Knockdown of Inhibitory Guanine Nucleotide Binding Protein Giα-2 by Antisense Oligodeoxynucleotides Attenuates the Development of Hypertension and Tachycardia in Spontaneously Hypertensive Rats

Yousra Ali El-Basyuni, MD, MSc; Yuan Li, MD, MSc; Madhu B. Anand-Srivastava, PhD

Background—We previously showed that the levels of both Giα-2 and Giα-3 proteins were augmented in spontaneously hypertensive rats (SHRs) before the onset of hypertension. In addition, intraperitoneal injection of pertussis toxin, which inactivates both Giα proteins, prevented the development of hypertension in SHRs. The aim of the present study was to determine the specific contributions of Giα-2 and Giα-3 proteins to the development of hypertension.

Methods and Results—Antisense oligodeoxynucleotide of Giα-2 and Giα-3 encapsulated in PEG/DOTAP/DOPE cationic liposomes were administered intravenously into 3-week-old prehypertensive SHRs and Wistar Kyoto rats, whereas the control Wistar Kyoto rats and SHRs received PBS, empty liposomes, or sense. The knockdown of Giα-2 but not Giα-3 protein attenuated tachycardia and prevented the development of hypertension up to age 6 weeks; thereafter, blood pressure started increasing and reached the same level as that of untreated SHRs at 9 weeks. Furthermore, Giα-2 and Giα-3 antisense oligodeoxynucleotide treatments significantly decreased the enhanced levels of Giα-2 and Giα-3 proteins, respectively, and enhanced levels of superoxide anion and NADPH oxidase activity in heart, aorta, and kidney and hyperproliferation of vascular smooth muscle cells from SHRs aged 6 weeks. In addition, antisense oligodeoxynucleotide treatment with Giα-2 but not Giα-3 restored enhanced inhibition of adenyl cyclase by oxotremorine to WKY levels.

Conclusions—These results suggested that the enhanced expression of Giα-2 but not Giα-3 protein plays an important role in the pathogenesis of hypertension and tachycardia in SHRs. (J Am Heart Assoc. 2016;5:e004594 doi: 10.1161/JAHA.116.004594)

Key Words: antisense • Giα-2 proteins • Giα-3 proteins • hypertension • oxidative stress • tachycardia

Guanine nucleotide binding proteins (G proteins) are a large family of guanosine triphosphate binding proteins that play a crucial regulatory role as transducers in a variety of cell-signaling pathways. G proteins are heterotrimeric proteins composed of 3 distinct subunits; α, β, and γ. All α subunits possess intrinsic GTPase activity and are responsible for specificity of receptor-effector interaction.1 According to amino acid homology, G proteins are classified into 4 major subfamilies and are represented as Gsα, Giα, Gqα/α11, and G12/α13. G proteins exert their action via several signal transduction pathways including adenyl cyclase and receptor-mediated activation of phospholipase C and A2.2–4 The inhibition and stimulation of adenyl cyclase is mediated via Giα and Gsα, respectively. Three distinct Giα forms (Giα-1, Giα-2, and Giα-3) are encoded by 3 different genes. All isoforms of Giα proteins inhibit adenyl cyclase and activate atrial K⁺ channels,5,6 whereas the 4 different isoforms of Gsα that were revealed by molecular cloning result from different splicing of 1 gene. Gsα is associated with adenyl cyclase stimulation and increased cyclic AMP (cAMP) production.6

Giα proteins and associated inhibition of adenyl cyclase signaling have been shown to be implicated in a variety of cellular functions, including vascular permeability,7,8 salt and water transport,9,10 and catecholamine release,11 all of which play key roles in the regulation of blood pressure (BP). Alteration in Giα protein levels is associated with impairment of cellular function that results in various pathological states including hypertension12 and heart failure.13,14 Enhanced expression of Giα-2 and Giα-3 proteins and their genes was also shown in cardiovascular tissues from several animal...
models of hypertension including spontaneously hypertensive rats (SHRs),\textsuperscript{15–24} whereas the levels of G\textsubscript{0}\textsubscript{0} and G\textsubscript{1} were not altered in hypertension.\textsuperscript{15,18,19} Moreover, high salt intake in salt-sensitive hypertensive Dahl rats was shown to enhance expression of G\textsubscript{x} proteins in heart failure, hypertension, and cardiac hypertrophy,\textsuperscript{25} whereas central G\textsubscript{x}-2 protein-gated pathways were reported to counter the development of salt-sensitive hypertension.\textsuperscript{26} Enhanced expression of G\textsubscript{x} proteins and the Gi protein–mediated pathway in cardiovascular tissues and kidney was shown to contribute to the maintenance of hypertension in SHRs.\textsuperscript{27,28} In contrast, G\textsubscript{x}-1 that is present predominantly in the brain and neural tissue and absent in heart and aorta\textsuperscript{23,29} does not appear to be implicated in the regulation of cardiovascular functions including BP in SHRs. The augmented expression of G\textsubscript{x} proteins has been reported to occur before the onset of hypertension in SHRs and DOCA–salt hypertensive rats\textsuperscript{30,31} and suggests that the enhanced expression of G\textsubscript{x}-2 and G\textsubscript{x}-3 proteins is a factor contributing to the development of hypertension rather than a consequence of hypertension. This was further supported by our study showing that a single intraperitoneal injection of pertussis toxin, which inactivates both G\textsubscript{x}-2 and G\textsubscript{x}-3 proteins, prevented the development of hypertension in prehypertensive SHRs aged 2 weeks.\textsuperscript{32} It is not clear whether the enhanced expression of G\textsubscript{x}-2 or G\textsubscript{x}-3 or both contributes to the development of hypertension in SHRs.

