G-Protein–Coupled Receptor Kinase Activity in Hypertension

Increased Vascular and Lymphocyte G-Protein Receptor Kinase-2 Protein Expression

Robert Gros, Jozef Chorazyczewski, Murray D. Meek, Jeffrey L. Benovic, Stephen S.G. Ferguson, Ross D. Feldman

Abstract—Impaired receptor-stimulated adenylyl cyclase activation has been observed in lymphocytes from hypertensive subjects and has been linked to an increase in lymphocyte G-protein receptor kinase-2 (GRK-2) protein expression. However, whether the increase in lymphocyte GRK-2 reflected an increase in vascular GRK-2 was unknown. Therefore, we compared GRK-2 protein expression in lymphocytes and aortas obtained from normotensive Wistar rats, Wistar-Kyoto rats (WKY), and spontaneously hypertensive rats (SHR) and from aortas of Dahl rats. Impaired β-adrenergic responsiveness was observed in lymphocytes and vascular tissues obtained from hypertensive SHR (10 and 15 weeks old) but not in those obtained from prehypertensive SHR (5 weeks old). Immunodetectable lymphocyte GRK-2 protein expression was increased in 10-week-old SHR (143 ± 10% of the expression in 10-week-old Wistar rats and 131 ± 11% of the expression in 10-week-old WKY, n = 5 in each group). Immunodetectable vascular smooth muscle cell GRK-2 was comparably increased (169 ± 14% of the expression in Wistar rats and 138 ± 7% of the expression in WKY, n = 5 in each group). Also, in hypertensive Dahl salt-sensitive rats, vascular GRK-2 protein expression was increased (185 ± 14% of the expression in Dahl salt-resistant rats, n = 5 in each group) compared with Dahl salt-resistant controls. These studies support a generalized defect in vascular GRK-2 protein expression in hypertension, which could be an important factor in the impairment of β-adrenergic–mediated vasodilation, characteristic of the hypertensive state. (Hypertension. 2000;35:38-42.)

Key Words: G protein ■ adrenergic receptors ■ adenylyl cyclase ■ hypertension, essential ■ cyclic AMP ■ rats

The basic hemodynamic abnormality in hypertension is increased peripheral resistance. This increase in peripheral resistance is due to both structural and functional factors. Functionally, peripheral resistance is the net balance between vasoconstrictor and vasodilator mechanisms. The physiological antagonism between vasoconstrictor and vasodilator mechanisms may be modified by defects in functional factors regulating these mechanisms. We and others have suggested that defects in vasodilator mechanisms shift this balance toward increased peripheral resistance (reviewed in Reference 1). Impaired receptor-mediated vasodilation has been reported in both human hypertension and animal models of hypertension. In rat models of hypertension, this impairment in receptor-mediated vasodilation has been reported for those receptors linked to activation of adenylyl cyclase, which include β-adrenergic receptors, adenosine receptors, prostanoïd receptors, and histamine receptors (reviewed in Reference 2). In human hypertension, impaired β-adrenergic–mediated vasodilation has also been observed, primarily in younger white borderline hypertensive subjects.3,4 To explain this defect in receptor-mediated vasodilation in hypertension, we and others have focused on the receptor/G-protein/adenylyl cyclase transmembrane signaling system. Impaired vascular and renal adenylyl cyclase responsiveness to hormones acting through receptors linked to the stimulatory G protein (Gs) has been reported in rat models of hypertension (reviewed in Reference 2).

In human hypertension, we have focused on the lymphocyte β-adrenoceptor/Gs/adenylyl cyclase complex as a model for the vascular β-adrenoceptor/Gs/adenylyl cyclase complex. We and others have shown that regulation of the lymphocyte β-adrenoceptor complex parallels the regulation of functional vascular β-adrenergic responsiveness.4,5 In a series of studies, we demonstrated that (1) lymphocyte β-adrenergic responsiveness was impaired in the hypertensive state, (2) this reflected an impairment in receptor/G-protein coupling,6–8

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and (3) the molecular basis of this defect in receptor/G-protein coupling may relate to an increase in expression of a G-protein–coupled receptor kinase (GRK).9,10 GRKs are a family of enzymes that specifically phosphorylate agonist-occupied G-protein–linked receptors.11–13

The present studies were performed to determine whether, in hypertension, the increase in lymphocyte GRK-2 expression was reflected by a parallel alteration in vascular GRK-2 expression. Data to be presented demonstrate that in both spontaneously hypertensive and Dahl models of hypertension, vascular GRK-2 protein expression is upregulated, providing a potential mechanism for the impairment in vascular responsiveness characteristic of the hypertensive state.

