Characteristics of Binding of Endothelin-1 and Endothelin-3 to Rat Hearts
Developmental Changes in Mechanical Responses and Receptor Subtypes

Tomohisa Ishikawa, Liming Li, Osamu Shinmi, Sadao Kimura, Masashi Yanagisawa, Katsushi Goto, and Tomohisa Masaki

Endothelin (ET) is potent vasoconstrictor peptide containing 21 amino acids, which was originally isolated from the conditioned medium of cultured endothelial cells.\(^1\) Recently, genomic DNA analysis has suggested that mammals, including humans, produce three distinct members of this peptide family, namely, ET-1 (identical to the originally isolated ET), ET-2, and ET-3.\(^2\) The biological activity of ET-2 is similar to that of ET-1, whereas the activity of ET-3 is somewhat different.\(^2\)\(^-\)\(^4\) Since the original report\(^1\) of the vasoconstricting capabilities of ET, ET-1 has been reported to initiate a wide variety of pharmacological activities in various tissues.\(^5\) In the heart, ET-1 produces positive inotropic and chronotropic effects.\(^6\)\(^-\)\(^9\) Specific binding sites for ET-1 have been identified in a variety of tissues and species, including humans.\(^10\) Binding to these sites is both saturable and temperature sensitive.\(^11\)\(^12\) Binding of \(^{125}\)I-ET-1 binding is competitively inhibited by unlabeled ET-1, ET-2, and ET-3 as well as sarafotoxin S6b, a chemically homologous toxin from the venom of the burrowing asp, Attractaspis engaddensis.\(^10\) Specific binding sites for ET-1 have also been identified in rat heart.\(^11\) Moreover, intriguing data have been presented that suggest that ischemia is accompanied by an increase in the density of \(^{125}\)I-ET-1 binding sites in the rat heart.\(^12\)

Many reports\(^13\)\(^-\)\(^17\) have described the developmental changes in the pharmacological and biochemical properties of various bioactive substances in the heart, such as \(\alpha\) and \(\beta\)-adrenoceptor agonists, muscarinic cholinoreceptor agonists, and \(\text{Ca}^{2+}\) channel antagonists. Although publications concerning the
actions of ET-1 on adult cardiac muscle preparations have appeared in recent years, developmental changes have not been clarified. Rats are known to be immature at birth with respect to many physiological functions; therefore, they have been used frequently as a model to study developmental physiology.

The objectives of the present study are to 1) compare two ET isoforms, ET-1 and ET-3, on the mechanical responses in the rat atria and ventricles, 2) characterize ET receptors in the rat atria and ventricles, and 3) investigate the developmental changes that occur in mechanical response and receptor binding with these two ET isoforms.

Materials and Methods

Materials

Drugs used were human ET-1 and ET-3 (Peptide Institute, Osaka, Japan); isoproterenol hydrochloride, bovine serum albumin (BSA, fraction V), and phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical Co., St. Louis, Mo.); aprotinin (Bayer, Leverkusen, FRG); chloramine T (Wako Pure Chemicals, Osaka, Japan); and MOPS (Dojin, Kumamoto, Japan). ET-1 and ET-3 were dissolved in a phosphate-buffered saline (pH 7.4) containing 0.05% BSA.

Measurement of Contraction and Beating Rate

Adult male (8-week-old) and neonatal (3–7–day-old) Wistar rats were anesthetized with pentobarbital sodium intraperitoneally, and the hearts were quickly removed and immersed in an ice-cold Krebs-Ringer solution of the following composition (mM): NaCl 113, KCl 4.8, CaCl₂ 2.2, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25, and glucose 5.5.

The beating rate and isometric contraction were measured as described previously. Briefly, spontaneously beating right atria of both the neonate and the adult, the left ventricular wall of the neonate, and the left atrium and a piece (2 x 15 mm) of the right ventricular wall of the adult were isolated. The right ventricular wall of the adult was used because constant contractions could not be obtained in the left one. This is probably because the wall of the left ventricle was too thick for oxygen to reach the center of the muscle. The preparations were suspended in siliconized glass organ baths containing Krebs-Ringer solution, which was maintained at 37°C and aerated with a mixture of 95% O₂–5% CO₂. The resting tensions applied were 0.25 g for the neonatal preparations and 1 g for the adult preparations. The left atrial and the ventricular preparations were placed between a pair of platinum electrodes and were electrically driven at 3.3 Hz (atria) or 1 Hz (ventricles) with square-wave pulses of 1-msec duration and of an intensity sufficient to elicit contractions (usually ~1 V). Contractions were not recorded from segments of isolated left atria of the neonate, since they were too small and easily torn off when they were tied with threads.

