Upregulation of G protein-linked receptor kinases with advancing age in rat aorta

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A WELL-DOCUMENTED AGE-RELATED change in cardiovascular physiology is the loss of β-adrenergic receptor (β-AR)-mediated vasorelaxation in the presence of intact receptor-mediated vasoconstriction (22). This shift in homeostasis toward vasoconstriction may be associated with hypertension, orthostatic hypotension, and atherosclerosis observed in the elderly (9).

β-ARs couple with stimulatory G proteins (Gs), which activate adenylyl cyclase. This interaction results in cAMP production, subsequent activation of protein kinase A, and, ultimately, vasorelaxation (26). Mechanistic studies to understand the age-related loss of β-AR-mediated vasorelaxation have been examined. It appears that there is little, if any, age-related changes in abundance of β-ARs (31), G proteins including Gs (17, 21, 28), the effector enzyme adenylyl cyclase (20), and protein kinase A (8). Together, these studies suggest the age-related loss of β-AR-mediated vasorelaxation may be directly related to β-AR desensitization (15).

Numerous reports indicate the primary mode of β-AR desensitization is phosphorylation via G protein-linked receptor kinases (GRKs), a family of serine/threonine protein kinases (as reviewed by Pitcher et al., Ref. 25). Desensitized β-ARs bind agonist poorly, and, therefore, their participation in downstream signal transduction is minimal (19). Once β-ARs are phosphorylated, they become targets for another class of proteins called β-arrestins, which mediate receptor downregulation (11).

To date, six different GRKs have been identified. Of interest, GRK-2, GRK-3, and GRK-5 (GRK-2 and GRK-3 are also known as β-AR kinases; β-ARK-1 and β-ARK-2, respectively) target β-ARs and are highly expressed in the cardiovascular system (25). Also, GRK-2 and GRK-3 predominantly reside in the cytosol (19), whereas GRK-5 is predominately plasma membrane bound (16). Functionally, GRKs preferentially phosphorylate agonist-occupied receptors and, on agonist-mediated β-AR activation, are targeted to the plasma membrane (7). GRKs are capable of β-AR phosphorylation only after plasma membrane targeting (30).

An age-related change in GRK activity or expression would implicate GRKs in the age-related decline in β-AR-mediated vasorelaxation. Therefore, in this study, we examine the hypothesis that age-related changes in β-AR-mediated vasorelaxation is caused by changes in GRK function. To that end, we examined age-related changes in total GRK activity in aorta from Fischer-344 rats ranging in age from 6 wk to 24 mo. We also examined similar aortic preparations for age-related changes in expression of GRK subtypes 2, 3, or 5 and β-arrestin. Last, we examined age-related changes...
in cellular localization (cytoplasmic vs. crude membrane preparations) of GRKs. Fischer-344 rats provide an animal model that exhibits similar vascular age-related changes to those documented in humans and are widely used in experimental aging research (23).

MATERIALS AND METHODS

Materials. Reagents were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Primary and secondary antibodies were obtained from Santa Cruz Biochemical (Santa Cruz, CA). Rhodopsin was prepared as described by Benovic et al. (1). The composition of PSS was (in mM) 114 NaCl, 4.7 KCl, 1.15 KH2PO4, 1.1 Na2HPO4, 1.18 MgSO4, 15 NaHCO3, 1.25 CaCl2, and 5 glucose. The composition of homogenization buffer (HB) was 25 mM Tris, 5 mM EDTA, 20 μg/ml leupeptin, 20 μg/ml benzamidine, and 40 μg/ml phenylmethylsulfonyl fluoride.

Male Fischer-344 rats (6 wk, 6 mo, 12 mo, and 24 mo old) obtained from Harlan Sprague Dawley (Indianapolis, IN) were killed by pentobarbital sodium sedation and exsanguination in accordance with the procedures approved by the Institutional Animal Care and Use Committee at the Portland Veterans Affairs Medical Center. Thoracic aortas were removed, cleaned of fat and connective tissue in ice-cold PSS, immediately frozen under liquid nitrogen, and stored at −80°C until use.

Tissue preparation. Frozen aortic tissue was pulverized, then thawed and homogenized in a glass-glass motor-driven tissue homogenizer in HB. Homogenates were centrifuged at 500 g for 15 min at 4°C, and the resulting supernatant was centrifuged at 100,000 g for 20 min at 4°C. Cytoplasmic supernatants were concentrated with Centricon-30 (Amicon, Beverly, MA) spin columns, whereas pellets (crude membrane fraction) were diluted in HB.

