Go protein subunit Go α and the secretory process of the natriuretic peptide hormones ANF and BNP

Cassandra Roeske¹, Amy Martinuk¹, Asna Choudhry¹, Geoffrey N Hendy³, Michael Gollob⁴, Qiuji Li⁴, Tina Georgalis¹ and Adolfo J de Bold^{1,2}

¹Cardiovascular Endocrinology Laboratory, University of Ottawa Heart Institute, 40 Ruskin Street, Ottawa, Ontario, Canada K1Y 4W7

²Department of Pathology and Laboratory Medicine, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada K1H 8M5

³Experimental Therapeutics and Metabolism, McGill University Health Centre-Research Institute, and Departments of Medicine, Physiology and Human Genetics, McGill University, Montreal, Quebec, Canada

⁴Toronto General Hospital, 200 Elizabeth Street, Toronto, Ontario, Canada M5G 2C4

Correspondence should be addressed to A J de Bold
Email
adebold@bell.net

Abstract

Expression of the G protein subunit Go α has been shown to be prominent in the atria of the rat heart and to be significantly associated with atrial natriuretic factor (ANF)-containing atrial-specific secretory granules by immunocytochemistry. In addition, differential expression profile analysis using oligonucleotide arrays has shown that the Go α isoform 1 (Go α 1) is 2.3-fold more abundant in the atria than it is in the ventricles. In the present report, we show protein–protein interaction between Go α and ANF by yeast two-hybrid and by immunoprecipitation. A cardiac conditional Go α knockout model developed for the present study showed a 90% decrease in Go α expression and decreased atrial expression and ANF and brain natriuretic peptides (BNP) content. Expression of chromogranin A, a specific atrial granule core constituent, was not affected. Morphometric assessment of atrial tissue showed a very significant decrease in atrial-specific granule density as well as granule core electron density. Atrial electrical activity was not affected. The results obtained are compatible with the suggestion that Go α plays a role in ANF sorting during intracellular vectorial transport and with the presence of a mechanism that preserves the molar relationship between cellular ANF and BNP stores in the face of the decreased production of these hormones.

Key Words

- ▶ endocrine heart
- ▶ ANF
- ▶ G proteins
- ▶ hormone storage
- ▶ hormone secretion

Journal of Molecular Endocrinology
(2015) **54**, 277–288

Introduction

At least three types of secretory mechanisms for the natriuretic peptides (NP) atrial natriuretic factor (ANF, ANP) and the brain natriuretic peptides (BNP) operate in atrial cardiomyocytes, and they include constitutive, regulated, and constitutive-like secretions (Ogawa *et al.* 1999). The stimulated secretion of NP involves G protein-associated mechanisms. Neuroendocrine agonists, such as endothelin-1 (ET-1), angiotensin II, and catecholamines, are

known to utilize receptors coupled to effectors by Gq proteins. On the other hand, ANF secretion promoted through atrial muscle stretch (stretch-secretion coupling) (Kuroski de Bold & de Bold 1991) utilizes a mechanism that is inhibited by pertussis toxin (PTX), a specific inhibitor of the Gi/o family of G proteins (Bensimon *et al.* 2004, McGrath & de Bold 2005). The stimulation of ANF secretion by ET-1 has been shown to proceed unimpaired in the atria from

PTX-treated rats, which shows that the Gq/11 and Gi/o pathways of signaling during stimulated ANF secretion are functionally independent and may function in parallel (Bensimon *et al.* 2004). This dual stimulatory pathway would be expected to be operational in hemodynamic overload conditions, such as chronic congestive heart failure, in which neuroendocrine activation coexists with increased intracardiac pressures that lead to increased atrial muscle stretch. Both agonist-mediated and stretch-mediated secretion of NP from atrial cardiomyocytes cause the release of newly transported peptides in preference over the older NP pool, as has been revealed by double-label pulse-chase studies (Mangat & de Bold 1993, Ogawa *et al.* 1999).

There is scant information regarding the details of G protein participation in agonist-promoted NP secretion. Expression of the G protein subunit Go α is prominent in the atria and is significantly associated with ANF-containing secretory granules, as has been shown by immunocytochemistry, and it is virtually absent in ventricular cardiomyocytes (Wolf *et al.* 1998, Bensimon *et al.* 2004). Differential expression profile analysis between rat atria and ventricles using oligonucleotide arrays revealed that the G protein Go α isoform 1 (Go α 1) is 2.3-fold more abundantly expressed in the atria than in the ventricles (McGrath & de Bold 2009).

