Isoprenaline Stimulates Gene Transcription of the Inhibitory G Protein α-Subunit G_{iα-2} in Rat Heart

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In vitro transcription reactions were performed with isolated ventricular nuclei of adult rats to investigate whether increased mRNA levels of the inhibitory G protein α-subunit G_{iα-2} after prolonged in vivo stimulation with the β-adrenoceptor agonist isoprenaline are caused by increased transcription. Rats were treated by a 4-day subcutaneous infusion of isoprenaline (2.4 mg/kg per day) or 0.9% NaCl as control. To avoid the influence of developmental expression patterns, adult rats were chosen for all experiments. Signals for G_{iα-2} and the stimulatory G protein α-subunit G_{α-} were specific and due to hybridization of nascent mRNA transcripts. In the isoprenaline group the transcriptional activity of G_{iα-2} gene increased to 140% of the control value, whereas gene specific hybridization for G_{α-} remained unchanged. These results show that increased G_{iα-2} mRNA levels after stimulation with isoprenaline are at least partially caused by enhanced transcription of G_{iα-2} mRNA. (*Circulation Research 1993;72:696–700*)

**KEY WORDS**  
• G protein α-subunit  
• in vitro transcription assay  
• isolated nuclei  
• rat heart  
• β-adrenergic stimulation

GTP-binding proteins (G proteins) play an important role in signal transduction in most organ systems and serve as coupling proteins between receptors and effectors such as adenylyl cyclase, enzymes of phosphatidylinositol metabolism, or ion channels.\(^1,2\) The heterogeneity of the α-subunits of heterotrimeric G proteins seems to be correlated with different functions of the G proteins; the functional significance of the heterogeneity in the βγ-subunits is not clear. In the heart, the adenylyl cyclase is coupled to stimulatory β\(_1\) and β\(_2\)-adrenoceptors by the stimulatory G protein (G\(_s\)) and coupled to inhibitory receptors such as the A\(_1\)-adenosine receptor or the M\(_2\)-cholinocceptor by the inhibitory G protein (G\(_i\)).\(^3\) Changes in G\(_s\) and/or G\(_i\) have been shown to correlate with alterations in adenylyl cyclase–dependent signal transduction both in vitro and in vivo.\(^4-7\) In failing human hearts of patients suffering from idiopathic dilated cardiomyopathy, the inhibitory G protein α-subunit (G_{α-}) and its mRNA are increased by 35–40% compared with nonfailing hearts, whereas the stimulatory G protein α-subunit (G_{α+}) remains unchanged.\(^8-13\) These findings led to the hypothesis that alterations of G proteins are involved in the pathogenesis of human heart failure with decreased responsiveness to β-adrenoceptor agonists and β-adrenoceptor–independent stimulators of the adenylyl cyclase. Since plasma noradrenaline levels are elevated in patients with end-stage heart failure,\(^14,15\) prolonged β-adrenergic stimulation might be responsible for the increased expression of G_{α-}. This assumption is supported by recent experiments demonstrating that a 4-day infusion of the β-adrenoceptor agonist isoprenaline in rats led to an increase in G_{α-} mRNA and pertussis toxin sensitive G proteins in the heart.\(^16,17\) It is not known whether the increased level of G_{α-} mRNA is due to enhanced transcription or prolonged stability of the mRNA under these conditions.

Therefore, in the present study, run-on transcription assays\(^18\) were performed with isolated ventricular nuclei of adult rats treated by a 4-day infusion of isoprenaline (2.4 mg/kg per day) or 0.9% NaCl as control to investigate the impact of prolonged in vivo β-adrenergic stimulation on transcriptional activity of the genes for G_{iα-2} and G_{α-}. Since experiments were done with ventricles of adult rats, results obtained with this method can be easily compared with previous studies on the G_{iα-2} and G_{α-} mRNA levels under the same experimental conditions without influence of developmental expression patterns.