GRK activity. Supernatants (100,000 g) prepared as described above were again centrifuged, and 300,000 g supernatants were collected. This fraction was purified through diethylaminoethyl Sephacel drip columns and concentrated with Centricon-10 spin columns. GRK activity of this concentrate was determined as described by Bliziotis et al. (3) using dark-adapted bovine rod outer segments (rhodopsin enriched). Briefly, 30 μg of rhodopsin (a GRK target protein) and 0.3 mM of γ-[32P]ATP (2.5 cpm/fmol) were mixed with 50 μg of protein from aortic preparations in HB + 10 mM MgCl2. This reaction was exposed to light and incubated at 30°C for 30 min. Then reactions were terminated by adding ice-cold HB + 10 mM MgCl2 to the mixture and centrifuged at 35,000 g for 15 min. The resultant pellet was resuspended in SDS-PAGE sample buffer containing 5% SDS and separated through a 12% polyacrylamide gel. After electrophoresis, the gel was dried and exposed to phosphor-sensitive film. Autoradiography was performed using a Storm Detection System (Molecular Dynamics).

Immunoblotting. Protein expression was determined as described (28) by immunoblotting polyvinylidene difluoride (PVDF) membranes with specific primary antibodies (1:750). Immunodetection was accomplished using an appropriate secondary antibody (1:5,000) and enhanced chemiluminescence Western blotting kit (Amersham). Image analysis was performed using a Storm Detection System. The PVDF membranes containing cytosolic proteins were then stripped of antibody with Re-Blot (Chemicon, Temecula, CA), reprobed with an anti-actin antibody, and visualized as described above.

Analysis. Differences between age groups were determined by one-way ANOVA with Bonferroni’s post hoc analysis. A value of P < 0.05 was considered significant. Uniform loading of proteins was validated with bicinchoninic acid (BCA) analysis compared with a BSA standard curve (Pierce Chemical, Rockford, IL) before immunodetection. Cytosolic proteins are expressed as a ratio of protein of interest per expression of α-actin. Particulate proteins are expressed as optical density (arbitrary units) normalized via the BCA analysis.

RESULTS

Alterations in total GRK protein activity. GRK activity in cytoplasmic aortic extracts from 6-wk, 6-mo, 12-mo, and 24-mo-old animals was assessed by phosphorylation of rhodopsin (Fig. 1). Total GRK activity increased with advancing age. In 6-wk-old animals, optical density was 81.3 ± 12.6 arbitrary units. This compared with 92.9 ± 8.3, 181.5 ± 17.2, and 169.9 ± 22.3 arbitrary units for 6-, 12-, and 24-mo-old animals, respectively. Therefore, if density of 6-wk and 6-mo-old animals is compared with density values of 12- and 24-mo-old animals, total GRK activity in aorta from 12- and 24-mo-old animals increased nearly 2.1-fold compared with aorta from 6-wk and 6-mo-old animals. The ability of cytoplasmic aortic preparations to phosphorylate rhodopsin was significantly reduced in the presence of heparin (a GRK inhibitor; 10 μg/ml) or when...
the reaction was maintained in the dark (data not shown).

**Alterations in GRK-2, -3, and β-arrestin protein expression from aortic cytoplasmic preparations.** The cytoplasmic fraction of Fischer-344 rat aorta contains GRK-2, GRK-3, and β-arrestin proteins. In general, expression of each of these proteins increased with age. Table 1 presents the absolute values for each protein and age. If expression values of 6-wk and 6-mo-old animals are compared with expression values of 12- and 24-mo-old animals, soluble GRK-2 expression in aorta from 12- and 24-mo-old animals increased nearly 3.6-fold compared with aorta from 6-wk and 6-mo-old animals (Fig. 2). Likewise, soluble GRK-3 expression in aorta from 12- and 24-mo-old animals increased ~3.8-fold compared with aorta from 6-wk and 6-mo-old animals (Fig. 3). Finally, β-arrestin expression in aorta from 12- and 24-mo-old animals increased ~1.6-fold compared with aorta from 6-wk and 6-mo-old animals (Fig. 4).

