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Redox Modulation of Gi Protein Expression and Adenylyl Cyclase Signaling: Role of Nitric Oxide

MADHU B. ANAND-SRIVASTAVA and FRANCESCO DI FUSCO

ABSTRACT

Nitric oxide (NO) has been shown to regulate a variety of physiological functions, including vascular tone. The inhibition of NO synthase by *N*ω-nitro-L-arginine methyl ester (L-NAME) has been reported to increase arterial blood pressure. The present studies were undertaken to investigate if the increased blood pressure by L-NAME is associated with enhanced expression of Gi proteins, implicated in the pathogenesis of hypertension. L-NAME was administered orally into Sprague–Dawley rats for a period of 4 weeks. Control rats were given plain tap water only. The systolic blood pressure was enhanced in L-NAME-treated rats as compared with control rats; however, the heart-to-body weight ratio was not different in the two groups. The levels of Giα-2 and Giα-3 proteins and their mRNA as determined by western and northern blotting, respectively, were significantly augmented in hearts from L-NAME-treated rats, whereas the levels of Gsα and Gβ were unaltered. In addition, the effect of low concentrations of GTPγS on forskolin-stimulated adenylyl cyclase activity (receptor-independent functions of Giα) was significantly enhanced, whereas the receptor-dependent inhibitions of adenylyl cyclase were completely attenuated in L-NAME-treated rats. Whereas cholera toxin-mediated stimulation of adenylyl cyclase was unaltered in both group of rats, the stimulatory effects of some agonists on adenylyl cyclase activity were diminished in L-NAME-treated rats. These results suggest the implication of NO in the modulation of Gi protein expression and associated adenylyl cyclase signaling. *Antioxid. Redox Signal.* 6, 385–392.

INTRODUCTION

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/cyclic AMP (cAMP) system. The adenylyl cyclase system is composed of three components: receptor, catalytic subunit, and stimulatory (Gs) and inhibitory (Gi) guanine nucleotide regulatory proteins (16, 38). The stimulation and inhibition of adenylyl cyclase by hormones are mediated by two distinct G proteins, Gs and Gi, respectively, that couple the receptor to the catalytic subunit. The G proteins are heterotrimeric and are composed of α, β, and γ subunits. The α subunits bind and hydrolyze GTP and confer specificity in receptor and effector interactions. Molecular cloning has revealed four different forms of Gsα resulting from the differential splicing of one gene (10, 36, 44) and three distinct forms of Giα, Giα-1, Giα-2, and Giα-3, encoded by three distinct genes (23). All three forms of Giα (Giα1–3) are implicated in adenylyl cyclase inhibition (23) and activation of atrial K^+ channels (53).

The adenylyl cyclase/cAMP system has been implicated in both the control of heart contractility and vascular smooth muscle tone (25, 49). The levels of cAMP are regulated by Gs and Gi proteins. Gi protein and associated adenylyl cyclase signaling have been shown to be implicated in a variety of cellular functions, including vascular permeability (20, 39), salt and water transport (15, 26), and catecholamine release (50), all of which play a key role in the regulation of blood pressure. Alterations in the levels of Gi proteins and cAMP that result in impaired cellular functions lead to various pathological states, including hypertension. Several abnormalities in

Department of Physiology, and Groupe de recherche sur le système nerveux autonome (GRSNA), Faculty of Medicine, University of Montreal, Montreal, Quebec, Canada.

G-protein expression, adenylyl cyclase activity, and cAMP levels have been reported in cardiovascular tissues from genetic [spontaneously hypertensive rats (SHR)] and different models of experimentally induced hypertensive rats (1, 2, 6, 7, 9, 48). An increased expression of Gi protein and Gi-protein mRNA in heart and aorta from SHR and hearts from DOCA-salt (deoxycorticosterone acetate) hypertensive rats with established hypertension has been reported (2, 7, 48). On the other hand, the levels of Gsα were shown to be unaltered in SHR, but were decreased in DOCA-salt hypertensive rats (2, 6, 7). We have recently shown that the increased expression of Giα proteins occurs before the onset of hypertension in SHR (34) and DOCA-salt hypertensive rats (35), suggesting that the enhanced levels of Giα proteins may be one of the contributing factors for the pathogenesis of hypertension. This was supported by our recent studies showing that inactivation of enhanced expression of Gi proteins by pertussis toxin treatment attenuates the development of hypertension in SHR (31).

The implication of free radicals in the pathogenesis of hypertension has been well established (27). Increased levels of superoxide anion ($O_2^{\bullet -}$) and hydrogen peroxide (H_2O_2) have been reported in hypertensive patients (28). The endothelium plays an important role in the regulation of cardiovascular functions by its ability to produce a variety of vasoactive agents, including nitric oxide (NO) (51). NO is synthesized from L-arginine by the action of NO synthase and regulates a variety of physiological processes, including vascular tone, platelet aggregation, neurotransmisson, cell differentiation, cell migration, hormone release, and apoptosis (30). Endothelial dysfunction has been shown to be an important contributor in the pathogeneis of hypertension due to the impaired generation of NO, which regulates vasodilation. Vitamin C, an antioxidant, has been reported to recover endothelial functions by restoring NO-mediated vasodilation of the endothelium in hypertensive patients (47). NO reacts with superoxide anion $O_2^{\bullet -}$ to form peroxynitrite ($ONOO^-$), which is a powerful oxidant and has been implicated in the vasoconstriction of vascular smooth muscle cells and atherosclerosis (37, 52). NO synthase has been shown to be inhibited by several arginine analogues, including *N*ω-nitro-L-arginine methyl ester (L-NAME) (42). Acute *in vivo* administration of L-NAME elicits marked arterial hypertension without hypertrophy (8), presumably as a consequence of an abrupt inhibition of NO synthesis (42) that results in the inhibition of vasorelaxation.

