GUANINE NUCLEOTIDE-BINDING INHIBITORY PROTEIN-MEDIATED INHIBITION OF ADENYLYL CYCLASE IS ENHANCED IN SPONTANEOUSLY HYPERTENSIVE RAT PREGLOMERULAR ARTERIOLAR SMOOTH MUSCLE CELLS

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ABSTRACT

The purpose of our study was to determine whether Gαi-mediated control over adenylyl cyclase in preglomerular arteriolar smooth muscle cells (PGASMC) is enhanced in the spontaneously hypertensive rat (SHR). PGASMC were cultured from preglomerular microvessels isolated from adult SHR (14-15 wk of age) and age-matched WKY rats. Confluent monolayers of cells in third passage were used for the experiments. cAMP released into the media (30 min) as well as cellular levels of cAMP were measured in the presence of a phosphodiesterase inhibitor, 1-isobutyl-3-methyl-xanthine (IBMX; 100 μM) and expressed as pmol/mg protein. Total (released + cellular) cAMP was significantly lower in SHR (14.19 ± 2.30 pmol/mg protein) as compared with WKY (28.3 ± 3.04 pmol/mg protein). Correspondingly, the released (4.6 ± 0.4 pmol/mg protein) as well as cellular (9.78 ± 2.18 pmol/mg protein) cAMP levels were also significantly lower in SHR when compared with WKY (8.85 ± 1.26 and 18.86 ± 2.0 pmol/mg protein, respectively). The steady-state levels of none of the Gαi subunits, namely Gαi1, Gαi2 and Gαi3, were higher in the SHR PGASMC. Pertussis toxin treatment (PTX; 100 ng/ml; 24 hr) caused complete ADP-ribosylation of Gαi subunits in both WKY and SHR PGASMC. The same treatment of PTX also produced a significant increase in total cAMP in SHR, but not in WKY, such that the total cAMP levels after PTX treatment were not significantly different between the two strains. Interestingly, PTX significantly increased the released (20.26 ± 0.90 pmol/mg protein) but not the cellular (13.63 ± 1.63 pmol/mg protein) cAMP in SHR. Forskolin (1 μM) induced similar increases in total cAMP and isoproterenol (1 μM) caused greater increases in total cAMP in SHR cells compared with WKY cells. These data strongly suggest that in SHR PGASMC total adenylyl cyclase activity is not altered. Furthermore, steady-state expressions of G αi1,G αi2 and G αi3 are not increased whereas Gαi-mediated inhibition of adenylyl cyclase is augmented in SHR PGASMC.

There is compelling evidence that there is a genetically determined and kidney-selective hyper-responsiveness to Ang II in SHR (Li and Jackson, 1989; Chatziantoniou and Arendshorst, 1991; Kost and Jackson, 1993; Vyas and Jackson, 1995). Ang II not only causes direct vasoconstriction by increasing free intracellular Ca2+ (Griendling et al., 1989) but also inhibits adenylyl cyclase via Gαi (Pobiner et al., 1991) and therefore decreases cAMP. It is likely that in the renal vasculature the adenylyl cyclase-cAMP pathway may be effectively modulated in SHR resulting in a decreased production of cAMP in these rats. This may be the cause for an impaired modulation of Ang II-induced renal vasoconstriction by agents that increase cAMP such as prostacyclin (Chatziantoniou and Arendshorst, 1992; Jackson and Herzer, 1994) and dopamine (Chatziantoniou et al., 1992) in these rats. We have recently demonstrated that the ability of Ang II to inhibit the stimulus-induced increase in cAMP release in SHR is, indeed, augmented (Vyas et al., 1996). We have also suggested that within the SHR kidney, the adenylyl cyclase-cAMP pathway in preglomerular microvessels may be the predominant target of Ang II (Vyas and Jackson, 1995). We propose that in SHR kidney, the ability of vasodilator hormones to stimulate cAMP synthesis is impaired...
because of amplified coupling between the Ang II receptors and adenylyl cyclase. Specifically, this may involve a general increase in the efficiency of the inhibitory guanine nucleotide-binding protein (G_i) in the SHR renal microvasculature. Our objective was to test this hypothesis by addressing the following specific aims using cultured PGASMC from adult SHR and age-matched WKY rats. 1) To determine whether pertussis toxin-catalyzed ADP ribosylation (inactivation) of G_i augments cAMP production more in SHR compared with WKY PGASMC and 2) To determine whether isoproterenol- and forskolin-induced increases in cAMP levels are attenuated in PGASMC from SHR.