In the present report, we further explore the association between Go α and atrial secretory function by assessing Go α protein–protein interactions in atrial muscle using yeast two-hybrid screening and the biochemistry and morphology of atrial cardiomyocyte phenotype in transgenic mice carrying a conditional cardiac Go α knockout. In the latter, we studied the expression of secretory granule components, including ANF, BNP, and chromogranin A (ChgA), as well as ANF processing and the effect of the knockout on ultra-structure and on cardiac electrophysiological activity.

Materials and methods

Experimental animals

All of the experiments involving animals were approved by the Animal Care Committee of the University of Ottawa and were carried out according to the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals.

Conditional Go α knockout

Go α -floxed mice with loxP sites flanking exons 5 and 6, which are common to both isoforms Go α_A and Go α_B (also

Go α_1 and Go α_2) were generously provided by Dr Lutz Birnbaumer. NIEHS mice, strain 129SvEv, were used. The process for disrupting of this gene has been previously described (Chamero *et al.* 2011). α -myosin-heavy chain (α -MHC)-Cre mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA), strain name B6.FVB-Tg(Myh6-cre)2182Mds/J, strain no. 011038. Expression of Cre, which is driven by the α -MHC (cardiac-specific) promoter, induces more than 90% recombination in cardiac muscle cells when two loxP sites are present.

Male mice that were obtained after a standard breeding scheme for F2 generation were used. Controls were C57BL/6 x129SvEv males.

Cre/lox generation

Standard Cre/lox tissue-specific breeding protocol was followed. Briefly, breeding a homozygous Go α -floxed mouse to a hemizygous α -MHC-Cre mouse produces 50% offspring that are heterozygous for the floxed allele that has been recombined in the presence of Cre to delete this allele in the heart (cGo α +/-). Breeding a homozygous Go α -floxed mouse to a hemizygous α -MHC-Cre produces 25% offspring that are homozygous for the floxed alleles that have both been recombined in the presence of Cre to delete both alleles in the heart (cGo α -/-).

All of the genotypes were produced in Mendelian frequencies. Table 1 shows all of the genotypes obtained from the Cre/lox mating scheme and their respective transgenic titles and genotypes. The homozygous floxed mouse Go α flx/flx was used as the control mouse (Zhao *et al.* 2010).

Genotyping and primers

At 21 days of age, a 0.1 cm tail clipping was taken from each mouse. DNA extraction was performed using a REDExtract-N-Amp Tissue PCR Kit (XNAT, Sigma–Aldrich), and subsequent PCR amplification was performed using a REDExtract-N-Amp PCR Mix (XNAT, Sigma–Aldrich) in an Eppendorf Mastercycler Thermal Cycler (Eppendorf, Mississauga, Ontario, Canada). PCR parameters for genotyping the Go α -floxed alleles, the Cre alleles, and control alleles are listed in Tables 2 and 3. Oligonucleotide sequences for genotyping are listed in Table 2. Amplicons were run on a 1% agarose gel using a 1 kb ladder (G571A, Promega) for calibration purposes, except for Cre products, which were run on a 1.5% agarose gel using a 50 bp ladder to calibrate the migrations of the

Table 1 Genotypes obtained from F1 and F2 Cre/lox generation breeding scheme

Go α genotype ^a	Transgenic title	Full genotype
C (conditional) Go α -/-	Homozygous conditional knockout	α MHC ^{Cre} ; Go α ^{Flx/Flx}
cGo α +/-	Heterozygous conditional knockout	α MHC ^{Cre} ; Go α ^{Flx/Wt}
cGo α +/+	Homozygous floxed control	Wt; Go α ^{Flx/Flx}
	Heterozygous floxed	Wt; Go α ^{Flx/Wt}
	Hemizygous Cre	α MHC ^{Cre} ; Go α ^{Wt/Wt}
	WT	Wt; Go α ^{Wt/Wt}

^aOnly the first and third genotypes were compared in the present report.

amplicons (10488, Invitrogen). Primers were commercially synthesized by Operon (www.operon.com/).

Chromogranin A knockout mice

The generation of the Chga knockout mice has been previously reported (Hendy *et al.* 2006). Controls were Balb/c. Only male knockout and control mice were used.

Yeast two-hybrid

The methodology followed for yeast two-hybrid screening has been previously published (Ogawa *et al.* 2009). Briefly, poly (A)+ RNA was isolated from rat atria using the Absolutely mRNA Purification Kit (Stratagene, Agilent Technologies, La Jolla, CA, USA). An atrial tissue library was constructed using a HybriZAP 2.1 XR Library Construction Kit and a HybriZAP 2.1 XR cDNA Synthesis Kit (Stratagene). cDNA inserts were cloned into the HybriZAP 2.1 vector, and the HybriZAP 2.1 library was converted to the pAD-GAL-4-2.1 library (target protein) by mass excision, as described in the manufacturer's instructions for the HybriZAP 2.1 XR Library Construction Kit and the HybriZAP 2.1 XR cDNA Synthesis Kit. DNA encoding amino acids 1–354 of the activated Q205L mutant of rat Go α 1 (kindly provided by Brad Denker, Harvard Medical School, Cambridge, MA, USA) was cloned into the pBD-GAL4 Cam phagemid 'bait' vector (Stratagene) and cotransformed with $\sim 20 \times 10^6$ atrial cDNA