**Materials and Methods**

*Animal Model*

Male Wistar rats (280 ± 6 g, n = 77) were treated by a 4-day subcutaneous infusion with 0.9% NaCl as control.
(Ctr, n=37) or with (±)-isoprenaline-HCl (Iso, n=40, 2.4 mg/kg per day, Boehringer Ingelheim, FRG) using osmotic minipumps (Alzet osmotic pumps type ML2, Alza Corporation, Palo Alto, Calif.). The minipumps were implanted in the neck under short ether anesthesia. The rats were fed standard laboratory diet and water ad libitum. Four days after implantation of the minipumps, rats were killed by a blow to the neck and decapitation. The hearts were rapidly removed, freed of atria and pericardium, and rinsed in 0.9% NaCl before being frozen in liquid nitrogen through the use of precooled Wollenberger clamps and stored at −80°C.

Preparation of Nuclei

All further steps were done on ice or at 4°C unless otherwise noted. Solutions and glass/plastic materials were sterilized by autoclaving or filtration to prevent ribonuclease activities. Ventricular nuclei were isolated with a modification of a procedure for preparation of nuclei from rat skeletal muscle described by Zahradka et al. Ventricles were homogenized with an Ultraturrax homogenizer (Janke & Kunkel, Staufen, FRG) for 10 seconds at maximal speed and with a Polytron homogenizer (PT 10-35, Kinematica, Lucerne, Switzerland) for 30 seconds at setting 5 in 25 ml lysis buffer (10 mM HEPES, pH 7.4; 5 mM KCl; 10 mM MgCl₂; and 5 mM 2-mercaptoethanol) containing 0.32 M sucrose and 0.1% Triton X-100. The homogenate was vacuum filtered through a nylon filter (200-μm mesh, Heidland, Gütersloh, FRG) and centrifuged at 2,000g for 10 minutes. Pellets were resuspended in 15 ml lysis buffer containing 2.2 M sucrose without detergent and centrifuged for 90 minutes at 100,000g (24,000 rpm) in a Kontron TST 28.38 rotor. Nuclear pellets were resuspended in 0.5 ml of the initial lysis buffer without Triton X-100. The nuclei of five ventricles were pooled and stored at −80°C. The DNA content was determined photometrically at 260 nm to estimate the yield of nuclei.

Nuclear Run-on Assay

Run-on transcription assays were performed by a modification of protocols of McKnight and Palmiter and McCully and Liew. The nuclei isolated from five ventricles (about 3,500 μg DNA) were incubated for 30 minutes at 30°C in a transcription reaction containing (final in 500 μl) 10% glycerol (vol/vol); 10 mM Tris (pH 8.0), 70 mM KCl; 2.5 mM MgCl₂; 0.5 mM MnCl₂; 7 mM 2-mercaptoethanol; 10 mM creatine phosphate; 0.1 mg/ml creatine kinase (Boehringer Mannheim, FRG); 160 units/ml ribonuclease inhibitor (Serva, New York); ATP, CTP, and GTP (1 mM each); and 500 μCi α-32P-UTP (3,000 Ci/mmol, Amersham-Buchler, Braunschweig, FRG). The reaction was stopped by centrifugation at 3,000g for 5 minutes. The nuclear pellet was treated with 1,500 Kunitz units DNase I, RNase-free (Sigma Chemical Co., St. Louis, Mo.), for 30 minutes at 37°C in the following buffer (final in 3.3 ml): 7% glycerol (vol/vol), 8 mM Tris (pH 8.0), 40 mM KCl, 1.7 mM MgCl₂, 0.3 mM MnCl₂, 5 mM 2-mercaptoethanol, 10 mM CaCl₂, and 360 mM NaCl. The reaction mixture was digested with 600 μg proteinase K (Boehringer Mannheim) in the presence of 0.1% sodium dodecyl sulfate (SDS) for 90 minutes. The 32P-labeled transcripts were purified by two phenol/chloroform extractions followed by one extraction with chloroform/isooamylic alcohol. The RNA was precipitated twice with 2.5 vol of 100% ethanol in the presence of 0.5 vol of 7.5 M ammonium acetate on dry ice for 30 minutes followed by centrifugation at 10,000g for 30 minutes at −10°C. The RNA pellet was washed with 70% ethanol and dissolved in 200 μl of 10 mM Tris and 1 mM EDTA (pH 7.8) after repeated centrifugation. Incorporation of 32P-UTP into total RNA was determined by precipitation of three aliquots of 3 μl with 10% trichloroacetic acid, vacuum filtration through GF-C glass fiber filter discs (Whatman, Maidstone, England), and scintillation counting.