**Alterations in GRK-2,-3, and -5 protein expression from aortic crude membrane preparations.** The crude membrane fraction prepared from Fischer-344 rat aorta contains GRK-2, GRK-3, and GRK-5 proteins. Age-related changes in expression varied with each GRK subtype. Table 1 presents the absolute values for each protein and age. When the expression values of

<table>
<thead>
<tr>
<th>Protein</th>
<th>Fraction</th>
<th>6 wk (n = 12)</th>
<th>6 mo (n = 12)</th>
<th>12 mo (n = 10)</th>
<th>24 mo (n = 7)</th>
<th>P Value; F Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRK-2</td>
<td>cytoplasmic</td>
<td>0.08 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>0.27 ± 0.02</td>
<td>0.29 ± 0.03</td>
<td>&lt;0.01; 22.7</td>
</tr>
<tr>
<td>GRK-2</td>
<td>membrane</td>
<td>164.3 ± 10.7</td>
<td>242.6 ± 25.3</td>
<td>235.1 ± 21.3</td>
<td>227.5 ± 15.9</td>
<td>&lt;0.05; 3.8</td>
</tr>
<tr>
<td>GRK-3</td>
<td>cytoplasmic</td>
<td>0.08 ± 0.02</td>
<td>0.11 ± 0.02</td>
<td>0.39 ± 0.03</td>
<td>0.42 ± 0.04</td>
<td>&lt;0.01; 38.8</td>
</tr>
<tr>
<td>GRK-3</td>
<td>membrane</td>
<td>36.2 ± 4.3</td>
<td>74.6 ± 7.7</td>
<td>71.1 ± 9.7</td>
<td>84.2 ± 12.8</td>
<td>&lt;0.05; 6.1</td>
</tr>
<tr>
<td>GRK-5</td>
<td>membrane</td>
<td>81.5 ± 10.9</td>
<td>92.3 ± 12.9</td>
<td>96.6 ± 8.1</td>
<td>101.2 ± 14.4</td>
<td>NS; 0.6</td>
</tr>
<tr>
<td>β-Arrestin</td>
<td>cytoplasmic</td>
<td>0.62 ± 0.07</td>
<td>0.59 ± 0.06</td>
<td>0.92 ± 0.07</td>
<td>0.95 ± 0.09</td>
<td>&lt;0.05; 6.6</td>
</tr>
</tbody>
</table>

Data are results in absolute values from immunoblot analysis of aortic protein extracts from indicated number of animals (n). Soluble fractions are optical density ratios between protein of interest per expression actin. Particulate fractions are optical density in arbitrary units normalized to BSA analysis. P value and F statistic are results of 1-way ANOVA. See MATERIALS AND METHODS for details. GRK, G protein receptor kinase.

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Fig. 2. GRK-2 expression in cytoplasmic aortic preparations from 6-wk, 6-mo, 12-mo, and 24-mo-old rats (see Table 1 for number of aorta per age and absolute values). Fifty micrograms of total aortic protein per lane was subjected to SDS-PAGE and immunoblotting with an anti-GRK-2 antibody. Immunoblots were analyzed by densitometry with a Storm fluorimager as described in MATERIALS AND METHODS. GRK-2 expression is presented as the ratio of intensity of GRK-2 bands compared with intensity of actin bands presented as means ± SE. *P < 0.05 vs. 6-wk-old animals. Inset: representative immunoblot for GRK-2 and actin.

Fig. 3. GRK-3 expression in cytoplasmic aortic preparations from 6-wk, 6-mo, 12-mo, and 24-mo-old rats (see Table 1 for number of aorta per age and absolute values). Fifty micrograms of total aortic protein per lane was subjected to SDS-PAGE and immunoblotting with an anti-GRK-3 antibody. Immunoblots were analyzed by densitometry with a Storm fluorimager as described in MATERIALS AND METHODS. GRK-3 expression is presented as the ratio of intensity of GRK-3 bands compared with intensity of actin bands presented as means ± SE. *P < 0.05 vs. 6-wk-old animals. Inset: representative immunoblot for GRK-3 and actin.
GRK-2 from aortic crude membrane preparations from 6-, 12-, and 24-mo-old animals are averaged, expression increased 1.5-fold compared with aorta from 6-wk-old animals (Fig. 5). Likewise, crude membrane localized GRK-3 expression in aorta from 6-, 12-, and 24-mo-old animals increased nearly 2.1-fold compared with aorta from 6-wk-old animals (Fig. 6). However, there was no age-related change in the expression of crude membrane localized GRK-5 (Fig. 7).

Fig. 4. β-Arrestin-1 expression in cytoplasmic aortic preparations from 6-wk, 6-mo, 12-mo, and 24-mo-old rats (see Table 1 for number of aorta per age and absolute values). Fifty micrograms of total aortic protein per lane was subjected to SDS-PAGE and immunoblotting with an anti-β-arrestin-1 antibody. Immunoblots were analyzed by densitometry with a Storm floiror imagert as described in MATERIALS AND METHODS. β-Arrestin-1 expression is presented as the ratio of intensity of β-arrestin-1 bands compared with intensity of actin bands presented as means ± SE. *P < 0.05 vs. 6-wk-old animals. Inset: representative immunoblot for β-arrestin-1 and actin.