Membrane Preparation

For each preparation, seven to 10 hearts from the adult or 32–64 hearts from the neonate were used. The isolated heart from the adult was initially perfused through the aorta with Krebs-Ringer solution to flush out any remaining blood. Membranes were prepared according to the protocol of Kwan et al with some modifications. The atria (except for that portion containing the sinoatrial node) or ventricular walls were removed from the hearts and immersed in an ice-cold homogenizing buffer of 10 mM MOPS, 0.25 M sucrose, 0.1% BSA, 0.1 mM PMSF, and 100 units/ml aprotinin (pH 7.2). A homogenate of the tissue was prepared with a polytron-type homogenizer at three-fourths maximum speed for two 30-second periods. After successive centrifugation of the homogenate at 600g for 10 minutes and at 8400g for 10 minutes, the supernatant was decanted and centrifuged at 100,400g for 60 minutes. All centrifugations were performed at 4°C. The resultant pellet was resuspended in the ice-cold homogenizing buffer. Membrane preparations were immediately used in binding assays without freezing. The protein content was determined by the method of Lowry et al with BSA as the standard.

Iodination of ET-1 and ET-3

ET-1 or ET-3 was radioiodinated using chloramine T. Briefly, ~2 nmol peptide and 1 mCi ¹²⁵I-labeled sodium (Amersham Corp., Arlington Heights, Ill.) were added to 100 µl sodium phosphate buffer (PB) (0.5 M, pH 7.4). Oxidation was started at room temperature by adding 20 µl freshly prepared chloramine T solution (26 mg/10 ml PB) and stirring for 10 seconds; oxidation was stopped by adding 50 µl sodium metabisulfite solution (37 mg/10 ml PB). After the addition of 1 ml of 0.5% BSA solution, the reaction mixture was loaded onto a Sep-Pak C₁₈ cartridge (Waters Associates, Milford, Mass.) and eluted with 5 ml of 0.1% trifluoroacetic acid containing 50% acetonitrile. The monoiiodinated ET-1 or ET-3 was purified by reverse-phase high-performance liquid chromatography (4.6 × 250-mm Chemosorb 50DS-H, Chemco). The elution time of ¹²⁵I-ET-3 used in the present study was identical with that of a commercially purchased (3,¹²⁵Iiodotyrosine)²⁰ET-3 (Amersham).

Binding Studies

¹²⁵I-ET-1 or ¹²⁵I-ET-3 binding was performed in siliconized glass tubes. The final assay volume of 250 µl contained incubation media (10 mM MOPS, 0.25 M sucrose, 10 mM MgCl₂, 0.1% BSA, 0.1 mM PMSF, and 100 units/ml aprotinin [pH 7.2]) and varying concentrations of radiolabeled ligands for saturation experiments. In competition experiments, 100 pM radiolabeled ligands was added to the incubation media along with varying concentrations of unlabeled ligands unless otherwise stated. The concentration of ¹²⁵I-ET-1 or ¹²⁵I-ET-3 used in the saturation experiments was ≤0.50 nM. Unlabeled ET-1 or ET-3 was
added to radiolabeled ligands in concentrations higher than those of ET-1 or ET-3. The binding reaction was initiated by the addition of the ventricular or atrial membrane preparation (50 μg protein/tube) to the incubation media. The reaction mixture was incubated for 2 hours at 25°C and then filtered under vacuum through GF/F filters (Whatman Inc., Clifton, N.J.) pretreated with 1% ethylenimine. The filters were washed four times with 5 ml ice-cold 10 mM Tris-HCl (pH 7.4) containing 0.5% BSA and counted for radioactivity in a gamma counter (model ARC-605-2, Aloka). Specific binding was defined as the difference in binding between total binding and nonspecific binding; the latter was determined as the radiolabeled ligand binding in the presence of a large excess of unlabeled ligand (1 μM).

