Inactivation of Enhanced Expression of G\textsubscript{i} Proteins by Pertussis Toxin Attenuates the Development of High Blood Pressure in Spontaneously Hypertensive Rats

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Abstract—We have previously shown that the enhanced expression of G\textsubscript{i} proteins in spontaneously hypertensive rats (SHR) that precedes the development of high blood pressure may be one of the contributing factors in the pathogenesis of hypertension. In the present study, we demonstrate that the inactivation of G\textsubscript{i} proteins by intraperitoneal injection of pertussis toxin (PT, 1.5 \( \mu \text{g} \)/100 g body wt) into 2-week-old prehypertensive SHR prevented the development of hypertension up to 4 weeks and that, thereafter, it started to increase and reached the same level found in untreated SHR after 6 weeks. A second injection of PT after 4 weeks delayed the increase in blood pressure for another week. The PT-induced decrease in blood pressure in 6-week-old SHR was associated with a decreased level of G\textsubscript{i}-2 and G\textsubscript{i}-3 proteins in the heart, as determined by in vitro ADP ribosylation and immunoblotting. The decreased level of G\textsubscript{i} proteins was reflected in decreased G\textsubscript{i} functions. Furthermore, an augmentation of blood pressure to the same level in PT-treated SHR as found in untreated SHR was associated with enhanced expression and function of G\textsubscript{s}. These results indicate that the inactivation of G\textsubscript{i} proteins by PT treatment in prehypertensive SHR attenuates the development of hypertension and suggest that the enhanced levels of G\textsubscript{i} proteins that result in the decreased levels of cAMP and associated impaired cellular functions may be contributing factors in the pathogenesis of hypertension in SHR. (Circ Res. 2002;91:247-254.)

Key Words: pertussis toxin ■ ADP ribosylation ■ G proteins ■ blood pressure ■ spontaneously hypertensive rats

Guanine nucleotide regulatory proteins (G proteins) are a family of GTP-binding proteins that play an important role in the regulation of a variety of signal transduction systems, including the adenylyl cyclase/cAMP system. The adenylyl cyclase system is composed of three components: receptor, catalytic subunit, and stimulatory (G\textsubscript{s}) and inhibitory (G\textsubscript{i}) guanine nucleotide regulatory proteins.\textsuperscript{1,2} The stimulation and inhibition of adenylyl cyclase by hormones are mediated by these two distinct G proteins (G\textsubscript{s} and G\textsubscript{i}, respectively), which couple the receptor to the catalytic subunit. The G proteins are heterotrimeric and are composed of \( \alpha \), \( \beta \), and \( \gamma \) subunits. The \( \alpha \) subunits bind and hydrolyze GTP and confer specificity in receptor and effector interactions. Molecular cloning has revealed four different forms of G\textsubscript{\alpha}, resulting from the differential splicing of one gene\textsuperscript{3-5} and three distinct forms of G\textsubscript{\alpha}(G\textsubscript{\alpha}-1, G\textsubscript{\alpha}-2, and G\textsubscript{\alpha}-3) encoded by three distinct genes.\textsuperscript{6} All three forms of G\textsubscript{\alpha}(G\textsubscript{\alpha}1 to G\textsubscript{\alpha}3) are implicated in adenylyl cyclase inhibition\textsuperscript{6} and in the activation of atrial K\textsuperscript{+} channels.\textsuperscript{7}