The present studies were undertaken to investigate if the increased blood pressure by L-NAME is associated with enhanced expression of Gi proteins and adenylyl cyclase signaling. We have shown that L-NAME treatment enhances the Gi protein expression and adenylyl cyclase signaling in rat heart, suggesting a role of NO in the modulation of Gi protein expression and thereby blood pressure.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats (200–250 g) purchased from Charles River Canada (St. Constant, Quebec, Canada) were used in the present studies. L-NAME (100 mg/kg/day) was administered orally into rats in their drinking tap water for a period of 4 weeks, whereas control rats received plain tap water only. The arterial blood pressure (mm Hg) was measured after 4 weeks of treatment by the tail-cuff method without anesthesia. After 4 weeks of treatment, the rats were killed by decapitation, and the hearts were removed for adenylyl cyclase activity determination and mRNA and protein quantification. All the animal procedures used in the present studies were reviewed and approved by the Comité de déontologie de l'expérimentation sur les animaux of the University of Montreal (no. 99050).

Preparation of heart particulate fraction

The heart particulate fraction was prepared as described previously (34, 35). Frozen hearts were quickly pulverized to a fine powder using mortar and pestle cooled in liquid N_2. The powder was stored at −80°C until assayed. The powder was homogenized (12 strokes) in a Teflon glass homogenizer, in a buffer containing 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 7.5. The homogenate was centrifuged at 1,000 *g* for 10 min. The supernatant fraction was discarded, and the pellet was finally resuspended and homogenized in a buffer containing 10 m*M* Tris-HCl and 1 m*M* EDTA, pH 7.5, and used for adenylyl cyclase activity determination and immunoblotting studies.

Cholera toxin (CT) treatment of heart particulate fraction

The heart particulate fraction was treated with CT as described earlier (34, 35). CT (500 μg/ml) was preactivated for 20 min at 37°C in a mixture containing 20 mmol/L dithiothreitol, 1 μg/ml bovine serum albumin, and 25 mmol/L KH_2PO_4, pH 8.0. To study the effect of CT on adenylyl cyclase activity, the heart particulate fraction was pretreated with and without CT for 30 min at 30°C in a reaction mixture containing 250 m*M* KH_2PO_4, pH 6.8, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, pH 8.0, 5 m*M* ATP, 15 m*M* thymidine, 0.15 m*M* GTP, 20 m*M* dithiothreitol, and 1 m*M* NAD. The particulate fractions were washed twice with buffer containing 10 m*M* Tris and 1 m*M* EDTA, pH 7.5, and finally suspended in the same buffer for adenylyl cyclase activity determination.

Adenylyl cyclase activity determination

Adenylyl cyclase activity was determined by measuring [^{32}P]cAMP formation from [α-^{32}P]ATP, as described previously (34, 35). The assay medium contained 50 m*M* glycylglycine, pH 7.5, 0.5 m*M* MgATP, [α-^{32}P]ATP ($1–1.5 \times 10^6$ cpm), 5 m*M* $MgCl_2$, 100 m*M* NaCl, 0.5 m*M* cAMP, 1 m*M* 3-isobutyl-1-methylxanthine, 0.1 m*M* EGTA, 10 μ*M* guanosine 5′-(γ-thio)triphosphate (GTPγS) (or otherwise as indicated) and an ATP regenerating system consisting of 2 m*M* phosphocreatine, 0.1 mg of creatine kinase/ml, and 0.1 mg of myokinase/ml in a final volume of 200 μL. Incubations were initiated by addition of the heart particulate fraction (50–100 μg) to the reaction mixture, which had been thermally preequilibrated for 2 min at 37°C. The reactions, conducted in triplicate for 10 min at 37°C, were terminated by the addition of 0.6 ml of 120 m*M* zinc acetate. cAMP was purified by coprecipitation of other nucleotides with $ZnCO_3$, addition of 0.5 ml of 144 m*M* Na_2CO_3, and subsequent chromatography by the double column system, as described by Salomon *et al.* (45). Under

the assay conditions used, adenylyl cyclase activity was linear with respect to protein concentration and time of incubation.

Protein was determined essentially as described by Lowry *et al.* (33), with crystalline bovine serum albumin as standard.

Immunoblotting

Immunoblotting of G proteins was performed as described previously (34, 35). After sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), the separated proteins were transferred electrophoretically to a nitrocellulose sheet (Schleicher and Schuell) with a semidry transblot apparatus (Bio-Rad) at 15 V for 45 min. After transfer, the membranes were washed twice in phosphate-bufferedsaline (PBS) and incubated in PBS containing 3% skim milk at room temperature for 2 h. The blots were then incubated with antibodies against G proteins in PBS containing 1.5% skim milk and 0.1% Tween 20 at room temperature overnight. The antigen–antibody complexes were detected by incubating the blots with goat anti-rabbit IgG (Bio-Rad) conjugated with horseradish peroxidase for 2 h at room temperature. The blots were then washed three times with PBS before reaction with enhanced chemiluminescence western blotting detection reagents (Amersham).

