Expression of Slow Skeletal Troponin I in Adult Mouse Heart Helps to Maintain the Left Ventricular Systolic Function During Respiratory Hypercapnia


Abstract—Compared with the adult, neonatal heart muscle is less sensitive to deactivation by acidic pH. We hypothesized that expression of slow skeletal troponin I (ssTnI), the embryonic isoform, in adult heart would help maintain left ventricular (LV) systolic function during respiratory hypercapnia. We assessed LV function by transthoracic 2D-targeted M-mode and pulsed Doppler echocardiography in transgenic (TG) mice in which cardiac TnI was replaced with ssTnI and in nontransgenic (NTG) littermates. Anesthetized mice were ventilated with either 100% oxygen or 35% CO2 balanced with oxygen. Arterial blood pH with 35% CO2 decreased to the same levels in both groups of animals. In the absence of propranolol, the LV fractional shortening was higher in TG compared with NTG mice throughout most of the experimental protocol. LV diastolic function was impaired in TG compared with NTG mice both at 100% oxygen and 35% CO2 because E-to-A wave ratio of mitral flow was significantly lower, and E-wave deceleration time and LV isovolumic relaxation time were longer in TG compared with NTG mice. When compensatory mechanisms that occur through stimulation of β-adrenergic receptors during hypercapnia were blocked by continuous perfusion with propranolol, we found that NTG mice died within 3 to 4 minutes after switching to 35% CO2, whereas TG mice survived. Our experiments demonstrate the first evidence that specific replacement of cardiac TnI with ssTnI has a protective effect on the LV systolic function during hypercapnic acidosis in situ. (Circ Res. 2005;97:0-0.)

Key Words: troponin I hypercapnia acidosis

It is known that acidosis alters cardiac contractility to a smaller degree in neonatal than adult heart.1,2 The lower sensitivity to deactivation by acidic pH during development may be attributable to differences in (1) isoforms of myofilament proteins expressed in neonatal and adult heart, (2) effects of acidosis on Ca2+ fluxes, and/or (3) intracellular pH and/or Ca2+ buffering. Based on experiments comparing adult and neonatal rat ventricular muscles, we have hypothesized that slow skeletal troponin I (ssTnI) is the major determinant of the differential response to acidic pH between adult and neonatal hearts.2 The ssTnI isoform is expressed during embryonic and early postnatal life, whereas cardiac TnI (cTnI) is expressed during adult life.3,4 The role of TnI isoforms and the role of specific molecular differences between these two isoforms have been extensively studied.1,2,5-10 We have reported that expression of ssTnI in adult cardiac papillary muscles is sufficient to prevent the force decline during hypercapnic acidosis in isolated papillary muscles.8 These data provide direct evidence for the important protective role of ssTnI during acidosis and most likely during ischemia/reperfusion. However, it is still unclear whether ssTnI has the same protective effect in the in situ ejecting heart during acidic conditions. In the present study, our objective was to test the protective effect of ssTnI on cardiac function during acidic conditions in vivo. An important rationale for our experiments comes from our recent data indicating that the role of TnI on cardiac function is best measured in ejecting, afterloaded hearts.11-13 Our experiments provide the first evidence that specific replacement of cTnI with ssTnI has a protective effect on the left ventricular (LV) systolic function during hypercapnic acidosis in the intact animal.

Materials and Methods

Animal Preparations

Adult 8- to 10-month old male and female transgenic (TG) and nontransgenic (NTG) mice that express ssTnI in the heart14 were used. Mice were anesthetized with 1.0% to 1.5% isoflurane mixed with the 100% oxygen delivered through a vaporizer connected to a rodent ventilator. Stroke volume was set at 0.25 to 0.30 mL, and a respiration rate was set at 136 breaths per minute.15 After 20 minutes of baseline conditions, 100% oxygen was switched to 35% CO2, whereas NTG mice survived. Our experiments demonstrate the first evidence that specific replacement of cardiac TnI with ssTnI has a protective effect on the LV systolic function during hypercapnic acidosis in situ. (Circ Res. 2005;97:0-0.)

