Ontogenesis of Sympathetic Responsiveness in Spontaneously Hypertensive Rats

II. Renal G Proteins in Male and Female Rats

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Abstract
Previously we have reported an increased renal \( \alpha_r \)- and \( \beta \)-adrenergic receptor expression in male spontaneously hypertensive rats that occurred ontogenetically in parallel with blood pressure elevation. However, increased receptor numbers were not accompanied by enhanced stimulation of inositol phosphate and cyclic AMP formation, respectively, indicating relative desensitization. We have now quantified \( \alpha \)-subunits of the G proteins \( G_\alpha \) (\( G_{\alpha \, \alpha} \) and \( G_{\alpha \, \beta} \)), \( G_\beta \), and \( G_\delta \) by immunoblotting and pertussis toxin-catalyzed ADP-ribosylation in renal membranes from 3-, 6-, 8-, and 28-week-old normotensive and spontaneously hypertensive male Wistar-Kyoto rats; additionally, 28-week-old female normotensive and spontaneously hypertensive rats were studied. During ontogeny of male normotensive rats, \( G_{\alpha \, \alpha} \) increased, \( G_{\beta \, \delta} \) remained unchanged, and \( G_\alpha \) and \( G_\delta \) decreased. In adult normotensive rats no sex differences were detected for \( G_{\alpha \, \alpha} \), \( G_{\beta \, \delta} \), and \( G_\alpha \). When male rats from the normotensive and spontaneously hypertensive strains were compared, all G protein \( \alpha \)-subunits were similar in the prehypertensive phase (3 weeks). In established hypertension (28 weeks), \( G_{\alpha \, \alpha} \) and \( G_\delta \) were reduced, whereas \( G_{\beta \, \delta} \) and \( G_\alpha \) remained unchanged. \( G_{\alpha \, \beta} \) was also reduced during the development of hypertension (6 and 8 weeks), whereas \( G_{\beta \, \delta} \) and \( G_\alpha \) were not consistently altered in this phase. The reduction in \( G_{\beta \, \delta} \) seen in male adult hypertensive rats was not detectable in female hypertensive rats. We conclude that reduced \( G_{\alpha \, \alpha} \) and \( G_\delta \) may explain the previously observed relative desensitization of adenylate cyclase and phospholipase C stimulation by \( \beta \)- and \( \alpha \)-adrenergic receptors, respectively, in male spontaneously hypertensive rats. Additionally, our data indicate sex-specific differences in G protein signaling in kidneys from hypertensive rats. (Hypertension. 1994;23:653-658.)

Key Words • sympathetic nervous system • receptors, adrenergic • G proteins • rats, inbred SHR • kidney

The kidney is the tissue with the greatest long-term impact on blood pressure control because of its infinite gain mechanism.1 On the other hand, chronically elevated arterial blood pressure affects renal function.2-3 Renal function is tightly controlled by neurotransmitters and hormones such as catecholamines, angiotensin II, endothelin, vasopressin, and neuropeptide Y, many of which act via G proteins.4-6 We have recently reported that in parallel with blood pressure elevations numbers of \( \alpha_r \)-, \( \alpha_\beta \)-, and \( \beta \)-adrenergic receptors increase in kidneys of spontaneously hypertensive rats (SHR) relative to those of age-matched normotensive Wistar-Kyoto (WKY) rats.7 Despite increased \( \alpha_r \)- and \( \beta \)-adrenergic receptor numbers, norepinephrine-stimulated inositol phosphate formation and isoprenaline-stimulated cyclic AMP (cAMP) formation remained unchanged.7 These data suggest the occurrence of a relative desensitization of renal \( \alpha_r \)- and \( \beta \)-adrenergic receptors during the development of hypertension in SHR.

