Targeted Inhibition of Cardiomyocyte Gi Signaling Enhances Susceptibility to Apoptotic Cell Death in Response to Ischemic Stress

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Background—A salient characteristic of dysfunctional myocardium progressing to heart failure is an upregulation of the adenylyl cyclase inhibitory guanine nucleotide (G) protein α subunit, Goα2. It has not been determined conclusively whether increased Gi activity in the heart is beneficial or deleterious in vivo. Gi signaling has been implicated in the mechanism of cardioprotective agents; however, no in vivo evidence exists that any of the Goα subunits are cardioprotective. We have created a novel molecular tool to specifically address the role of Gi proteins in normal and dysfunctional myocardium.

Methods and Results—We have developed a class-specific Gi inhibitor peptide, GiCT, composed of the region of Goα2 that interacts specifically with G protein–coupled receptors. GiCT inhibits Gi signals specifically in vitro and in vivo, whereas Gs and Gq signals are not affected. In vivo expression of GiCT in transgenic mice effectively causes a “functional knockout” of cardiac Goα2 signaling. Inducible, cardiac-specific GiCT transgenic mice display a baseline phenotype consistent with nontransgenic mice. However, when subjected to ischemia/reperfusion injury, GiCT transgenic mice demonstrate a significant increase in infarct size compared with nontransgenic mice (from 36.9±2.5% to 50.9±4.3%). Mechanistically, this post-ischemia/reperfusion phenotype includes increased myocardial apoptosis and resultant decreased contractile performance.

Conclusions—Overall, our results demonstrate the in vivo utility of GiCT to dissect specific mechanisms attributed to Gi signaling in stressed myocardium. Our results with GiCT indicate that upregulation of Goα2 is an adaptive protective response after ischemia to shield myocytes from apoptosis. (Circulation. 2008;117:1378-1387.)

Key Words: apoptosis ■ heart diseases ■ myocardial infarction ■ signal transduction

Apoptosis has been proposed as a mechanism for a significant amount of cell death occurring during acute myocardial infarction1–6 and is thought to contribute significantly to the progressive loss of functional myocardium in the failing heart.7,8 A variety of cardioprotective agents used in clinical practice to manage patients with acute myocardial infarction such as β-adrenergic receptor (β-AR) blockers, adenosine, and morphine are thought to be cardioprotective because of altered signaling through their cognate G protein–coupled receptor (GPCR). Importantly, these ligands have been demonstrated to reduce apoptotic myocyte cell death in animal models of ischemic injury,9–11 a finding that supports their clinical use as cardioprotective agents. Of note, the GPCRs acted on by these drugs can couple to the adenylyl cyclase inhibitory G protein α subunit (Gαι).12,13

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Heterotrimeric G proteins exist as Go subunits linked to Gβγ subunits. On ligand binding, GPCRs undergo a conformational change, which facilitates an interaction with the G protein heterotrimer, facilitating the dissociation of Go and Gβγ subunits that transduce intracellular signaling pathways, leading to specific GPCR-mediated cellular events.14 The Go

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The online-only Data Supplement, which contains expanded Methods, can be found with this article at http://circ.ahajournals.org/cgi/content/full/CIRCULATIONAHA.107.752618/DC1.

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subunits can be used to classify heterotrimeric G proteins into 4 primary families: Gαs, Gαi/o, Gαq/11, and Gα12/13. Gαi proteins are ubiquitously expressed and have been classically associated with the inhibition of adenylyl cyclase, whereas Gβγ has a variety of cellular effectors, including phosphoinositol-3 kinase/Akt, Ras, GPCR kinases, and phospholipase C-β.15 Of the Gαi isoforms (Gαi1, Gαi2, Gαi3), Gαi2 is the predominant isoform in cardiac myocytes; along with Gαi3, it has been shown to couple to cardiac β2-ARs.16