Methods

Male SHR and WKY rats (13-14 wk of age) were ordered from Taconic Farms (Germantown, NY) and were housed at the University of Pittsburgh Central Animal Facility with controlled temperature, relative humidity and light cycle (22°C, 55% and 7 A.M. to 7 P.M., respectively). Mean arterial blood pressures of a parallel group of conscious male SHR (13-14 wk of age), determined by telemetry, were consistently observed to be above 150 mm Hg whereas mean arterial pressures of age matched WKY rats were observed to be below 120 mm Hg. Animals were treated in accordance with institutional guidelines. The rats were maintained on Wayne Rodent Diet 5004 (sodium, 135 mmol/kg; potassium, 254 mmol/kg) (Madison, WI). The studies were conducted with prior approval from the Institutional Animal Care and Use Committee.

Isolation and culture of renal PGASMC. Renal PGASMC were cultured from explants of renal preglomerular arterioles isolated by a slight modification of method described earlier (Dubey et al., 1992). Briefly, for each culture, three rats from each strain were used. Rats were anesthetized with pentobarbital (45 mg/kg, i.p.), and 10 ml of a 5% w/v suspension of iron oxide particles (ferroso-ferric oxide, black, were anesthetized with pentobarbital (45 mg/kg, i.p.), and 10 ml of a slight modification of method described earlier (Dubey et al., 1992). Briefly, the kidneys were decapsulated and the purity of the microvessels was established by light microscopy. Only microvessels free of glomeruli and tubules and ≤100 μm in diameter were used for cell culture. For culture, aliquots (5 ml) of this suspension were plated in tissue culture flasks and incubated overnight at 37°C under 5% CO_2 and room air and 98% humidity. Additional culture medium II was added on the second day. On the sixth day of incubation, the medium was completely replaced with culture medium III (DMEM culture medium, penicillin (100 U/ml), streptomycin (100 μg/ml), NaHCO_3 (13 mM), HEPES (25 mM) and fetal calf serum (20%)). Thereafter, the medium was changed every other day until confluence was attained. PGASMC growing out from the arterioles were enriched while removing the possibility of contamination with fibroblasts by the method described by Aviv et al. (1983). Briefly, PGASMC were suspended by incubating in calcium- and magnesium-free Hanks’ balanced salt solution (GIBCO) in the presence of trypsin (0.06%) for 2 to 3 min. Suspended PGASMC were separated from iron-laden arterioles with a magnet. The microves- sel-free cell suspension was plated, and 20 min later the unattached cells (primarily PGASMC) were aspirated with the media and trans- ferred to another culture flask. This enrichment procedure was re- peated four to five times and followed by an overnight incubation. The purity of the PGASMC was ascertained by criteria described earlier (Dubey et al., 1992), such as characteristic morphology and positive immunofluorescence staining with monoclonal antibodies to smooth muscle-specific α- and γ-isoactin, myosin and desmin. PGASMC (1-3X passage; 2 × 10^5 cells/flask) were plated in culture well plate as required. Experiments were conducted at confluence.