**Methods**

**Animal Protocol**

Male spontaneously hypertensive rats (SHR), normotensive Wistar rats, normotensive Wistar Kyoto rats (WKY), and Rapp Dahl salt-sensitive and salt-resistant rats (100 to 350 g, Harlan Sprague Dawley, Inc, Indianapolis, Ind) were used. The rats were cared for in accordance with the Canadian Council on Animal Care guidelines and housed under a 12-hour light/dark cycle with free access to standard laboratory rat chow and drinking water. For both Dahl salt-sensitive and salt-resistant rats, laboratory chow was supplemented with 8% NaCl. Indirect tail-cuff measurements of systolic blood pressure (SBP) were obtained in lightly anesthetized rats (1% to 1.5% halothane in a mixture of 50% nitrous oxide and 50% oxygen) by use of a Harvard rat tail monitor. Mean SBP in 5-week-old WKY was 102±5 mm Hg. In 5-week-old SHR, mean SBP was 98±4 mm Hg. In all 10-week-old SHR studied, SBP was >125 (mean SBP 138±2 mm Hg). In all 10-week-old Wistar rats and WKY studied, SBP was <110 (mean SBP 96±4 mm Hg [Wistar] and 94±2 mm Hg [WKY]). In 15-week-old SHR, mean SBP was 154±5 mm Hg (with no pressure <135 mm Hg). In all 15-week-old WKY, SBP was <115 (mean SBP 108±3 mm Hg). In Dahl salt-sensitive rats after 4 weeks of NaCl-supplemented diet, mean SBP was 156±7 mm Hg (with no pressure <140 mm Hg). In Dahl salt-resistant rats, mean SBP was 122±2 mm Hg (with no pressure >130 mm Hg). All terminal procedures were performed after anesthesia with 5% halothane.

**Lymphocyte Preparation**

Whole blood was obtained from rats by cardiac puncture, and mononuclear leukocytes were separated from heparin-anticoagulated whole blood by the method of Böyum.14 For assessment of adenylyl cyclase activity in rat lymphocytes, permeable cells were prepared as previously described.9 For assessment of GRK-2 protein expression, whole-cell lymphocyte samples were obtained as described above, pelleted, and frozen at −80°C.

**Preparation of Isolated Aortic Vascular Smooth Muscle Cells and Proteins**

Freshly isolated thoracic aortas (prepared with use of our previously published techniques15) were placed into a Petri dish containing ice-cold physiological salt solution (mmol/L: NaCl 130, HEPES 20, d-glucose 10, KCl 5, CaCl2 1, and MgCl2 1, pH 7.4) and extensively cleaned of adventitial fat and connective tissue by use of fine forceps and scissors. The thoracic aortas were cut into longitudinal strips ~20 to 30 mm long and 5 mm wide. The aortic strips were then transferred to 2 mL of dissociation solution (physiological salt solution plus 0.25 mmol/L EDTA, 1.2 mg/mL BSA, 1 mg/mL papain, 0.8 mg/mL Sigma blend collagenase, and 0.09 mg/mL dithiothreitol, pH 7.0) and incubated at 37°C for 1 hour. Tissue was teased apart by triturating with a Pasteur pipette. Undigested material was removed with forceps. The vascular preparation was centrifuged at 500g for 10 minutes at 4°C. The pellet was resuspended in physiological salt solution and transferred to microtube tubes and frozen at −80°C until needed. As an alternative to isolated vascular smooth muscle cells, we prepared thoracic aortic lystases as follows: clean thoracic aortic strips were placed in microtube tubes with 400 µL lysis buffer (100 mmol/L NaCl, 1% Triton X-100, 10% glycerol, 50 mmol/L HEPES [pH 7.4], and 1 mmol/L EDTA) and incubated at 4°C for 1 hour. After incubation, microtube tubes and tissues were frozen at −80°C until needed.