The present study was undertaken to examine the specific contributions of G\textsubscript{x}-2 and G\textsubscript{x}-3 to the development of hypertension in SHRs by using an antisense oligodeoxynucleotide approach. Antisense oligodeoxynucleotide is composed of short fragments of single-strand DNA (13–25 nucleotides). The main concept underlying antisense therapy is simple: Use of a certain DNA sequence that is complementary to a specific mRNA will inhibit its transcription/gene expression and consequently inhibit protein synthesis.\textsuperscript{33,34} The antisense approach has been used extensively and successfully in hypertension research. Antisense targeting angiotensin receptor type 1, angiotensinogen, thyrotropin-releasing hormone, epidermal growth factor receptor, and insulin-like growth factor 1 receptor\textsuperscript{35–39} attenuated BP in SHRs. Moreover, antisense targeting \( \beta \)-adrenergic receptor exhibited profound and prolonged reduction in BP.\textsuperscript{40} Because antisense oligodeoxynucleotide is not serum stable and can be degraded easily by exonuclease and endonuclease,\textsuperscript{41,42} it was necessary to use the delivery system. The recent advancement in nanotechnology was positively reflected in the improvement of the liposomes as drug-delivery vectors. Liposome-based nanomedicines offer an interesting approach for delivery of gene therapeutic agents like antisense.

This study provided evidence that knockdown of G\textsubscript{x}-2 but not G\textsubscript{x}-3 protein using antisense oligodeoxynucleotide encapsulated in PEG/DOTAP/DOPE cationic liposomes attenuated the development of hypertension and tachycardia and suggested that overexpression of G\textsubscript{x}-2 but not G\textsubscript{x}-3 protein plays an important role in tachycardia and the development of hypertension in SHRs.

\section*{Methods}

\subsection*{Antisense Oligodeoxynucleotide}

Antisense and the inverted-form sense oligomer targeting G\textsubscript{x}-2 and G\textsubscript{x}-3 proteins were purchased from Alpha DNA. These oligodeoxynucleotides were modified by phosphothioation of 3 nucleotides on both sides. The following sequences were used: G\textsubscript{x}-2 antisense, 5'-C*T*T*GTCGATCATCTTA*G*A*3'; G\textsubscript{x}-3 antisense, 5'A*A*G*TTGCGGTCGATC*A*T*3'; G\textsubscript{x}-2 sense, 5'T*C*T*AAGATGATCGACA*A*G*3'; and G\textsubscript{x}-3 sense, 5'-A*T*G*ATC-GACCGCAAC*T*T*3'.

\subsection*{Liposomes}

Cationic lipid DOTAP and helper lipid DOPE were purchased from Avanti Polar Lipids and mixed at a 1:1-M ratio. In addition, 10% DSPE-PEG(2000) (Avanti Polar Lipids) was incorporated in the lipid mixture to increase liposome stability, to prolong circulation time in the blood stream, and to prevent leakage and aggregation of liposomes, as described previously.\textsuperscript{43}

\subsection*{Formulation of Lipoplexes}

DOTAP, DOPE, and PEG were dissolved in chloroform. Dried lipid film was formulated by exposing the lipid mixture to a slow N\textsubscript{2} stream for evaporation of chloroform and then to a vacuum system overnight to ensure complete removal of chloroform traces. The lipid film was hydrated using sterile PBS with pH 7.4 at room temperature. The suspension was left at room temperature for 1 hour to allow assembly and formulation of liposomes. Liposomes were exposed to freeze/thaw cycles (7–9 cycles) to facilitate size management using liquid nitrogen and warm tap water. The size was homogenized using manual graded extrusion with 800-, 400-, and 100-nm polycarbonate membranes (LiposoFast; Avestin, Inc). The final size of the liposomes was 100 to 200 nm with a polydispersity index of 0.1 measured using a dynamic light scattering technique (Malvern Zetasizer; Malvern Instruments). Antisense or sense of G\textsubscript{x}-2 or G\textsubscript{x}-3 was added to the final concentration of 200 \( \mu \)g/mL in sterile PBS with pH 7.4 and an N:P ratio of 2 calculated according to the Felgner equation.\textsuperscript{44} Lipoplexes were left for 1 hour at room temperature to allow encapsulation of antisense or sense. To ensure that antisense was encapsulated inside the liposome, fluorescently labeled antisense was used.
Animals

Male SHRs aged 2 weeks and age-matched normotensive Wistar Kyoto (WKY) rats were purchased from Charles River Laboratories International, Inc. Animals were maintained at room temperature with free access to water and regular rat chow in 12-hour light/dark cycles. Rats were left for 1 week for adaptation. SHRs and WKY rats were divided into 6 groups (control, empty liposomes, Gi-2 antisense, Gi-2 sense, Gi-3 antisense, Gi-3 sense; 6–10 rats per group). Antisense or sense of Gi-2 or Gi-3 (1 mg/kg body weight) and empty liposomes were injected intravenously via tail vein once into prehypertensive SHRs aged 3 weeks and age-matched WKY rats. BP was monitored every week by the tail-cuff method without anesthesia, using the CODA standard noninvasive BP system (Kent Scientific Corp). After measurement of BP, heart rate, and body weight, groups of rats (WKY and SHRs with and without different treatments, as indicated above) aged 6 and 9 weeks were euthanized by decapitation after CO exposure. Thoracic aortas, hearts, and kidneys were removed by dissection and used for determination of Gi protein levels, oxidative stress, and other biochemical assays. All animal procedures used in the present study were approved by the Ethics Committee for experimentation on animals of the University of Montreal (approval 99050). The investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the National Institutes of Health.

BP and Heart Rate Measurement

Rats were left for 1 week for adaptation. BP was measured weekly using the CODA noninvasive tail-cuff method, according to the recommendation of American Heart Association. The CODA tail-cuff BP system uses volume pressure recording sensor technology to measure tail BP. Volume pressure recording is clinically validated and provides 99% correlation with telemetry and direct BP and heart rate measurements. BP was expressed as milligrams of mercury and heart rate as beats per minute.