The role of Gi signaling in the context of myocardial ischemia and the progression toward heart failure is not straightforward.17,18 One salient characteristic of the failing heart is upregulation of Gαi2 at the protein and transcript levels19; to date, however, there has been controversy surrounding the beneficial or maladaptive nature of this upregulation.16,20 To address Gi signaling in this regard, we have developed a Gi-selective inhibitory peptide to “functionally knock out” all GPCR-Gi–induced signals in the heart. This peptide, called GiCT, comprises the carboxyl-terminal 63 amino acids of human Gαi2, which shares >90% similarity with other Gαi subunit family members, and is modeled after specific Gq and Gs inhibitory peptides previously described by our laboratory.21,22 The carboxy-terminal region of Gαi2 facilitates the interaction of the ligand-activated receptor and G protein.23 Therefore, because GiCT is composed of this region, targeting the intracellular GPCR and G protein interface, both Gαi and Gβγ signals are inhibited. Mice harboring the GiCT transgene specifically in myocytes have allowed us to directly address the role of Gi signaling in response to stress in the form of myocardial ischemia, a causative agent in the development of heart failure, without knocking out or overexpressing any endogenous genes.

Methods

For an expanded Methods section, please refer to the online-only Data Supplement.

Construction of Plasmid, Adenovirus, and Transgenic Mice

The carboxyl-terminal 63 amino acids of human Gαi2 (Gαi2 293 through 355) were inserted into the pCMV-HA vector (Clontech, Mountain View, Calif) to generate GiCT. Recombinant GiCT adenoviruses were generated with the Adeasy XL Adenoviral Vector System (Stratagene, La Jolla, Calif). For transgenic mouse generation, the bitransgenic system initially described by Sanbe and colleagues24 was used. The stable tetracycline analog doxycycline was administered to mice at 300 mg/kg of mouse diet (Bio-Serv, Frenchtown, NJ) to repress GiCT transcription as described.25

RNA Isolation and Semiquantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction was carried out with iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, Calif) and the following gene-specific oligonucleotides: 28s, Gαi2, Gαs, Gαq, and HA-GICT (sequences available on request).

cAMP Production Assay

Mouse cardiac myocytes were isolated using a standard retrograde perfusion method from GiCT transgenic (Tg-GiCT) and nontransgenic mice. Myocytes were treated with 0.5 mmol/L 3-isobutyl-1-methylxanthine to inhibit phosphodiesterases and then stimulated as indicated. cAMP accumulation was quantified with a cAMP ELISA-based assay according to the manufacturer’s protocol (Sigma-Aldrich, St Louis, Mo).

Transthoracic Echocardiographic Analysis

Transthoracic 2-dimensional echocardiography in mice anesthetized with an intraperitoneal dose of Avertin (8 µL/g) with spontaneous respiration was performed with a 12-MHz probe as described previously.26

Ischemia/Reperfusion Injury Model

Surgical procedures were carried out according to National Institutes of Health Guidelines on the Use of Laboratory Animals, and all procedures were approved by the Thomas Jefferson University Committee on Animal Care. The ischemia/reperfusion (I/R) injury model was performed as previously described with minor modifications.27

Hemodynamic Analysis of Cardiac Function

Hemodynamic analysis was conducted as described previously.26

Determination of Left Ventricular Infarct Size and Area at Risk

Left ventricular (LV) infarct size and area at risk were assessed as previously described with slight modifications.27

Assessment of Myocardial Apoptosis and Apoptotic Signaling

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed as previously described.27 DNA fragmentation was assessed with the Gentra Puregene Tissue DNA Isolation Kit (Qiagen, Valencia, Calif). Caspase-3 cleavage was assessed by immunoblotting with cleaved caspase-3 antibody (Calbiochem, San Diego, Calif).

Isolation and Primary Culture of Neonatal Rat Ventricular Cardiomyocytes

Ventricular cardiomyocytes from 1- to 2-day–old rat neonatal hearts (neonatal rat ventricular myocytes [NRVMs]) were prepared as published in detail elsewhere.28 On day 2 of culture, NRVMs were infected with the indicated adenoviruses at a multiplicity of infection of 100, and experiments were performed within 24 hours of infection.