**Assessment of GRK Protein Expression**

Assessment of GRK protein expression was determined by immunoblotting (as previously described in References 9 and 10). GRK-2 protein expression was determined by using a 1:100 dilution (for lymphocyte studies) or a 1:10 dilution (for vascular smooth muscle studies) of a mouse monoclonal antibody 3A10 raised against purified recombinant bovine GRK-2 (as previously described in Reference 9). Immunoreactivity was detected with the enhanced chemiluminescence detection system (ECL, Amersham Corp). Submaximally exposed autoradiographs were assessed densitometrically by using NIH Image 1.6 software. Initial studies determined that under these conditions the assay was linear in the range of protein concentrations used.

**Assays of Adenylyl Cyclase Activity in Permeabilized Lymphocytes**

Adenylyl cyclase activity was determined in permeabilized lymphocytes according to our previously published methods.8 β-Adrenoceptor stimulation of adenylyl cyclase activity was assessed in the presence of isoproterenol (100 µmol/L) and GTP (100 µmol/L). Maximal catalytic activity was assessed with forskolin (10 µmol/L). 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.8–10

**Assessment of Tension in the Aortic Ring Segments**

Assessment of tension in the aortic ring segments was performed according to our previously described methods.15 After the equilibration, rings were maximally preconstricted with phenylephrine (3 µmol/L) and allowed to reach a plateau. Relaxation was assessed in response to a single addition of isoproterenol (10 µmol/L) or sodium nitroprusside (10 nmol/L). Relaxation response with isoproterenol and nitroprusside was quantified by determination of the area above the relaxation response curve by use of a trapezoidal method of analysis as previously described.15

**Analysis**

Those parameters expressed as a ratio (eg, isoproterenol-stimulated adenylyl cyclase activity expressed relative to GTP-stimulated activity) are log-normally distributed.16 Therefore, these data were log-transformed for statistical analysis (t test, correlations). A value of P<0.05 on a 2-tailed 2-group test was used as a minimum level of significance.

**Results**

**Alterations in SHR Lymphocyte β-Adrenergic–Stimulated Adenylyl Cyclase Activity**

Isoproterenol-stimulated adenylyl cyclase activity was significantly reduced in lymphocytes from 10-week-old SHR compared with 10-week-old Wistar rats, to an extent comparable to that previously seen in borderline/mildly hypertensive human subjects (Figure 1A). In contrast, GTP-stimulated adenylyl cyclase activity was not significantly altered (2.98±0.87 pmol/min per 10⁶ cells [SHR] and 2.57±0.59
pmol/min per 10^6 cells [Wistar]). Additionally, forskolin-stimulated activity was not significantly altered in lymphocytes from SHR (Figure 1B).

Alterations in Lymphocyte GRK-2 Protein Expression in SHR

Paralleling the decrease in isoproterenol-stimulated adenylyl cyclase activity in lymphocytes from 10-week-old SHR, there was a significant increase in GRK-2 protein expression compared with either 10-week-old Wistar rats (143±10% of expression in Wistar rats, *P*, 0.05, n=5 in each group; Figure 2) or WKY (131±11% of expression in WKY, *P*, 0.05, n=5 in each group).

Alterations in Isoproterenol-Mediated Relaxation in SHR Aortic Ring Segments

In aortic ring segments isolated from 10-week-old SHR, Wistar rats, and WKY, there was an age-dependent and blood pressure–dependent impairment in vascular β-adrenergic–mediated response in SHR. Maximal phenylephrine-mediated constriction was not significantly different between WKY or Wistar rats and SHR (data not shown). In phenylephrine-precontracted aortic ring segments, the addition of isoproterenol resulted in a rapid relaxation (reaching a nadir by 5 minutes). The extent of maximal relaxation was significantly attenuated in SHR compared with Wistar rats and WKY (Table). In contrast, responses to doses of nitroprusside that mediated an extent of relaxation comparable to that seen with isoproterenol were not altered between SHR and Wistar rats (Table).

Alterations in Vascular GRK-2 Protein Expression

GRK-2 protein expression was increased in vascular smooth muscle cells of 10-week-old SHR versus either 10-week-old Wistar rats or WKY, with both expressed on an absolute basis (Figures 3 and 4) and as a proportion of α-actin expression (GRK-2/α-actin ratio 1.00±0.18 normalized arbitrary units [Wistar] and 1.79±0.17 normalized arbitrary units [SHR], *P*, 0.015). Relative α-actin expression was not significantly altered in SHR (data not shown).