Cell Culture

Aortic vascular smooth muscle cells (VSMCs) from SHRs aged 6 or 9 weeks and age-matched WKY rats (control group) and empty liposome, sense-, and antisense-treated SHRs and WKY rats were cultured, as described previously. The purity of the cells was checked with an immunofluorescence technique using α-actin, as described previously. These cells were found to contain high levels of smooth muscle–specific actin. The cells were plated in 75-cm² flasks and incubated at 37°C in 95% air and 5% CO₂ humidified atmosphere in DMEM (with glucose, l-glutamine, and sodium bicarbonate) containing antibiotics and 10% heat-inactivated FBS. Confluent cell cultures were starved by incubation for 3 hours in DMEM without FBS at 37°C to reduce the interference by growth factors present in the serum. The cell lysates were prepared and used for Western blotting and adenylyl cyclase activity determination.

Preparation of Heart, Aorta and Kidney Particulate Fraction

Heart, aorta, and kidney particulate fractions were prepared, as described previously. After rats were sacrificed, hearts, aortas, and kidneys were removed by dissection, quickly frozen in liquid nitrogen, and then pulverized into fine powder using a mortar and pestle precooled in liquid nitrogen. The powder was stored at −80°C until assayed. The powder was homogenized (12 strokes) in a glass homogenizer with a buffer containing 25 mmol/L Tris-HCl (pH 7.5), 25 mmol/L NaCl, 1 mmol/L sodium orthovanadate, 10 mmol/L sodium fluoride, 10 mmol/L sodium pyrophosphate, 2 mol/L benzamidine, 2 mmol/L ethylene-bis(oxy-ethylenenitrolo)tetraacetic acid, 2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, 1% Triton X-100, 0.1% sodium dodecyl sulfate, and 0.5 μg/mL leupeptin. The homogenate was centrifuged at 12 000g for 15 minutes at 4°C. The supernatants were transferred to a fresh microcentrifuge tube without disturbing the pellet. Protein concentration was determined by Bradford assay. The supernatant was used for Western blotting.

Western Blotting

Western blotting of Gi proteins was performed using specific antibodies, as described previously. After SDS-PAGE, the separated proteins were electrophoretically transferred to a nitrocellulose membrane with a semi–dry transfer blot apparatus (Bio-Rad Laboratories) at 15 V for 45 minutes. After transfer, the membranes were washed twice in PBS and were incubated in PBS containing 5% skim milk at room temperature for 1 hour. The blots were then incubated with the following specific antibodies: Gi-2 (L5), Gi-3 (C-10), and dynein (74-1; Santa Cruz Biotechnology) incubated in PBS containing 0.1% Tween 20 overnight at 4°C. The antigen–antibody complexes were detected by incubating the blots with goat anti–rabbit immunoglobulin G (Bio-Rad Laboratories) conjugated with horseradish peroxidase for 1 hour at room temperature. The blots were then washed 3 times with PBS before reacting with enhanced chemiluminescence Western blotting detection reagents (Santa Cruz Biotechnology). Quantitative analysis of the protein was performed by densitometric scanning of the autoradiographs using the enhanced laser densitometer LKB Ultroscan XL and quantified...
using the Gelscan XL evaluation software (version 2.1) from Pharmacia.

**Determination of Adenylyl Cyclase Activity**

Adenylyl cyclase activity was determined by measuring 32P-labeled cAMP formation from α-32P-labeled ATP, as described previously. Briefly, the assay medium containing 50 mmol/L glycylglycine (pH 7.5), 0.5 mmol/L MgATP, α-32P-labeled ATP ([1.5–10]×10⁶ count per minute), 5 mmol/L MgCl₂ (in excess of the ATP concentration), 100 mmol/L NaCl, 0.5 mmol/L cAMP, 1 mmol/L IBMX, 0.1 μmol/L EGTA, 10 μmol/L GTP₇S, and an ATP-regenerating system consisting 2 mmol/L phosphocreatine, 0.1 mg creatine kinase per milliliter, and 0.1 mg myokinase per milliliter in a volume of 200 μL was preincubated for 2 minutes at 37°C. The reaction was initiated by the addition of the membrane proteins (20–30 μg) to the reaction mixture. The reactions were terminated after 10 minutes by the addition of 0.6 mL of 120 mmol/L zinc acetate. The cAMP was purified by coprecipitation of other nucleotides with ZnCO₃, addition of 0.5 mL of 120 mmol/L sodium carbonate, and subsequent chromatography by the double-column system, as described by Salomon.

**Determination of Cell Proliferation**

Cell proliferation was quantified by DNA synthesis, which was evaluated by incorporation of 3H-labeled thymidine into cells, as described previously. Subconfluent VSMCs from control and antisense-treated SHRs and WKY rats were plated in 6-well plates for 24 hours and were serum deprived for 24 hours to induce cell quiescence. The 3H-labeled thymidine (1 μCi/mL) was added and further incubated for 4 hours before the cells were harvested. The cells were rinsed twice with ice-cold PBS and incubated with 5% trichloroacetic acid for 1 hour at 4°C. After being washed twice with ice-cold water, the cells were incubated with 0.4 N sodium hydroxide solution for 30 minutes at room temperature, and radioactivity was determined using a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science). Cell viability was checked with the trypan blue exclusion technique and indicated that >90% to 95% of cells were viable.

**Determination of Superoxide Anion Production and NADPH Oxidase Activity**

Basal superoxide anion production and NADPH oxidase activity in heart, aorta, and kidney were measured using the lucigenin-enhanced chemiluminescence method with a low concentration (5 μmol/L) of lucigenin, as described previously. The heart, aorta, and kidney tissues from control and antisense-treated SHRs and WKY rats were washed in oxygenated Krebs HEPES buffer and placed in scintillation vials containing lucigenin solution, and the emitted luminescence was measured with a liquid scintillation counter (Wallac 1409; Perkin Elmer Life Science) for 5 minutes. The average luminescence value was estimated, the background value was subtracted, and the result was divided by the total weight of tissue in each sample.