clones contained within the pAD-GAL-4-2.1 'prey' vector using yeast strain YRG-2 (Stratagene). Plasmid recovered from 92 positive clones that grew on triple dropout (-Trp/-Leu/-His) plates were sequenced. The yeast transformation was performed by the S. C. Easy Comp Transformation Kit (Invitrogen) according to the manufacturer's instructions.

Plasma collection

Trunk blood was collected in 1.5 ml tubes that had previously been rinsed with 15% EDTA and centrifuged at 2000 g for 20 min at 4 °C, and the plasma was stored at -80 °C. The plasma samples were acidified with 100 μ l 0.1 M HCl/ml plasma and were passed three times through Sep-Pak C18 cartridges (Millipore-Waters Corp., Milford, MA, USA) pre-wetted with 5 ml 80% acetonitrile (ACN) in 0.1% trifluoroacetic acid (TFA), followed by 20 ml of 0.1% TFA. After processing, the cartridges that had received the plasmas were then eluted with 3 ml 80% ACN in 0.1% TFA. Samples were then freeze-dried and reconstituted in 220 μ l of RIA buffer (0.1 M sodium phosphate, 0.05 M NaCl, 0.01% sodium azide, 0.1% Triton X-100, and 0.1% heat-treated BSA).

RNA extraction and RT

Atrial appendages and the apical portions of the ventricles were immersed in 1 and 3 ml respectively of TRIzol Reagent (Invitrogen) immediately after removal and were stored at

Table 2 Primers used for genotyping

Name	Forward primer (5'-3')	Reverse primer (5'-3')
Go α -floxed	AAGAATAGAACCTAGGACTGGAGG	GCAGACAAGTGAACAAGTGAAACCC
α -MHC-Cre	ATGACAGACAGATCCCTCTATCTCC	CTCATCACTCGTTGCATCATCGAC
TCR- α control	CAAATGTTGCTTGCTGGTG	GTCAGTCGAGTGCACAGTTT
ANF WT	CTGTCCAACACAGATCTGATG	CTGTTGCAGCCTAGTCCACT
ANF mutant	CTGTCCAACACAGATCTGATG	CCTTCTATCGCTTCTTGACG
ChgA WT	GATGAAGTGCCTGGAAGTCATCTCCGA	CTTGGAGAGCCAGGTCTTGAAGTTCCTCA
ChgA mutant	ACAACAGACAATCGGCTGCTC	CCATGGGTACGACGAGATC

Table 3 Oligonucleotide primer sequences for quantitative real-time PCR

Name	Forward primer (5'-3')	Reverse primer (5'-3')
BNP	CAGCTCTTGAAGGACCAAGG	AGACCCAGGCAGAGTCAGAA
Go α	TGCACGAGTCTCTCATGCTCT	AGATGGTCAAGGGTGACTTCT
ANF	GCCGGTAGAAGATGAGGTCA	GGGCTCCAATCCTGTCAATC
G6PD	CCAGCCTCCTACAAGCACCTCA	AATAGCCCCACGACCCTCAGTA

–80 °C. Further processing was done by homogenizing using a Polytron (PT10-35, Kinematica, Inc., Bohemia, NY, USA) at 70% power for 15–20 s and completing total RNA extraction according to the manufacturer's instructions. The quality of the RNA extracted was assessed using a 2100 Bioanalyzer (Agilent Technologies) with an RNA 6000 Nano Kit (Agilent Technologies). cDNA was synthesized by a Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science) following the manufacturer's instructions using 1 μ g of total RNA.

Quantitative real-time PCR

Quantitative real-time RT-PCR was performed using Light-Cycler 480 SYBR Green I Master Mix and analyzed using the LightCycler 480 SW 1.5 quantification software (Roche Applied Science) according to the manufacturer's instructions.

Primer nucleotide sequences were as published (Niu *et al.* 2005) or obtained from the literature and validated using Primer3 Input version 0.4.0 (<http://frodo.wi.mit.edu>). Primer nucleotide sequences are listed in Table 3. Glucose-6-phosphate dehydrogenase (G6PD) was used as the reference gene, and concentration ratios were normalized to the calibrator and corrected using primer efficiency. Analyses of each gene of interest were performed in triplicate. A standard curve was constructed in triplicate for each primer pair.