Mitochondrial Membrane Potential

To assess mitochondrial membrane potential (∆ψm), we used tetramethylrhodamine ethyl ester (Molecular Probes, Eugene, Ore), which accumulates in mitochondria in response to the ∆ψm. Experiments were performed as described elsewhere.29

Statistical Analysis

All values in the text and figures are presented as mean±SEM from independent experiments given numbers. Statistical significance of multiple treatments was determined by 1-way or 2-way ANOVA followed by the Bonferroni post hoc test when appropriate. Statistical significance between 2 groups was determined by use of the 2-tailed Student t test. Values of P<0.05 were considered significant.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.
Results

Expression and Gi Protein Selectivity of GiCT

The 63 amino acids of the Gαi2 carboxyl-terminus were ligated in frame into pCMV-HA to generate an amino-terminus hemaglutinin-tagged GiCT construct (Figure 1A), after which cells were subjected to immunoblotting for hemaglutinin tag (HA). Immunoblot for GAPDH is shown as a loading control. COS-7 cells were transfected with β1-AR, α1-AR, HA-GICT, or control vector and then stimulated with 10 μmol/L isoproterenol (ISO), 1 μmol/L phenylephrine (PE), and lysophosphatidic acid (LPA) 10 μmol/L, as indicated for 5 minutes; then, cell lysates were subjected to immunoblotting for detection of phospho- and total p42/44 ERK levels and HA. D, Quantification of ERK phosphorylation. E, Representative reverse-transcription polymerase chain reaction detection of HA-GICT transgene in nontransgenic (nTg) and transgenic HA-GICT (Tg-GICT) mice, from breeding pairs (Bps) 2, 3, and 5, from cDNA generated from whole-heart total RNA. F, Representative immunoblots of HA-GICT transgene from myocardial, liver, and lung extracts of nTg and Tg-GICT mice. *P<0.05 vs control; n=3.

Generation and Functional Characterization of Mice Expressing GiCT Specifically in Cardiomyocytes

Founder transgenic mice that express GiCT under the control of the cardiac-specific tetracycline-responsive α-myosin heavy chain promoter described by Sanbe et al24 were crossbred with homozygous mice possessing an α-myosin heavy chain–driven tetracycline transactivator construct. The resultant pups from this
crossing consisted of mice possessing both the GiCT and tetracycline transactivator transgenes, which will express the GiCT construct when doxycycline is removed from the food supply. These mice are referred to as Tg-GiCT mice, whereas mice possessing only the tetracycline transactivator transgene are referred to as nontransgenic mice and are used as control for functional and biochemical characterization. Tg-GiCT mice demonstrated robust expression of the ~10-kDa HA epitope-tagged transgene at both the transcript and protein levels specifically in the heart (Figure 1E and 1F).

To assess the baseline functional consequences of our Gi inhibitor peptide at the whole-heart level, 6- to 8-week-old mice were subjected to transthoracic echocardiography under Avertin anesthesia. Tg-GiCT mice demonstrated no significant differences with respect to heart rate, wall thickness as assessed by LV posterior wall thickness and LV septal wall thickness, chamber dilatation as assessed by LV end-diastolic diameter, or contractile function as assessed by ejection fraction and fractional shortening (the Table). Tg-GiCT mice demonstrated no significant differences in organ morphometry as assessed by the ratios of heart weight to body weight and HW/TL, ratio of heart weight to tibial length.