Preliminary studies were performed to determine optimal binding conditions. The binding of 125I-ET-1 and 125I-ET-3 to the microsomal preparation increased linearly with protein concentrations between 25 and 100 μg/250 μl and was unchanged between 1.5 and 3 hours of incubation at 25°C (data not shown). Based on these data, all binding assays were carried out for 2 hours at 25°C with 50 μg membrane proteins in 250 μl incubation media.

Data Analysis

Saturation binding experiments were analyzed with Scatchard plots for determination of the dissociation constant (Kd) and the number of binding sites (Bmax). A weighted, nonlinear least-squares program, which was programmed using the NLIN procedure (SAS Institute Inc., Cary, N.C.) was used to fit a two-site model. The data are expressed as mean±SEM. Statistical significance was determined using an unpaired Student’s t test. A value of p<0.05 was considered statistically significant.

Mechanical Response

ET-1 and ET-3 caused positive inotropic and chronotropic responses in spontaneously beating right atria isolated from both adult and neonatal rats (Figures 1 and 2). Both ET-1 and ET-3 began to elicit a chronotropic response at low concentrations (i.e., ~10 pM). The potencies of the chronotropic effects of ET-1 and ET-3 were not significantly different. The maximum chronotropic responses with ET-1 and ET-3 were quite small (only 16–32% of the maximum response to isoproterenol) and were attained at 30 nM of either agonist. With concentrations of ET-1 and ET-3 >30 nM, there was actually less enhancement of the chronotropic response. The inotropy of ET-1 and ET-3 in the right atria was not analyzed statistically, since myocardial contractility is known to change with changes in beating rate.

ET-1 and ET-3 induced concentration-dependent, positive inotropic responses in the electrically driven left atria of the adult (Figures 1 and 3C). These responses developed slowly and continued for at least an hour after repeated washing with fresh Krebs-Ringer solution. The maximal responses to ET-1 and ET-3 were 59.4±5.7% and 46.3±3.5%, respectively,
of the maximal response to isoproterenol. The half-maximal effective concentration (EC\textsubscript{50}) of ET-1 was 0.47 nM, which was about fivefold lower than that of ET-3 (2.2 nM).

In the electrically driven adult right ventricular preparation, concentrations of ET-1 and ET-3 ≤300 nM caused very weak (maximum response 7–20\% of that to isoproterenol) and no positive inotropic response, respectively (Figures 1 and 3B). In contrast, in the neonatal left ventricle, both ET-1 and ET-3 caused marked positive inotropic responses (Figures 1 and 3A). Although the concentration–response curves for ET-1 and ET-3 in neonatal ventricles plotted in Figure 3A continue to rise for concentrations ≤300 nM, it was confirmed in some experiments that a higher concentration (500 nM) of either ET did not produce a further increment in contraction. Thus, 300 nM was estimated to be a maximal concentration of either ET in neonatal ventricle. The EC\textsubscript{50} value of ET-1 (23 nM) was significantly lower than that of ET-3 (52 nM).

**Endothelin Binding on Neonatal Preparations**

Saturation studies of \textsuperscript{125}I-ET-1 and \textsuperscript{125}I-ET-3 binding to the atrial and ventricular membranes from the neonate are shown in Figure 4. Binding in each case approached saturation (Figures 4A and 4C). \textsuperscript{125}I-ET-1 binding differed from \textsuperscript{125}I-ET-3 binding in that the former resulted in a linear Scatchard plot (Figure 4B), whereas the latter resulted in a curvilinear plot (Figure 4D). This was true for both the atrial and the ventricular membranes. Computer analysis of the
$^{125}$I-ET-3 binding yielded an optimal fit to a two-site model with high- and low-affinity sites. The $K_d$ for $^{125}$I-ET-1 fell in between the high- and low-affinity sites for $^{125}$I-ET-3. The $B_{max}$ values for $^{125}$I-ET-1 in atria and ventricles were comparable to those of the $^{125}$I-ET-3 low-affinity site, whereas the $B_{max}$ values for the $^{125}$I-ET-3 high-affinity site were significantly less (Table 1).

The results of competitive binding inhibition studies for the atrial and ventricular membranes from the neonate are shown in Figure 5. The $^{125}$I-ET-1 binding in both atria and ventricles was completely displaced by either unlabeled ET-1 or ET-3 in a concentration-dependent manner, although ET-1 was more potent than ET-3 (Figures 5A and 5C). Unlabeled ET-1 and ET-3 also inhibited $^{125}$I-ET-3 binding in both the atria and the ventricles. In this case, however, ET-1 and ET-3 exhibited a similar potency for displacing $^{125}$I-ET-3 (Figures 5B and 5D).