We also obtained aortas from Dahl salt-sensitive and -resistant rats after 4 weeks of a high salt diet. Vascular

### Table: Age-Dependent Alterations in Vascular Responsiveness

<table>
<thead>
<tr>
<th>Age</th>
<th>ISO-Mediated Relaxation</th>
<th>SNP-Mediated Relaxation</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>397±17</td>
<td>379±21</td>
</tr>
<tr>
<td>SHR</td>
<td>401±25</td>
<td>404±19</td>
</tr>
<tr>
<td>10 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>358±14</td>
<td>345±32</td>
</tr>
<tr>
<td>Wistar</td>
<td>332±9</td>
<td>394±12</td>
</tr>
<tr>
<td>SHR</td>
<td>276±14*</td>
<td>364±23</td>
</tr>
<tr>
<td>15 wk</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>197±14†</td>
<td>229±16†</td>
</tr>
<tr>
<td>SHR</td>
<td>132±17†</td>
<td>227±13†</td>
</tr>
</tbody>
</table>

Values are mean±SEM and are expressed as percent - min (area above relaxation curve) (n=5 to 8 rats in each group). ISO indicates isoproterenol; SNP, sodium nitroprusside.

* *P*, 0.02 vs normotensive control rats; † *P*, 0.02 vs 5- and 10-wk-old rats.
mediated constriction was not significantly different between 5-, 10-, and 15-week-old rats, maximal phenylephrine-pertensive), 10 weeks, and 15 weeks of age. In all age groups
pressure, we examined SHR and WKY at 5 weeks (prehypertensive, 10 weeks, and 15 weeks of age). In all age groups
5 rats in each group). * 
SEM (n = the mean 

P

5, 10-, and 15-week-old SHR and WKY. The symbols represent
the mean ± SEM (n = 5 rats in each group). * P < 0.03 vs normo-
tensive WKY; # P < 0.01 vs 10- and 15-week-old WKY.

GRK-2 protein expression was significantly increased (185 ± 14% of expression in Dahl salt-resistant rats, P < 0.05)
in aortic tissue lysates obtained from Dahl salt-sensitive rats compared with Dahl salt-resistant rats. Relative α-actin ex-
pression was not different (data not shown).

Developmental Regulation of Vascular Reactivity
and GRK-2 Expression
To examine the developmental relation among vascular β-adrenergic responsiveness, GRK-2 expression, and blood pressure, we examined SHR and WKY at 5 weeks (prehypertensive), 10 weeks, and 15 weeks of age. In all age groups (5-, 10-, and 15-week-old rats), maximal phenylephrine-mediated constriction was not significantly different between SHR and WKY (data not shown). In contrast to the impair-
ment in β-adrenergic–mediated relaxation in adult (10-week-old) SHR, no alterations in isoproterenol-mediated relaxation were observed in 5-week-old rats (Table). Notably, compared with 5-week-old and 10-week-old WKY, 15-week-old SHR and WKY exhibited a parallel impairment in both sodium nitroprusside–mediated and isoproterenol-mediated relaxation (P < 0.02, Table). The impairment in both sodium nitroprusside–mediated and isoproterenol-mediated relaxation is consistent with a defect “downstream” from the receptor/G-protein/enzyme complex. However, even when normalized for the impairment in sodium nitroprusside–mediated vasodilation, an impairment in isoproterenol-mediated relaxation was still evident in 15-week-old SHR compared with WKY (Table).

To examine the developmental regulation of vascular GRK-2 protein expression, we examined aortic tissue lysates obtained from 5-, 10-, and 15-week-old SHR versus WKY. GRK-2 protein expression was comparably increased in hypertensive rats at both 10 and 15 weeks of age in SHR versus WKY (138 ± 7% of the expression in 10-week-old WKY and 147 ± 11% of expression in 15-week-old WKY, Figure 4). Notably, GRK-2 protein expression was reduced in 5-week-old (prehypertensive) SHR compared with 5-week-old WKY (81 ± 10% of the GRK-2 expression observed in 5-week-old WKY, Figure 4). Overall, in WKY, vascular GRK-2 protein expression decreased with increasing age, whereas vascular GRK-2 protein levels in SHR did not demonstrate significant alteration with increasing age (Figure 4). Relative α-actin protein expression was not significantly altered between SHR and WKY and did not change from 5 to 15 weeks of age (data not shown).