Fig. 5. GRK-2 expression from crude membrane preparations of aortic from 6-wk, 6-mo, 12-mo, and 24-mo-old rats (see Table 1 for number of aorta per age and absolute values). Ten micrograms of total aortic protein per lane was subjected to SDS-PAGE and immunoblotting with an anti-GRK-2 antibody. Immunoblots were analyzed by densitometry with a Storm floiror imagert as described in MATERIALS AND METHODS. Results are presented as means ± SE. *P < 0.05 vs. 6-wk-old animals. Inset: representative immunoblot for GRK-2.

Fig. 6. GRK-3 expression from crude membrane preparations of aortic from 6-wk, 6-mo, 12-mo, and 24-mo-old rats (see Table 1 for number of aorta per age and absolute values). Ten micrograms of total aortic protein per lane was subjected to SDS-PAGE and immunoblotting with an anti-GRK-3 antibody. Immunoblots were analyzed by densitometry with a Storm floiror imagert as described in MATERIALS AND METHODS. Results are presented as means ± SE. *P < 0.05 vs. 6-wk-old animals. Inset: representative immunoblot for GRK-3.

Fig. 7. GRK-5 expression from crude membrane preparations of aortic from 6-wk, 6-mo, 12-mo, and 24-mo-old rats (see Table 1 for number of aorta per age and absolute values). Ten micrograms of total aortic protein per lane was subjected to SDS-PAGE and immunoblotting with an anti-GRK-5 antibody. Immunoblots were analyzed by densitometry with a Storm floiror imagert as described in MATERIALS AND METHODS. Results are presented as means ± SE. *P < 0.05 vs. 6-wk-old animals. Inset: representative immunoblot for GRK-5.
DISCUSSION

The present study examines age-related changes in GRK activity, expression, and subcellular localization in whole aorta from male Fischer-344 rats. These studies were undertaken because β-AR-mediated vasorelaxation declines with advancing age (4), whereas β-AR density is maintained (31) in whole aortic tissue. Furthermore, an age-related increase in aortic low-affinity β-AR has been documented (15), and β-AR phosphorylation is the primary mode for producing low-affinity receptors (2). Our results show that in similar whole aortic tissue preparations, measures of GRK activity or expression increase with advancing age (Table 1). Total GRK activity increases as animals age (Fig. 1) as does expression of cytoplasmic GRK-2 (Fig. 2), GRK-3 (Fig. 3), and β-arrestin (Fig. 4). Likewise, expression of crude membrane localized GRK-2 (Fig. 5) and GRK-3 (Fig. 6) increase with advancing age, whereas crude membrane localized GRK-5 expression (Fig. 7) remains unchanged.

Aging is associated with a pronounced decline in β-AR-stimulated cAMP production and subsequent function (9, 15). Generally, there is an age-related decrease in β-AR responsiveness in blood vessels, heart, brain, parotid gland, and lung to both circulating and pharmacological β-AR agonists (22). Experiments with human vessels, including in vivo studies of the dorsal hand vein and in vitro studies of the saphenous vein, found decreased β-AR-mediated relaxation with age (24).

Aorta from Fischer-344 rats exhibit impaired isoproterenol (a β-AR agonist)-mediated vasorelaxation with age, whereas forskolin (directly activates adenyl cyclase)-mediated relaxation is normal (4), thus providing an animal model that mimics changes in human physiology. Although the aorta is not a resistance vessel, it is widely used in studies of this nature because it exhibits similar physiology and pharmacology to resistance vessels, it is also easily collected, and it provides a relatively large quantity of tissue for experimental manipulation. Also, much of the physiological and biochemical data collected in regard to age-related changes in β-AR signaling to date has been from aorta (5). Aortic cAMP accumulation to isoproterenol stimulation is proportional to relaxation in young and old age groups, and both forskolin and dibutylryl-cAMP (a membrane-permeant derivative of cAMP) relax both ages of vessels normally. The age-related loss of vasorelaxation appears to be explained by a decrease in cAMP production, but not protein kinase A activity (8).

Tsujimoto and associates (31) reported that β-AR density in whole artery preparations was unaltered with age, whereas Gurdal et al. (15) reported a slight age-related decrease in β-AR density. However, this slight β-AR downregulation was found to be in conjunction with complete loss of high-affinity receptors in aortic tissue from older rats. Together, these findings suggest that the age-related decline in β-AR-mediated signaling may be due only slightly to β-AR downregulation but significantly due to changes in β-AR affinity state. Therefore, it could be concluded that age-impaired vasorelaxation is related to β-AR desensitization but not downregulation.