Northern analysis

Total RNA was isolated by the guanidinium thiocyanate–phenol–chloroform method described by Chomczynski and Sacchi (12). cDNA inserts encoding for Giα-2, Giα-3, Gsα, and catalytic subunit V of adenylyl cyclase were radiolabeled with [α-^{32}P]dCTP by random priming essentially as described by Feinberg and Vogelstein (13). Dimethyl sulfoxide/glyoxal-treated total RNA was resolved on 1% agarose gels and transferred to nylon membrane as described by Sambrook *et al.* (46). Filters, after prehybridization at 65°C for 6 h in hybridization solution (600 m*M* NaCl, 8 m*M* EDTA, 120 m*M* Tris, pH 7.4) containing 0.1% sodium pyrophosphate, 0.2% SDS, and heparin (500 U/ml), were hybridized overnight in hybridization solution containing 10% (wt/vol) dextran sulfate and cDNA probe (1–3 × 10^6 cpm/ml). Filters were then rinsed at 65°C for 2 × 30 min in 300 m*M* NaCl, 4 m*M* EDTA, 60 m*M* Tris, pH 7.4, and 0.1% SDS. Autoradiography was performed with *x*-ray films at −70°C. To assess the possibility of any variations in the amounts of total RNA in individual samples applied to the gel, each filter was hybridized with the ^{32}P end-labeled oligonucleotide, which recognizes a highly conserved region of 28S ribosomal RNA. The blots that had been probed with the G-protein cDNA were dehybridized by washing for 1 h at 65°C in 50% formamide, 300 m*M* NaCl, 4 m*M* EDTA, and 60 m*M* Tris, pH 7.4, and rehybridized overnight at room temperature with the oligonucleotide. Quantitative analysis of the hybridization of probes bound was performed by densitometric scanning of the autoradiographic film using the enhanced laser densitometer, LKB Utroscan XL, and quantified using the gel scan XL evaluation software (v. 2.1) from Amersham Pharmacia Biotech (Baie d'Urfe, PQ, Canada).

Data analysis

Results are expressed as means ± SEM. Comparisons between groups (control and L-NAME-treated rats) were made with Student's *t* test for unpaired samples, whereas ANOVA followed by Dunnett's multiple comparison tests or Newman–Keuls multiple comparison tests were performed for the dose-response curve of GTPγS on forskolin (FSK)-stimulated adenylyl cyclase activity. $p < 0.05$ was considered statistically significant.

RESULTS

Treatment of rats with L-NAME for 4 weeks significantly increased the arterial systolic blood pressure (190 ± 9.2 mm Hg versus 121 ± 6.3 mm Hg); however, it did not affect the heart-to-body weight ratio (2.8 ± 0.36 versus 2.7 ± 0.26 mg/g).

Effect of L-NAME treatment on Gi protein levels

To investigate if the increased blood pressure by L-NAME treatment is associated with increased expression of Giα proteins, the levels of Gi proteins were determined in hearts from control and L-NAME-treated rats by immunoblotting techniques using specific antibodies AS/7 against Giα-1 and Giα-2 and EC/2 against Giα-3. The results depicted in Fig. 1 show that the AS/7 recognized a single protein of M_r 40 kDa referred to as Giα-2 [Giα-1 has been shown to be absent in heart (24)], whereas EC/2 antibodies recognized a single protein of 41 kDa referred to as Giα-3 in hearts from control and L-NAME-treated hearts; however, the amounts of immunodetectable Giα-2 and Giα-3 protein were increased in L-NAME-treated rats by 38.6 ± 5.9% and 87.9 ± 9.7% ($n = 4$), respectively, as determined by densitometric scanning. In addition, the levels of Gsα and Gβ were also determined by using antibodies RM/1 and SW/1 against Gsα and Gβ (common), respectively; however, no differences in the amounts of immunodetectable Gsα or Gβ protein were detected between the two groups (data not shown).

We extended our studies further to investigate if mRNA levels of G proteins change concomitantly with protein levels and determined the mRNA content of G proteins from hearts from control and L-NAME-treated rats using cDNA probes encoding for Giα-2, Giα-3, and Gsα. The results depicted in Fig. 2 show that the Giα-2 and Giα-3 probes detected a message of 2.3 and 3.5 kb, respectively, in both control and L-NAME-treated heart; however, the Giα-2 and Giα-3 mRNA levels were increased by 40 ± 3.6% ($n = 4$) and 66 ± 4.2% ($n = 4$), respectively, in hearts from L-NAME-treated rats as compared with their control rats as determined by densitometric scanning. On the other hand, the cDNA probe encoding Gsα detected a message of 1.8 kb in both control and L-NAME-treated rats; however, no difference in Gsα mRNA levels was detected between the groups (data not shown). The alterations in Giα-2 and Giα-3 mRNA in L-NAME-treated hearts as compared with control rats may not be attributed to the variation in the amounts of total RNA applied to the gels, because hybridization with 32-mer oligonucleotide, which recognizes a highly conserved region of 28S rRNA, showed a similar amount of 28S rRNA loaded from control and L-NAME-treated rats onto the gels (data not shown).

Effect of L-NAME treatment on Gi protein functions

To investigate if the enhanced expression of Gi proteins was reflected in an increased function of Gi proteins, the ef-

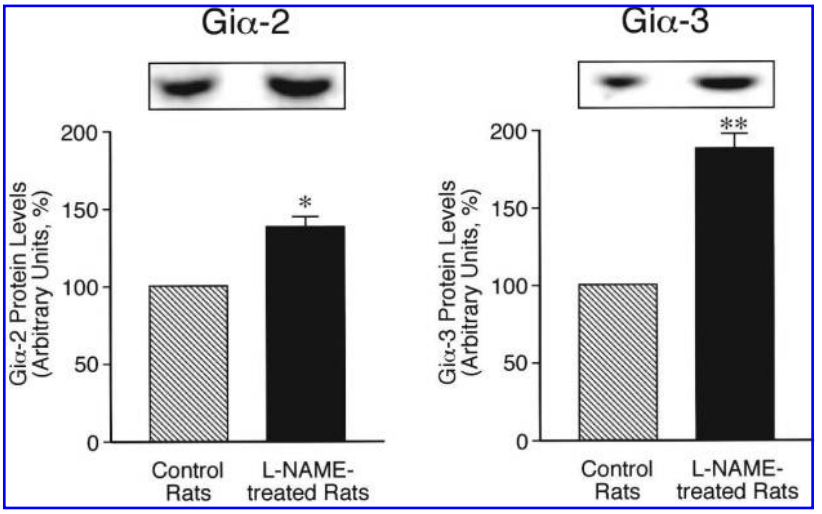

FIG. 1. Quantification of G proteins by immunoblotting in hearts from control and L-NAME-treated rats. (Upper panel) The membrane proteins (50 μg) from control and L-NAME-treated rats were separated by SDS–PAGE electrophoresis and transferred to nitrocellulose, which was then immunoblotted using antibody AS/7 for Giα-1 and Giα-2 and antibody EC/2 for Giα-3 as described in Materials and Methods. The autoradiogram is representative of four separate experiments. The detection of different G proteins was performed by using enhanced chemiluminescence western blotting detection reagents. **(Lower panel)** Densitometric scanning of G proteins from control and L-NAME-treated rats. The results are expressed as arbitrary units and values from control rats were taken as 100%. Values are means ± SEM of four separate experiments. *$p < 0.05$.

