A Decrease in the Amount and Function of Inhibitory GTP-Binding Protein in the Resistance Small Artery From Spontaneously Hypertensive Rats

Motomaru Masutani, Mitsumasa Ohyanagi, Jun Shibuya, Yasuhiro Ishigami and Tadaaki Iwasaki

First Department of Internal Medicine, Hyogo College of Medicine, 1–1, Mukogawa-cho, Nishinomiya 663–8501, Japan

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ABSTRACT—The inhibitory GTP-binding protein (Gi protein) plays an important role in regulation of vascular tone. Many studies have implicated the role of Gi protein in conduit vessels. However, the physiological role of Gi protein in the control of peripheral microvascular tone in hypertension has not been established yet. Therefore, we investigated the concentration of Gi protein in the peripheral resistance arteries and aorta in the spontaneously hypertensive rats (SHR), normotensive Wistar-Kyoto rats (WKY) and renovascular hypertensive rats (RHR), using immunohistochemical methods semiquantitatively. Changes in the function of Gi protein in relation to $\alpha_2$-adrenoceptor were also investigated by microcannulation techniques. We have shown that the amount of $\alpha_2$ subunits of Gi protein in the cremaster small artery was significantly lower in SHR aged 4 weeks and older than in age-matched WKY and that there were no significant differences between RHR and WKY. We also demonstrated that the function of Gi protein in relation to $\alpha_2$-adrenoceptor was already lower in SHR before the onset of hypertension. The quantitative and functional decline in Gi protein in the smooth muscle cells of peripheral small arteries were observed in SHR even before the onset of hypertension, whereas rats with secondary hypertension did not exhibit this finding.

Keywords: Gi protein, Spontaneously hypertensive rats, Resistance artery, Immunohistochemistry, $\alpha_2$-Adrenoceptor

A functional impairment in regulation of vascular tone may be important in the pathogenesis and/or maintenance of elevated peripheral vascular resistance in hypertension. G proteins are important in signal transduction in many organs. The inhibitory GTP-binding protein (Gi protein) conducts stimuli to the $\alpha_2$-adrenoceptor and the M2-muscarinic receptors in arterial smooth muscle cells and plays an important role in regulation of vascular tone and vasoconstriction (1). Many studies have implicated a role of Gi protein in conduit vessels (2–11). However, those results are diverse: an increase in the levels of Giα2 mRNA and protein in the aorta from SHR (4–6), no changes in the levels of Giα2 in the aorta from Milan hypertensive rats (7) and spontaneously hypertensive rats (SHR) (8, 9), and a reduction in Gi protein in aortic smooth muscles (10, 11). The physiological role of Gi proteins in the control of peripheral microvascular tone in hypertension has not been established yet. Notably, no studies address possible differences in the amount and reactivity of Gi protein in the resistance small arteries in hypertensive and normotensive rats.

We therefore investigated the localization and concentration of Gi protein in the peripheral resistance arteries and large vessels in SHR, normotensive Wistar-Kyoto rats (WKY), and rats with secondary hypertension, using immunohistochemical methods. Changes in Gi function in relation to $\alpha_2$-adrenoceptor were also investigated.

MATERIALS AND METHODS

Animals

SHR aged 4, 10 and 20 weeks (n=7 in each group) and age-matched WKY (n=7) were the descendents of animals provided by Prof. Kouzo Okamoto (Kinki University, Osaka-Sayama). The genetic background for our SHR was screened by the Division of Genetics, International Council for Laboratory Animal Science Monitoring Center using biochemical genetic markers and class 1 major histocompatibility complex (RT1) typing.
Results were as follows: hemoglobin β-chain (a), amylase, pancreatic (a), seminal vesicle protein-1 (a), major urinary protein-1 (a), group-specific component (a), esterase (Es)-6 (a), Es-1 (a), Es-2 (a), Es-3 (b), Es-4 (a), Es-7 (b), Es-8 (b), Es-9 (a), Es-10 (a), Es-14 (not tested), kidney alkaline phosphatase-1 (b), serum alkaline phosphatase-1 (a), cell surface aldoantigen-2 (b), major histocompatibility antigen-1 A; RT1A (k). (Matsumoto et al. (12) showed that three genetic markers, Es-3b, Es-4a and PT1k, were identified as specific markers of SHR. The genetic background for our SHR was consistent with those results and highly homologous to SHR/NCrj). As the secondary hypertensive animal model (renovascular hypertensive rats: RHR), WKY aged 7 weeks in which the right kidney had been removed and the left renal artery was constricted with a silver clip (13) were studied (n = 5). Increase in systolic blood pressure over 180 mmHg was confirmed in these animals 3 weeks after surgery. Animals received humane care and were maintained in accordance with the rules for animal experimentation of Hyogo College of Medicine. The systolic blood pressure of rats was measured by the tail-cuff method using an electro-sphygmomanometer (PE-300; Tokai Irika, Tokyo). The temperature and duration of pre-heating at systolic blood pressure measurement were 40°C and 10 min, respectively.