cAMP Accumulation in Isolated Tg-GiCT Cardiomyocytes
To establish the ex vivo biochemical phenotype of cardiomyocytes from Tg-GiCT mice, cAMP levels were measured from isolated myocytes from 8- to 10-week-old mice at baseline in response to the Gi-coupled agonist carbachol, which will inhibit cAMP production; the Gs-coupled agonist isoproterenol alone, which will stimulate cAMP production; and isoproterenol after carbachol pretreatment nearly completely inhibited cAMP accumulation in response to isoproterenol, an effect that was significantly blocked in Tg-GiCT myocytes, which demonstrate a significant reduction in Gi-mediated response to carbachol pretreatment (113.7±19.4% for nontransgenic compared with 201.9±26.9% for Tg-GiCT myocytes; Figure 2). Furthermore, as expected, Tg-GiCT mice demonstrated a trend toward a smaller reduction in baseline cAMP in response to carbachol treatment alone, and no significant difference was observed between cAMP accumulation in nontransgenic and Tg-GiCT mice in response to the Gs-coupled agonist isoproterenol, further confirming the Gi-selective nature of the GiCT transgene ex vivo.

| Table. Functional and Morphological Characterization of Tg-GiCT Mice |
|---------------------|---------------------|
|                     | nTg                | Tg-GiCT             |
| HR, bpm             | 407±24             | 379±15              |
| LVPWT, mm           | 0.90±0.08          | 0.86±0.07           |
| LVAWT, mm           | 0.83±0.04          | 0.81±0.04           |
| LVEDD, mm           | 3.23±0.11          | 3.40±0.13           |
| EF, %               | 73.4±4.0           | 72.5±2.9            |
| FS, %               | 43.3±3.9           | 41.4±2.4            |
| HW/BW, mg/g         | 4.36±0.10          | 4.31±0.10           |
| HW/TL, mg/mm        | 6.21±0.22          | 6.22±0.20           |

nTg indicates nontransgenic; HR, heart rate; LVPWT, LV posterior wall thickness; LVAWT, LV anterior wall thickness; LVEDD, LV end-diastolic diameter; EF, ejection fraction; FS, fractional shortening; HW/BW, ratio of heart weight to body weight; and HW/TL, ratio of heart weight to tibial length.

Altered Gα Subunit Expression After I/R Injury
To explore the potential for I/R injury to regulate the mRNA expression of G protein α subunits, we isolated mRNA from the area at risk of wild-type mice subjected to 30 minutes of ischemia followed by 72 hours of reperfusion and sham-operated mice. Semi-quantitative reverse-transcription polymerase chain reaction conducted on these samples demonstrated a 3-fold upregulation of cardiac Gαq compared with sham-operated mice (Figure 3A), whereas no significant change occurred in the mRNA levels of Gαi1 or Gαi3 (Figure 3B and 3C). Levels of Gαi1 and Gαi3 also were investigated. Gαi3 mRNA levels were not detectable, whereas Gαi1 mRNA expression was increased after I/R, but the expression was significantly (~20-fold) less compared with Gαq (data not shown). These data indicate that stress in the form of I/R injury results in changes in the signal transduction milieu, ultimately resulting in the regulation of Gi signaling at the mRNA level.
Effect of Myocyte-Specific Inhibition of Gi Signaling on Susceptibility to I/R Injury

Given that Gi is regulated in this model of myocardial dysfunction, we investigated the role of Gi signal inhibition on susceptibility to myocardial I/R injury. We subjected 8- to 10-week–old Tg-GiCT and nontransgenic mice maintained on a standard or doxycycline-supplemented diet to 30 minutes of myocardial ischemia followed by 24 hours of reperfusion, at which time infarct sizing was conducted by a combined Evan’s Blue/triphenyltetrazolium chloride staining method (Figure 4B). It is important to note that Tg-GiCT mice maintained on a doxycycline-supplemented diet do not express a detectable amount of GiCT transgene at the protein level (Figure 4A). The infarct size of Tg-GiCT mice, represented as the percentage of the area at risk, was significantly increased by 14.2% (36.9 ± 2.5% for nontransgenic and 50.9 ± 4.3% for Tg-GiCT; Figure 4C), although the area at risk was not significantly changed between groups (53.2 ± 1.5% for nontransgenic and 51.5 ± 2.1% for Tg-GiCT; Figure 4D), thus indicating that Gi signal inhibition, specifically in myocytes, results in increased susceptibility to I/R injury. Importantly, the addition of doxycycline completely ameliorates the susceptibility for I/R injury of Tg-GiCT mice (Figure 4B through 4D). Tg-GiCT and nontransgenic mice maintained on doxycycline were subjected to the same I/R protocol, which resulted in a normalization of infarct sizes between groups consistent with that of nontransgenic mice subjected to I/R, thus indicating that “turning off” the expression of the GiCT transgene normalizes susceptibility to I/R injury.