Key Words: troponin I hypercapnia acidosis

Materials and Methods

Animal Preparations

Adult 8- to 10-month old male and female transgenic (TG) and nontransgenic (NTG) mice that express ssTnI in the heart14 were used. Mice were anesthetized with 1.0% to 1.5% isoflurane mixed with the 100% oxygen delivered through a vaporizer connected to a rodent ventilator. Stroke volume was set at 0.25 to 0.30 mL, and a respiration rate was set at 136 breaths per minute.15 After 20 minutes of baseline conditions, 100% oxygen was switched to 35% CO2, whereas NTG mice survived. Our experiments demonstrate the first evidence that specific replacement of cardiac TnI with ssTnI has a protective effect on the LV systolic function during hypercapnic acidosis in situ. (Circ Res. 2005;97:0-0.)

Key Words: troponin I hypercapnia acidosis

Original received January 26, 2005; revision received June 3, 2005; accepted June 6, 2005.

From the Center for Cardiovascular Research, Department of Physiology and Biophysics (D.U., L.A.W., R.J.S., B.M.W.) and Department of Medicine (F.A.L.D., J.R.P., L.A.W., B.M.W.), Section of Cardiology, University of Illinois at Chicago.

Correspondence to Dr Beata M. Wolska, University of Illinois at Chicago, Dept of Medicine, Section of Cardiology, 840 S Wood St (MC 715), Chicago, IL 60612. E-mail bwolska@uic.edu

© 2005 American Heart Association, Inc.

Circulation Research is available at http://www.circresaha.org DOI: 10.1161/01.RES.0000173849.68636.1e
40 minutes. In some mice, the right femoral vein was isolated and catheterized as previously described, and propranolol (34 ng/g body weight/min) was continuously infused using a pump.

**Echocardiography**

Transathoracic 2D-targeted M-mode and pulsed Doppler echocardiography (ECHO) were performed with a 15-MHz linear array transducer (Acuson Sequoia C256 system). The transducer was placed over a layer of acoustic coupling gel that was applied to the left hemithorax; adequate contact was maintained while avoiding excessive pressure on the chest. Mice were imaged in a shallow left lateral decubitus position. M-mode images of the left ventricle were obtained from the parasternal short axis view at the level of the papillary muscles. Interventricular septal and LV posterior wall thicknesses and LV internal dimensions at the end of diastole and systole were measured by the American Society of Echocardiography leading-edge method on the M-mode tracings. Fractional shortening of the left ventricle, a measure of LV systolic function, was calculated from digital images as: LV fractional shortening (FS) (%)=(LVDD−LVIsd)/LVDD×100, where LVDD is the internal diastolic dimension of the LV, and LVIsd is the internal systolic dimension of the LV.

Diastolic transmission inflow recordings were acquired from apical four-chamber view using 7 MHz pulsed Doppler ECHO. The probe was positioned subcostally at the xiphoid applying minimal pressure. The Doppler range gate depth was set at 4 mm to obtain optimal signals from the LV inflow and outflow tracks. The sample volume was positioned along the long axis in the middle of the mitral ring at the tips of the opened cusps of the mitral valve. Three parameters of the LV diastolic function were evaluated: (1) E/A ratio = ratio of the maximal velocity of E (early LV filling) and A (atrial contraction) waves; (2) E-wave deceleration time (DT) = the time from the peak of the E wave to the intersection of the deceleration slope of the E wave with the baseline; and (3) LV isovolumic relaxation time (IVRT), which was measured from the aortic valve closure to the mitral valve opening. The M-mode and Doppler tracings were conducted with a paper speed of 200 mm/sec.

**Blood Gas Analysis**

Blood gas analysis was performed using IL Synthesis Systems (Instrumentation Laboratory, 1998, Milano, Italy). The right carotid artery or left femoral artery was dissected, and a polyethylene catheter (PE-10) was inserted into the artery up to the aortic arch. A catheter was placed in the left ventricle for measurement of arterial blood pH after 40 minutes of exposure to 100% O2, and three mice in each group were ventilated with 35% CO2. Approximately 0.3 mL of blood was drawn into a syringe for measurement of arterial blood pH after 40 minutes of ventilation with either 100% oxygen or 35% CO2 balanced with oxygen. During the exposure to either gas, LV function was monitored by ECHO.