Immunohistochemical analysis

Animals were anesthetized with diethyl ether and then perfused with Zamboni solution containing 2% paraformaldehyde, 0.2% picric acid and 0.1 M sodium phosphate (pH 7.5). Subsequent procedures were performed at 4°C unless otherwise indicated. The abdominal aorta and the cremaster artery were removed, immersed overnight in the same fixative, incubated in a series of solutions of increasing sucrose concentration [10%, 15%, 20% and 25% (W/V)] and then mounted in OCT embedding medium (Miles, Kankakee, IL, USA). Specimens were rapidly frozen and sectioned with a cryostat. Frozen sections, 10-μm-thick, were mounted on APS-coated glass slides (Matsunami, Tokyo) and washed three times with 50 mM Tris (pH 7.5) containing 0.2 M NaCl (Tris-buffered saline, TBS) for 10 min. Sections were incubated with methanol for 30 min to inhibit endogenous peroxidase, with 0.03% H₂O₂ in 20 mM sodium phosphate (pH 7.5) containing 0.15 M NaCl (phosphate-buffered saline, PBS) for 30 min and with TBS containing 5% (W/V) bovine serum albumin for 30 min. Sections were then incubated for 48 hr with polyclonal antibodies to the α-subunits of Gl2 (Glα2; Dupont, Wilmington, DE, USA) (14, 15) at a final dilution of 1:50 in TBS. As the control, sections were incubated with nonimmune rabbit immunoglobulin G. After being washed three times with PBS, sections were incubated for 16 hr with horseradish peroxidase-labeled goat antibodies to rabbit immunoglobulin G (Amersham, Buckinghamshire, UK) at a final dilution of 1:20 in TBS. After being washed three times with PBS, sections were incubated with 50 mM Tris (pH 7.5) containing 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Dojindo, Tokyo) for 30 min at room temperature and then with the same solution containing 0.01% H₂O₂ for 10 min. The sections were then washed twice with PBS for 10 min, embedded in 50% (V/V) glycerol in PBS and examined by light microscopy.

An interactive build analysis system (Kontron, Munich, Germany) was used to generate 256-step gray images, and the wall thickness/lumen diameter ratio was measured. The gray scale levels of the images were displayed. Since it is difficult to measure all sections under exactly the same conditions, the gray scale index was calculated by subtracting the mean gray scale level of 10 background sites (the size of each site was 5 pixels) from the mean gray scale level of 10 immunoreactive sites (the size of the site was 5 pixels) in vascular smooth muscle (the center of the media). The fibrotic tissues around the small artery were regarded as the background sites. The mean gray scale levels of 5 sections in each rat were regarded as the gray scale index. This gray scale index was regarded as the relative concentration level of the immunoreactive concentration.

Functional analysis (in vitro study)