The NADPH oxidase activity in the samples was assessed by adding 10 to 4 mol/L NADH (Sigma-Aldrich) in the vials before counting. Basal superoxide–induced luminescence was then subtracted from the luminescence value induced by NADH.

**Statistical Analysis**

Data are expressed as mean±SD and were analyzed using 1-way ANOVA in conjunction with the Newman–Keuls test with GraphPad Prism 5 software (GraphPad Software). A mean difference between groups was considered statistically significant at P<0.05.

**Results**

**Role of Liposome Quality on Antisense Transfection Efficiency**

In the current study, we used freshly formulated liposomes composed of monocationic lipid DOTAP and neutral helper lipid DOPE at a 1:1-M ratio. A novel upgrade in antisense/liposome-based hypertension research was the incorporation of 10% DSPE-PEG(2000) to increase the circulation time of the liposomes. Other important factors affecting circulation time and thus transfection efficiency are size and polydispersity index. Manual extrusion was used to ensure size control and homogeneity. Liposome size was 100 to 200 nm with a polydispersity index of 0.1 measured using dynamic light scattering. Figure 1 shows that antisense was efficiently encapsulated in liposomes, as determined using fluorescently labeled antisense (FITC). Figure 1A shows fluorescently labeled Gix-2 antisense encapsulated inside liposomes that appeared as green circles under fluorescent microscopy. Figure 1B shows measurement of the size of the PEG-cationic liposomes and the polydispersity index using dynamic light scattering. The size of the freshly formulated liposomes ranged from 100 to 200 nm with a polydispersity index of 0.1.

**Effect of Gix-2 and Gix-3 Knockdown on BP and Heart Rate**

To investigate the contribution of enhanced expression of Gix-2 on BP, the effect of Gix-2 knockdown on BP was examined.
by single intravenous injection of Gi\textsubscript{a}-2 antisense into prehypertensive rats aged 3 weeks, and the results are shown in Figure 2. Systolic BP (SBP) (Figure 2A) was not different in SHRs aged 3 weeks compared with age-matched WKY rats and started to increase from the age of 4 weeks. Treatment of SHRs with Gi\textsubscript{a}-2 antisense and not with empty liposomes or Gi\textsubscript{a}-2 sense prevented the increase in SBP up to age 6 weeks (123±2.9 mm Hg); thereafter, SBP started to increase and reached the same level as that of SHR control groups at 9 weeks (190±5 mm Hg). A second injection of Gi\textsubscript{a}-2 antisense at that time point again decreased BP significantly but not to the level in control WKY rats. In contrast, Gi\textsubscript{a}-2 antisense treatment did not have any significant effect on the SBP in WKY rats (121.8±1.6 versus 123.8±2 mm Hg). In addition, diastolic BP (Figure 2B) and mean arterial BP (Figure 2C) were slightly higher in all SHR groups compared with WKY groups at the age of 3 weeks. The knockdown of Gi\textsubscript{a}-2 protein by antisense prevented any further increase in diastolic and mean BP up to age 6 weeks; however, at age 9 weeks, like SBP, diastolic and mean arterial BPs reached the same levels as those of SHR control groups.

To investigate whether enhanced expression of Gi\textsubscript{a}-3 also contributes to the development of hypertension in SHRs, the effect of Gi\textsubscript{a}-3 knockdown on BP was examined using Gi\textsubscript{a}-3 antisense, and the BP profile is shown in Figure 2D through 2F. SBP (Figure 2D), diastolic BP (Figure 2E), and mean arterial BP (Figure 2F) started to increase at the age of 4 weeks in all groups of control SHRs (SHR control, empty liposome, and Gi\textsubscript{a}-3 sense group); however, BP was slightly decreased in the SHR group treated with Gi\textsubscript{a}-3 antisense compared with the control SHR groups, but this decrease was not significant (mean arterial BP: control SHRs 148.5±4.2 mm Hg; Gi\textsubscript{a}-3 antisense–treated SHRs, 139.7±4.5 mm Hg). At 9 weeks, the Gi\textsubscript{a}-3 antisense–treated SHR group had the same BP as that of SHR control groups. In contrast, Gi\textsubscript{a}-3 antisense treatment did not have any significant effect on the mean arterial BP in treated WKY rats (95.6±4.9 versus 98.2±2 mm Hg).

Both Gi\textsubscript{a}-2 and Gi\textsubscript{a}-3 antisense treatments did not show any adverse side effects on the health of rats because all rats treated with antisense gained weight during the study period (body weights were 142.5±4.5 g for WKY control rats, 144±3 g for Gi\textsubscript{a}-2 antisense–treated WKY rats, 141±5 g for Gi\textsubscript{a}-3 antisense–treated WKY rats, 135±3.5 g for control SHRs, 139.2±3.9 g for Gi\textsubscript{a}-2–antisense–treated SHRs, and 136.0±6.0 g for Gi\textsubscript{a}-3 antisense–treated SHRs). In addition, the ratio of heart weight:body weight was not different in SHRs aged 6 and 9 weeks compared with age-matched WKY rats, and it was not affected by the antisense treatment, as reported previously.}\footnote{31}
Prehypertensive tachycardia has been reported in SHRs.\textsuperscript{52,53} To further examine the contribution of enhanced expression of Gi\textsubscript{a}-2 and Gi\textsubscript{a}-3 proteins to the regulation of heart rate in SHRs, the effect of antisense of Gi\textsubscript{a}-2 and Gi\textsubscript{a}-3 treatment on heart rate was examined in SHRs and WKY rats at age 6 and 9 weeks (Figure 3). At age 6 weeks (Figure 3A), all SHR control groups had higher heart rates than age-matched WKY rats by \(\pm\)25\%. Treatment of SHRs with Gi\textsubscript{a}-2 antisense significantly attenuated increased heart rate by \(\pm\)15\%. In contrast, Gi\textsubscript{a}-3 antisense treatment did not have any significant effect on the heart rate. Furthermore, treatment of rats with empty liposomes, Gi\textsubscript{a}-2 sense, and Gi\textsubscript{a}-3 sense had no impact on heart rate in SHR and WKY groups. Moreover, the increased heart rate in SHRs at age 9 weeks compared with age-matched WKY rats was not attenuated by these treatments (Figure 3B).