Hybridization and Quantification of Signals

The plasmids (pGEM-2) with cDNA inserts for rat Gαs (1,750 bp), Gα13 (3,070 bp), and Gαi2 (1,740 bp) were kind gifts from Dr. R. R. Reed. The specificity of the G protein cDNAs and the absence of cross-hybridization under identical conditions of hybridization and washing have been demonstrated by Northern-blot techniques. The plasmids were immobilized onto nitrocellulose according to a modification of a procedure of Kafatos et al. DNA was denatured in 0.1N NaOH, heated to 100°C for 5 minutes, neutralized with 1 vol of 2 M ammonium acetate, and rapidly cooled on ice for additional 5 minutes. Five micrograms of DNA in 50 μl solution were dotted on 1-cm² nitrocellulose filter discs (Bio-Rad, Richmond, Calif.) and allowed to air dry before they were baked at 80°C for 1 hour. The filters were prehybridized for 4 hours at 42°C in a solution containing (final in 2 ml) 50% formamide, 5× Denhardt (Ficoll, polyvinylpyrrolidone, and BSA, 1 mg/ml each), 0.9 M NaCl, 60 mM Na₂HPO₄, 6 mM EDTA, and 0.5% SDS. 32P-labeled transcripts (3×10⁶ dpm) were hybridized with the prehybridized filters in 3 ml fresh hybridization solution (prehybridization solution containing 375 μg yeast tRNA). The reaction was overlaid with 1 ml light paraffin oil and incubated for 24–36 hours at 42°C. After hybridization, filters were washed at a final stringency of 0.2× SSC, 0.1% SDS at 60°C, including a 30-minutes digestion with 1 μg/ml RNase A and 10 units/ml RNase T₁ at 37°C to digest any single-stranded RNA not hybridized to DNA. The filters were treated with 500 μl of 40 mM NaOH to release the RNA and neutralized with 100 μl of 0.1 M acetic acid. The radioactivity was determined after the addition of 15 ml scintillation liquid (Picofluor-15, Packard) by scintillation counting in a full-spectrum dual-label mode. The Packard Tricarb 2000CA scintillation counter had a background of 40 cpm for 3H and 15 cpm for 32P. Because much of the data relies on samples having 100 cpm or less, the sample was counted for 60 minutes to reduce counting error. In each hybridization reaction the nonspecific background was determined by hybridization to pGEM-2 without insert and was 1±0.3 ppm in both groups. Since hybridization efficiencies may vary with each probe because of the competition of endogenous and nonlabeled mRNA that cannot be completely removed during preparation of nuclei, hybridization efficiencies were determined in each reaction by hybridization of known amounts of 3H-labeled cRNAs coding for Gαs and Gαi2 synthesized in vitro from the sequences inserted in pGEM-2 using an in vitro transcription kit (Boehringer Mannheim) and [5-3H]-
UTP (Amersham-Buchler). Hybridization efficiencies were on average 11% under the washing conditions used and did not differ in both groups. For calculation of gene-specific hybridization in parts per million, in each experiment the counts per minute hybridized to each probe were corrected for the nonspecific background and for 100% hybridization efficiency before they were divided by the amount of labeled RNA that was included in the respective hybridization reaction. All values were expressed as mean±SEM. Statistical significance was determined using Student's t test for unpaired values. A value of p<0.05 was considered significant.

Run-on assays with ventricular nuclei of nontreated rats were done either in the absence or presence of 1 μg/ml α-amanitin to control the specificity of the signals. At this concentration, α-amanitin is a selective inhibitor of RNA-polymerase II, the enzyme responsible for transcribing nascent mRNA.33

Results

Signal Specificity

Purified cardiac transcripts isolated from nuclear run-on assays performed in either the absence or presence of α-amanitin were hybridized against pGEM-2 and plasmids containing cDNA probes specific for G-protein α-subunits immobilized on nitrocellulose membranes (Figure 1). RNA transcribed in the absence of α-amanitin contained transcripts that hybridized to the probes specific for Gia-2, Gsa, and Gia. Signals for Gia and Gsa were easily detectable by autoradiography, whereas those for Gia were very weak. No positive signal to pGEM-2 could be detected under these conditions demonstrating the specificity of the signals for the G protein transcripts and the absence of nonspecific binding. In the presence of α-amanitin at a concentration that specifically inhibits RNA polymerase II, incorporation of 35P-UTP into nascent transcripts decreased by about 50% and abolished the positive signals for Gia and Gsa, respectively. The hybridized transcripts seen in the absence of α-amanitin, therefore, were caused by labeled mRNA and not tRNA or rRNA.