Enhanced Myocardial Apoptosis in Tg-GiCT Mice in Response to I/R Injury

To ascertain the mechanism for the increased infarct size in response to ischemic injury in Tg-GiCT mice, we subjected 8- to 10-week–old mice to 30 minutes of ischemia followed by 3 hours of reperfusion, at which point hearts were embedded in paraffin and processed for TUNEL staining. Tg-GiCT mice demonstrate an ~2.5-fold increase in the number of TUNEL-positive nuclei compared with nontransgenic control mice (Figure 5A and 5B), indicating increased apoptotic cell death in Tg-GiCT mice in response to ischemic stress. No difference between groups was observed with respect to baseline TUNEL-positive nuclei because positive TUNEL staining was rarely observed in either sham-operated group.

To independently confirm the finding of increased apoptotic cell death in mice with cardiomyocyte-specific expression of GiCT, we subjected 8- to 10-week–old mice to the same I/R protocol. Tg-GiCT and nontransgenic mice subjected to the same I/R protocol, which resulted in a normalization of infarct sizes between groups consistent with that of nontransgenic mice subjected to I/R, thus indicating that “turning off” the expression of the GiCT transgene normalizes susceptibility to I/R injury.
of GiCT, we investigated DNA fragmentation and caspase-3 cleavage in these mice. Preparation of genomic DNA from the area at risk demonstrated apparent DNA fragmentation in nontransgenic control mice after I/R; however, the degree of DNA fragmentation was markedly more evident in Tg-GiCT mice (Figure 5C). Furthermore, caspase-3 cleavage was significantly increased, as evidenced by the increased detection of the ~17-kDa caspase-3 large cleavage product in the myocardial extracts from the area at risk of Tg-GiCT mice subjected to 30 minutes of ischemia followed by 1.5 hours of reperfusion compared with nontransgenic control mice (Figure 5D).

**Effect of Myocardial Expression of GiCT on Cardiac Function in Response to I/R Injury**

To measure cardiac function in Tg-GiCT and nontransgenic control mice subjected to stress in the form of I/R injury or sham-operative procedure, hemodynamic parameters were recorded after LV catheterization at baseline and in response to increasing doses of isoproterenol. Under sham conditions, both at baseline and in response to β-AR stimulation, no difference was detectable between Tg-GiCT and nontransgenic control mice with respect to heart rate, cardiac contractility as assessed by LV ± dP/dt, LV end-diastolic pressure, and mean arterial blood pressure (Figure 6). However, Tg-GiCT mice subjected to 30 minutes of ischemia and 72 hours of reperfusion demonstrated a significant reduction in cardiac contractile performance compared with both sham-operated mice and I/R-operated nontransgenic control mice (Figure 6). In addition, Tg-GiCT mice exhibited a trend toward increased LV end-diastolic pressure. These data indicate an overall worsening of contractile performance in Tg-GiCT in response to stress in the form of I/R injury and represent a functional correlate to our data indicating increased infarct size and apoptosis in Tg-GiCT mice.