**Figure 2.** Effect of Gi\textsubscript{a}-2 and Gi\textsubscript{a}-3 knockdown on the development of high blood pressure (BP). Antisense (AS) or sense of Gi\textsubscript{a}-2 and Gi\textsubscript{a}-3 encapsulated in PEG-cationic liposomes (1 mg/kg body weight) and empty liposomes were injected intravenously via tail vein once into prehypertensive spontaneously hypertensive rats (SHRs) aged 3 weeks and age-matched Wistar Kyoto (WKY) rats. A second injection of AS Gi\textsubscript{a}-2 (1 mg/kg body weight) was given at 8 weeks to the AS-treated SHR group. Systolic BP (A and D), diastolic BP (B and E), and mean arterial BP (C and F) were monitored weekly using the CODA system until rats were aged 9 weeks. Values are mean\(\pm\)SD of 6–10 rats in each group. ***\(P<0.001\) vs WKY control, **\(P<0.001\) vs SHR control.

Effect of Gi\textsubscript{a}-2 and Gi\textsubscript{a}-3 Knockdown on Gi\textsubscript{a} Protein Levels in Aorta and Aortic VSMCs From SHRs and WKY Rats

To examine the relationship between BP and Gi\textsubscript{a} protein expression, we determined the levels of Gi\textsubscript{a} proteins by Western blotting in aortas and VSMCs from untreated control SHRs and age-matched WKY rats and those treated with different interventions at age 6 weeks, when the increase in BP was prevented by Gi\textsubscript{a}-2 antisense treatment, and age 9 weeks, when BP in antisense-treated SHRs was augmented.
Figure 3. A and B, Effect of Giα-2 and Giα-3 knockdown on heart rate in spontaneously hypertensive rats (SHRs) and age-matched Wistar Kyoto (WKY) rats. Antisense (AS) or sense of Giα-2 and Giα-3 encapsulated in PEG-cationic liposomes (1 mg/kg body weight) and empty liposomes were injected intravenously via tail vein once into prehypertensive SHRs aged 3 weeks and age-matched WKY rats. Heart rate was monitored weekly using the CODA system. A, Effect of Giα-2 and Giα-3 AS, sense, and empty liposomes on heart rate in 6-week-old SHRs and WKY rats. B, Effect of Giα-2 and Giα-3 AS, sense, and empty liposomes on heart rate in 9-week-old SHRs and WKY rats. Values are mean±SD of 6–10 rats in each group. ***P<0.001 vs WKY control (CTL), ###P<0.001 vs SHR CTL. C through F, Effect of Giα-2 and Giα-3 knockdown on the expression of Giα-2 or Giα-3 proteins in aorta and vascular smooth muscle cells (VSMCs) from SHRs aged 6 weeks and age-matched WKY rats. AS or sense of Giα-2 and Giα-3 encapsulated in PEG-cationic liposomes (1 mg/kg body weight) and empty liposomes were injected intravenously via tail vein once into prehypertensive SHRs aged 3 weeks and age-matched WKY rats. Aorta (C and D) and VSMC lysates (E and F) from 6-week-old SHRs and WKY rats with or without treatments were subjected to Western blotting using antibodies against Giα-2 (C and E) and Giα-3 (D and F). The protein bands were quantified by densitometric scanning. The results are expressed as ratio of Gi protein:dynamin of WKY rats taken as 100%. Values are mean±SD of 4–6 separate experiments using different rats or cell populations in each group. **P<0.01, ***P<0.001 vs WKY CTL; ###P<0.001 vs SHR CTL.
to the same level as that of control SHRs. Results indicated that, as reported previously,\textsuperscript{15,17} expression of G\textsubscript{i-2} (Figure 3C and 3E) and G\textsubscript{i-3} (Figure 3D and 3F) proteins was significantly enhanced in aortas (Figure 3C and 3D) and VSMCs (Figure 3E and 3F) from rats aged 6 weeks by \(\approx 50\%\) to \(\approx 55\%\) in all SHR groups compared with WKY rats. In addition, the treatment of rats with G\textsubscript{i-2} antisense but not with sense or empty liposomes completely abolished enhanced expression of G\textsubscript{i-2} in aorta (Figure 3C); inhibition of \(\approx 70\%\) to \(80\%\) was observed in VSMCs (Figure 3E). Similarly, G\textsubscript{i-3} antisense but not sense or empty liposomes also attenuated enhanced expression of G\textsubscript{i-3} protein in aortas (Figure 3D) and VSMCs (Figure 3F) from SHRs aged 6 weeks by \(\approx 90\%\) and \(60\%\), respectively. In addition, G\textsubscript{i-2} and G\textsubscript{i-3} antisense also attenuated expression of G\textsubscript{i-2} and G\textsubscript{i-3}, respectively, in aortas and VSMCs from SHRs aged 9 weeks by \(\approx 20\%\) to \(40\%\). In contrast, the enhanced expression of G\textsubscript{i-2} or G\textsubscript{i-3} was not attenuated by G\textsubscript{i-2} or G\textsubscript{i-3} antisense treatments in aortas from SHRs aged 9 weeks (data not shown).