**Endothelin Binding on Adult Preparations**

The concentration-dependent, saturable binding of $^{125}$I-ET-1 and $^{125}$I-ET-3 to the atrial and ventricular membranes from the adult are shown in Figure 6. The results of the Scatchard analyses for radioligand binding in the adult atrial membranes are similar to those in the neonatal membranes, a linear plot for $^{125}$I-ET-1 binding and a curvilinear plot for $^{125}$I-ET-3.

**Table 1. Scatchard Analyses from Saturation Assays of Binding Sites for Endothelin-1 and Endothelin-3 on Atrial and Ventricular Membranes of Adult and Neonatal Rats**

<table>
<thead>
<tr>
<th></th>
<th>$^{125}$I-Endothelin-1</th>
<th>$^{125}$I-Endothelin-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_d$ (pM)</td>
<td>$B_{max}$ (fmol/mg protein)</td>
</tr>
<tr>
<td>Adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atria</td>
<td>442±40</td>
<td>1,267±120</td>
</tr>
<tr>
<td>Ventricles</td>
<td>59.3±6.6</td>
<td>330±18</td>
</tr>
<tr>
<td>Neonate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atria</td>
<td>655±25</td>
<td>3,561±120</td>
</tr>
<tr>
<td>Ventricles</td>
<td>603±26</td>
<td>2,936±80</td>
</tr>
</tbody>
</table>

Values are mean±SEM of three to five separate experiments. High, high-affinity site; Low, low-affinity site.

**Figure 4.** Saturation analysis of $^{125}$I-endothelin-1 ($^{125}$I-ET-1, panels A and B) and $^{125}$I-endothelin-3 ($^{125}$I-ET-3, panels C and D) in atrial (●) and ventricular (○) membranes of the neonatal rat. Specific binding of $^{125}$I-ET-1 and $^{125}$I-ET-3 is plotted in panels A and C, respectively (nonspecific binding as defined in “Materials and Methods” has been subtracted). In panels B and D, Scatchard plots of the data in panels A and C, respectively, are shown, with B/F indicating the bound/free ratio. These plots are representative of three separate preparations, each performed in duplicate. $K_d$ and $B_{max}$ are summarized in Table 1.
binding. Computer analysis of the $^{125}$I-ET-3 binding yielded an optimal fit to a two-site model with high- and low-affinity sites (Figures 6B and 6D). The adult ventricular membranes differed from the other preparations studied in that both $^{125}$I-ET-1 binding and $^{125}$I-ET-3 binding resulted in linear Scatchard plots (Figures 6B and 6D). The $K_d$ for binding of $^{125}$I-ET-1 to adult ventricular muscle was significantly less than that for $^{125}$I-ET-3 binding, whereas their $B_{max}$ values were almost the same (Table 1).

Competition experiments demonstrated that $^{125}$I-ET-1 binding to the adult atrial and ventricular membranes could be completely displaced by both unlabeled ET-1 and ET-3. $^{125}$I-ET-3 binding was also completely displaced by both ET-1 and ET-3 (data not shown). The displacing activities of ET-1 for both $^{125}$I-ET-1 and $^{125}$I-ET-3 binding sites were more potent than those of ET-3 in both preparations.

**Discussion**

Several studies\(^6\)-\(^9\) have demonstrated that ET-1 induces positive chronotropic and inotropic responses in isolated atrial preparations. The present studies indicate that not only ET-1 but also ET-3 exerts positive inotropic and chronotropic effects on the rat atria. The inotropic action of ET-3 was less potent than that of ET-1, whereas the chronotropic action of ET-3 was equipotent to that of ET-1, suggesting the possibility that the ET receptor mediating the chronotropic response is different from that mediating the inotropic response. The inotropic response to ET-1 in the ventricles of the adult rat was also observed, but it was weak compared with that in the atria, which is in agreement with the previous studies in rats and humans.\(^8\),\(^9\) No inotropic effect of ET-3 was observed in the adult rat ventricles.