Experimental protocol. The effects of pertussis toxin-catalyzed ADP-ribosylation of G_i on cAMP production in cultured PGASMC from WKY and SHR were examined in our study. On the day of the experiment, culture medium from some wells was replaced with medium containing 100 ng/ml pertussis toxin to allow ADP-ribosylation of G_i protein in the cell membranes. In the remaining wells the medium was replaced with fresh medium containing only an equiv- alent volume of vehicle for pertussis toxin (25 μl of 10% glycerol). The plates were incubated at 37°C for 24 hr. After the 24-hr treatment with or without pertussis toxin, confluent monolayers of PGASMC were divided into two sets. One set of these cells was washed thoroughly with PBS and frozen at -70°C to determine the steady-state expressions of G_ia and G_is under basal conditions, and the effects of PTX thereupon, using Western immunoblotting method as described in "Methods." Cells from the second set were washed three times with 500 μl of Dulbecco’s PBS containing calcium chloride (0.1 g/liter) and magnesium chloride (0.1 g/liter). Finally, 500 μl of pre- warmed (37°C) and oxygenated (95% O_2 + 5% CO_2) PBS containing IBMX (100 μM), a phosphodiesterase inhibitor, was added to each of the wells. IBMX was included in the PBS to amplify the cAMP signal. The cells were incubated in the presence of IBMX for 30 min. In some experiment, the effects of isoproterenol (1 μM; 30 min) or forskolin (1 μM; 30 min) on cAMP levels were determined in PGASMC in the presence of IBMX. Appropriate controls were conducted for all these experiments. Parallel experiments were performed in PGASMC ob- tained from WKY rats. The PBS containing released cAMP was then rapidly collected on dry ice and immediately stored at -70°C until assayed for cAMP. cAMP accumulated in the cells was extracted with propanol by a method described earlier (Goossens et al., 1994). The propanol extracts were dried using a SpeedVac concentrator, and the residue was resuspended in PBS and stored immediately at -70°C. The cell debris in the wells was dissolved in 500 μl of 0.1 M NaOH (0.1% SDS) at 37°C for 30 min, and the protein content in each well was determined using the bicinchoninic acid method (Brown et al., 1988). cAMP contents in the cell extracts as well as media (released cAMP) were determined by high-pressure liquid chromatography coupled with fluorometric detection as described below. The total cAMP in each well is calculated as the sum of released and extracted (cellular) cAMP and is presented as pmol/mg protein.

Measurement of cAMP. cAMP was measured by a high pressure liquid chromatographic-fluorometric assay by a method described by us recently (Vyas et al., 1996). Briefly, 200-μl aliquots of thawed sample were placed in polypropylene microials. Ten μl of 0.5 mol/
liter acetate-buffer, 10 μl of 1 mmol/liter adenine 9-β-D-arabinoside (internal standard) and 10 μl of 50% chloroacetaldehyde (aqueous solution) were added to the sample. The vials were capped, vortexed and placed in an oven (80°C) for 1 hr for complete derivatization of cAMP. A total of 80 μl of the derivatized sample was injected into an ISCO (Lincoln, NE) HPLC system (pump model 2350, gradient programmer model 2360, 4.6 × 250 mm C18 reverse-phase column with 5 μm particle size; ChemResearch Data Management System, Lincoln, NE). The fluorometric detection was achieved at an excitation wavelength of 275 nm and an emission wavelength of 420 nm using a Waters 470 fluorescence detector. The proteins were then centrifuged at 2000 rpm at 4°C on Beckman J2-MI centrifuge for 15 min. The supernatants, containing the membrane fraction, were transferred to fresh microfuge tubes and centrifuged at 17,000 rpm at 4°C for 15 min. The pellets containing plasma membranes were suspended in 10 μl of Tris-EDTA (10 mM, pH 7.4) buffer containing protease inhibitors (antipain, 2 μg/ml; aprotinin, 1 μg/ml; leupeptin, 2 μg/ml). Each homogenate was passed through a fresh fine-gauge (25 G) needle and then centrifuged at 2000 rpm at 4°C on Beckman J2-MI centrifuge for 15 min. The supernatants, containing the membrane fraction, were transferred to fresh microfuge tubes and centrifuged at 17,000 rpm at 4°C for 15 min. The pellets containing plasma membranes were suspended in 150 μl of Tris-EDTA buffer and the suspensions were centrifuged at 17,000 rpm at 4°C for 15 min. The supernatants were discarded and the pellets, containing the membrane fraction, were resuspended in 100 μl of Tris-EDTA buffer for approximately 30 min at 4°C. These suspensions were then heated at 85°C for 2 min on a heat block. The protein contents in these membranes were determined using Bio-Rad Dye (Bio-Rad Labs, Hercules, CA) Bradford assay (1976) as described by Blewett et al. (1996). The membranes were then suspended in a lysis buffer to provide a final concentration of 1 μg protein/μl.