fect of low concentrations of GTPγS on FSK-stimulated adenylyl cyclase activity was examined, and the results are shown in Fig. 3. GTPγS inhibited FSK-stimulated adenylyl cyclase activity in a concentration-dependent manner in both groups; however, the inhibition of FSK-stimulated adenylyl cyclase by GTPγS was significantly enhanced in L-NAME-treated rats as compared with their control rats. For example, GTPγS at 1 n*M* inhibited FSK-stimulated enzyme activity by ~20% in control rats and ~50% in L-NAME-treated rats.

As Gi proteins couple the inhibitory hormone receptors to adenylyl cyclase and mediate the inhibitory responses of hormones on adenylyl cyclase activity, it was interesting to determine if enhanced expression and functions of Giα were also associated with enhanced inhibition of adenylyl cyclase by inhibitory hormones. To explore this, the effects of inhibitory hormones on adnylyl cyclase activity were examined in hearts from control and L-NAME-treated rats. Figure 4 shows that oxotremorine, C-ANP_{4-23} (a ring-deleted analogue of atrial natriuretic peptide), and angiotensin II (Ang II) inhibited adenylyl cyclase activity to various degrees in control rats, but the inhibition was completely attenuated in L-NAME-treated rats.

Effect of L-NAME treatment on Gs protein functions

To corroborate our results of Gsα levels with Gsα function, we analyzed the effect of CT treatment on GTP or GTPγS-sensitive adenylyl cyclase in heart membranes from both control and L-NAME-treated rats. CT-treatment augmented GTP or GTPγS-sensitive adenylyl cyclase activity in both groups, however, the percentage stimulation was not significantly different in L-NAME-treated rats as compared with control rats (data not shown), indicating that Gsα functions were also not altered in L-NAME-treated rats. As Giα has been shown to modulate Gsα functions (11, 14), it was of interest to examine if increased Giα levels in L-NAME-treated rats could affect Gsα-mediated hormonal stimulations of adenylyl cyclase. As shown in Table 1, isoproterenol, *N*-ethylcarboxamido adenosine (NECA), and glucagon stimulated adenylyl cyclase ac-

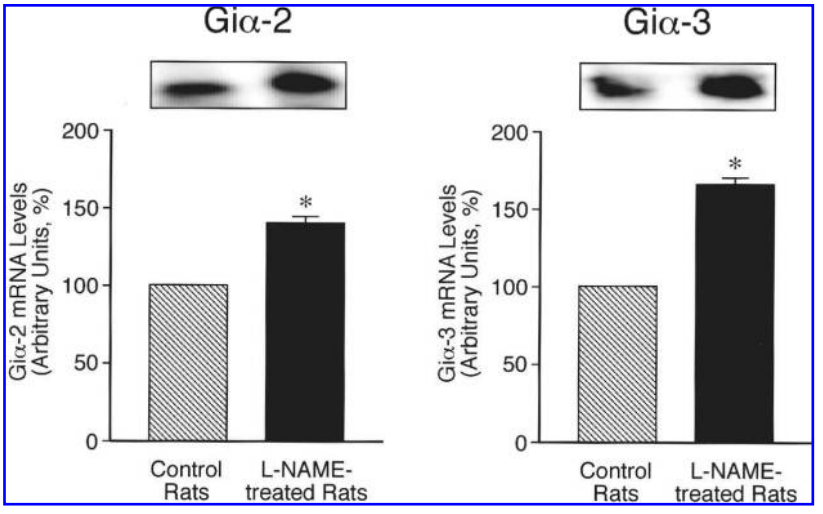

FIG. 2. mRNA expression of G proteins by northern blot in hearts from control and L-NAME hypertensive rats. (Upper panel) Total RNA (10 μg) extracted from heart from control and L-NAME hypertensive rats was separated on 1% agarose and transferred to nylon membranes, which was then hybridized with full-length cDNA probe encoding Giα-2 and Giα-3 as described in Materials and Methods. The autoradiogram is representative of four separate experiments. **(Lower panel)** Densitometric scanning of G-protein mRNA from control and L-NAME-treated rats. The results are expressed as arbitrary units, and values from control rats were taken as 100%. Values are means ± SEM of four separate experiments. *$p < 0.05$.