The adenylyl cyclase/cAMP system has been implicated in both the control of heart contractility and vascular smooth muscle tone.\textsuperscript{8,9} The levels of cAMP are regulated by G\textsubscript{s} and G\textsubscript{i} proteins. G\textsubscript{s} protein and associated adenylyl cyclase signaling has been shown to be implicated in a variety of cellular functions, including vascular permeability,\textsuperscript{10,11} salt and water transport,\textsuperscript{12,13} and catecholamine release,\textsuperscript{14} all of which play a key role in the regulation of blood pressure (BP). Alterations in the levels of G\textsubscript{s} proteins and cAMP levels that result in the impaired cellular functions lead to various pathological states, including hypertension. Several abnormalities in G-protein expression, adenylyl cyclase activity, and cAMP levels have been reported in cardiovascular tissues from genetic models (spontaneously hypertensive rats [SHR]) and different models of experimentally induced hypertensive rats.\textsuperscript{15-20} An increased expression of G\textsubscript{s} protein and G\textsubscript{i} protein mRNA in hearts and aortas from SHR and in hearts from deoxycorticosterone acetate (DOCA)-salt hypertensive rats with established hypertension has been reported.\textsuperscript{15,17,19} On the other hand, the levels of G\textsubscript{\alpha} were shown to be unaltered in SHR but were decreased in DOCA-salt hypertensive rats.\textsuperscript{15-17} The increased levels of G\textsubscript{\alpha} were shown to be associated with hypertension and not with hypertrophy,\textsuperscript{21} whereas the decreased levels of G\textsubscript{\alpha} were associated with hypertrophy and not with hypertension.\textsuperscript{22} We have recently shown that the increased expression of G\textsubscript{\alpha} proteins occurs before the onset of hypertension in SHR\textsuperscript{23} and in DOCA-salt hypertensive rats.
rath, suggesting that the enhanced levels of $G_i$ proteins may be one of the contributing factors in the pathogenesis of hypertension. The present studies were undertaken to investigate the effect of inactivation of $G_i$ protein by pertussis toxin (PT) treatment on the development of high BP in SHR. We have provided the first evidence that the inactivation of $G_i$ protein in 2-week-old SHR (prehypertensive state) attenuates the development of high BP in SHR and that $G_i$ proteins may be one of the possible factors in the regulation of BP.

Materials and Methods

Materials

AS7 and EC2 antibodies and [$\alpha$-32P]NAD$^+$ were purchased from DuPont Canada. The C-atrial natriuretic peptide fragment 4-23 (C-ANP, 23) was purchased from Peninsula Laboratories, and PT was from List Biochemicals. All other materials were purchased from commercial sources and were of the highest purity available.

Animal Treatment

Male SHR (2 weeks old) and age-matched Wistar-Kyoto (WKY) rats were purchased from Charles River Canada (St-Constant, Quebec, Canada) and housed at the University of Montreal for 2 days. PT (1.5 $\mu$g/100 g body wt) or vehicle was injected into 2-week-old SHR and their age-matched WKY rats, respectively, as described earlier. A second injection of PT (1.5 $\mu$g/100 g body wt) was given to one group of PT-treated SHR at 6 weeks and to another group at 8 weeks. The BP was measured weekly up to 9 weeks by the tail-cuff method without anesthesia. The rats were euthanized by decapitation at 6, 8, and 9 weeks of age. The hearts and mesenteric arteries were dissected out, frozen immediately in liquid nitrogen, and stored at −80°C. All biochemical studies were performed in 6- and 8-week-old control and PT-treated SHR and WKY rats that received a single injection of PT or vehicle, respectively, at 2 weeks of age.

Preparation of Heart and Mesenteric Artery Particulate Fraction

Heart and mesenteric artery particulate fractions were prepared as described previously. 21,23

PT-Catalyzed ADP Ribosylation

ADP ribosylation of heart membranes by PT was performed as described previously. 15 The heart membranes were solubilized with Lubrol PX (0.3%; Sigma) at 25°C for 10 minutes and were centrifuged at 10,000 g for 1 hour. The solubilized fractions were incubated in 25 mmol/L glycylglycine buffer, pH 7.5, containing 15 $\mu$mol/L [$\alpha$-32P]NAD$^+$ (20 $\mu$Ci/mL), 0.4 mmol/L GTP, 15 mmol/L thymidine, 10 mmol/L dithiothreitol, and 0.1 mg/mL ovalbumin with or without PT (5 $\mu$g/mL) for 30 minutes at 30°C in total volume of 100 $\mu$L. The reaction was terminated by the addition of 20 $\mu$L stop mixture containing 5% SDS and 50% $\beta$-mercaptoethanol. The proteins were analyzed by SDS-PAGE and subsequently quantified as described previously. 15