**Effect of G\textsubscript{i-2} and G\textsubscript{i-3} Knockdown on the Expression of G\textsubscript{i} Proteins in Hearts and Kidney From SHRs and WKY Rats**

To examine the relationship between heart rate and levels of G\textsubscript{i} proteins, we determined the levels of G\textsubscript{i-2} and G\textsubscript{i-3} in hearts from SHRs and WKY rats with and without antisense treatment (Figure 4). As reported previously,\textsuperscript{15,17} the expression of G\textsubscript{i-2} (Figure 4A) and G\textsubscript{i-3} (Figure 4B) was significantly enhanced by \(75\%\) and \(55\%\), respectively, in hearts from SHRs compared with WKY rats. In addition, antisense of both G\textsubscript{i-2} and G\textsubscript{i-3} restored the enhanced expression of G\textsubscript{i-2} and G\textsubscript{i-3} proteins, respectively, to levels in WKY controls. G\textsubscript{i-2} and G\textsubscript{i-3} antisense also attenuated the expression of G\textsubscript{i-2} and G\textsubscript{i-3}, respectively, in hearts from WKY rats by \(\approx 50\%\) and \(25\%\), respectively. In contrast, levels of G\textsubscript{i-2} (Figure 4C) and G\textsubscript{i-3} (Figure 4D) proteins that were significantly enhanced by \(\approx 50\%\) in hearts from SHRs aged 9 weeks compared with WKY rats were not attenuated by G\textsubscript{i-2} or G\textsubscript{i-3} antisense treatments. These results suggested a role of enhanced levels of G\textsubscript{i-2} but not G\textsubscript{i-3} protein in increased heart rate in SHRs.

In kidneys from SHRs and WKY rats, we examined the effect of G\textsubscript{i-2} and G\textsubscript{i-3} antisense on the expression of G\textsubscript{i-2} and G\textsubscript{i-3} proteins, respectively, which were shown to play an important role in the regulation of renal vascular tone in SHRs.\textsuperscript{27,54} Results shown in Figure 4 indicated that the expression of G\textsubscript{i-2} (Figure 4E) and G\textsubscript{i-3} (Figure 4F) proteins in kidneys from SHRs was significantly augmented by \(\approx 40\%\) compared with WKY rats, and this increase was completely restored to levels in WKY rats by antisense treatment. In addition, G\textsubscript{i-2} and G\textsubscript{i-3} antisense also attenuated the expression of G\textsubscript{i-2} and G\textsubscript{i-3}, respectively, in kidneys from WKY rats by \(\approx 25\%\).

**Effect of G\textsubscript{i-2} Knockdown on G\textsubscript{i-3} Expression and Vice Versa**

To examine the specificity of the antisense oligonucleotides, the effect of G\textsubscript{i-2} antisense treatment on G\textsubscript{i-3} protein expression and the effect of G\textsubscript{i-3} antisense treatment on G\textsubscript{i-2} protein expression was assessed in the hearts (Figure 5A and 5B) and aortas (Figure 5C and 5D) from SHRs aged 6 weeks and age-matched WKY rats. Enhanced expression of G\textsubscript{i-2} was not attenuated by the antisense of G\textsubscript{i-3} (Figure 5A and 5C), and enhanced expression of G\textsubscript{i-3} was not attenuated by the antisense of G\textsubscript{i-2} (Figure 5B and 5D). In addition, the antisense of G\textsubscript{i-3} and G\textsubscript{i-2} was also ineffective in attenuating the expression of G\textsubscript{i-2} and G\textsubscript{i-3} proteins, respectively, in hearts and aortas from WKY rats.

**Effect of G\textsubscript{i-2} and G\textsubscript{i-3} Knockdown on Adenyllyl Cyclase Inhibition in Hearts From SHRs and WKY Rats**

To investigate whether knockdown of G\textsubscript{i-2} and G\textsubscript{i-3} by antisense treatment was reflected in attenuation of Gi-mediated functions, we examined the effect of antisense treatment on the inhibitory effects of oxotremorine, which interacts with muscarinic receptors coupled to adenylyl cyclase inhibition through G\textsubscript{i} proteins.\textsuperscript{55} Results shown in Figure 6 indicated that oxotremorine inhibited adenylyl cyclase activity in hearts from SHRs and WKY rats aged 6 weeks; however, as reported previously,\textsuperscript{32} the extent of inhibition was significantly greater in SHRs. Oxotremorine, for example, inhibited adenylyl cyclase activity by \(\approx 30\%\) in WKY rats and \(\approx 45\%\), in SHRs. Knockdown of G\textsubscript{i-2} by G\textsubscript{i-2} antisense attenuated oxotremorine-mediated inhibition of adenylyl cyclase by \(\approx 55\%\) (13.5\% versus 29\%\% 2.0\% in WKY rats and \(\approx 35\%\) (28.0\% versus 43.7\% in SHRs. In contrast, the knockdown of G\textsubscript{i-3} by G\textsubscript{i-3} antisense did not have any effect on the percentage of inhibition of adenylyl cyclase by oxotremorine in SHRs and WKY rats (29.0\% versus 28.5\%\% 1.5\% versus 41.7\% in SHRs).

**Effect of G\textsubscript{i-2} and G\textsubscript{i-3} Knockdown on Proliferation of VSMCs From SHRs and WKY Rats**

Because enhanced expression of G\textsubscript{i} proteins is implicated in hyperproliferation of VSMCs from SHRs,\textsuperscript{56} it was of interest to examine whether knocking down of G\textsubscript{i} proteins by antisense
Treatment would also result in attenuation of hyperproliferation of VSMCs from SHRs. Results shown in Figure 7 indicated that the proliferation of VSMCs from SHRs was significantly augmented by $\approx 170\%$ compared with WKY rats, and Gi$\alpha$-2 and Gi$\alpha$-3 antisense treatment decreased hyperproliferation by $\approx 70\%$ and $20\%$, respectively. In addition, the

**Figure 4.** Effect of Gi$\alpha$-2 and Gi$\alpha$-3 knockdown on the expression of Gi$\alpha$-2 or Gi$\alpha$-3 proteins in hearts from 6- or 9-week-old and in kidney from spontaneously hypertensive rats (SHRs) aged 6 weeks and age-matched Wistar Kyoto (WKY) rats. Proteins in heart membrane (30 μg) from SHRs aged 6 weeks (A and B) and 9 weeks (C and D) and in kidney (E and F) from 6-week-old SHRs and WKY rats with or without treatment were subjected to Western blotting using antibodies against Gi$\alpha$-2 (A, C, and E) and Gi$\alpha$-3 (B, D, and F). The protein bands were quantified by densitometric scanning. The results are expressed as the ratio of Gi protein:dynein of WKY rats taken as 100%. Values are mean±SD of 4–6 separate experiments using different rats from each group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs WKY control (CTL); ###$P<0.001$ vs SHR CTL.
proliferation of VSMCs from WKY rats was attenuated significantly by both Giα-2 and Giα-3 antisense treatments.