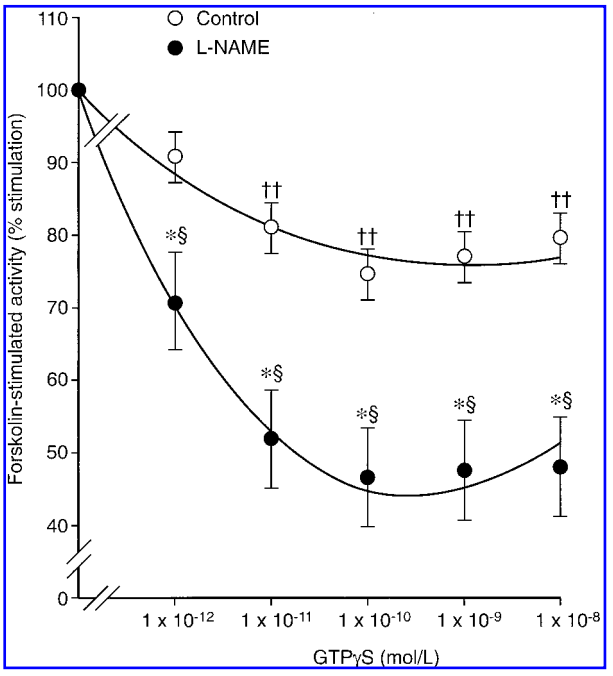

FIG. 3. Effect of GTPγS on FSK-stimulated adenylyl cyclase activity in heart membranes from control and L-NAME-treated rats. Adenylyl cyclase activity in the presence of 100 μ*M* FSK was determined in the absence (basal) or presence of increasing concentrations of GTPγS in control (◯) and L-NAME hypertensive rats (●) as described in Materials and Methods. The values are means ± SEM of three separate experiments. Adenylyl cyclase activities in the presence of 100 μ*M* FSK in control and L-NAME-treated rats in the absence of GTPγS were 2,934.5 ± 84.8 and 3,072.5 ± 37.7 pmol of cAMP/mg of protein/10 min, respectively. Statistical analysis was performed by ANOVA followed by Dunnett's test for comparison between basal and all other doses of GTPγS (§$p < 0.01$, ††$p < 0.05$), whereas Newman–Keuls test was used for comparison between control and L-NAME-treated rats (*$p < 0.001$).

tivity to various degrees in heart membranes from both groups; however, the extent of stimulation of adenylyl cyclase was significantly diminished in L-NAME-treated rats as compared with control rats. In addition, FSK- and NaF-stimulated adenylyl cyclase activities were also significantly diminished in L-NAME-treated rats as compared with control rats. To investigate if the decreased stimulation of adenylyl cyclase by FSK in L-NAME-treated rats is attributed to the decreased levels of catalytic subunit, the mRNA levels of type V enzyme were determined in hearts using a cDNA probe encoding type V enzyme. However, no difference in the amount of mRNA type V was observed between the two groups as quantified by densitometric scanning (data not shown).

DISCUSSION

In the present studies, we have shown that inhibition of NO levels by L-NAME treatment of rats for 4 weeks increases the levels of Gi proteins, as well as blood pressure.

The hearts from L-NAME-treated rats exhibited an increased expression of Gi proteins as compared with control rats as demonstrated by immunoblotting studies. These data suggest that the reduction of NO levels by L-NAME that results in the augmentation of Gi proteins may be responsible for the enhanced blood pressure in L-NAME-treated rats. This notion is supported by our earlier studies showing the implication of enhanced expression of Gi proteins and associated functions in the pathogenesis of hypertension (31, 34, 35). However, L-NAME treatment of rats did not affect the levels of Gs proteins, as well as Gs mRNA. In addition, the functions of Gs were not altered by L-NAME treatment, suggesting that NO may not play a role in the modulation of Gs protein expression and Gs-mediated functions.

The increased expression of Giα-2 and Giα-3 in L-NAME-treated rats was also reflected in Giα functions as was shown by the inhibition of FSK-stimulated adenylyl cyclase activity by GTPγS, which was greater in L-NAME-treated rats as compared with control rats. These results are in agreement with the previous studies reported in SHR and other models of hypertension showing a relationship between enhanced expression of Gi proteins and Gi functions (2, 17, 34, 35). However, a complete attenuation of adenylyl cyclase inhibition by inhibitory hormones such as oxotremorine, C-ANP$_{4-23}$, and Ang II in hearts from L-NAME-treated rats is in contrast with other studies conducted in genetic and experimental hypertensive rats, where a correlation between the enhanced levels of Gi

FIG. 4. Effect of inhibitory hormones on adenylyl cyclase activity in heart membranes from control and L-NAME-treated rats. Adenylyl cyclase was determined in the presence of GTPγS alone (basal) or in combination with 10 μ*M* Ang II, 50 μ*M* oxotremorine (OXO), or 0.1 μ*M* C-ANP$_{4-23}$ as described in Materials and Methods. The values are means ± SEM of three separate experiments. The basal adenylyl cyclase activities in control and L-NAME-treated rats in the presence of 10 μ*M* GTPγS were 481.2 ± 10.4 and 460.5 ± 11.2 pmol of cAMP/mg of protein/10 min), respectively. *$p < 0.05$, **$p < 0.01$.

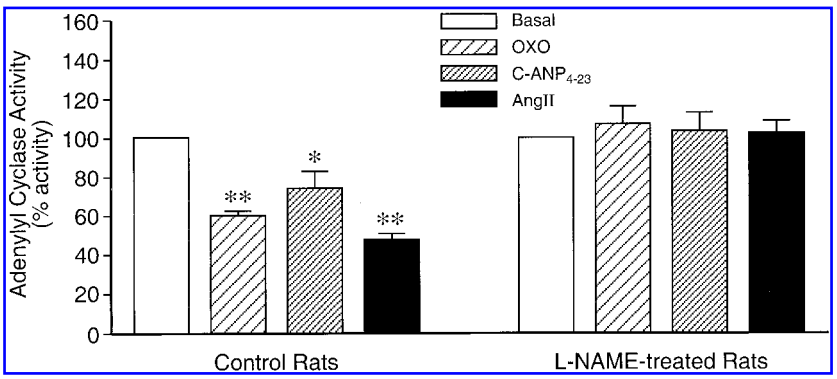

TABLE 1. EFFECT OF SOME AGONISTS ON ADENYLYL CYCLASE ACTIVITY IN HEART MEMBRANES FROM CONTROL AND L-NAME-TREATED RATS

	Adenylyl cyclase activity (pmol of cAMP/mg of protein/10 min)	
Addition	*Control rats (% stimulation)*	*L-NAME-treated rats (% stimulation)*
None	32.4 ± 2.4	34.2 ± 2.8
GTP	66.6 ± 1.4	68.3 ± 2.8
GTP + ISO	373.0 ± 33.4 (460)	293.7 ± 22.5* (330)
GTP + GLU	266.4 ± 26.4 (300)	218.6 ± 6.83* (220)
GTP + NECA	326.3 ± 26.6 (390)	266.4 ± 17.1* (290)
FSK	2,332.8 ± 194.4 (7,200)	1,949.4 ± 239* (5,800)
NaF	421.2 ± 36.5 (1,300)	324.9 ± 30.8† (950)