Immunoblotting

Immunoblotting of $G_i$ proteins in heart particulate fractions was performed using specific antibodies against different $G_i$ proteins and the enhanced chemiluminescence kit (Amersham), as described previously. 15

Adenylyl Cyclase Assay

Adenylyl cyclase activity was determined by measuring [$\alpha$-32P]AMP formation from [$\alpha$-32P]ATP, as described previously. 15,21-24

Results

Effect of In Vivo PT Treatment on Development of High BP

The BP profile is shown in Figure 1. Mean arterial BP was significantly different in 3-week-old SHR compared with age-matched WKY; however, BP started to increase from 4 weeks in SHR. On the other hand, PT-treated SHR did not show any increase in BP up to 6 weeks of age; thereafter, BP started to increase and reached the same level as that of untreated SHR at 8 weeks. A second injection of PT at this time point again decreased the BP significantly but not to the control WKY level. However, a second injection of PT at 6 weeks into PT-treated SHR delayed further the increase in BP for another week. After that, it started to go up but was always lower than the BP of untreated SHR. On the other hand, PT treatment did not significantly affect the BP in WKY rats. PT (1.5 $\mu$g/100 g body wt) did not appear to have adverse effects on the health of animals in the study, because all rats treated with PT maintained or gained weight during the period of the studies (body weights were as follows: for WKY rats, 160±7.8 g; for PT-treated WKY rats, 154±7.4 g; for SHR, 150±7.0 g; and for PT-treated SHR, 149±3.9 g).

In addition, as reported earlier, the ratio of heart weight to body weight was not different in 6- and 8-week-old SHR.
compared with their age-matched WKY rats and was not affected by PT treatment (data not shown).

**Effect of In Vivo PT Treatment on PT-Catalyzed ADP Ribosylation of G Proteins**

The in vitro ADP ribosylation studies were conducted to evaluate the effectiveness of the in vivo treatment with PT on the premise that the G proteins that were ADP-ribosylated in vivo by PT treatment would not be subjected to ADP ribosylation in vitro. The results indicated in Figure 2 show PT-catalyzed ADP ribosylation of Gi/Go proteins in hearts from 6- and 8-week-old control and PT-treated SHR and WKY rats. As reported earlier, PT in the presence of [α-32P]NAD catalyzed the ADP ribosylation of a protein band of 40/41-kDa referred to as Gi/Go in heart membranes solubilized by Lubrol PX at a final concentration of 0.3% from control untreated and PT-treated 6- and 8-week-old SHR and WKY rats (Figure 2A); however, it was significantly enhanced by ~45% in 6-week-old SHR compared with age-matched WKY rats, as determined by densitometric scanning (Figure 2B). On the other hand, the extent of ADP ribosylation of Gi/Go was significantly lower in 6-week-old PT-treated SHR and WKY rats compared with untreated SHR and WKY rats, respectively. However, the extent of ADP ribosylation of Gi/Go in 8-week-old PT-treated SHR was similar to that in untreated 8-week-old SHR, suggesting that the effect of PT had worn off at 8 weeks (6 weeks after the treatment).

**Effect of PT Treatment on G-Protein Levels**

Figure 3 shows the effect of PT treatment on the levels of Gi-2 and Gi-3 proteins. On the top, blots show heart membrane proteins (20 μg) from 6- and 8-week-old SHR and WKY rats with or without PT treatment that were resolved by SDS-PAGE and transferred to nitrocellulose, which was then immunoblotted with antibody AS/7 for Gi-2 (A) or antibody EC/2 for Gi-3 (B) as described previously. Autoradiogram is representative of 3 or 4 separate experiments. The graphs on the bottom show quantification of protein bands by densitometric scanning. The results are expressed as percentage of WKY control at 6 weeks, which has been taken as 100%. Values are mean±SEM of 3 or 4 separate experiments. NS indicates not significant. *P<0.05; **P<0.01; and ***P<0.001.