**Electrophoresis and Western Blot Analysis**

**TnI Phosphorylation**

The apex of the left ventricle was placed directly into 10% TCA in acetone (−70°C) and placed in the −70°C freezer for a minimum of 16 hours. Samples were slowly warmed to room temperature (1 hour at −20°C, 1 hour at 4°C, and 1 hour at room temperature) to allow acetone substitution of the tissue, preserving the phosphorylation status of the proteins. TnI phosphorylation was analyzed by non-equilibrium 1D isoelectric focusing as described previously. Percentage phosphorylation was expressed as TnI (phosphorylated)/TnI (dephosphorylated) × 100%.

**Expression of SERCA2 and Phospholamban Phosphorylation**

Hearts were frozen in liquid nitrogen and stored at −80°C. Samples were homogenized in modified radioimmunoprecipitation assay buffer with protease and phosphates inhibitors (Upstate). SDS-PAGE was performed using 8% acrylamide gels (SERCA2a) and 4% to 20% acrylamide gradient gels (phospholamban [PLB]). Proteins were transferred to nitrocellulose membranes, blocked with 5% nonfat milk, and incubated with anti-PLB (1:2000; Upstate), anti-phospho-PLB Ser16 (Upstate; 1:1000), anti-phospho-PLB Thr17 (1:2500; Badrilla-UK) or anti-SERCA2a (1:2000; ABR) antibodies. Following incubation with primary antibody, membranes were incubated in horseradish peroxidase–coupled secondary antibodies (Amer sham) (1:5000 for anti-phospho-PLB and 1:10000 for anti-PLB and anti-SERCA2a) followed by ECL detection (Pierce). Membranes incubated initially in anti-phospho-PLB antibody were stripped using a commercial stripping buffer (Pierce), and the same membranes were probed using an anti-PLB antibody. To assess the levels of expression of PLB and SERCA2a, densitometric analysis (Personal densitometer; Amersham Biosciences) was performed using the DataQuaNT software.

**Statistical Analysis**

All results are presented as mean ± SE. The statistical significance of differences between groups of NTG and TG mice was determined by one-way or one-way repeated-measures ANOVA followed by the Student–Newman–Keuls test, as appropriate. A value of P < 0.05 was considered significant.

**Results**

**Effect of Hypercapnic Acidosis on LV Systolic and Diastolic Function in the Absence of Propranolol**

Figure 1A shows a summary of the changes in FS in NTG (n = 5) and TG (n = 5) mice during the baseline conditions (100% O2), hypercapnic acidosis (35% CO2), and recovery (100% O2) assessed by ECHO. FS was significantly higher in
TG compared with NTG mice throughout most of the experimental protocol. In NTG mice during hypercapnic acidosis, FS dropped from 47.3±2.1% at baseline (20 minutes of 100% O₂) to 27.8±1.0% at 5 minutes of CO₂ and returned to the baseline level at 20 minutes of CO₂ (43.4±3.0%). During recovery, there was a transient increase in FS that reached 63.0±2.2% at 5 minutes of recovery time.

In TG mice, hypercapnic acidosis resulted in an increase in FS throughout the exposure to CO₂. During baseline conditions, FS was 51.0±2.4% (20 minutes of 100% O₂) and increased to 74.2±1.0% at 20 minutes of hypercapnic acidosis. Moreover, FS stayed significantly elevated during the recovery time in TG mice. At 20 minutes of recovery, FS was 70.4±4.6% in TG and 55.6±4.0% in NTG mice. Figure 1B shows representative M-mode ECHO recordings of the LV in NTG and TG mice at 20 minutes of 100% oxygen (baseline conditions) and at 5 minutes of 35% CO₂. Under baseline conditions, FS was 50% in NTG and 48% in TG hearts; however, after 5 minutes exposure to CO₂, FS dropped to 29% in NTG and increased to 72% in TG hearts in this experiment.