Effect of Giα-2 Knockdown on the Production of Superoxide Anion and NADPH Oxidase Activity in SHRs Aged 6 Weeks and Age-Matched WKY Rats

Oxidative stress caused by the overproduction of reactive oxygen species contributes to the pathophysiology of cardiovascular diseases including hypertension. We previously reported the implication of enhanced expression of Giα proteins in enhanced oxidative stress exhibited by VSMCs from SHRs. To investigate whether knockdown of enhanced levels of Giα-2 and Giα-3 proteins in SHRs by antisense treatments also resulted in attenuation of oxidative stress, the effect of antisense treatments on superoxide anion and NADPH oxidase activity was examined in hearts, aortas, and kidneys from SHRs aged 6 weeks and age-matched WKY rats (Figure 8). As reported previously, the production of superoxide anion that was increased by about ≈35%, 25%, and 45% in hearts, aortas, and kidneys, respectively, from SHRs compared with WKY rats (Figure 8A through 8C) was significantly attenuated by Giα-2 and Giα-3 antisense treatment. In addition, NADPH oxidase activity was also significantly enhanced by ≈70%, 100%, and 45% in hearts, aortas, and kidneys, respectively, from SHRs compared with WKY rats (Figure 8D through 8F), and Giα-2 and Giα-3 antisense treatments almost completely restored the enhanced activity to levels in WKY controls. In contrast, Giα-2 and Giα-3 antisense decreased the production of superoxide anion and NADPH oxidase activity in these tissues from WKY rats.

Discussion

We previously showed that enhanced expression of Giα proteins may be the contributing factor in the pathogenesis of hypertension in SHRs. This finding was supported by our study showing that the intraperitoneal injection of pertussis toxin, which inactivates both Giα-2 and Giα-3, in prehypertensive SHRs aged 2 weeks attenuated the development of high BP. Furthermore, we showed recently that treatment of SHRs with natriuretic peptide receptor C agonist...
C-ANP-4-23, which inhibits the enhanced expression of both Giα-2 and Giα-3 proteins in VSMCs from SHRs,12,59 attenuated the development of hypertension.12 Several studies using knockout mice have shown a role of Giα-2 in physiological functions.50-55 Nagata et al showed that ablation of Giα-2 in mice resulted in attenuation of muscarinic M2 receptor-mediated antiadrenergic effect on β-adrenergic receptor–induced contractility and calcium currents in adult cardiomyocytes.64 The disruption of the Giα-2 gene in mice was also shown to result in impaired platelet activation and aggregation,61 ulcerative colitis, and adenocarcinoma of the colon.65 Furthermore, a lack of Giα-2 proteins has been shown to result in dilated cardiomyopathy and increased mortality in β1-adrenergic receptor-overexpressing mice62 and in increased infarct size in the heart in myocardial ischemia reperfusion injury.63 In addition, transgenic mice overexpressing Gi inhibitor peptide, which causes functional knockout of cardiac Giα-2 signaling when subjected to ischemia reperfusion, were shown to have increased infarct size.60 In the present study, by using antisense oligodeoxynucleotide encapsulated in PEG-cationic liposomes to knock down the Giα-2 and Giα-3 proteins in SHRs, we reported for the first time that enhanced expression of Giα-2 and not Giα-3 plays a major role in the development of hypertension.

We found that treatment of prehypertensive SHRs aged 3 weeks with a single intravenous injection of 1 mg/kg body weight of Giα-2 antisense prevented the development of hypertension up to age 6 weeks and was associated with attenuation of enhanced expression of Giα-2 protein, which has been implicated as a contributing factor in the pathogenesis of hypertension.15,17 In contrast, the increase in BP at age 9 weeks may be due to the elimination of antisense from the system or to the de novo synthesis of Giα proteins. This notion was supported by our results showing that Giα-2 protein expression that was attenuated by antisense treatment in SHRs aged 6 weeks was enhanced in SHRs aged 9 weeks and was not attenuated by antisense treatment and thus contributed to increased BP. Our results are in accordance with earlier studies showing that the effect of antisense of β1-adrenergic receptor on BP had worn off after 20 days.40 In contrast, antisense treatment decreased the expression of Giα-2 proteins but not BP in WKY rats, suggesting that enhanced expression of Giα protein is implicated in the development of high BP in SHRs. Our results are consistent with our previous studies showing that pertussis toxin, which attenuated the expression of Giα proteins in WKY rats, did not affect BP.32 We also showed that BP in all SHR groups was slightly higher compared with WKY groups at age 3 weeks. This result may be attributed to enhanced expression of Giα-2 protein and vascular changes that occur in the prehypertensive state.31,66,67 Alternatively, knockdown of Giα-3 protein in SHRs using Giα-3 antisense, which decreased the enhanced levels of Giα-3 proteins, did not significantly decrease BP. It is important to note that the extent of attenuation of enhanced expression of Giα-2 and Giα-3 proteins by Giα-2 and Giα-3

Figure 6. Effect of knockdown of Giα-2 antisense (AS) on M2 receptor-dependent Gi functions. Adenylyl cyclase activity was determined in the absence or presence of 5 μmol/L oxotremorine in heart particulate fractions from control (CTL) and Giα-2 AS-treated 6-week-old spontaneously hypertensive rats (SHRs) and Wistar Kyoto (WKY) rats. Values are mean±SD of 4 separate experiments using different rats from each group. ***P<0.001 vs WKY CTL; ###P<0.001 vs SHR CTL. NS indicates not significant.