Figure 2. Effect of in vivo PT treatment on in vitro PT-catalyzed ADP ribosylation of G proteins. The solubilized heart particulate fractions were incubated with [α-32P]NAD in the presence of 5 μg/mL PT at 30°C for 30 minutes, as described in Materials and Methods. The 32P-labeled proteins were analyzed by SDS-PAGE, followed by autoradiography. A, Autoradiograms are representative of 3 or 4 separate experiments. B, Quantification of protein bands by densitometric scanning is shown. The results are expressed as percentage of WKY control at 6 weeks, which has been taken as 100%. Values are mean±SEM of 3 or 4 separate experiments. NS indicates not significant. **P<0.01; ***P<0.001.

Figure 3. Effect of in vivo PT treatment on levels of Gi-2 and Gi-3 proteins. On the top, blots show heart membrane proteins (20 μg) from 6- and 8-week-old SHR and WKY rats with or without PT treatment that were resolved by SDS-PAGE and transferred to nitrocellulose, which was then immunoblotted with antibody AS/7 for Gi-2 (A) or antibody EC/2 for Gi-3 (B) as described previously. Autoradiogram is representative of 3 or 4 separate experiments. The graphs on the bottom show quantification of protein bands by densitometric scanning. The results are expressed as percentage of WKY control at 6 weeks, which has been taken as 100%. Values are mean±SEM of 3 or 4 separate experiments. NS indicates not significant. *P<0.05; **P<0.01; and ***P<0.001.
(Gα-1 has been shown to be absent from heart), on immunobots of heart membranes from 6- and 8-week-old SHR and WKY rats. However, as reported earlier, the relative amount of immunodetectable Gα-2 was significantly enhanced by ≈40% in SHR compared with WKY rats, as determined by densitometric scanning. On the other hand, the levels of Gα-2 were significantly reduced in 6-week-old PT-treated SHR and WKY rats compared with untreated SHR and WKY rats; whereas the levels of Gα-2 were similar in 8-week-old PT-treated and untreated SHR. Similarly, the EC2 antibody recognized a single protein of 41 kDa referred to as Gα-3 in hearts from 6- and 8-week-old SHR and WKY rats (Figure 3B); however, the relative amount of immunodetectable Gα-3 was significantly augmented by ≈20% to 25% in SHR compared with WKY rats, as determined by densitometric scanning. On the other hand, the levels of Gα-3 were significantly decreased in 6-week-old PT-treated SHR and WKY rats compared with control untreated SHR and WKY rats, respectively, whereas the levels of Gα-3 were similar in 8-week-old PT-treated and untreated SHR.

**Effect of PT Treatment on Gα Functions**

To investigate whether the inactivation of Gα proteins by PT-catalyzed ADP ribosylation was reflected in Gα functions, the receptor-independent and -dependent Gα functions were determined in hearts from 6- and 8-week-old SHR and WKY rats without and with PT treatment. Figure 4 shows the effect of varying concentrations of GTPγS on forskolin (FSK)-stimulated adenylyl cyclase activity (receptor-independent Gα functions) in 6- and 8-week-old control and PT-treated SHR and WKY rats. GTPγS inhibited FSK-stimulated adenylyl cyclase activity in a concentration-dependent manner in hearts (Figure 4A) and mesenteric arteries (Figure 4B) from 6-week-old SHR and WKY rats; however, the extent of inhibition was significantly increased in SHR compared with WKY rats (≈20% in WKY rats and 30% in SHR). On the other hand, PT treatment reduced the extent of inhibition of FSK-stimulated adenylyl cyclase by GTPγS in SHR and almost completely attenuated the effect in WKY rats.