Diastolic function was assessed using the pulsed Doppler ECHO. Figure 2 shows the E-to-A wave ratios (E/A) (Figure 2A), E-wave DTs (Figure 2B) and LV isovolumic relaxation times (IVRT) (Figure 2C) at 20 minutes of baseline conditions, 20 minutes of hypercapnic acidosis, and 20 minutes of recovery. At all time points during the experiments, the E/A ratios were significantly lower in TG compared with NTG hearts. In TG mice, the E-wave DT was prolonged at 20 minutes of baseline conditions and 20 minutes of hypercapnic acidosis (Figure 2B). LV IVRT was the same in NTG and TG mice during baseline conditions but was longer in TG compared with NTG at 20 minutes of 35% CO₂ (Figure 2C). In TG hearts, LV IVRT was longer at 20 minutes of CO₂ than
in baseline and recovery conditions. Figure 2D shows representative examples of pulsed Doppler mitral flow recordings in NTG and TG mice at baseline level and at 20 minutes of 35% CO₂. At baseline the E/A ratios were 1.3 in NTG and 1.0 in TG hearts and at 20 minutes of CO₂, decreased to 1.2 in NTG and 0.9 in TG hearts, respectively. E-wave DT was 25 ms in NTG and 31 ms in TG hearts at baseline and decreased to 21 ms in NTG and 29 ms in TG hearts at 20 minutes of CO₂. LV IVRTs were the same at baseline (15 ms in NTG and 15 ms in TG hearts) but at 20 minutes of CO₂, increased only in TG heart to 23 ms.

**Effect of Hypercapnic Acidosis on LV Systolic and Diastolic Functions in the Presence of Propranolol**

To determine whether the response from the sympathetic nervous system is critical for the survival of NTG and TG mice during the hypercapnic conditions, we blocked the β-adrenergic receptors by continuous infusion of propranolol. As illustrated in Figure 3A, in the presence of propranolol (at 20 minutes of propranolol), FS decreased from a baseline (20 minutes of O₂) value 42.0 ± 1.9% to 25.2 ± 1.9% in NTG mice (n=5) and from 51.6 ± 3.4% to 27.6 ± 2.8% in TG mice (n=5). In NTG mice, switching to 35% CO₂ resulted in a precipitous, sudden decrease in FS, and by 4 minutes all five NTG mice had died. However, in TG mice, FS decreased only slightly initially, and during 15 to 20 minutes of hypercapnic acidosis, they recovered fully to the baseline level. After switching back to 100% oxygen, FS transiently increased to 68.0 ± 1.5% (5 minutes of recovery) and then returned to a level slightly below FS in baseline conditions. Figure 3B presents M-mode ECHO recordings of the LV in NTG and TG mice during exposure to hypercapnic acidosis in the presence of propranolol. The image of the LV in the NTG mouse was taken at the moment of death, 4 minutes after switching to 35% CO₂. Under the same conditions, however, there was normal LV systolic function in TG mouse. The M-mode ECHO recordings of LV in TG mouse is presented 7 minutes after the onset of ventilation with 35% CO₂.

The effects of propranolol on LV diastolic function of TG mice are presented in Figure 4. There was no difference in the
Infusion of propranolol resulted in significant decreases in HRs in NTG and TG mice.

Table 1 summarizes the effect of hypercapnic acidosis on heart rate (HR) in the presence and absence of propranolol. In surviving mice, HR was not significantly different between NTG and TG animals (Table 2). Hypercapnic acidosis resulted in a significant decrease in blood pH, an increase in PCO2, and a decrease in PO2 in both groups of animals (Table 2), but there was no significant difference between NTG and TG animals.

Effect of Hypercapnic Acidosis on TnI and PLB Phosphorylation

Because hypercapnia is associated with stimulation of the sympathetic nervous system and activation of protein kinases, in the next series of experiments we determined the states of TnI and PLB phosphorylation in NTG and TG groups of mice.

Blood Gas Analysis During Hypercapnic Acidosis

Under baseline conditions blood pH, Pco2, and PO2 were not different between NTG and TG animals (Table 2). Hypercapnic acidosis resulted in a significant decrease in blood pH, an increase in Pco2, and a decrease in PO2 in both groups of animals (Table 2), but there was no significant difference between NTG and TG animals.