Adenylyl cyclase activity in heart membranes from control and L-NAME-treated rats was determined in the absence or presence of 50 μ*M* FSK, 10 m*M* NaF, or 10 μ*M* GTP alone or in combination with 50 μ*M* isoproterenol (ISO), 1 μ*M* glucagon (GLU), or 10 μ*M* NECA as described in Materials and Methods. The values are means ± SEM of three separate experiments.

*$p < 0.05$.

†$p < 0.01$.

protein and functions has been reported (17, 34, 35). However, the reason for the lack of correlation between the enhanced levels of Gi proteins and receptor-dependent Gi functions in the present studies is not clear. It may be possible that the hormone receptors are down-regulated by increased activity of the renin–angiotensin system induced by L-NAME treatment (43). In this regard, we have previously shown an attenuation of C-ANP_{4-23} and Ang II-mediated inhibition of adenylyl cyclase by Ang II treatment in A10 smooth muscle cells (40).

We have also shown that L-NAME treatment of rats resulted in decreased Gsα-mediated stimulations of adenylyl cyclase by NECA, glucagon, and isoproterenol as compared with control rats, which may be attributed to down-regulation of hormone receptors (32) or decreased levels of Gsα. However, L-NAME treatment did not alter the levels of Gsα protein and functions, suggesting that Gsα may not be responsible for the observed attenuated responsiveness of adenylyl cyclase to stimulatory hormones. On the other hand, the levels of plasma catecholamines have been shown to be enhanced by L-NAME treatment (54), which may be responsible for the desensitization of β-adrenergic receptors in L-NAME-treated rats. However, Laflamme *et al.* (29) did not observe any changes in density and affinity of cardiac β-adrenergic receptors in L-NAME-induced hypertensive rats as compared with sham-operated control rats. The modulation of Gsα functions by Giα has been reported by several investigators (11, 14). An increased expression of Giα-2 and Giα-3 has been shown to be associated with attenuated responsiveness of adenylyl cyclase to stimulatory hormones (2, 34, 35), whereas a decreased expression of Giα-2 resulted in the augmentation of stimulatory hormones on adenylyl cyclase (3, 4). Taken together, it may be possible that the enhanced levels of Giα-2 and Giα-3 in hearts by L-NAME treatment may be responsible for the diminished sensitivity of adenylyl cyclase to isoproterenol, NECA, and glucagon stimulation.

The decreased stimulation of adenylyl cyclase by FSK in L-NAME-treated rats may be due to the impaired catalytic subunit of adenylyl cyclase or to the overexpression of Giα or both. However, as the mRNA expression of type V enzyme was not decreased in hearts by L-NAME treatment and the levels of type VI enzyme were not determined, the overexpression of Giα-2 and Giα-3 proteins induced by L-NAME and type VI enzyme that may be impaired by L-NAME treatment may be responsible for the decreased sensitivity of adenylyl cyclase to FSK stimulation. In this regard, the role of Giα in FSK-mediated stimulation for adenylyl cyclase has been reported (5). In addition, the requirement of Gsα and guanine nucleotides for FSK activation of adenylyl cyclase has also been shown (21). As the levels and functions of Gsα were not altered in hearts by L-NAME treatment, the role of Gsα in eliciting decreased FSK stimulation of adenylyl cyclase cannot be attributed to the impaired Gsα.

In conclusion, we have demonstrated that L-NAME treatment of rats that results in decreased levels of NO increased the expression of Giα-2 and Giα-3 genes and translated proteins, whereas the levels and functions of Gsα were not altered by such treatment.

NO has also been shown to play a crucial role as mediator of ischemic preconditioning (18). In this regard, a natural antioxidant, resveratrol, found in grapes and wine has been reported to protect ischemic myocardium through NO, because L-NAME, which inhibits the production of NO, abolished resveratrol-mediated cardioprotective effects (19). Furthermore, resveratrol was also unable to precondition hearts from inducible NO synthase knockout mice (22). A role of Gi proteins in ischemic preconditioning has also been reported (41). Taken together, it may be suggested that NO, by modulating the expression of Giα proteins, may be responsible for ischemia preconditioning.

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ABBREVIATIONS

Ang II, angiotensin II; cAMP, cyclic AMP; C-ANP$_{4-23}$, a ring-deleted analogue of atrial natriuretic peptide, ANP (des-[Gln^{18},Ser^{19},Gln^{20},Leu^{21},Gly^{22}]ANP$_{4-23}$-NH$_2$]); CT, cholera toxin; FSK, forskolin; G protein, guanine nucleotide regulatory protein; Gi, inhibitory guanine nucleotide regulatory protein; Gs, stimulatory guanine nucleotide regulatory protein; GTPγS, guanosine 5′-(γ-thio)triphosphate; L-NAME, N^{ω}-nitro-L-arginine methyl ester, NECA, *N*-ethylcarboxamido adenosine; NO, nitric oxide; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SHR, spontaneously hypertensive rats.