**Effect of GiCT on Determining Mitochondrial Response to Oxidative Stress In Vitro**

To assess the effect of in vitro blockade of Gi signaling on cardiomyocyte response to oxidative stress, a key component of ischemia/reperfusion injury, we measured Δψm. Loss of Δψm represents an initial step in the mitochondrial apoptotic cascade, followed by release of cytochrome c and activation of caspases. NRVMs infected with the GiCT adenovirus demonstrated no difference in Δψm compared with LacZ-infected NRVMs at baseline, and expression of GiCT in NRVMs was confirmed by immunoblot (Figure 7A). In response to 100 µmol/L H2O2, both LacZ- and GiCT-infected NRVMs displayed a significant loss of Δψm; however, this effect was significantly enhanced in GiCT-infected NRVMs (Figure 7B), indicating that the Gi pathway protects cardiomyocytes from cell death at the level of the mitochondria.

Importantly, differential survival and apoptotic signals have been reported for cardiac β-ARs, with β1-ARs being proapoptotic (via Gs) and β2-ARs being primarily prosurvival via the apparent ability of this receptor to couple to Gi proteins. In fact, data have shown that stimulation of β2-AR signaling can prevent reactive oxygen species-induced cell death through its ability to couple to Gi. Thus, in these experiments, we stimulated myocytes with the β2-AR agonist zinterol. Zinterol alone resulted in a significant increase in Δψm in LacZ-infected NRVMs (Figure 7B). However, cells expressing the GiCT showed no response to zinterol from baseline Δψm (Figure 7B).
Importantly, the addition of zinterol in the absence of tetramethylrhodamine ethyl ester did not alter baseline fluorescence (data not shown). In addition, expression of GiCT blocked the protective effect of zinterol pretreatment in response to H₂O₂-induced cell death. This indicates that the Gi component of β₂-AR signaling provides survival signals that are blocked by GiCT, resulting in a loss of Δψₘ followed by release of deleterious factors from mitochondria, activation of the caspase cascade, and death of cardiomyocytes. Blockade of prosurvival pathways by expression of GiCT in NRVMs was confirmed by assessment of Akt phosphorylation. Short-term exposure to reactive oxygen species has previously been shown to increase the phosphorylation and thus activation of the serine/threonine kinase Akt, leading to decreased apoptotic cell death. Exposure to 100 μmol/L H₂O₂ for 60 minutes resulted in a significant increase in Akt phosphorylation in LacZ-infected NRVMs that was significantly inhibited in GiCT-infected NRVMs (Figure 7C and 7D), further establishing that Gi signals are a key component to survival signaling in response to reactive oxygen species, a key contributor to myocardial injury in response to I/R.

Figure 6. Effect of myocyte-specific Gi inhibition on cardiac function and hemodynamic parameters after 30 minutes of ischemia and 72 hours of reperfusion. Hemodynamic parameters, including heart rate (HR), maximum and minimum LV pressure derivatives (+dP/dtₘₐₓ, −dP/dtₘᵟᵣₚ), LV end-diastolic pressure (LVEDP), and mean arterial blood pressure (MABP), were recorded at baseline and in response to increasing doses of isoproterenol (Iso). *P<0.05 vs sham; †P<0.05 vs nontransgenic (nTg) I/R; n=8 to 12.

Figure 7. Effect of GiCT-mediated Gi inhibition on response to oxidative stress in cultured NRVMs in vitro. A, Representative immunoblot demonstrating infection of NRVMs treated with an adenovirus encoding LacZ or HA-GiCT. NRVMs were infected with adenovirus at a dose of 100 multiplicities of infection. B, Assessment of mitochondrial membrane potential via tetramethylrhodamine ethyl ester (TMRE) uptake in NRVMs subjected to 100 μmol/L H₂O₂ for 60 minutes in the presence and absence of pretreatment with the β₂AR agonist zinterol. *P<0.05 vs Ad-LacZ basal; †P<0.05 vs Ad-LacZ; n=3. C, Assessment of Akt phosphorylation in NRVMs after treatment with 100 μmol/L H₂O₂ for 60 minutes in LacZ- and GiCT-infected NRVMs. D, Quantification of Akt phosphorylation. *P<0.05 vs basal; n=3.
Discussion