\( \beta \)-Adrenergic receptor coupling to cAMP formation by adenylate cyclase occurs via the stimulatory G protein \( G_\beta \) and can be attenuated via the inhibitory G protein \( G_{\alpha \, \delta} \); additionally, one or more subtypes of \( \alpha_r \)-adrenergic receptors may couple to inositol phosphate formation via a pertussis toxin-sensitive G protein, possibly \( G_{\alpha \, \delta} \).8-10 We hypothesized that altered expression of \( G_{\alpha \, \delta} \), \( G_\beta \), and/or \( G_{\alpha \, \alpha} \) might explain the previously observed relative desensitization of renal \( \alpha_r \)- and \( \beta \)-adrenergic receptors in SHR. Thus, we have determined \( \alpha \)-subunits of \( G_{\alpha \, \alpha} \), \( G_{\beta \, \delta} \), \( G_\alpha \), and \( G_\delta \) by quantitative immunoblotting in renal membranes from 3-week-old ("prehypertensive" phase), 6- and 8-week-old (developing hypertension), and 28-week-old (established hypertension) WKY and SHR. In addition, pertussis toxin-catalyzed ADP-ribosylation was assessed to quantify \( G_{\alpha \, \delta} \). The renal membranes for the present study were prepared from the same animals in which we previously observed a relative desensitization of renal \( \alpha_r \)- and \( \beta \)-adrenergic receptors.7 Because the pathophysiology of hypertension may differ between males and females but too little is known about the specific pathophysiology of female hypertension,11 we also studied adult female WKY and SHR.

Methods

Animals and Tissue Preparation

The tissues used for the present study were obtained from the same animals as those used in our previous study on \( \alpha_r \)- and \( \beta \)-adrenergic receptors.7 Briefly, WKY and SHR were obtained from Mollegard. Male WKY and SHR were used at the ages of 3, 6, 8, and 28 weeks. Additionally, we used female rats at the age of 28 weeks that had served as breeders in our previous study. Body weight, kidney weight, kidney-to-body weight ratio, and systolic blood pressure of all male rats have been described.7 In female SHR, body weight (WKY: 258±1 g, n=29; SHR: 223±2 g, n=39; P<.0001) and kidney weight (WKY: 779±59 mg; SHR: 705±43 mg; P<.0001) were lower than in female WKY; in contrast, kidney-to-body weight ratio

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(WKY: 2.91±0.24 g/kg; SHR: 3.16±0.19 g/kg; P<.0001) and systolic blood pressure (WKY: 93±2 mm Hg; SHR: 169±2 mm Hg; n=6 each, P<.001) were significantly greater in female SHR than WKY. Both strains had slightly lower body weights, kidney weights, kidney-to-body weight ratios, and systolic blood pressures than the age- and strain-matched male rats reported earlier.7 After removal, the kidneys were macroscopically freed from adjacent connective tissue, rapidly frozen in liquid nitrogen, and stored at -80°C. The experimental protocol was approved by the state review board on animal experimentation (Tierschutzkommission beim Regierungspräsidenten Düsseldorf).

Kidneys were thawed in ice-cold 20 mmol/L NaHCO3 buffer, minced with scissors, and homogenized with an Ultra Turrax (Janke & Kunkel) once for 10 seconds at full speed and twice for 20 seconds at two-thirds speed. The homogenates were filtered through four layers of gauze and centrifuged for 20 minutes at 4°C at 50,000g. Protein content was determined by the method of Bradford12 using bovine immunoglobulin G as the standard.

Immunoblotting

G protein subunits were measured by quantitative immunoblotting as described by Burnette13 with minor modifications.14,15 Briefly, aliquots of the membrane preparations (=100 µg protein per sample) were separated on sodium dodecyl sulfate/polyacrylamide gels (10% acrylamide in the running gel) and thereafter blotted overnight to nitrocellulose membranes (Hybond ECL, Amersham) at a constant voltage of 55 V. The blots were washed and incubated overnight at 4°C with a 1:500 dilution of the indicated antiserum. The blots were washed twice again and then incubated for 1 hour at room temperature with [32P]protein A solution (8.5 µCi/µg, 129 µCi/mL). After another four washes the blots were used for autoradiography at -80°C. Using the autoradiograms, the molecular weights of the specific bands were identified. Corresponding sections were cut from the blot and counted in a γ-counter. Protein dependency was established for each antibody. To allow the detection of possible increases or decreases, protein amounts corresponding to the middle of the linear part of the protein-dependency curves were used.