**Western blot analyses.** A total of 40 μg of the membrane proteins from SHR and WKY controls as well as PTX treated cells were resolved using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). The proteins were then electroblotted on to a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA). Immunoblotting was performed using the methods described earlier (Chinoy et al., 1998). Briefly, the membranes were blocked with 5% milk for 1 hr and incubated for 2 hr at room temperature with Gαs,1-2 or Gαs,3 antibodies (Calbiotech, San Diego, CA) at a dilution of 1:1000 each in 2% milk in Tris-buffered saline containing Triton x 100. The membranes were then washed in Tris-buffered saline containing Triton and incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG secondary antibody (Amersham, Arlington Heights, IL) at 1:2500 dilution. The antigen-antibody complexes were detected using an enhanced chemiluminescence kit for immunodetection (Amersham). Densitometric analyses were done on a 100A Molecular Dynamics Densitometer using the Protein Data Basis Incorporation Software. To determine the extent of ADP-ribosylation of the Gαs subunits, gradient gels were prepared with 10% SDS and 4 to 8 M urea. The 8 M urea gel mixture was poured in the front chamber and 4 M urea gel mixture was poured in the back chamber of the Bio-Rad Gradient Former. As described above, 40 μg of the membrane protein from each sample were loaded and the Western blotting was carried out as described above.

**Statistical analysis.** Data are presented as mean (densitometric analyses) or mean ± S.E.M. (cAMP). Statistical analyses were performed on a personal computer with Number Cruncher Statistical System software package (Kaysville, UT). All data were subjected to D’Agostino test for verifying the distribution pattern before performing analyses using parametric statistics. Data for released, cellular and total (release + cellular) cAMP were analyzed using 2-factor (factor 1: strain; factor 2: pertussis toxin, isoproterenol or forskolin, as applicable) analysis of variance model followed by Duncan’s post hoc test for multiple comparisons. P < .05 denotes statistical significance. The specific statistical applications are indicated in text and figure legends.

**Results**

Total cAMP, in the presence of 100 μM IBMX, in PGASMC from SHR (14.19 ± 2.30 pmol/mg protein) was significantly lower as compared with WKY PGASMC (28.30 ± 3.04 pmol/mg protein) (fig. 1). Similarly, the released (4.60 ± 0.40 pmol/mg protein) as well as cellular (9.78 ± 2.18 pmol/mg protein) cAMP in SHR PGASMC were significantly lower than WKY PGASMC (8.85 ± 1.26 and 18.86 ± 2.00 pmol/mg protein, respectively) (fig. 2). Pertussis toxin produced significant increases in total cAMP in SHR PGASMC but not in WKY PGASMC (fig. 1). With regard to total cAMP, a 2-factor analysis of variance revealed a significant strain x pertussis toxin interaction. After PTX treatment the total cAMP levels in SHR and WKY PGASMC were not significantly different (fig. 1). Despite producing a significant increase in total cAMP in SHR PGASMC, pertussis toxin did not significantly increase the cellular cAMP in these cells (fig. 2; lower panel). Interestingly, however, pertussis toxin did significantly increase the released cAMP in these same cells (fig. 2; upper panel). Therefore, almost all of the increment in cAMP after pertussis toxin treatment was exported out of SHR PGASMC. A 2-factor analysis of variance showed a highly significant strain (P = .0028) and strain x pertussis toxin interaction (P < .0001) with regard to released cAMP. However, no significant strain x pertussis toxin interaction was observed. The specific statistical applications are indicated in text and figure legends.
noted for the cellular cAMP levels. Neither released nor cellular cAMP in WKY PGASMC was significantly altered by pertussis toxin.

In a separate set of experiments, ISO (1 mM; 30 min) produced significant increases in total cAMP in both WKY and SHR PGASMC (fig. 3). The stimulatory effect of ISO, however, was significantly greater in SHR cells as compared with WKY cells as evidenced by a significant strain x ISO interaction (fig. 3). As shown in figure 3, forskolin (1 mM; 30 min) produced significant increases in cAMP in PGASMC from both strains. The magnitude of this effect was not significantly different between SHR and WKY cells as indicated by a nonsignificant strain x forskolin interaction (fig. 3). The differential effects of ISO and forskolin on released as well as cellular cAMP are depicted in figure 4. ISO produced significantly (strain x ISO; P < .0001) greater (nearly 10-fold) increase in released cAMP in SHR (162 times control) as compared with WKY (17 times control). Forskolin, however, produced significant (P < .001) and comparable increases in released cAMP in SHR (20 times control) and WKY (15 times control). ISO-induced increases in cellular cAMP levels in SHR and WKY preglomerular ASMC were not statistically significant. Forskolin increased cellular levels of cAMP in both WKY and SHR PGASMC (fig. 4). The magnitudes of these increases in cellular cAMP levels, based on the resting levels, however, were noted to be comparable in SHR (59 times control) and WKY (54 times control) PGASMC.