Table 2. Blood Gas Analysis

<table>
<thead>
<tr>
<th>Gas</th>
<th>NTG</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (100% O2)</td>
<td>4</td>
<td>7.30±0.05</td>
</tr>
<tr>
<td>Hypercapnic acidosis (35% CO2)</td>
<td>4</td>
<td>7.44±0.05</td>
</tr>
</tbody>
</table>

Hypercapnic acidosis (35% CO2)

<table>
<thead>
<tr>
<th>NTG</th>
<th>3</th>
<th>6.61±0.03*</th>
<th>269.2±67.4*</th>
<th>237.0±89.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG</td>
<td>3</td>
<td>6.63±0.02*</td>
<td>247.6±20.1*</td>
<td>282.7±39.5*</td>
</tr>
</tbody>
</table>

*Significant difference vs baseline within group (P<0.05). All data are presented as mean±SE.
mice expressing cTnI. To the best of our knowledge, our work is also the first to show that effects of hypercapnic acidosis can be studied directly in vivo.

Differences in the response to acidic pH among various types of muscles lie in the isofrom of myofilament proteins. Donaldson et al.²⁰ reported the first evidence that effects of acidosis on Ca²⁺-force relations of isolated myofilaments differ among fast, slow, and cardiac muscle. Early studies also demonstrated that Ca²⁺ activation of neonatal heart myofilaments is relatively insensitive to deactivation by acidic pH, when compared with adult myofilaments,² and that this differential effect is localized in thin filament proteins.¹ With identification of ssTnI as the neonatal isoform,²¹ we set out to determine whether this isoform switch is responsible for the differential effect of acidosis.⁷ Definitive evidence that this is indeed the case came from investigations of myofilaments from TG mice in which ssTnI completely replaced cTnI.⁸ Wolska et al.²² reported that Ca²⁺-force relations of skinned fiber bundles from hearts of the ssTnI-TG mice were less right shifted in acidosis than the NTG controls. With acidosis, there was also a significant and sustained fall in tension of electrically stimulated NTG papillary muscles but no effect in the TG muscles.

Various regions of TnI have been proposed to be responsible for the differential response to acidic pH between ssTnI and cTnI. Studies using different chimeras composed of regions of cTnI and ssTnI suggest that a pH-sensitive domain may reside in the carboxyl terminus⁵ or in the inhibitory portion of TnI.¹⁰ However, more recent studies have localized a specific amino acid (Ala162) of cTnI as largely responsible for the differential response to acidosis.⁵ When Ala162 was replaced with His (the corresponding ssTnI amino acid), the Ca²⁺ sensitivity of ATPase activity of reconstituted myofilament preparations at pH 6.5 was restored to that at pH 7.0. An important question, which we have addressed here, is the relative significance of these changes in hearts in vivo. Apart from the differences between ssTnI and cTnI in their response to acidosis, there are other differences between the two isoforms that may play a role in its ability to protect the myocardium from deactivation by acidic pH. One major difference between cTnI and ssTnI is the presence of a 32-amino acid N-terminal extension in cTnI.²² This extension contains two serines at positions 22 and 23, which are phosphorylated by protein kinase A.²³ Phosphorylation of these sites results in a decrease in myofilament sensitivity to Ca²⁺ and contributes to the enhanced rate of relaxation in situ during β-adrenergic stimulation.¹²,¹⁴,²⁴,²⁵

During acidosis, in the absence of the β-adrenergic blocker propranolol, we observed the complete recovery of FS in NTG hearts and an increase in FS in TG hearts. The mechanical recovery during acidosis has been previously reported by us⁸ and others,⁶,²⁵,²⁶ and can be explained by partial recovery of intracellular pH and/or an increase in Ca²⁺ transient amplitude attributable to increased sarcoplasmic reticulum (SR) Ca²⁺ load as a consequence of increased activity of Na/H exchanger, inhibition of Na/K pump, and phosphorylation of PLB. Our current data demonstrate that acidosis in the absence of propranolol significantly increases the level of phosphorylation of PLB (Figure 6). The phosphorylation of PLB releases SERCA2a inhibition and results in an increased SR Ca²⁺ uptake and increased Ca²⁺ transient. However, at the same time, acidic pH directly suppresses the SR CaATPase activity; therefore, the net effect of SERCA2a activity depends on multiple factors. It has been reported in isolated cells that the Ca²⁺ transient is significantly increased not only during acidosis but also stays elevated after switching to solutions with normal pH.⁶,²⁷ Small transient increases above the baseline value (before acidosis) in intracellular pH have also been reported during the first few minutes after switching to control solution.⁶,²⁷,²⁸ These alterations in pH and Ca²⁺ transients can significantly influence the recovery kinetics of FS and may have more profound effects on TG mice because their myofilaments are more sensitive to Ca²⁺.⁸ During recovery, intracellular pH comes to a baseline value faster than dephosphorylation of PLB and myofilament regulatory proteins that were phosphorylated during hypercapnic acidosis. In Langendorff perfused hearts stimulated with isoproterenol, cTnI stayed phosphorylated after 15 minutes perfusion with drug-free solution, whereas, at the same time, PLB was almost completely dephosphorylated.²⁹