ADP-Ribosylation

Renal membranes were extracted with 2% cholate for 1 hour at 4°C, and 1:10 diluted extracts were used for all ADP-ribosylation experiments. Pertussis toxin-catalyzed ADP-ribosylation was assessed under conditions yielding maximal ribosylation as previously described16 with minor modifications.14 Briefly, cell membranes were incubated with 1 µmol/L [32P]nicotinamide adenine dinucleotide (NAD) and 0.2 µg pertussis toxin for 1 hour at 30°C. The ribosylation reaction was stopped by addition of sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 125 mmol/L Tris-HCl, pH 8.0) and subsequent boiling. The reaction products were separated by overnight electrophoresis on SDS gels containing 17% polyacrylamide. Specific phosphorylation bands were identified on autoradiograms of the dried gels. [32P] incorporation was quantified by cutting appropriate bands from the gels and counting them in a scintillation counter. Basal [32P] incorporation was estimated in separate lanes loaded with membranes that had been incubated with [32P] in the absence of pertussis toxin. To obtain specific [32P] incorporation, basal incorporation was subtracted from total incorporation. Protein dependency of ADP-ribosylation was assessed. Gels were loaded with protein amounts that were in the middle of the linear part of the protein-dependency curves.

Chemicals

The antisera AS/7 and RM/1, which specifically recognize Gα11 and Gα13 respectively, were obtained from New England Nuclear. The antisera Z808-12, which specifically recognizes the α-subunit of Gq and G11, was a kind gift of Dr Paul Sternweis (Dallas, Tex). [32P]NAD (specific activity, 30 Ci/mmol) and [32P]protein A (specific activity, 8.5 µCi/µg) were obtained from New England Nuclear. Pertussis toxin was from List, and highly purified sodium cholate ("Ultrapure") was from Calbiochem.

Data Analysis

Data are presented as mean±SEM of "n" animals. Data on immunoreactive G protein subunits in SHR are expressed as percent of the mean value obtained with a similar number of WKY in the same experiments. Statistical significance of differences was assessed by unpaired two-tailed t tests when two groups were compared. When multiple groups were compared, one-way ANOVA was used; if this indicated that the variance between groups was greater than that within groups, individual groups were compared by t tests with Bonferroni corrections for multiple comparisons. A value of P<.05 was considered significant. All statistical calculations were performed with the Instat program (GraphPAD Software).

Results

To determine renal G protein α-subunits we have used quantitative immunoblotting and the following specific antisera: Gα11 was detected with the antisera RM/1, which recognized two specific bands with apparent molecular weights of 42 and 45 kD (Fig 1) referred to as Gα11 Short and Gα11 Long in this manuscript; both bands were excised and analyzed separately. Gα11 was detected with the antisera AS/7, which recognized a specific band with an apparent molecular weight of 40 kD (Fig 1); in some blots the AS/7 antisera detected an additional band with an apparent molecular weight of 46 kD. The following data refer to the 40-kD band only; the 46-kD band was not included in our analysis because its presence was not specific for an age group, strain, or sex, and samples from the same animal exhibited this band in some but not other blots (data not shown). The intensity of the 46-kD band tended to increase with age but did not significantly differ between male WKY and SHR at 3 and 6 weeks of age or between female adult WKY and SHR (data not shown). Additionally, we quantified Gα13 with pertussis toxin-catalyzed ADP-ribosylation that detected the incorporation of [32P] into a specific band with an apparent molecular weight of 40 to 41 kD. Finally, we quantified α-subunits of Gα13 and Gα14; with the antisera Z808-12, which had been raised against the common carboxyl terminals of both α-subunits; the Z808-12 antisera recognized a specific band with an apparent molecular weight of 41 kD (Fig 1).

We first investigated the regulation of renal G protein α-subunits during ontogeny in male WKY, ie, between the ages of 3 and 28 weeks. Immunodetectable Gα11 sharply increased markedly between 3 and 6 weeks of age and tended to increase further, with values at 28 weeks being ~2.5 times as high as those in 3-week-old WKY (Fig 2). In contrast, the amount of immunodetectable Gα11 was similar in all four age groups. Immunodetectable Gα13 sharply decreased in rats between the ages of 3 and 6 weeks and remained at this level in older age groups (Fig 2); thus, at 28 weeks immunodetectable Gα13 was considerably lower than at 3 weeks, although this difference barely missed statistical significance (P=.0582). Therefore, we confirmed our immunoblot findings by pertussis toxin-
Figu 1. Representative autoradiograms show immunoblots of renal Ga, Gi, and Gq11a from male adult normotensive Wistar-Kyoto and spontaneously hypertensive rats. Each lane represents a normotensive (W) or spontaneously hypertensive (S) animal. Ga, Gi, and Gq11a were significantly reduced in renal membranes from 28-week-old relative to those from 3-week-old male WKY (Fig 3). The amount of immunodetectable renal Ga was also significantly lower in 28-week-old relative to 3-week-old male WKY (Fig 4). The abundance of immunodetectable Gt, Gi, and Ga was similar in membranes from male and female 28-week-old WKY (data not shown), indicating that no major sex-specific differences exist for this parameter.

