertension. *Nephron.* 58:413–417.

27. Paietta, E., and J.D. Schwarzmeier. 1983. Beta-adrenergic responsiveness of human peripheral lymphocytes after mitogenic transformation with phytohemagglutinin. *Biochem. Pharmacol.* 32:3085–3089.

28. Laxminarayana, D., and G.M. Kammer. 1996. Activation of type l protein kinase A during receptor-mediated human T lymphocyte activation. *J. Immunol.* 156:497–506.

29. Mills, P.J., C.C. Berry, J.E. Dimsdale, M.G. Ziegler, R.A. Nelesen, and B.P. Kennedy. 1995. Lymphocyte subset redistribution in response to acute experimental stress: effects of gender, ethnicity, hypertension, and the sympathetic nervous system. *Brain Behav. Immun.* 9:61–69.

30. Stiles, G.L. 1991. Adrenergic receptor responsiveness and congestive heart failure. *Am. J. Cardiol.* 67:13C–17C.

31. Kiuchi, K., R.P. Shannon, K. Komamura, D.J. Cohen, C. Bianchi, C.J. Homcy, S.F. Vatner, and D.E. Vatner. 1993. Myocardial β-adrenergic receptor function during the development of pacing-induced heart failure. *J. Clin. Invest.* 91:907–914.

32. Colucci, W.S., J.P. Ribeiro, M.B. Rocco, R.J. Quigg, M.A. Creager, J.D. Marsh, D.F. Gauthier, and L.H. Hartley. 1989. Impaired chronotrophic response to exercise in patients with congestive heart failure. Role of postsynaptic β-adrenergic desensitization. *Circulation.* 80:314–323.

33. Maisel, A.S., C. Phillips, M.C. Michel, M.G. Ziegler, and S.M. Carter. 1989. Regulation of cardiac β-adrenergic receptors by captopril. Implications for congestive heart failure. *Circulation.* 80:669–675.

34. Goldstein, D.S. 1981. Plasma norepinephrine in essential hypertension. A study of the studies. *Hypertension (Dallas.).* 3:48–52.