Our data indicate that during hypercapnic conditions in situ, reflex activation of the sympathetic nervous system is critical for survival of NTG mice. Because we used propranolol, a specific β-adrenergic receptor blocker, the increase in the FS during hypercapnic acidosis in TG mice is most likely
attributable to stimulation of α-adrenergic receptors, an increase in Ca\(^{2+}\) transient amplitude, and partial recovery of intracellular pH. Stimulation of α-adrenergic receptors by phenylephrine in Langendorff perfused mouse hearts in the presence of a β-blocker results in positive inotropic effect.\(^{30,31}\) Moreover, it has been demonstrated in swine that acute hypercapnia was associated with release of both nor-epinephrine and epinephrine.\(^{32}\) Plasma norepinephrine concentration increased 3.4-fold and epinephrine increased 1.8-fold compared with basal concentrations. A similar increase in catecholamine levels was most likely present in our experimental conditions. Our functional (ECHO) and biochemical (TnI and PLB phosphorylation) data indicate that during hypercapnic conditions, there is a significant release of catecholamines that stimulate both adrenergic receptors in the absence of propranolol and α-adrenergic receptors in the presence of propranolol. In NTG hearts, which demonstrate a more significant reduction of myofilament sensitivity to Ca\(^{2+}\) during acidosis than TG hearts, the contribution of α-receptors is apparently too small to prevent mice from death. Interestingly, in the presence of propranolol, acidosis decreases HR in TG mice, despite an increase in FS. In these conditions, HR is a net result of at least two opposite effects: stimulation of α-adrenergic receptors and acidification, which is known to decrease the frequency of spontaneous beating of the sino-atrial node.\(^{33}\)

The rate of cardiac relaxation is influenced by both myofilaments properties and Ca\(^{2+}\) decay. We have previously shown in vitro that myofilaments from TG mice expressing ssTnI demonstrated higher myofilament sensitivity to Ca\(^{2+}\) than control mice, which resulted in an impairment of diastolic function in TG mice.\(^{8,14}\) This was confirmed in the experiments reported here and manifested by a smaller E/A ratio and longer E-wave DT in TG mice. Our data also support the more general hypothesis that alteration in myofilament response to acidosis is the major factor responsible for depression of contractility in acidosis. These data indicate that ssTnI, which is expressed in fetal and neonatal myocardium, plays a critical function in protecting immature hearts in acidic conditions from significant decreases in contractility caused by myofilament desensitization. Our data also support the hypothesis that alteration in myofilament response to Ca\(^{2+}\) is a major variable affecting cardiac function.

**Acknowledgments**

This research was supported by NIH research grants RO1 HL-64209 (to B.M.W.), R37 HL-22231 (to R.J.S.), and PO1 HL-62426 (to R.J.S.). B.M.W. is an Established Investigator of the American Heart Association.

**References**


Expression of Slow Skeletal Troponin I in Adult Mouse Heart Helps to Maintain the Left Ventricular Systolic Function During Respiratory Hypercapnia
Dalia Urboniene, Fernando A.L. Dias, James R. Peña, Lori A. Walker, R. John Solaro and Beata M. Wolska

Circ Res. published online June 16, 2005;

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circres.ahajournals.org/content/early/2005/06/16/01.RES.0000173849.68636.1e.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/