Western blot

Freshly obtained tissues were placed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0), 1% Nonidet P-40, and 0.5% sodium deoxycholate) and 1:100 dilution of protease inhibitor cocktail (P8340, Sigma) and stored at –80 °C. The tissues were then homogenized using a Polytron at 70% power for 15–20 s, and the homogenate was centrifuged at 9300 *g* for 10 min at 4 °C. Protein quantification in the supernatant was determined using a Pierce BCA Protein Assay Kit (Pierce, Thermo Scientific, Ottawa, Ontario, Canada).

For electrophoretic separation, the samples were prepared by adding 7 μ g of atrial protein to 3X Blue loading dye (7722, Cell Signaling Technology, Danvers, MA, USA) and 10% dithiothreitol (DTT) and boiling for 2 min at 95 °C. A 30 μ l sample was loaded into a 10% Mini-PROTEAN TGX precast gel (Bio-Rad Laboratories, Inc.) and run at 200 V for 45 min, along with a biotinylated ladder (Precision-Plus Protein Western C, Bio-Rad Laboratories, Inc.) and mouse brain extract (sc-2253, Santa Cruz Biotechnology, Inc.). The gel was transferred to a 0.45 μ m PVDF membrane (Millipore-Waters Corp.) at 100 V for 1 h and blocked for 1 h in a buffer containing 5% fat-free milk in Tris-buffered saline, 0.1% Tween-20 (TBST). The membranes were then rinsed three times with TBST and incubated overnight at 4 °C with antibodies diluted in blocking buffer (Go α 1:2000 (polyclonal anti-Go α , 551, MBL Int., Woburn, MA, USA), α -tubulin 1:400 (sc-8035, Santa Cruz Biotechnology, Inc.)). This Go α antibody cross-reacts with rat and human Go α , but it does not bind Go α_2 or Go α_3 . Membranes were rinsed three times with TBST and incubated for 1 h at room temperature with secondary antibodies conjugated to HRP diluted in blocking buffer (goat anti-rabbit 1:5000 (sc-2004, Santa

Table 4 Identification of molecules associated with Go α 1 (Q205L) by yeast two-hybrid

Name	Accession number	% identity
Intermediate conductance calcium-activated potassium channel	AF190458	95
Natriuretic peptide precursor type A (eight clones)	NM_012612	99
Ankyrin repeat domain 1	BC072699	98
Myosin, heavy polypeptide 6, cardiac muscle	NM_017239	94
Ankyrin repeat domain 1 (cardiac muscle)	NM_013220	95
Actin-binding Rho-activating protein	NM_175844	97
Clusterin	BC061534	99
Ubiquitin A-52 residue ribosomal protein fusion	NM_031687	99

Figure 1

Western blot analysis following electrophoresis of immunoprecipitates from atrial or ventricular homogenates using anti-ANF antibodies.

Cruz Biotechnology, Inc.), goat anti-mouse 1:5000 (1858413, Pierce, Thermo Scientific), and Precision Protein StrepTactin 1:5000 (161-0380, Bio-Rad Laboratories, Inc.)). Membranes were rinsed three times with TBST and were visualized using the FluroChem Alpha Ease ECL imaging system (Alpha Innotech Corp., San Leandro, CA, USA) following enhancement with Luminata Forte chemiluminescence (Millipore-Waters Corp.).

Electron microscopy

Tissues were finely minced in saline and fixed in Karnovsky's fixative (4% paraformaldehyde, 5% glutaraldehyde in 0.2 M phosphate buffer (pH 7.4)) overnight; they were then washed three times in washing solution (0.1 M Sorensen's phosphate buffer (pH 7.4), 10% sucrose, 1% calcium chloride) and post-fixed in 2% osmium tetroxide in phosphate buffer. The tissues were then

dehydrated in a graded series of alcohols and embedded in Spurr resin. Tissue sections mounted in copper grids were stained with lead citrate and uranyl acetate and then viewed in a JEM-1230 electron microscope. Granule counting was carried out in randomly chosen fields at 3000 $\times$ magnification.

NP extraction

Tissues were extracted in 3 ml of a solution containing 0.1 M HCl, 1.0 M acetic acid, and 1% NaCl using a Polytron at 70% power for 15–20 s. The homogenates were centrifuged at 10 000 *g* for 30 min at 4 °C, and the supernatants were processed using Sep-Pak C18 (Millipore-Waters Corp.) cartridges as described in the previous sections, separated into two aliquots, and freeze-dried. The freeze-dried samples were solubilized in either 0.1% TFA or RIA buffer for HPLC and RIA respectively.