In a parallel set of experiments, steady-state expressions of various isoforms of G_i in SHR and WKY PGASMC were measured by Western blotting. In the same set of experiments, the relative extent of ADP-ribosylation of G_i in the presence of PTX (100 ng/ml; 24 hr) was also examined in PGASMC from both strains. As shown in figure 5, steady-state expressions of G_iα1 and G_iα2, and G_iα3 in SHR (1.6 and 4.8 OD x mm, respectively) PGASMC were not higher than in WKY (4.2 and 7.4 OD x mm, respectively) PGASMC. Also, 24 hr of treatment with pertussis toxin (100 ng/ml) completely ADP-ribosylated all three G_iα isoforms as indicated by an absence of G_iα bands in Western immunoblots of protein extracts from pertussis toxin-treated PGASMC (fig. 6). These observations corroborate findings of other investigators who have previously shown maximal ADP-ribosylation in cell cultures using exactly the same protocol for pertussis toxin treatment (Katada and Ui, 1980). When combined, these data from Western blots clearly indicate that enhanced cAMP levels in SHR PGASMC in response to pertussis toxin treatment are not dependent on a greater extent of ADP-ribosylation of G_i in these cells.

Discussion

Some endogenous vasodilator agents such as prostacyclin (Veis et al., 1990) and dopamine (Alkadhi et al., 1986) mediate their vascular effects in part by stimulating the adenylyl cyclase-cAMP pathway. However, some vasoconstrictors such as Ang II inhibit adenylyl cyclase via G_i (Pobiner et al., 1991) and, therefore, decrease cAMP levels. We have recently
demonstrated that the ability of Ang II to inhibit isoproterenol-induced cAMP production in the SHR renal vasculature is significantly enhanced (Vyas et al., 1996). Such an augmented inhibitory influence on cAMP production may be closely associated with the enhanced renovascular responsiveness to Ang II observed in adult (Li and Jackson, 1989; Chatziantoniou and Arendshorst, 1991; Kost and Jackson, 1993) as well as young prehypertensive (Chatziantoniou et al., 1990; Vyas and Jackson, 1995) SHR. In our study we evaluated the hypothesis that Gi-mediated inhibition of adenylyl cyclase is enhanced in SHR renal arteriolar smooth muscle cells. The use of cultured PGASMC allowed evaluation of this hypothesis in that segment of the renal vasculature that contributes importantly to the effects of Ang II on renal vascular cAMP production and vascular resistance (Vyas and Jackson, 1995). Our findings support the hypothesis that Gi-mediated inhibition of adenylyl cyclase is augmented in SHR renal arteriolar smooth muscle cells.

The basal levels of cAMP were significantly lower in PGASMC from SHR when compared with WKY. This was a highly consistent observation in all the experiments conducted in PGASMC from the present study as well as from other ongoing studies in our laboratory. This finding lends direct support to the idea that the basal adenylyl cyclase activity in PGASMC from genetically hypertensive rats is under a greater inhibitory control. In a recent study, in perfused kidneys, we found that the basal cAMP release was higher in SHR kidneys as compared with WKY (Vyas et al., 1996). The different results obtained from the two studies in this regard is most likely due to the presence of captopril in the perfusate in the earlier study (Vyas et al., 1996). Captopril, by blocking the production of Ang II, may have unmasked a compensatory increase in basal adenylyl cyclase activity in SHR kidneys. In addition, differences in basal cAMP production could be related to varying regulatory mechanisms in the two different models.