We then studied renal G protein α-subunits during the development of hypertension in SHR. Our technique of immunoblotting assesses relative rather than absolute amounts of G proteins. To facilitate comparison between WKY and SHR in various age groups, we determined each α-subunit in similar numbers of age- and sex-matched WKY and SHR in parallel; we expressed our SHR data as percent of the mean WKY value found in the same experiment. In the prehypertensive phase (3 weeks), immunodetectable renal Gt, Gi, and Ga (Fig 5), and Ga, (Fig 4) were similar in male WKY and SHR. In contrast, catalyzed ADP-ribosylation. Pertussis toxin substrates were significantly reduced in renal membranes from 28-week-old relative to those from 3-week-old male WKY.
tested in a separate experiment in which eight to 10 age-matched male normotensive Wistar-Kyoto (WKY) rats were compared with WKY (Fig 4 and 5), whereas immunodetectable $G_{\alpha_{s}}$ short and $G_{\alpha_{o}}$ (Fig 5) as well as pertussis toxin substrates (Fig 3) were similar in both strains. During the development of hypertension (6 and 8 weeks), $G_{i/0}$ and $G_{\alpha_{i}}$ were significantly reduced in SHR compared with WKY (Figs 4 and 5), whereas immunodetectable $G_{\alpha_{s}}$ short and $G_{\alpha_{o}}$ (Fig 5) as well as pertussis toxin substrates (Fig 3) were similar in both strains. During the development of hypertension (6 and 8 weeks), $G_{i/0}$ and $G_{\alpha_{o}}$ were significantly reduced in SHR compared with WKY (Fig 5), whereas immunodetectable $G_{\alpha_{s}}$ short and $G_{\alpha_{o}}$ were also significantly reduced (Fig 5). Immunodetectable $G_{i/0}$ short and $G_{\alpha_{o}}$ were not consistently altered, with $G_{i/0}$ short being significantly reduced at 6 but not 8 weeks and $G_{\alpha_{o}}$ being significantly reduced at 8 but not at 6 weeks (Fig 5). The amount of immunodetectable $G_{i/0}$ short was not significantly altered in female SHR compared with WKY (data not shown); immunodetectable $G_{i}$ and $G_{\alpha_{s}}$ short were also similar in 28-week-old female rats of both strains (data not shown).

**Discussion**

Renal $\alpha_{s}$- and $\beta$-adrenergic receptors mediate effects of sympathetic outflow to the kidneys such as alterations of renin release, intrarenal vascular tone, and tubular sodium handling. We have previously demonstrated that the number of renal $\alpha_{s}$- and $\beta$-adrenergic receptors is elevated in SHR starting at 6 weeks of age. Similar elevations of $\alpha_{s}$- and $\beta$-adrenergic receptors in SHR kidney have been repeatedly been reported by many investigators, although they were not detectable in a few other studies (for review see Reference 2). Previous studies on SHR kidney have reported either unchanged $\alpha_{s}$-adrenergic receptor numbers accompanied by reduced inositol phosphate formation or increased receptor numbers accompanied by unchanged inositol phosphate formation. Thus, the amount of inositol phosphates formed per $\alpha_{s}$-adrenergic receptor was reduced in SHR relative to WKY kidney in all studies, indicating a relative desensitization. Previous studies on $\beta$-adrenergic receptor–mediated cAMP formation in hypertensive kidney have reported increased, unchanged, or decreased values. Because almost all investigators agree that renal $\beta$-adrenergic receptor number is elevated in SHR and other models of hypertension, there also appears to be a relative desensitization of renal $\beta$-adrenergic receptors, at least in our set of animals.

Therefore, we hypothesized that alterations in the G proteins mediating $\alpha_{s}$- and $\beta$-adrenergic receptor coupling to phospholipase C and adenylyl cyclase, respectively, might be involved in this relative desensitization. Previous studies have detected the presence of $G_{i/0}$ short, $G_{i/0}$ long, and three forms of $G_{o}$ on the protein and messenger RNA level in rat kidney, whereas $G_{\alpha_{o}}$ was not detectable. Individual G protein $\alpha$-subunits exhibit a distinct pattern of distribution along the nephron. Overall analysis of the renal cortex has indicated that G proteins are equally expressed on apical and basolateral membranes, whereas separate analysis of renal cell types has indicated that individual G protein subunits are differentially distributed between brush border and basolateral membranes. This heterogeneity should be considered when interpreting the present findings, which were obtained in crude membranes prepared from whole kidney.