Figure 4C shows the effect of GTPγS on FSK-stimulated adenylyl cyclase activity in hearts from 8-week-old control and PT-treated SHR and WKY rats. As shown above, the extent of GTPγS-mediated inhibition of FSK-stimulated adenylyl cyclase was also greater in 8-week-old SHR compared with age-matched WKY rats. However, PT treatment was ineffective in altering the increased inhibition of FSK-stimulated adenylyl cyclase by GTPγS in SHR.

Figure 5A shows the relationship between PT-induced inactivation of Gα proteins with receptor-dependent functions. Angiotensin II (Ang II), C-ANP, and oxotremorine, which interact with Ang II type 1, ANP-C, and muscarinic receptor, respectively, and inhibit adenylyl cyclase activity through Gα proteins, inhibited adenylyl cyclase activity in hearts from 6-week-old SHR and WKY rats; however, as reported earlier, the extent of inhibition was significantly greater in SHR. For example, Ang II, C-ANP, and oxotremorine inhibited adenylyl cyclase activity by ≈15%, 20%, and 30%, respectively, in WKY rats and ≈25%, 30%, and 45%, respectively, in SHR. PT

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receptor-dependent Gi function. Adenylyl cyclase activity was compared with untreated SHR.

whereas the percent inhibition of adenylyl cyclase by these hormones was significantly decreased in PT-treated SHR, suggesting the implication of enhanced expression of Gi, Ang II, and resultant decreased levels of cAMP in response to various hormones, including Ang II, may be one of the contributing factors in the pathogenesis of hypertension.

In the present study, we report that the inactivation of Gi proteins by a single intraperitoneal injection of PT at 2-week-old prehypertensive SHR attenuates the development of high BP up to 6 weeks of age, after which BP starts increasing and reaches the same level as found in SHR at 8 weeks, which may be due to the possibility that PT is no longer effective or may be eliminated from the system. This notion is further supported by the fact that a second injection of PT at 6 weeks delays the increase in BP for another week, whereas a second injection at 8 weeks decreases the BP significantly but not to the control WKY level. On the other hand, no significant reduction in BP was evoked by PT in WKY rats. The reduction in BP by PT treatment in 12-week-old SHR has been reported. In addition, Kost et al have also shown that PT treatment reduced BP in adult SHR (4 to 7 months old) with established hypertension but not in WKY rats; however, this is the first study showing the attenuation of the development of high BP in SHR by PT treatment.

PT has been reported to ADP-ribosylate and inactivate Gi proteins as well as Gai proteins; these occurrences result in the inhibition of a variety of Gi-mediated hormonal and peptidergic effects. The inactivation of Gai protein attenuates the GTP-dependent and hormone receptor-mediated inhibition of adenylyl cyclase and results in the augmentation of cAMP levels.

In the present study, we have shown that intraperitoneal injection of PT into 2-week-old prehypertensive SHR and their age-matched WKY rats results in similar in vivo ADP ribosylation of Gi proteins as well as in the decreased levels of active unribosylated Gai-2 and Gai-3 proteins in 6-week-old rats. These data suggested that the distribution of toxin was not different in the two strains. However, the attenuation of BP by PT treatment was observed only in SHR and not in WKY rats, suggesting the implication of enhanced expression of Gai protein in the development of high BP in SHR. Our results are in accordance with the studies of other investigators, who also did not observe any effect of PT on BP in

treatment, on the other hand, almost completely attenuated Ang II–mediated, C-ANP4-23–mediated, and oxotremorine-mediated inhibition of adenylyl cyclase in WKY rats, whereas the percent inhibition of adenylyl cyclase by these hormones was significantly decreased in PT-treated SHR compared with untreated SHR.

In addition, Ang II, C-ANP4-23, and oxotremorine inhibited adenylyl cyclase activity to the same degree in untreated and PT-treated SHR at 8 weeks compared with WKY rats (Figure 5B).