SHR (aged 4 weeks, n = 6) and age-matched WKY (n = 6) were anesthetized with phenobarbital (0.1 mg/g, i.m.). After the scrotum was opened, the right cremaster muscle was separated from the subdermal connective tissue and incised along the ventral surface, and the testicle was removed. The cremaster was then quickly excised, rinsed in cold saline, and placed in a temperature-controlled (4°C) dissection chamber containing sterile physiological salt solution of the following composition: 145 mM NaCl, 4.7 mM KCl, 2.0 mM CaCl₂·2H₂O, 1.2 mM MgSO₄·7H₂O, 5.0 mM glucose, 2.0 mM pyruvate, 3.0 mM MOPS [3-(N-morpholino) propanesulfonic acid], 0.02 mM EDTA, 1.2 mM NaH₂PO₄, 0.5 mM verapamil: HCl (pH 7.4) and 1% bovine serum albumin. Techniques used for the micro cannulation of resistance vessels resembled those described previously (16–19). The cremaster muscle was pinned flat, and a 1- to 1.5-mm length of the cremaster small artery (diameter of 100 μm) in the center of the tissue that was free of side branches was identified with a dissection microscope (magnification × 50–125). The vessel was separated from the surrounding tissue with ultrathin scissors and forceps (Fine Science Tools, Inc., Foster City, CA, USA) and was transferred to a water-jacketed tissue bath with attached micropipette micromanipulators. The vessel was cannu-
lated at both ends with fluid-filled glass micropipette (60-μm outside diameter) and 11-0 ophthalmic suture (18 μm). The pipette and the vessel were filled with a modified Krebs solution of the following composition: 118.5 mM NaCl, 4.7 mM KCl, 2.55 mM CaCl₂, 2H₂O, 1.19 mM MgSO₄·7H₂O, 1.19 mM KH₂PO₄, 19.9 mM NaHCO₃ and 11.6 mM dextrose. One pipette was connected to a Krebs reservoir whose height could be adjusted within 1–2 sec to produce changes in intraluminal pressure that were measured with a pressure transducer in parallel with the pipette perfusion line. The second pipette was closed to eliminate any flow-related vasoactive stimuli. The covered 5-ml tissue bath was continuously exchanged (4 ml/min) without recirculation from a Krebs reservoir where pH, temperature and gases were adjusted to maintain the tissue bath at pH 7.35–7.45, PaO₂ 65–75 mmHg and PaCO₂ 35–40 mmHg. Other drugs were applied to the tissue bath by means of syringe infusion pumps connected to the Krebs perfusion line, using drug infusion rates of 8–15 ml/min. The vessel preparation was then transferred to the stage of an inverted microscope (Diaphot; Nikon, Tokyo). The vessel was imaged with an ×20, 0.5 NA planapochromat (Nikon) and a CCD camera (C2400; Hamamatsu Photonix, Tokyo). The diameter of the lumen was measured with a digital image analysis system (Argus 10) that was calibrated with a stage micrometer. To evaluate the viability and stability of the smooth muscle preparation, the temperature of the bath was gradually increased over 90 min to 34°C at an in vivo pressure of 40 mmHg. During this period of equilibration, the vessel developed an intrinsic tone. Any vessels that failed to develop intrinsic tone and myogenic response were discarded (19, 20). Propranolol (1 μM), α₁-normetanephrine (10 μM) and desipramine (0.01 μM) were added to the Krebs solution to block the β-adrenoceptors, the non-neuronal and neuronal catecholamine uptake mechanisms, respectively (21). Indomethacin (3 μM) was added to the Krebs solution to block prostaglandine and Nω-nitro-L-arginine methyl ester (300 mM) was added to block endothelium-derived relaxing factor (EDRF). Since in vitro conditions selectively attenuate α₂-adrenoceptor sensitivity, and subthreshold angiotensin II restores α₂-sensitivity in vivo values, we also added angiotensin II (10 nM) to the Krebs solution (22).

We compared the concentration response curves produced by exposure to UK-14,304 (α₂-adrenoceptor agonist, 10⁻³–10⁻⁵ M) between SHR and WKY. After washout at 20 min, pertussis toxin (PTX; 100 ng/ml, 1 hr) (23) was given as a treatment, and we then compared the concentration response curves produced by exposure to UK-14,304 in the same arteries in both strains. (Preliminary results indicated that increasing the incubation time to 3 hr did not alter the inhibitory effect of PTX.)

**Statistical analyses**

Data are expressed as means±S.D. and were analyzed by the unpaired t-test, as appropriate. Bonferroni procedures were used in making multiple comparisons among the concentration response data. A level of P<0.05 was accepted as statistically significant.