Reverse-phase HPLC

Reverse-phase HPLC (RP-HPLC) analysis was carried out in a C₁₈ column using a linear gradient elution profile from 15 to 85% of 80% ACN in 0.1% TFA at 1.5 ml/min with monitoring at 275 nm over a total period of 80 min. Fractions were collected every 2 min. One hundred microliters of an aqueous solution of 1 mg/ml BSA was added to each fraction before it was freeze-dried.

RIA

RIAs for ANF and BNP were performed as previously described (Sarda *et al.* 1989, Bensimon *et al.* 2004).

Immunoprecipitation of Go α /ANF complexes

Immunoprecipitation of Go α /ANF complexes was carried out in atrial or ventricular homogenates that were obtained by homogenizing ~50 mg of tissue in 0.01 M phosphate buffer (pH 6.5) containing 1 μ M GDP,

Table 5 ECG parameters in mice. Values are averages of the same time point (1600–1900 h or 0400–0700 h) over 3 days. *n* = 3

Phase/time	Genotype	RR-I (ms)	HR (bpm)	R-amp (mV)	P-amp (mV)	QRS-dur (ms)	PR-I (ms)
Rest 1600–1900 h	Control	107.4864	569.1028	0.725167	0.087531	15.43949	34.4616
Rest 1600–1900 h	cGo α –/–	106.8187	572.3695	0.852373	0.076315	14.30988	33.22616
Awake 0400–0700 h	Control	107.071	576.5367	0.735165	0.083329	15.08417	33.66375
Awake 0400–0700 h	cGo α –/–	106.3489	576.0036	0.84694	0.079072	14.34907	33.42748

RR-I, interval between R waves; HR, heart rate; R-amp, amplitude of the R wave; P-amp, amplitude of the P wave; QRS-dur, duration of the QRS complex; PR-I, interval between the P wave and the R wave.

Figure 2

(A) mRNA expression levels of Go α in control and cGo α -/- genotypes through RT-PCR. Values are means $\pm$ s.e.m.; $n=6$; *** $P<0.001$. (B) Protein expression of Go α in atrial tissue. Western blot analysis using a specific Go α polyclonal antibody in control and cGo α -/- mice. Representative band of $n=4$.

30 μ M AlF_4^- (30 μ M AlCl_3 , 1.2 mM NaF), 6 mM MgCl_2 , and 1% NaCl. Following centrifugation, the pellet was re-homogenized, and the supernatants were combined. After incubation overnight with 1 μ l of undiluted ANF antibody, 20 μ l of Protein A/G Plus Agarose (Santa Cruz Biotechnology, Inc.) were added and mixed by inversion for 1 h at 4 $^\circ\text{C}$. Following centrifugation at 400 g for 30–60 s, the resulting pellet was washed twice with 0.01 M phosphate buffer containing 1 μ M GDP, 30 μ M AlF_4^- , 6 mM MgCl_2 , and 1% NaCl. Electrophoretic separation of samples and western blotting was carried out as described above. Go α (polyclonal anti-Go α , 551, MBL Int.) and ANF RyB8 antibody (an ANF-specific polyclonal antibody produced in our laboratory) were used at a concentration of 1:1333 and 1:2000 respectively. This Go α antibody cross-reacts with rat and human Go α , but it does not bind Gi2 α and Gi3 α .

Electrocardiography

ECG transmitters (ETA-F20, Data Science International, San Paul, MN, USA) were implanted in mice pretreated with s.c. buprenorphine (0.05 mg/kg) and anesthetized with isoflurane (1.5–2%). A 2–3 cm midline abdominal incision was made, and the telemetry unit was placed within the abdominal cavity. Telemetry data gathering began on post-surgery day 3 using the software supplied with the telemetry unit.

Statistical analysis

Data are reported as means $\pm$ s.e.m. Student's t -tests were performed to determine statistical significance between groups. $P\leq 0.05$ was considered significant.

Results

Yeast two-hybrid and co-immunoprecipitation

To determine whether the previously observed microscopic co-localization of Go α and ANF could reflect protein–protein interaction at the cellular level, two-hybrid and co-immunoprecipitation studies were conducted. Screening of a rat atrial cDNA library with a mutationally active form of Go α 1 (Q205L) identified several cDNA clones of interest, including eight clones that contained open reading frames corresponding to proANF. No clones containing BNP sequences were found, and only single clones were detected for the other species listed in Table 4.

Immunoprecipitation of atrial tissue homogenates using anti-ANF antibodies produced complexes that upon electrophoretic and western blot analysis yielded bands consistent with protein–protein interaction between Go α and ANF. Immunoprecipitates isolated from ventricular tissue homogenates did not reveal bands for either ANF or Go α (Fig. 1).

These results support the view that activated forms of Go α and ANF interact to form a complex *in vivo* (Table 5).