REFERENCES

1. Anand-Srivastava MB. Altered responsiveness of adenylate cyclase to adenosine and other agents in the myocardial sarcolemma and aorta of spontaneously-hypertensive rats. *Biochem Pharmacol* 37: 3017–3022, 1988.
2. Anand-Srivastava MB. Enhanced expression of inhibitory guanine nucleotide regulatory protein in spontaneously hypertensive rats. Relationship to adenylate cyclase inhibition. *Biochem J* 288: 79–85, 1992.
3. Anand-Srivastava MB. Platelets from spontaneously hypertensive rats exhibit decreased expression of inhibitory guanine nucleotide regulatory protein: relation with adenylyl cyclase activity. *Circ Res* 73: 1032–1039, 1993.
4. Anand-Srivastava MB. Downregulation of atrial natriuretic peptide ANP-C receptor is associated with alterations in G-protein expression in A-10 smooth muscle cells. *Biochemistry* 39: 6503–6513, 2000.
5. Anand-Srivastava MB, Srivastava AK, and Cantin M. Pertussis toxin attenuates atrial natriuretic factor-mediated inhibition of adenylate cyclase. Involvement of inhibitory guanine nucleotide regulatory protein. *J Biol Chem* 262: 4931–4934, 1987.
6. Anand-Srivastava MB, Picard S, and Thibault C. Altered expression of inhibitory guanine nucleotide regulatory proteins (Gi alpha) in spontaneously hypertensive rats. *Am J Hypertens* 4: 840–843, 1991.
7. Anand-Srivastava MB, de Champlain J, and Thibault C. DOCA-salt hypertensive rat hearts exhibit altered expression of G-proteins. *Am J Hypertens* 6: 72–75, 1993.
8. Arnal JF, Elamrani AI, Chatellier G, Menard J, and Michel JB. Cardiac weight in hypertension induced by nitric oxide synthase blockade. *Hypertension* 22: 380–387, 1993.
9. Bohm M, Gierschik P, Knorr A, Weismann K, and Erdmann E. Desensitization of adenylate cyclase and increase of Gi alpha in cardiac hypertrophy due to acquired hypertension. *Hypertension* 20: 103–112, 1992.
10. Bray P, Carter A, Simons C, Guo V, Puckett C, Kamholz J, Spiegel A, and Nirenberg M. Human cDNA clones for four species of G alphas signal transduction protein. *Proc Natl Acad Sci U S A* 83: 8893–8897, 1986.
11. Cerione RA, Staniszewski C, Caron MG, Lefkowitz RJ, Codina J, and Birnbaumer L. A role for NO in the hormonal stimulation of adenylate cyclase. *Nature* 318: 293–295, 1985.
12. Chomczynski F and Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* 162: 156–159, 1987.
13. Feinberg AP and Vogelstein B. A technique for radiolabelling DNA restriction-endonuclease fragment to high specific activity. *Anal Biochem* 132: 6–13, 1983.
14. Feldman AM, Cates AE, Veazey WB, Hershberger RE, Bristow MR, Baughman KL, Baumgartner WA, and Dop CV. Increase of the 40,000 mol wt pertussis toxin substrate (G-protein) in the failing human heart. *J Clin Invest* 82: 189–197, 1988.
15. Feraille E and Doucet A. Sodium-potassium adenosine triphosphatase-dependent sodium transport in the kidney: hormonal control. *Physiol Rev* 81: 345–418, 2001.
16. Fleming JW, Wisler PL, and Watanabe AM. Signal transduction by G proteins in cardiac tissues. *Circulation* 85: 420–433, 1995.
17. Ge C, Garcia R, and Anand-Srivastava MB. Altered expression of Gi-protein and adenylyl cyclase activity in hearts from one kidney one clip hypertensive rats: effect of captopril. *J Hypertens* 17: 1617–1626, 1999.
18. Guo Y, Jones WK, Xuan YT, Tang XL, Bao W, Wu WJ, Han H, Laubach VE, Ping P, Yang Z, Qiu Y, and Bolli R. The late phase of ischemic preconditioning is abrogated by targeted disruption of the inducible NO synthase gene. *Proc Natl Acad Sci U S A* 96: 11507–11512, 1999.
19. Hattori R, Otani H, Maulik N, and Das DK. Pharmacological preconditioning with resveratrol: role of nitric oxide. *Am J Physiol Heart Circ Physiol* 282: H1988–H1995, 2002.
20. Hempel A, Noll T, Bach C, Piper HM, Willenbrock R, Hohnel K, Haller H, and Luft FC. Atrial natriuretic peptide clearance receptor participates in modulating endothelial permeability. *Am J Physiol* 275: H1818–H1825, 1998.
21. Hildebrandt JD, Hanoune J, and Birnbaumer L. Guanine nucleotide inhibition of cyc-S49 mouse lymphoma cell membrane adenylyl cyclase *J Biol Chem* 257: 14723–14725, 1982.
22. Imammura G, Bertelli AA, Bertelli A, Otani H, Maulik N, and Das DK. Pharmacological preconditioning with resveratrol: an insight with iNOS knockout mice. *Am J Physiol Heart Circ Physiol* 282: H1996–H2003, 2002.
23. Itoh H, Toyama R, Kozasa T, Tsukamoto T, Matsuoka M, and Kaziro Y. Presence of three distinct molecular species of Gi protein alpha subunit. Structure of rat cDNAs and human genomic DNAs. *J Biol Chem* 263: 6656–6664, 1988.
24. Jones DT and Reed R. Molecular cloning of five GTP-binding protein cDNA species from rat olfactory neuroepithelium. *J Biol Chem* 262: 14241–14249, 1987.
25. Katz AM, Tada M, and Kirshberger MA. Control of calcium transport in the myocardium by the cyclic AMP-protein kinase system. *Adv Cyclic Nucleotide Res* 5: 453–472, 1975.
26. Kinoshita S, Sidhu A, and Felder RA. Defective dopamine-1 receptor adenylate cyclase coupling in the proximal con-