**RESULTS**

The systolic blood pressure of SHR and WKY, aged 4, 10 and 20 weeks, is shown in Table 1. The ratio of wall thickness to lumen diameter in the resistance artery (internal diameter, 70 to 80 μm) did not differ significantly between SHR and WKY at 4 or 10 weeks of age. However, the ratio in SHR at 20 weeks of age was significantly (P<0.001) greater than that in age-matched WKY or in SHR at 4 or 10 weeks of age (Fig. 1). The

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Data are each mean±S.D. **P<0.01 vs age-matched WKY.**

**Fig. 1.** Ratio between wall thickness to lumen diameter in the small arteries of SHR (□) and WKY (●) aged 4 to 20 weeks. Values are means±S.D. (n=7). **P<0.001 vs age-matched WKY and SHR at 4 or 10 weeks of age.
systolic blood pressure of RHR 3 weeks after surgery was $184 \pm 14$ mmHg as compared with $108 \pm 10$ mmHg for WKY aged 10 weeks. The ratio of wall thickness to lumen diameter in the resistance artery did not differ significantly between RHR ($0.13 \pm 0.02$) and WKY ($0.12 \pm 0.03$).

**Immunoreactivity in the cremaster small arteries from SHR and WKY**

The Gi-like immunoreactivity (Gi-LIR) of the vascular smooth muscle cells of the cremaster small artery from WKY and SHR at 4, 10 and 20 weeks of age is shown in Fig. 2. Gi-LIR was detected in the vascular smooth muscle of both and it was less abundant in SHR than in the

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**Fig. 2.** Immunoreactivity for Gi protein in the cremaster artery of WKY (A to C) and SHR (D to F) at 4 (A and D), 10 (B and E) and 20 (C and F) weeks of age. Scale bar, 100 μm.
Fig. 3. Gray scale index of immunoreactivity for Gi protein in smooth muscle of the cremaster artery of WKY and SHR at 4, 10 and 20 weeks of age. Values are means ± S.D. (n = 7). **P < 0.001 vs age-matched WKY. Pattern = WKY, solid = SHR.

age-matched WKY.

The gray scale indexes of Gi-LIR in SHR at 4, 10 and 20 weeks of age were significantly (P < 0.001) lower than the corresponding values for age-matched WKY (Fig. 3).

**Immunoreactivity in the aorta from SHR and WKY**

Gi-LIR was detected in the vascular smooth muscle cells of the aorta in both SHR and WKY, with no apparent difference in the level of either immunoreactivity between the two strains (Fig. 4). The gray scale indexes for either Gi-LIR did not differ significantly between SHR and WKY at 4, 10 and 20 weeks of age (Fig. 5).

**Immunoreactivity in the cremaster artery from RHR and WKY**

There was no apparent difference in the pattern of Gi-LIR in the cremaster artery between RHR and WKY (Fig. 6, upper panel). The gray scale indexes of Gi-LIR in the vascular smooth muscle of the cremaster artery did not differ significantly between RHR and WKY (Fig. 6, lower panel).

**In vitro functional study**

Before the administration of PTX, the UK-14,304 concentration-response curves of SHR were significantly less than that of WKY. After the PTX treatment, the UK-14,304 concentration-response curves were attenuated to the same level in both strains (Fig. 7).

**DISCUSSION**

This is the first report to describe the concentration of Gi protein in the resistance small arteries and aorta evaluated semiquantitatively immunohistochemically. We have shown that the amount of Gi protein in peripheral arterial smooth muscle cells was significantly lower in SHR aged 4 weeks and older than in age-matched WKY. The concentration of Gi protein in aortic smooth muscle cells did not differ significantly between SHR and WKY. No significant differences were observed in the amount of Gi protein in the peripheral arterial smooth muscle cells of RHR (secondary hypertension) and WKY. By use of vascular responsiveness, the present study demonstrated that the function of Gi protein in relation to α2-adrenoceptor was already lower in SHR before the onset of hypertension.

Many studies have implicated the role of Gi protein in conduit vessels, but the results are diverse. In terms of the mRNA level, an increase in Gi protein was reported in SHR aortic smooth muscle and Gαi was unchanged (4–6). Concerning the protein levels, there are reports of an increase in the level of Gi protein and an increase in the adenylate cyclase (AC) activity in SHR aortic smooth muscle (4, 6). In contrast, a study found that the levels of Gi2α and Gqα/G11α were similar in the Milan hypertensive and control rats, while the levels of Gsα- and the β-subunit were significantly reduced in the hypertensive strain rats (7). Other studies found no difference in Gi protein in the mesenteric arteries between SHR and WKY, whereas the AC activity was decreased in SHR (8, 24). The extent of PTX ADP-ribosylation of Gαi was similar in SHR and WKY cells (9). Moreover, a reduction in Gi protein has also been reported in aortic smooth muscles from SHR (3), and secondary hypertension (10). Reduced function of Gi proteins has been reported in platelets from SHR (11).