Discussion
We recently demonstrated that in hypertensive subjects an increase in lymphocyte GRK activity and GRK-2 expression parallels the reduction in lymphocyte β-adrenergic–mediated adenylyl cyclase activity.9,10 The present study demonstrates that lymphocyte β-adrenergic–stimulated adenylyl cyclase activity in SHR is reduced (comparable to that previously seen in lymphocytes from human hypertensive subjects) and that this parallels an increase in lymphocyte GRK-2 expression. Furthermore, the increase in GRK-2 protein expression is also evident in vascular smooth muscle cells from SHR with developed hypertension and Dahl salt-sensitive hypertensive rats, and this is associated with an impairment in β-adrenergic–mediated vascular relaxation (again comparable to that previously seen in human hypertension). Moreover, we demonstrate an age-dependent decrease in vascular GRK-2 protein expression in normotensive WKY. This decrease in vascular GRK-2 does not appear to occur in SHR.

We have previously speculated that the increase in lymphocyte GRK-2 expression in human hypertensive subjects was likely not restricted to lymphocytes (ie, might be general-
ized to vascular smooth muscle cells), and if so, it might account for the reduction in vascular β-adrenergic respon-
siveness demonstrated in both human hypertension9 and in rat models of hypertension.2 The present studies support this hypothesis. SHR have been used commonly for the study of β-adrenergic receptor systems in hypertension (reviewed in
Reference 2). Impaired isoproterenol-mediated vasodilation and impaired isoproterenol-stimulated adenylyl cyclase activation in vascular tissues have been previously demonstrated in this model. In the present study, we demonstrate that β-adrenergic–mediated adenylyl cyclase activity in lymphocytes from SHR is impaired to an extent comparable to that previously reported in studies of vascular smooth muscle cells (reviewed in Reference 2) and similar to the extent of impairment seen in lymphocytes from human hypertensive subjects.9,13 Thus, this observation reiterates the utility of the lymphocyte as a model for the vascular β-adrenoceptor complex in hypertension.

The reduction in β-adrenergic–mediated adenylyl cyclase activity was associated with an increase in GRK-2 protein expression in both lymphocytes and vascular smooth muscle cells. This relative increase in GRK-2 protein expression in hypertensive rats is comparable to the increase in GRK-2 protein expression in lymphocytes from hypertensive subjects. In total, these studies suggest a more generalized defect in GRK-2 expression both in animal models of hypertension and human hypertensive subjects as well as in lymphocytes and myocytes.

The linkage between increased GRK-2 protein expression and increased blood pressure has yet to be determined. In this regard, we examined age-dependent changes in both blood pressure and GRK-2 protein expression in SHR and WKY. The relative increase in GRK-2 protein expression in SHR was apparent only in conjunction with established hypertension and was not apparent in the prehypertensive stage (up to 5 weeks of age). Notably, we observed an age-dependent decrease in vascular GRK-2 expression in normotensive WKY. In contrast, vascular GRK-2 protein expression did not decrease with increasing age in SHR. The reason for the observed developmental decrease in vascular GRK-2 protein expression with age, apparent in normotensive rats, is unknown. However, in this context, the relative increase in GRK-2 protein expression in SHR might be seen as a failure in the developmental downregulation that occurs in normotensive animals.

The mechanisms regulating GRK-2 protein expression are unclear. Our previous studies have suggested that the increase in GRK-2 protein expression was not due to an increase in GRK-2 mRNA expression. Developmental regulation of GRK-2 has been reported neonatal rats. In addition, recent data have shown that GRK-2 protein expression is regulated via a proteosomal degradation pathway. Whether this pathway is altered in hypertension (or with age) is unknown. However, if this pathway is important in developmental regulation of GRK-2 expression and is defective or absent in SHR, the net result might be a relative increase in GRK-2 protein expression in aging SHR compared with aging WKY. This hypothesis is the focus of ongoing studies.

In conclusion, our studies in SHR demonstrate that the increase in GRK-2 expression is evident in both lymphocytes and vascular smooth muscle cells and parallels an impairment of β-adrenergic–mediated adenylyl cyclase activity (comparable to that seen in human hypertension) and β-adrenergic–mediated vasodilation. In addition, increased vascular GRK-2 protein expression was also observed in hypertensive Dahl rats, suggesting a conserved change in GRK-2 protein expression across different rat models of hypertension. Overall, our findings support the hypothesis that an alteration in GRK function could underlie the defect in G-protein–coupled receptor-mediated adenylyl cyclase activation contributing to the impairment in vascular function characteristic of the hypertensive state.

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