Effect of Treatment

The effects of isoprenaline infusions on physiological parameters have been characterized previously,10,11 and ISO delivery by osmotic minipumps can be adequately controlled. Compared with treatment with 0.9% NaCl (Ctr), a 4-day infusion of ISO (2.4 mg/kg per day) increased ventricular wet weight by 34% (Ctr: 928±25 mg, n=37; ISO: 1,243±31 mg, n=40; p<0.05) and ventricular-weight-to-body-weight ratio by 45% (Ctr: 3.1±0.04 mg/g, n=37; ISO: 4.5±0.07 mg/g, n=40; p<0.05). The body weight was not changed significantly after treatment in both groups. The yield of nuclei, as measured by the DNA content per heart, did not differ in the two groups (Ctr: 694±29 μg, seven preparations from 37 hearts; ISO: 667±34 μg, seven preparations from 40 hearts). No difference in 35P-UTP incorporation into nascent transcripts was observed (Ctr: 10.8±4 mio dpm, n=7; ISO: 8.6±2 mio dpm, n=7). The effect of treatment with ISO on transcriptional activity of Gia and Gsa is shown in Figure 2. The most striking result was that ISO increased the incorporation of 35P-UTP into nascent transcripts encoding Gia by about 40% compared with control (Ctr: 27±2 ppm, n=7; ISO: 38±4 ppm; n=7; p<0.05). These ppm values were calculated in each experiment by dividing the radioactivity bound to the specific probe by the radioactivity of the RNA included in the respective hybridization reaction after correction for 100% hybridization efficiency as described above; the amounts of labeled transcripts included in the hybridization reactions were on average 5.4±1.1 mio dpm (Ctr) and 5.3±1.1 mio dpm (ISO), and we measured on average 140±30 dpm for Gia in the Ctr group and 187±35 dpm in the ISO group. Values were corrected for the corresponding hybridization efficiencies, which were on average 11.8±1.6% for all experi-
The present study shows that the β-adrenoceptor agonist Iso increased transcriptional activity of the \(G_{\text{in}}\) gene by 40% in rat myocardium, whereas the activity of the \(G_{\alpha}\) gene was unchanged. Compared with members of the immediate early response gene family, e.g., fos and jun, which are rapidly induced and increase by several hundredfold above basal activity, the modulation of transcriptional activity of the \(G_{\text{in}}\) gene by prolonged β-adrenergic stimulation was very moderate. The \(G_{\text{in}}\) gene has distinct consensus “housekeeping” sequences\(^{24}\) and would not be expected to be highly “induced” or “repressed.” In fact, all studies on regulation of \(G_{\text{in}}\) protein \(\alpha\)-subunits consistently report increases of cellular \(G_{\text{in}}\) protein or mRNA levels of between 30% and 200% of control values,\(^ {8\text{--}9,11,13,16}\) which correspond well with the 40% increase in transcriptional activity reported here. This strongly suggests that transcriptional control plays an important role in the β-adrenoceptor mediated upregulation of \(G_{\text{in}}\) in the heart. Data concerning the upregulation of \(G_{\beta}\) adrenoceptors in response to short-term agonist exposure also demonstrate the importance of transcriptional activation in this process.\(^ {25}\) These data provide intriguing evidence that components of G protein–mediated signal transduction are under transcriptional control. The molecular events between β-adrenoceptor activation on one hand and transcriptional activation of the \(G_{\text{in}}\) gene on the other are not known in detail. There is, however, evidence that upregulation of \(G_{\text{in}}\) depends on the presence of intact cAMP-dependent protein kinase A\(^ {26}\) and that the \(G_{\alpha}\) gene contains possible consensus sequences of a cAMP response element.\(^ {24}\) The result of the present study strengthens the hypothesis that Iso infusion causes chronic activation of protein kinase A via stimulation of adenyl cyclase and enhanced transcription of the \(G_{\text{in}}\) gene via binding of a cAMP response element binding protein (CREB). This concept is supported by the lack of Iso-induced transcriptional activation of the \(G_{\alpha}\) gene which does not contain a cAMP response element.\(^ {27}\) However, there are theoretical concerns and data on discrepancies between \(G_{\alpha}\) mRNA and protein levels suggesting that this is not the only mechanism determining cellular levels of \(G_{\alpha}\) and that posttranslational modifications may be involved in this process.\(^ {7}\)