A notable finding from our study is that treatment with pertussis toxin produces a distinct and highly significant increase in basal cAMP in SHR PGASMC but fails to do so in PGASMC from WKY. Pertussis toxin prevents inhibition of adenylyl cyclase by heterotrimeric inhibitory G proteins. Therefore, the fact that pertussis toxin increases basal cAMP in SHR PGASMC strongly corroborates the idea that Gi-mediated inhibition of adenylyl cyclase is exaggerated in renal arteriolar vascular smooth muscle cells from SHR. It is important to note that the differential effect of pertussis toxin on cAMP levels in PGASMC from the two strains does not depend on a higher extent of ADP-ribosylation in SHR cells as evident from figure 6. That increases in Gi-mediated mechanisms may play important role in maintenance of high blood pressure is supported by the fact that a single injection of pertussis toxin (10 µg/kg, i.v.) causes a significant reduc-

Fig. 4. Differential effects of isoproterenol (ISO; 1 µM) and forskolin (FORSK; 1 µM) on cAMP release (upper panel) and accumulation (lower panel) of cAMP in preglomerular arteriolar smooth muscle cells (PGASMC). Data are presented as mean ± S.E.M. (six to nine wells). P represents probability value from 2 factor (factor 1: strain; factor 2: isoproterenol or forskolin) analysis of variance. *Denotes P < .05 vs. BASAL within strain and † denotes P < .05 vs. other strain (Duncan's post hoc test).

Fig. 5. Western immunoblot for Gi isoforms in SHR and WKY preglomerular arteriolar smooth muscle cells (PGASMC). PGASMC from adult (14-15 wk of age) male SHR and age-matched WKY rats were cultured as described in “Methods.” Cells from both strains were incubated at 37°C in the presence or absence of pertussis toxin (100 ng/ml; 24 hr). Cells were collected at the end of the 24 hr and membranes were isolated. A total of 40 µg of membrane protein from each group was resolved using 10% SDS-PAGE. Immunoblots for these runs for Giα1 and Giα2 (top) and Giα3 (bottom) are shown. Lanes (left to right) are as follows: 1, SHR control; 2, SHR pertussis toxin treatment; 3, WKY control; 4, WKY pertussis toxin treatment. None of the Giisoforms appears to be higher in SHR PGASMC.
In conclusion, the findings from our study demonstrate that the Gi pathway-mediated inhibition of adenylyl cyclase is increased in SHR renal arteriolar smooth muscle cells; whereas Gi pathway-mediated stimulation of cAMP synthesis is not defective in these cells. The possibility that export of cAMP may be altered in vascular cells in hypertension must be duly considered and needs to be evaluated carefully. Enhanced extrusion of cAMP out of vascular cells may have associations as a causal factor in hypertension or it could merely be an epiphenomenon of hypertension. We do know, now, that an extracellular cAMP-adenosine pathway exists which controls vascular cell growth (Dubey et al., 1996). Furthermore, there is at least one other study that shows that active cAMP export may be enhanced in lymphocytes from hypertensive subjects (Mills et al., 1994).

In our study, forskolin increased the release of cAMP in a similar fashion in SHR and WKY PGASMC. More importantly, we observed that forskolin reduced, by more than 50%, the released/total ratio of cAMP levels in SHR (0.14) and WKY (0.12) PGASMC. It is worth noting here that cellular cAMP levels were not significantly altered by isoproterenol in either WKY or SHR cells. The possibility that export of cAMP may be altered in vascular cells in hypertension must be duly considered and needs to be evaluated carefully. Enhanced extrusion of cAMP out of vascular cells may have associations as a causal factor in hypertension or it could merely be an epiphenomenon of hypertension. We do know, now, that an extracellular cAMP-adenosine pathway exists which controls vascular cell growth (Dubey et al., 1996). Furthermore, there is at least one other study that shows that active cAMP export may be enhanced in lymphocytes from hypertensive subjects (Mills et al., 1994).

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In conclusion, the findings from our study demonstrate that the Gi pathway-mediated inhibition of adenylyl cyclase is increased in SHR renal arteriolar smooth muscle cells; whereas Gi pathway-mediated stimulation of cAMP synthesis is not defective in these cells. An increased receptor-Gi-adenyl cyclase coupling may play an important role in genetic hypertension.
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