voluted tubule from spontaneously hypertensive rats. *J Clin Invest* 84: 1849–1856, 1989.
27. Kumar KV and Das UN. Are free radicals involved in the pathobiology of human essential hypertension? *Free Radic Res Commun* 19: 59–66, 1993.
28. Lacy F, O'Connor DT, and Schmid-Schonbein GW. Plasma hydrogen peroxide production in hypertensive and normotensive subjects at genetic risk of hypertension. *J Hypertens* 16: 291–300, 1998.
29. Laflamme AK, Foucart S, Moreau P, Lambert C, Cardinal R, and de Champlain J. Sympathetic functions in N^G-nitro-L-arginine-methyl-ester-induced hypertension: modulation by the renin–angiotensin system. *J Hypertens* 16: 63–76, 1998.
30. Lane P and Gross SS. Cell signaling by nitric oxide. *Semin Nephrol* 19: 215–229, 1999.
31. Li Y and Anand-Srivastava MB. Inactivation of enhanced expression of Gi proteins by pertussis toxin attenuates the development of blood pressure in spontaneously hypertensive rats. *Circ Res* 91: 247–254, 2002.
32. Limas C and Limas CJ. Reduced number of β-adrenergic receptors in the myocardium of spontaneously hypertensive rats. *Biochem Biophys Res Commun* 83: 710–714, 1978.
33. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951.
34. Marcil J, Thibault C, and Anand-Srivastava MB. Enhanced expression of Gi-protein precedes the development of blood pressure in spontaneously hypertensive rats. *J Mol Cell Cardiol* 29: 1009–1022, 1997.
35. Marcil J, de Champlain J, and Anand-Srivastava MB. Overexpression of Gi-proteins precedes the development of DOCA-salt-induced hypertension: relationship with adenylyl cyclase. *Cardiovasc Res* 39: 492–505, 1998.
36. Murakami T and Yasuda H. Rat heart cell membranes contain three substrates for cholera toxin-catalyzed ADP-ribosylation and a single substrate for pertussis toxin-catalyzed ADP-ribosylation. *Biochem Biophys Res Commun* 138: 1355–1361, 1986.
37. Nakazono K, Watanabe N, Matsuno K, Sasaki J, Sato T, and Inoue M. Does superoxide underlie the pathogenesis of hypertension? *Proc Natl Acad Sci U S A* 88: 10045–10048, 1991.
38. Neer EJ. Heterotrimeric G proteins: organizers of transmembrane signals. *Cell* 80: 249–257, 1995.
39. Noll T, Hempel A, and Piper NM. Neuropeptide Y reduces macromolecule permeability of coronary endothelial monolayers. *Am J Physiol* 271: H1878–H1883, 1996.
40. Palaparti A, Ge C, and Anand-Srivastava MB. Angiotensin II enhances the expression of Giα-2 in A10 cells (smooth muscle): relationship with adenylyl cyclase activity. *Arch Biochem Biophys* 365: 113–122, 1999.
41. Piacentini L, Wainwright CL, and Parratt JR. The antiarrhythmic effect of ischaemic preconditioning in isolated rat heart involves a pertussis toxin sensitive mechanism. *Cardiovasc Res* 27: 674–680, 1993.
42. Rees DD, Palmer RMJ, and Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci U S A* 86: 3375–3378, 1998.
43. Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, and Zatz R. Chronic inhibition of nitric oxide synthesis: a new model of arterial hypertension. *Hypertension* 20: 298–303, 1992.
44. Robishaw JD, Smigel MD, and Gilman AG. Molecular basis for two forms of the G protein that stimulates adenylate cyclase. *J Biol Chem* 261: 9587–9590, 1986.
45. Salomon Y, Londos C, and Rodbell M. A highly sensitive adenylyl cyclase assay. *Anal Biochem* 58: 541–548, 1974.
46. Sambrook J, Fritisch EF, and Maniatis T. Labeling the 5′ terminus of DNA with bacteriophage T4 polynucleotide kinase. In: *Molecular Cloning. A Laboratory Manual,* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989, pp. 10.59–10.61.
47. Taddei S, Virdis A, Ghiadoni L, Magagna A, and Salvetti A. Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. *Circulation* 97: 2222–2229, 1998.
48. Thibault C and Anand-Srivastava MB. Altered expression of G-protein mRNA in spontaneously hypertensive rats. *FEBS Lett* 313: 160–164, 1992.
49. Triner L, Vulliemoz Y, Verosky M, Habif DU, and Nahas GG. Adenylyl cyclase-phosphodiesterase system in arterial smooth muscle. *Life Sci* 11: 817–824, 1972.
50. Tsuda K, Tsuda S, Nishio I, Goldstein M, and Masuyama Y. Modulation of [^{3}H]dopamine release by neuropeptide Y in rat striatal slices. *Eur J Pharmacol* 321: 5–11, 1997.
51. Vanhoutte PM. Endothelium and control of vascular function. *Hypertension* 13: 658–667, 1989.
52. White CR, Brock TA, Chang LY, Crapo J, Brisco P, Ku D, Bradley WA, Gianturco SH, Gore J, and Freeman BA. Superoxide and peroxynitrite in atherosclerosis *Proc Natl Acad Sci U S A* 91: 1044–1048, 1994.
53. Yatani A, Mattera R, Codina J, Graf R, Okabe K, Padrell E, Iyengar R, Brown AM, and Birnbaumer L. The G protein-gated atrial K^+ channel is stimulated by three distinct Gi alpha-subunits. *Nature* 336: 680–682, 1988.
54. Zanchi A, Schad NC, Osterheld MC, Growzmann E, Nussberger J, Brunner HR, and Waeber B. Effect of NO synthase inhibition in rats on renin–angiotensin system and sympathetic nervous system. *Am J Physiol* 37: H2267–H2273, 1995.

Address reprint requests to:
Dr. Madhu B. Anand-Srivastava
Department of Physiology
Faculty of Medicine
University of Montreal
C.P. 6128, Succ. Centre-ville
Montreal, Quebec, Canada H3C 3J7

E-mail: anandsrm@physio.umontreal.ca

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