In the present study, run-on assays were performed with ventricular nuclei of adult rats. Although transcriptional activity of ventricular nuclei decreases with age,\(^ {20}\) adult rats were used for these experiments to avoid effects of developmental expression patterns; however, the present study demonstrates by hybridization to cDNAs coding for \(G_{\text{in}}\) and \(G_{\alpha}\) that transcriptional activity can be reproducibly determined as gene-specific hybridization in ventricular nuclei of adult rats. The total transcriptional activity as determined by total \(^{32}\text{P}-\text{UTP}\) incorporation in RNA was about 100 dpm per assay (about 0.24 dpm per nucleus) and was the same in both groups. This value is comparable with results obtained by McCully and Liew\(^ {28}\) on the general transcriptional activity of ventricular nuclei of adult rats as determined by total \(^{3}\text{H}-\text{UTP}\) incorporation. Our data are presented in parts per million, as previously described by McKnight and Palmiter\(^ {18}\) and Long et al.,\(^ {29}\) as opposed to counts per minute, because counts per minute values by themselves are dependent on a number of variables, including the amount of radioactivity incorporated into the total RNA added to each hybridization and the fact that the hybridization efficiency may vary with each probe. Nonspecific hybridization to the vector was excluded in each experiment. Nonspecific hybridization of tRNA and rRNA could be ruled out, because of the signal sensitivity to α-amanitin. To test the possible contribution from nonmyocardial nuclei in eight control experiments, transcripts were hybridized with a fibronectin cDNA. No specific signals for fibronectin transcripts were detectable. As previously shown with the same clone,\(^ {29}\) fibronectin is not expressed in cardiac isolated nuclei but is expressed in nonmyocardial nuclei and thus in a tissue specific manner. Furthermore, transcripts of both groups were hybridized with a 500-bp fragment in pUC18 vector containing exon II of the human β-myosin heavy chain gene (kind gift of Dr. H.P. Vosberg, Heidelberg, FRG). With this muscle-specific probe, we found specific signals that did not differ in groups (Ctr: 35 ± 12 ppm, \(n=3\)); Iso: 29 ± 10 ppm, \(n=3\)). This indicates that the majority of the hybridization signals may have come from myocyte-derived nuclei, although a contribution from other nuclei cannot

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**Figure 2.** Bar graphs showing the effect of a 4-day infusion of isoproterenol (Iso; 2.4 mg/kg per day) on transcriptional activity (ppm) of \(G_{\text{in}}\) and \(G_{\alpha}\) genes compared with control (Ctr; 4-day infusion of 0.9% NaCl). Purified transcripts synthesized in isolated rat ventricular nuclei were hybridized with cDNAs encoding \(G_{\text{in}}\) and \(G_{\alpha}\) immobilized onto nitrocellulose filter discs. Filters were washed under stringent conditions and radioactivity was determined by scintillation counting (see “Materials and Methods”).
be completely ruled out. In summary, the present study demonstrates that in vivo prolonged β-adrenergic stimulation leads to transcriptional activation of the \( G_{\alpha2} \) gene but not of the \( G_{\alpha} \) gene. The results support the hypothesis that increased levels of \( G_{\alpha} \) and \( G_{\alpha2} \) mRNA in human end-stage heart failure are at least in part caused by increased transcriptional activity secondary to elevated catecholamine levels. Further investigations with this method should give more insights into the regulation of \( G_{\alpha2} \) expression and the possible role of the cAMP system in this process.

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Circ Res. 1993;72:696-700
doi: 10.1161/01.RES.72.3.696
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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