**Effect of PT Treatment on Gi-Mediated Hormonal Stimulations of Adenylyl Cyclase Activity**

Figure 6 shows the effects of inactivation of Gai proteins by PT treatment on the stimulatory effects of some agonists on adenylyl cyclase activity in 6- and 8-week-old SHR. Glucagon, isoproterenol, and 5′-N-ethylcarboxamidoadenosine stimulated adenylyl cyclase activity in hearts from 6- and 8-week-old SHR and WKY rats; however, as reported earli-
WKY rats. However, G proteins that are also subjected to ADP ribosylation by PT may not be implicated in the reduction of BP, because their levels were shown not to be altered in SHR. Our results are consistent with studies reported earlier showing that intraperitoneal injection of PT into rats decreased the level of G proteins in the pancreas after 24 and 48 hours of PT treatment and that this decrease was accompanied by the appearance of a band that was an inactive ADP-ribosylated form of G with less mobility (2-kDa shift in molecular weight). However, we were unable to detect the inactivated ADP-ribosylated form of G proteins in the hearts of 6-week-old SHR compared with untreated SHR has been demonstrated by decreased inhibition of FSK-stimulated adenylyl cyclase activity by GTPγS as well as by decreased inhibition of adenylyl cyclase by Ang II, C-ANP 4-23, and oxotremorine. Our results are in agreement with previous studies showing the attenuation of enhanced renal vascular responsiveness to Ang II in SHR by PT treatment. In addition, PT-induced inactivation of G proteins has also been shown to affect renal functions in SHR, as has been indicated by increased renal blood flow and decreased renal vascular resistance in SHR; these findings suggest that a G-mediated pathway may contribute to increased vascular tone in the SHR kidney. The role of cAMP in diuresis and natriuresis has been demonstrated. Proximal tubules from SHR have been shown to generate decreased levels of cAMP in response to dopamine and Ang II, which may contribute to decreased diuresis and increased sodium retention in the SHR. Taken together, it may be possible that intraperitoneal injection of PT, which inactivates G proteins.
and therefore increases the formation of cAMP to hormonal stimuli in the proximal tubule, results in increased diuresis and decreased sodium retention and may thus contribute to the attenuation of the development of high BP at 6 weeks in SHR. However, Kost et al. did not observe any significant increase in urine volume and sodium excretion after intravenous injection of PT into adult SHR. The reason for lack of a PT effect on natriuresis and diuresis is not clear in their studies and may be due to the possibility that the duration of PT treatment was too short (3 to 5 days) to observe these effects. Thus, it may be suggested that the inactivation of Gi proteins in various target tissues (including mesenteric artery resistance vessels, renal vasculature, and proximal tubules) by intraperitoneal injection of PT and the resultant increased levels of cAMP may contribute (by decreasing vascular resistance and salt and water retention and increasing vascular permeability) to the attenuation of development of high BP in SHR up to 6 weeks.

On the other hand, the levels of Giα-2 and Giα-3 in PT-treated 8-week-old SHR that were increased to the same extent as levels found in untreated SHR may be due to the possibility that PT at 8 weeks was ineffective to ADP-ribosylate and thereby decrease the enhanced levels of Gi proteins. The enhanced expressions/functions of Gi proteins in the heart (leading to decreased levels of cAMP) may not contribute to the pathogenesis of hypertension but may be implicated in decreased cardiac function (contractility) in hypertension, which may lead to the development of heart failure. However, the increased levels of Giα-2 and Giα-3 and the resultant decreased levels of cAMP in various target tissues in PT-treated SHR at 8 weeks may be responsible for impaired cellular functions, including increased vascular resistance, increased salt and water retention, and decreased capillary permeability, all of which may contribute to the augmentation of BP.

In conclusion, we have provided the first evidence showing that inactivation of Giα proteins by PT in prehypertensive SHR attenuates the development of high BP. From these studies, it can be suggested that the novel strategies that target Gi proteins could be designed to treat hypertension.

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References


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