Cardiac Go α knockout

Given the association between Go α and ANF that was found by yeast two-hybrid and co-immunoprecipitation,

Figure 3

ANF relative expression levels in atrial tissue. Expression levels of ANF in control and cGo α -/- genotypes. Values are means $\pm$ s.e.m.; $n=6$; * $P<0.05$.

Figure 4

BNP relative expression levels in atrial tissue. Expression levels of BNP in control and cGo α -/- genotypes. Values are means $\pm$ S.E.M.; $n=6$; ** $P<0.01$.

we examined the role of Go α in atrial cardiomyocyte phenotype using a conditional Go α -floxed mouse in which two loxP sites exist in both alleles flanking the fifth and sixth exons common to the two variants (Go α 1 and Go α 2). By expressing Cre, the loxP sites recombine to delete the fifth and sixth exons of Go α , which thereby produces a truncated mRNA sequence and deletes the C-terminal functional amino acids (Jiang *et al.* 1997, Chamero *et al.* 2011). The N-terminus of Go α lacks the functional binding domains to its signaling pathways with adenylyl-cyclase and phospholipase C as well as its binding to the G $\beta\gamma$ complex (Birnbaumer 2007, Zhao *et al.* 2010).

Through the standard breeding of Go α -floxed mice and α -MHC-Cre mice, heart-specific conditional Go α knockout mice were derived after two generations of mating. Continuous breeding of α -MHC-Cre mice to Go α -floxed mice produced six genotypes of mice (Table 1). No Cre toxicity was observed in these mice (Baba *et al.* 2005), because no two animals expressing α -MHC-Cre were mated.

Genotyping of tail, ventricular, or atrial tissue using Go α -floxed primers to distinguish between the WT, heterozygous, and homozygous Go α -floxed and Go α -deleted genotypes showed a 2142 bp PCR product that represented the Go α allele and contained two loxP sites in the Go α gene (Go α -floxed alleles), an 1868 bp PCR product that represented the Go α WT allele, and a 442 bp PCR product that represented the Go α allele after recombination by Cre recombinase, which had thereby produced a deleted allele and truncated band.

Genotyping of tail, ventricular, and atrial tissue using α -MHC-Cre primers to distinguish between the mice

carrying the Cre enzyme and those without the Cre enzyme showed a 300 bp PCR product that represented the presence of Cre in the genome (one Cre allele) and a 200 bp PCR product that represented the internal control in a sample without the presence of Cre.

Go α , NP, and ChgA mRNA and protein expression

In the atria of cGo α -/- mice, Go α mRNA was reduced by 90% ($P<0.001$) as compared to control mice (Fig. 2A). In line with these results, Go α protein expression was much less abundant in the atria of cGo α -/- mice as compared to control mice (Fig. 2B). ANF was knocked down by 23% in the atria of cGo α -/- mice as compared to controls ($P<0.05$; Fig. 3). BNP had a more significant reduction in expression (63%) than ANF did ($P<0.01$; Fig. 4).

ChgA knockout mice did not show any changes in either ANF or BNP transcript levels in the atria (data not shown).

The quantitation of peptide content by RIA in atrial tissue extracts of control and cGo α -/- mice showed that the atrial ANF content in cGo α -/- mice (16 489 pg/mg of tissue) was not statistically different from that in control mice (18 546 pg/mg of tissue) (Fig. 5). Tissue BNP content was significantly lower in the atria of cGo α -/- mice. Control mice had an average BNP concentration of 118 pg/mg of tissue, whereas the cGo α -/- mice had an average BNP concentration of 84 pg/mg of tissue ($P<0.05$) (Fig. 6). ChgA knockout mice did not show any changes in either ANF or BNP peptide levels in the atria or plasma (data not shown).

Figure 5

Concentration of ANF peptide in atrial tissue. Values are means $\pm$ S.E.M.; $n=6$.

Figure 6

Concentration of BNP peptide in atrial tissue. Values are means $\pm$ S.E.M.; $n=6$; * $P<0.05$.

HPLC analysis of ANF tissue stores

Molecular forms of intracellular ANF in atrial tissue extracts were examined by RP-HPLC to determine the ratio content of low molecular weight processed ANF (ANF₉₉₋₁₂₆) and high molecular weight proANF (ANF₁₋₁₂₆). RP-HPLC profiles showed the presence of both forms of ANF in each genotype, with similar ratios of proANF to processed ANF (Fig. 7A and B).

ANF peptide content in plasma

Plasma ANF concentration did not differ between cGo α ^{-/-} mice and control mice (Fig. 8). BNP plasma level concentration was below assay sensitivity for normal and transgenic animals.