Here, we have developed a novel molecular tool, GiCT, for selectively inhibiting signals induced by Gi activation. We have found that Gi class–specific inhibition in vivo in the heart via GiCT expression worsens outcome after myocardial ischemia via increased myocyte apoptosis. Thus, GiCT serves as a tool to delineate the role of Gi upregulation in dysfunctional myocardium, and we have created inducible transgenic mice that effectively represent a functional knockout of cardiac Gi proteins without deleting a gene or overexpressing endogenous genes. Our findings reveal that after I/R injury the increased Gi signaling resulting from Gi upregulation appears to be adaptive and promotes cellular protection after ischemia.

We have previously demonstrated the utility of specific G protein α subunit carboxyl-terminal peptides to serve as molecular tools to dissect G protein–selective intracellular signaling in vitro and in vivo.21,22 Indeed, using a peptide (Gq1) specific for GPCR-Gq activation, we determined that Gi signals are the final common trigger for induction of pressure-overload ventricular hypertrophy.21 Moreover, we developed a similar tool for the specific inhibition of Gs-coupled receptor signaling in cells.22 These studies have revealed that class-specific G protein inhibition is possible and may have therapeutic potential for combating certain cardiovascular diseases such as maladaptive hypertrophy or hypertension.33,34 In this study, we find that it is possible to selectively inhibit Gi-mediated signals in vivo with GiCT expression, and using this tool, we have begun to study more in depth the role of Gi in dysfunctional myocardium.

Importantly, we found no basal cardiac phenotype resulting from transgenic GiCT expression, showing that Gi inhibition in the adult heart leads to no overt structural or functional myocardial alterations. This is similar to what was observed in mice expressing a Gq-inhibitory peptide in myocytes21 and is encouraging for any potential therapeutic applications of these peptides. We did confirm that cardiac Gi signals were blocked in Tg-GiCT mice because myocytes were isolated and inhibition of cAMP production resulting from the Gi-coupled receptor agonist carbachol was attenuated compared with myocytes from control mice. Moreover, apoptotic signaling was enhanced in vivo after ischemia via I/R injury and in vitro in myocytes exposed to oxidative stress when Gi signals were blocked by GiCT expression. Thus, GiCT appears to be effective at Gi blockade, and signaling via Gi activation after myocardial ischemia appears to be prosurvival. This is apparently enhanced even when myocardium is stressed with I/R; we found enhanced Gaβ3 transcript levels after I/R, which indicates that myocytes adapt to injury with signaling molecules promoting survival. Thus, GiCT expression blocks even enhanced levels of Gaβ3 and targeted inhibition of Gi results in increased myocardial infarct size and myocardial apoptosis, as evidenced by enhanced TUNEL staining, DNA fragmentation, and activation of caspases. This translated into Tg-GiCT mice having worsened in vivo LV contractile function after I/R, most probably as a result of an increased loss of viable myocardium.

The cell death induced by I/R injury is a combination of apoptotic and necrotic processes. Our goal in the present work was to assess the role of Gi signaling in vivo on the susceptibility to apoptosis in response to stress. In doing so, however, we are not excluding a potential role of necrosis in the increased infarct size seen in Tg-GiCT mice. Importantly, we have assessed necrosis in vitro in NRVMs after exposure to H2O2 at concentrations and times that we have shown to elicit enhanced apoptosis in GiCT-infected NRVMs, and GiCT expression did not appear to alter necrotic cell death (data not shown).