β-AR phosphorylation causes desensitization and thus profound decreases in the receptor’s ability to transduce signal in response to agonist binding (10, 29). GRKs are a superfamily of kinases that phosphorylate and desensitize G protein-linked coupled receptors (25). Three GRKs, GRK-2, GRK-3 (also known as β-ARK-1 and -2), and GRK-5 rapidly phosphorylate and desensitize not only β-ARs but also many other Gs-linked receptors on agonist binding in numerous tissues, including the cardiovascular system (6).

GRKs, similar to all proteins, are synthesized in the cytoplasm. However, their targets, in this case, β-ARs, are located at the cell membrane. Therefore, GRKs can phosphorylate and desensitize receptor targets only after their translocation to the membrane (30). In the present study, we investigate age-related changes in GRK expression in both cytoplasmic and crude membrane fractions of aortic tissue extracts. The changes documented in the cytoplasmic fractions (Figs. 2–4) suggest that there is, in general, an age-related increase in the quantity of GRKs. Similarly, the changes documented in the crude membrane fractions (Figs. 5–7) suggest that the membrane localization of GRK is likewise increased with advancing age. There is an age-related increase in the concentration of GRKs at membrane, where β-ARs are located. Therefore, we propose increased age-related GRK expression in the cytoplasm may allow increased desensitization and the documented age-related increase in low-affinity β-ARs at the membrane (15).

Numerous reports discuss GRK function in disease states. Ungerer et al. (32) found significant increases in expression and activity of GRKs in left ventricle heart samples from humans with dilated or ischemic cardiomyopathy. In other studies, overexpression of GRK-2 (18) or GRK-5 (27) significantly attenuated cardiac β-AR signaling in transgenic mice. Similarly, transgenic mice that expressed a GRK-2 inhibitory peptide had an enhanced β-AR-mediated signaling (18) as well as reduced β-AR desensitization in response to induced pressure overload hypertrophy (6). Ishizaka et al. (16) determined that agonist-induced GRK-5 expression was higher in aortic cultured vascular smooth muscle cells from hypertensive rats compared with normal controls. Finally, Gros and associates (12–14) showed increases in GRK-2 expression and activity in lymphocytes of hypertensive humans and rats.

The present study is the first, to our knowledge, to examine GRK activity and expression with age in aorta. Few studies have been performed to specifically understand age-related changes in GRKs in the cardiovascular system. Xiao and associates (33) determined that neither GRK-2, -5, nor soluble GRK activity increased with age in hearts of Wistar rats. Gros et al. (12) did not detect any age-related changes in GRK activity or GRK-2 and -5 expression in lymphocytes of humans. Herein, we determined that, with both maturation and advancing age, GRK activity increases.

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This increase in activity is coupled with increased expression of GRK-2 (cytoplasmic and crude membrane localized), GRK-3 (cytoplasmic and crude membrane localized), and β-arrestin (cytoplasmic) but not GRK-5 (crude membrane localized). Therefore, enhanced GRK activity and expression may be related to the age-related declines in β-AR-mediated signaling. Further studies that directly examine age-related changes in phosphorylation of β-AR in vascular tissue will further this hypothesis.

**Perspectives**

Herein, we present an important finding that may provide a mechanism to explain results of previous studies investigating age-related changes in vascular reactivity. It is well known that β-AR-mediated vasorelaxation declines with advancing age, and this decline is associated with an age-related decrease in cAMP production without a change in β-AR density. The decline in observed physiology and biochemistry has been associated with an age-related decrease in β-AR affinity for agonist. The receptors desensitize with advancing age without downregulation. Blood vessels from old animals contain β-ARs that are all almost entirely in the low-affinity state and therefore do not respond to agonist, regardless of its circulating concentration. Comparatively, blood vessels from young animals contain predominantly high-affinity β-ARs, and thus these receptors can respond to agonist stimulation and ultimately yield vasorelaxation. Our results implicate GRKs in this etiology as both total GRK activity and cellular expression of specific isoforms of GRKs increase with advancing age. Furthermore, other groups have shown an association between GRKs and cardiovascular diseases such as congestive heart failure. Therefore, our results, as well as those from other laboratories, strongly suggest that GRK function plays an important role in cardiovascular physiology. Additional study is required to elucidate the molecular basis of its action. To that end, GRKs, or at least a specific GRK isoform, may provide a therapeutic target for medicinal intervention for numerous cardiovascular disorders.

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