Microscopy

Figure 9A and B shows medium-power images of atrial cardiomyocytes by conventional transmission electron microscopy. The general morphological features of both transgenic and control atrial cardiomyocytes did not show obvious morphological differences except for an apparent loss of electron density in the atrial granule's core in transgenic animals (Fig. 9B). However, even by simple inspection, it was obvious that cardiomyocytes of the Go α ^{-/-} mice contained fewer granules than the controls did. Quantification of the number of granules per square micron of tissue section from Go α conditional knockouts showed a near halving in the number of granules as compared to controls (Fig. 10).

Electrocardiography

In order to determine differences in the electrical conductance of cGo α ^{-/-} mice, telemetry recordings were obtained via an implantable telemetry unit. A 3-day continuous recording period was carried out in three mice per genotype during daytime hours (at rest) and during nighttime hours (while awake). The parameters analyzed in these mice included RR intervals, heart rate, R-wave amplitude, P-wave amplitude, QRS duration, P-wave duration, and PR interval. Both genotypes had similar recordings, and none of the aforementioned parameters was significantly different in the cGo α ^{-/-} mice as compared to the control mice (Table 3). An ECG tracing that represents four cardiac cycles is shown in Fig. 11.

Discussion

The present study was designed to determine the role of the Go protein α subunit Go α in determining atrial cardiomyocyte phenotype, given previous indications that it is involved with atrial secretory granules and with ANF secretion. Generally, heterotrimeric G proteins are associated with secretory granule biogenesis in other

Figure 7

RP-HPLC profiles of atrial tissue ANF in (A) control mice and (B) cGo α ^{-/-}.

Figure 8

ANF concentration in plasma of control or cGo α -/- genotypes. Values are means $\pm$ S.E.M.; $n=4$ for control; $n=5$ for cGo α -/-.

endocrine organs (Barr *et al.* 1991, Burgoyne & Morgan 1993, Gasman *et al.* 1997). The Go α subunit has been implicated in various parts of this pathway (Zhao *et al.* 2010). Far greater amounts of Go α are found within the atria of the heart as compared to the ventricles (Eschenhagen *et al.* 1995). In addition, G proteins such as Go, are stretch-sensitive (Van Wagoner 1993), and they are involved in several secretory processes, including ANF secretion, together with K⁺ channels (Ogawa *et al.* 2009). Both light and electron microscopy immunocytochemistry have shown that Go α co-localizes in atrial granules with ANF with a preferential distribution in the granule membrane (Wolf *et al.* 1998, Bensimon *et al.* 2004, Muth *et al.* 2004). The bulk of ventricular cardiomyocytes do not display Go α immunoreactivity.

An additional indication of Gi/o involvement in ANF secretion is the fact that PTX can completely abolish stretch-secretion coupling (Kuroski de Bold & de Bold 1991, Bensimon *et al.* 2004). Finally, a comparison of gene expression profiles using oligonucleotide arrays has shown a uniquely high expression of Go α 1 in the atria (McGrath & de Bold 2009). The results in the present study, which were obtained by yeast two-hybrid and immunoprecipitation techniques further suggest an association between ANF and Go α .

ANF exists in the form of proANF within atrial secretory granules. The latter appears to play a master regulatory role in the formation of secretory granules, as is suggested by the fact that ANF knockout mice lack secretory granules (John *et al.* 1995, Dikeakos & Reudelhuber 2007). Given the association of ANF with Go α , it is reasonable to postulate a sorting role for membrane-bound Go α during granule maturation, a model that was proposed by Arvan & Halban (2004). This model, which

was denominated 'sorting by retention', suggests that proteins within secretory vesicles (e.g., proANF) that arise from the TGN and are destined to be secreted by a regulatory mechanism are retained in secretory granules (e.g., by interaction with Go α), whereas other proteins are sorted into other compartments, such as lysosomes or endosomes.

In all, these previous studies do not provide a clear view of how Go α participates in the biogenesis of

Figure 9

Electron microscopy images of atrial cardiomyocytes. Atrial muscle cell in control mice (A) and cGo α -/- mice (B). SG, secretory granules; G, Golgi complex; N, nucleus; M, mitochondria; My, myofibrils. Magnification 5000 $\times$.

Figure 10

Specific atrial granule density in atrial cardiomyocytes. *n* per group = 3 animals/genotype $\times$ 2 blocks/animal $\times$ 1 section/block $\times$ 95 or 98 fields/section for control or cGo α $-/-$ mice respectively. Values are means $\pm$ S.E.M.; ****P* < 0.001.

ANF-containing granules. Therefore, we postulated that the suppression of Go α expression would contribute information in this respect. A complication in taking this approach is that the homologous recombinant knockout for the *Gnao1* gene induces severe neurological complications and very early lethality (Jiang *et al.* 1997). For this reason, we generated a conditional, heart-specific knockout of Go α in mice according to published breeding protocols for Cre/lox mice (Niu *et al.* 2005). In the present study, heart-specific Go α knockout mice were produced in Mendelian frequencies without any severe motor or neurological symptoms nor any gross morphological features in the heart associated with this deletion. These mice appeared normal and lived into adulthood.