The use of GiCT as a molecular tool to study the functional consequences of in vivo myocyte-specific Gi blockade represents an advancement over previous approaches using pertussis toxin (PTx) or global Gi isoform knockout mice. Conclusions derived from in vivo application of PTx, derived from Bordetella pertussis, the causative agent of whooping cough, are difficult to establish definitively given the array of noncardiac side effects of intraperitoneal injection of the toxin.35 Furthermore, ex vivo application of PTx through a modified Langendorff procedure may affect cardiac function; however, the respective contribution of cardiac myocytes compared with nonmyocytes is difficult to ascertain.36,37 Importantly, in cells, we found that GiCT inhibited Gi signaling as effectively as PTx (data not shown). Thus, GiCT appears to have key advantages over PTx because it can be expressed in a myocyte-specific manner and is nontoxic to basal cellular homeostasis.

Global Gαi knockout mice also are subject to similar concerns. Gαi2 knockout mice display a phenotype resembling histopathological features of ulcerative colitis and adenocarcinoma of the colon with premature death, potentially masking any gross cardiac phenotype.38 In addition, the effect of Gαi3 or any Gi α subunit knockout does not identify any specific signals attributed to Giβγ dissociation. Because GiCT competes with endogenous Gi proteins with agonist-occupied GPCRs, preventing Gα activation and subsequent Gα and Giβγ dissociation, it is a blocker of total Gi signaling, much like PTx. Therefore, GiCT has an advantage over classic methods of knocking out a Gαi gene. Moreover, Tg-GiCT mice express this Gi inhibitor in a manner temporally controlled by doxycycline, and importantly, we demonstrate that the worsening of myocardial cell death after I/R is absent in these mice if they are maintained on doxycycline and GiCT expression is silenced.

The utility of GiCT allowed us to specifically investigate in myocytes the potential mechanistic role of Gi in the prosurvival signaling that has been attributed to β2-AR activation. Previously, using PTx and the βARKct peptide as a Giβγ inhibitor has implicated Giβγ-mediated phosphoinositide-3 kinase activation in promoting antiapoptotic pathways in myocytes.17 In this study, we were able to use a single molecular tool, GiCT, to attenuate the β2-AR–mediated protective effects on Δψm. As mentioned, GiCT effectively inhibits both Gai and Giβγ signals, and these results are consistent with that notion.

Overall, our results indicate that the upregulation of Gαi2 in ischemic myocardium appears to be protective in
inducing cell survival pathways to prevent myocyte death in response to I/R injury. This may also provide insight into the role of upregulation of cardiac Goi in human heart failure, which may provide survival signals that prevent apoptotic cell death induced by the toxic levels of catecholamines and calcium overload present in chronically dysfunctional myocardium.39

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Disclosures

None.

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### CLINICAL PERSPECTIVE

A variety of cardioprotective agents used in clinical practice for the management of patients with acute myocardial infarction such as β-adrenergic receptor blockers, adenosine, and morphine are thought to be cardioprotective because of altered signaling through their cognate G protein–coupled receptor (GPCR). Of note, the GPCRs acted on by these drugs can couple to the adenylyl cyclase inhibitory G protein α subunit (Gi). Furthermore, Gi is upregulated in failing myocardium at the protein and transcript levels; however, the role of Gi signaling in the context of myocardial ischemia and the progression toward heart failure is controversial. To address Gi signaling in this regard, we have developed a Gi-selective inhibitory peptide to “functionally knock out” all GPCR-Gi–induced signals in the heart. This peptide, called GiCT, can block the interaction of the ligand-activated GPCR and G protein, thus selectively inhibiting signals induced by Gi activation. Mice harboring the GiCT transgene specifically in myocytes have allowed us to directly address the role of Gi signaling in response to stress in the form of myocardial ischemia, a causative agent in the development of heart failure. This study demonstrates a marked enhancement of myocardial infarct size and apoptotic signaling after transient myocardial ischemia and reperfusion when Gi signaling is blocked, thus indicating that signaling via Gi activation appears to be prosurvival in the context of myocardial ischemic injury. Our work suggests that the upregulation of Go(αi2) in human heart failure may provide survival signals that prevent apoptotic cell death induced by toxic levels of catecholamines present in dysfunctional myocardium.
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