The responsiveness of G protein–coupled receptors can change with age, but these changes are not always explained by alterations in receptor number and may involve altered G protein expression. Previous studies looking at different age ranges and varying tissues of multiple species (eg, rat heart and brown adipose tissue, rabbit liver, human platelets) have not provided a conclusive evaluation of this hypothesis. The present study has found increased $G_{i/0}$ short, unchanged $G_{i/0}$ long, and reduced $G_{o}$ and $G_i$ in adult compared with 3-week-old rats (Table). Thus, during this phase of development the balance between $G_i$ and $G_i/0$-mediated pathways may shift.
Desensitization of renal α2-adrenergic receptor coupling to phospholipase C has repeatedly been reported from SHR kidney.7,12,24,53 α2-Adrenergic receptors preferentially couple to phospholipase stimulation via Go.9,54 Although the involvement of a pertussis toxin-sensitive G protein, possibly Goi, has been postulated for some α2-adrenergic receptor subtypes,10 a fraction of rat renal α2-adrenergic receptors may also stimulate inositol phosphate formation via a pertussis toxin-sensitive G protein (R. Büscher, W. Erdbürgä, O.-E. Brodde, and M.C. Michel, unpublished observations, 1993). The reduction in renal Go/ in adult SHR in the present study (≈18%) is similar to previously described increases in α2-adrenergic receptors (≈18%) and thus may well explain the unchanged inositol phosphate formation despite increased receptor number.5 It should be noted, however, that reduction of Go/ does not occur in all tissues of adult SHR as it was not detected in heart15 and vascular smooth muscle,39,40 indicating tissue-specific regulation of G protein expression.

Two lines of evidence suggest that renal G protein alterations are not a primary feature of the SHR model of hypertension. First, reductions of Goi and Go/ were not detectable in prehypertensive SHR. Second, cardiac G protein alterations can occur in various secondary forms of hypertension, including renovascular and mineralocorticoid-induced hypertension.41 Thus, the observed changes may rather be a consequence of elevated blood pressure and may possibly occur secondary to hypertensive renal damage. On the other hand, all forms of hypertension may be accompanied by the same G protein alterations. For example, angiotensin II infusion--induced hypertension increases Gi/ in glomerular membranes without affecting the abundance of Goi.42

The present data also allow indirect conclusions regarding renal α2-adrenergic receptor function in SHR. Renal α2-adrenergic receptor number has repeatedly been reported to be increased in SHR kidney (for review see References 22 and 28). Renal α2-adrenergic receptor signaling occurs via a pertussis toxin-sensitive G protein,4 most likely a form of Gi, as Gi is not expressed in the kidney (see above). Unfortunately, we7 and others43 have not been able to quantify α2-adrenergic receptor signaling in SHR kidney. However, the repeated finding of elevated renal α2-adrenergic receptors22,28 together with the present finding of a shift in the overall Gi/Gi ratio toward Gi may indicate that this receptor, which is the most abundant adrenergic receptor in rat kidney,7 may not be desensitized and thus also functionally activated in SHR kidney.

Taken together, our data demonstrate that the abundance of renal Gi/ and Go/ is reduced in male SHR relative to WKY, whereas Go/ and Gi is not consistently altered (Table). This reduction does not precede the development of hypertension and may thus occur secondary to blood pressure elevations. Although the present study was designed to elucidate our previous observation of a relative desensitization of renal α1- and β-adrenergic receptors,7 it may also be relevant for the understanding of the effects of other hormones in SHR kidney, including angiotensin II,5 endothelin,6 neuropetide Y,18 or parathyroid hormone,7,26 all of which also act via the G proteins investigated here. Given the already apparent differences in G protein regulation in SHR between kidney (present study), heart,15,41-44 and vascular smooth muscle,39,40 caution should be applied in extrapolation of the present data to other tissues. Apart from hypertension, our data are of interest because to the best of our knowledge they provide the first report of pathophysiological regulation of Go/ and differential regulation of Go/ and Gi/.
References

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