The yeast two-hybrid experiments and the immunoprecipitation studies carried out as part of the present investigation clearly show an association of Go α with ANF in a fashion that suggests that Go α plays a role in proANF sorting into storage vesicles, which is in accordance with the sorting by retention model proposed by Arvan & Halban (2004).

No protein-protein interaction was detected between BNP and Go α , but atrial stores of BNP nonetheless decreased. The finding that BNP decreased more significantly than ANF did may reflect the fact that the determination of ANF introduces a larger variance than the determination of BNP does because of the large dilutions required to perform the RIA. Nevertheless, the finding suggests that a mechanism exists to keep the molecular ratios of these two hormones constant within the storage granules. A second suggestion brought about by the present observations is that the association between

proANF and Go α protects the former molecule but not BNP from proteolytic processing, which thus explains why the storage forms of NP in the atria consist of proANF and processed BNP. In addition, the fact that Chga expression was not affected in the knockouts suggests that Go α function is restricted to the NP.

We have previously reported on the relationship between ANF secretion, K⁺ channels, and Go α based on evidence obtained through oligonucleotide array analysis and yeast two-hybrid analysis (McGrath & de Bold 2009, Ogawa *et al.* 2009). Pharmacological interventions with drugs known to pharmacologically affect the function of specific K⁺ channels, including adenosine triphosphate-sensitive K⁺ channels, TWIK-related K⁺ channel-1 (TREK-1), and the Ca²⁺-activated intermediate conductance K⁺ channel (SK4) modified ANF secretion to various extents. SK4 was expressed specifically in the atria, and the yeast two-hybrid analysis specifically demonstrated an interaction of this channel with Go α . However, in the present investigation, we found no changes in cardiac electrophysiological activity in cGo α $-/-$ animals.

The most notable phenotypic change that was observed in atrial cardiomyocytes of Go α $-/-$ animals was a loss of electron density of the granule's core and

Figure 11

ECG tracings of four cardiac cycles in mice. Four cardiac cycles are shown in control mice (A) and cGo α $-/-$ mice (B). P, P wave; Q, Q wave; R, R wave; S, S wave. *n* = 3.

a significant reduction in the number of granules. The conditional Go α deletion led to a 90% loss of Go α mRNA, so these mice had an average 10% genetic expression of Go α , which was reflected by the small but significant amount of Go α protein evident in the western blots. This remaining protein likely represents cells in which Cre-induced deletion may not have been completed on both chromosomes.

Whether a total deletion would have led to a complete disruption of the formation of granules cannot be determined from the present investigations. Together with the changes in phenotype revealed by electron microscopy, it was evident that both ANF and BNP atrial content decreased (albeit not significantly for ANF) in the transgenic animals. That stored ANF content would diminish alongside a decrease in its interacting partner, Go α , was not surprising, but the significant decrease in BNP was not expected, and it hints at a mechanism that preserves a predefined molar relationship of stored NP in both normal and transgenic hearts.

The present investigation was designed to determine cardiomyocyte secretory phenotype changes rather than changes in the contractile phenotype, cardiac function, or other parameters in the animal as a whole. Therefore, we did not attempt to determine if the knockout had whole-animal consequences (e.g., effects on blood pressure, cardiac remodeling, etc.). This point should be addressed in future investigations.

Go α regulates insulin granule dynamics by acting as a repressor and inhibitor of vesicular docking in pancreatic β cells during normal physiological functions (Zhao *et al.* 2010). In pancreatic-specific conditional Go α knockout mice, 35–100% more insulin vesicles are docked at the plasma membrane as compared to control β cells (Zhao *et al.* 2010). Therefore, in the pancreatic β -cell model, Go α acts as a key regulator of granule docking by interacting at either the docking or the priming station of cells to prevent the oversecretion of insulin (Zhao *et al.* 2010). In chromaffin cells, Go α inhibits granule fusion to the plasma membrane by controlling the priming of granules, which therefore regulates catecholamine secretion (Gasman *et al.* 1997). The most prominent result from the present study was the 51% significant decrease in granule numbers and changes in the electron density of the granules in cGo α ^{−/−} mice as compared to control mice. A finding similar to this was not reported in the previous studies on β cells or chromaffin cells, which suggests that either Go α plays various roles in the vectorial transport of secretory products within secretory cells or that it plays different roles in different cell types.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the Heart and Stroke Foundation of Ontario (grant number NA 6992).

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Received in final form 15 April 2015

Accepted 24 April 2015

Accepted Preprint published online 27 April 2015