However, the physiological role of Gi proteins in the control of peripheral microvascular tone in hypertension has not been established yet. There has been no study using Western blotting of Gi protein in the peripheral small arteries, because the low level of such protein in the small arteries makes it difficult to use this method of analysis. We now ascertained the localization of Gi protein in peripheral small arteries by using immunohistochemical procedures and assessed it semiquantitatively. Our data showed a reduction in immunoreactive Gi protein in the peripheral small arterial smooth muscle in SHR but not in that of RHR. Functionally, the vasoconstrictor response to UK-14,304 (a selective α2-adrenoceptor agonist) before treatment with PTX were significantly lower in 4-week-old SHR as compared with WKY. After the PTX treatment, the UK-14,304 concentration-response curves were attenuated to the same level in both strains, and the significant difference disappeared. These findings suggest that the function of Gi protein in relation to α2-adrenoceptor was already lower in SHR before the onset of hypertension. Immunohistochemical staining suggests that the smaller amount of Gi protein may play a role in
the smaller function of Gi protein. Since the level of Gi protein was not smaller in the smooth muscle cells of the resistance vessels of rats with secondary hypertension, the reduction in the concentration of Gi protein in the resistance vascular smooth muscle of SHR was likely to be attributable to genetic factors rather than being secondary to hypertension. The level of Gi protein was not reduced in aortic smooth muscle cells from SHR in the present study. These findings are consistent with those in the previous studies (8, 9). Since the peripheral resistance small arteries play an important role in the regulation of blood pressure but not in the aorta, the mechanisms for the regulation of Gi protein may differ between the peripheral small artery and aorta. While an increase in Gi mRNA and protein in the aorta of 2-week-old SHR has been reported (4), the present study

Fig. 4. Immunoreactivity for Gi protein in the aorta of WKY (A to C) and SHR (D to F) at 4 (A and D), 10 (B and E) and 20 (C and F) weeks of age. Scale bar, 100 μm.
showed no significant difference in the aorta of 4-week-old SHR vs WKY. Since these disparate findings could be attributable to a difference in the measurement, further studies are required for clarification.

A decline in the function of Gi protein in relation to $\alpha_2$-adrenoceptor inhibits vasoconstriction in the peripheral small arteries and, therefore, is not thought to be the cause of hypertension. In fact, no significant difference in blood pressure was observed between the 4-week-old SHR and WKY. This reduced function of Gi protein before the onset of hypertension may not play an important role in the pathogenesis of hypertension in SHR. However, this attenuated vasoconstriction via $\alpha_2$-adrenoceptor-Gi protein systems may protect against the decrease in the perfusion of peripheral tissues in SHR.

Study limitation: Since there are Gi proteins of the sympathetic nerve endings in small arterial vascular

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**Fig. 5.** Gray scale index of Gi protein immunoreactivity in the aortic smooth muscle of WKY and SHR at 4, 10 and 20 weeks of age. Values are means ± S.D. (n=7). Pattern = WKY, solid = SHR.

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**Fig. 6.** Immunoreactivity in the cremaster artery from RHR and WKY. Upper panel: Immunohistochemistry of Gi protein in the cremaster artery of WKY (A) and RHR (B). Scale bar, 100 μm. Lower panel: Gray scale index of immunoreactivity of Gi protein in vascular smooth muscles in the small arteries of RHR and WKY. Values are means ± S.D. (n=7). Pattern = WKY, solid = RHR (renovascular hypertensive rats).
smooth muscle, we cannot exclude the possible contamination by Gi proteins of the sympathetic nerve ending from vascular smooth muscle cells in these immunohistochemical methods.

In summary, the quantitative and functional decline in Gi protein in the peripheral small arterial smooth muscles was observed in SHR even before the onset of hypertension, whereas rats with secondary hypertension did not exhibit this finding.

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