Figure 7. Effect of knockdown Giα-2 and Giα-3 antisense (AS) on thymidine incorporation in vascular smooth muscle cells (VSMCs) from spontaneously hypertensive rats (SHRs) aged 6 weeks and age-matched Wistar Kyoto (WKY) rats. Thymidine incorporation in confluent VSMCs from control (CTL) and AS-treated SHRs and WKY rats was determined. Results are expressed as percentage of WKY CTL (taken as 100%). Values are mean±SD of 4 separate experiments using different rats from each group. ***/P<0.001 vs WKY CTL; ###P<0.001 vs SHR CTL.
antisense treatment, respectively, was almost the same, whereas high BP was decreased by knocking down only Giα-2 and not Giα-3 proteins. This result suggests the contribution of enhanced expression of Giα-2 protein to the development of hypertension.

Several studies have shown that knocking out one isoform of Giα protein upregulates the expression of other isoforms of Giα protein in knockout mice.62,68 In the current study, however, the effect of antisense was very specific because Giα-2 antisense attenuated the expression of the Giα-2 protein without affecting the expression of Giα-3 protein, and Giα-3 antisense attenuated the expression of Giα-3 and not Giα-2 protein. Consequently, the lack of effect of Giα-3 antisense on attenuation of high BP may not be caused by upregulation of Giα-2 and further suggests a role for enhanced expression of Giα-2 in the development of high BP in SHRs.

We also reported for the first time that enhanced expression of Giα-2 protein in SHRs contributes to increased heart rate in SHRs because the knockdown of Giα-2 protein attenuated the enhanced heart rate in SHRs aged 6 weeks.

Augmented expression of Giα-3 protein did not appear to play a role in the regulation of heart rate because the knockdown of Giα-3 protein by antisense treatment was ineffective in attenuating the enhanced heart rate in SHRs. Consequently, it may be suggested that the Giα-2 protein may be a major contributor to the regulation of heart rate in SHRs. The molecular mechanism responsible for Giα-2-mediated regulation of heart rate appears to involve cAMP because knockdown of Giα-2 protein by Giα-2-antisense, which attenuated increased heart rate, also attenuated enhanced inhibition of adenylyl cyclase by the Giα-2–coupled M2 receptor agonist oxotremorine in hearts from SHRs. In this regard, several G protein–coupled receptors have been shown to regulate cardiac contractility via cAMP and the Ca2+ pathway.69–71 Whether Giα-2 antisense also attenuates intracellular levels of Ca2+ needs to be investigated. Our results, however, agree with the study by Nagata et al, who showed a role for Giα-2 but not Giα-3 in the regulation of cardiac contractility and calcium currents by muscarinic receptor in adult cardiomyocytes.64

Figure 8. Effect of knockdown of Giα-2 and Giα-3 antisense (AS) on the production of superoxide anion (O2−) production and NADPH oxidative activity in heart, aorta, and kidney from spontaneously hypertensive rats (SHRs) aged 6 weeks and age–matched Wistar Kyoto (WKY) rats. The O2− production and NADPH oxidase activity were determined in heart (A and D), aorta (B and E), and kidney (C and F) from SHRs aged 6 weeks and age-matched WKY rats with or without AS treatment. Values are mean±SD of 4 separate experiments by using different rats from each group. *P<0.05, **P<0.01, ***P<0.001 vs WKY control (CTL); ##P<0.01, ###P<0.001 vs SHR CTL. CPM indicates count per minute.
In the present study, we demonstrated that Giα-2 and Giα-3 antisense treatment also attenuated enhanced expression of Giα-2 and Giα-3 proteins in kidney, a target tissue involved in the regulation of BP. Because enhanced expression of Giα proteins in kidneys of SHRs has been shown to contribute to increased renal vascular resistance and decreased renal blood flow that results in augmentation of BP in SHRs, it may be suggested that Giα-2 antisense-induced attenuation of enhanced expression of Giα-2 proteins in kidney may also participate in attenuation of high BP in SHRs by decreasing renal vascular resistance and increasing renal blood flow.

The attenuation of high BP by knockdown of Giα-2 is also associated with inhibition of enhanced oxidative stress and hyperproliferation of VSMCs, suggesting that attenuation of oxidative stress and hyperproliferation of VSMCs may be a mechanism contributing to the attenuation of high BP and tachycardia in SHRs. In this regard, increased oxidative stress caused by overproduction of reactive oxygen species and hyperproliferation of VSMCs have been shown to contribute to the pathophysiology of cardiovascular diseases such as hypertension and atherosclerosis. Alternatively, knockdown of Giα-3 in SHRs by Giα-3 antisense treatment also resulted in attenuation of enhanced oxidative stress and hyperproliferation of VSMCs in SHRs. It appears, however, that inhibition of enhanced oxidative stress and hyperproliferation induced by Giα-3 antisense may not play a role in decreasing high BP in SHRs.

**Perspectives**

Hypertension is a multifactorial disease. The present study demonstrated an important role for Giα-2 protein in the regulation of BP. Moreover, it showed for the first time that a single intravenous injection of Giα-2 antisense, and not Giα-3 antisense, encapsulated in PEG-cationic liposomes prevents the development of high BP and attenuates tachycardia in SHRs. Based on these findings, it may be suggested that the highly specific gene therapeutic agents encapsulated in nanoliposomes targeting Giα-2 protein may have the potential for use in the treatment and/or prevention of hypertension and tachycardia.

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**Disclosures**

None.

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