ET-1 and ET-3 also exhibited positive inotropic and chronotropic effects on the neonatal right atria. The chronotropic response in atria did not change during development; that is, the potencies of ET-1 and ET-3 in both the neonate and the adult were very similar, and the effective concentration ranges in the neonate were almost equal to those in the adult. These results suggest that ET-1 and ET-3 bind with similar affinity to ET receptors that mediate the chronotropic response and that these receptors change little with development. The sinoatrial node (which initiates the chronotropic response in the intact tissue) was not included in membrane preparations in the present study; thus, the results of binding experiments do not provide any direct evidence with respect to the receptor responsible for the chronotropic actions of ET-1 and ET-3 in the isolated right atria.
In the neonate, the binding of ET-1 and ET-3 in the atria and the ventricles showed similar characteristics. The Scatchard analyses produced a linear plot for ET-1 binding and a curvilinear plot for ET-3 binding. On the other hand, in competitive experiments ET-1 was approximately equipotent with ET-3 in displacing 0.1 nM of 125I-ET-3 binding, most of which would bind to the high-affinity binding site, in both the atria and ventricles. The binding of a higher concentration of 125I-ET-3 (1 nM) is also completely displaced by ET-1 in neonatal ventricular membranes (authors’ unpublished observations). Interestingly, displacement of the higher concentration of 125I-ET-3 by ET-1 produced a biphasic inhibition curve that would be compatible with the presence of two different ET binding sites. Although ET-1 and ET-3 were equipotent in displacing 125I-ET-3 in the present study, ET-1 was found to be more potent than ET-3 at displacing 125I-ET-1 binding sites. Thus, in the neonate, ET-1 and ET-3 both appear to bind to the same two sites, but they do so with different affinities. Taken together, we propose the following hypothesis: The neonatal cardiac membranes (both atria and ventricles) have two distinct binding sites for ET. One site has low affinity for ET isoforms and a high B<sub>max</sub>. This site binds ET-1 more avidly than ET-3. The other site has higher binding affinity for ET isoforms and a lower B<sub>max</sub>. This site binds ET-1 and ET-3 with similar potency. The existence of ET receptor subtypes with similar characteristics has been reported in renal glomerular cells. We have no complete explanation for why 125I-ET-1 binding to the neonatal membranes resulted in a linear Scatchard plot suggesting a single binding site. One possibility is that a high-affinity binding site for 125I-ET-1 may not have been detected with our assay; that is, there may have been insufficient points in our Scatchard plots at low concentrations of ET-1 to resolve into a two-site model. This same limitation may be applicable to the binding data with adult atrial membranes, where once again only one binding site for 125I-ET-1 was detectable from Scatchard plot analysis. Finally, unlike findings in the chick heart, our findings in the rat heart could provide no evidence for a third ET receptor subtype at which ET-3 had the highest affinity.

We have proposed two distinct receptor subtypes in rat hearts. However, it is also possible that the two binding sites detected here may be ascribed to two conformational states of a single receptor that is coupled to a GTP-binding protein (i.e., G protein). It is well known that the receptors that couple with G proteins, such as α<sub>1</sub>-adrenoceptors and angiotensin II receptors, can show two different affinity states (i.e., low- and high-affinity states). The low-affinity state is associated with the coupling of GTP to the G

**FIGURE 6.** Graphs showing saturation analysis of 125I-endothelin-1 (125I-ET-1, panels A and B) and 125I-endothelin-3 (125I-ET-3, panels C and D) in atrial (●) and ventricular (○) membranes of the adult rat. Specific binding for 125I-ET-1 and 125I-ET-3 are plotted in panels A and C, respectively (nonspecific binding as defined in “Materials and Methods” has been subtracted). In panels B and D, Scatchard plots of the data in panels A and C, respectively, are shown, with B/F indicating the bound/free ratio. These figures are representative of five (ET-1) and four (ET-3) separate preparations, each performed in duplicate. K<sub>d</sub> and B<sub>max</sub> are summarized in Table 1.
protein–receptor complex. Thus, when these receptors are coupled to nonhydrolyzable analogues of GTP, the agonist affinity is reduced.26–29 There is also evidence that G proteins are involved in the coupling of various Ca\(^{2+}\)-mobilizing receptors to phosphatidylinositol hydrolysis.30 Numerous reports31–36 have shown that ET-1 increases phospholipase C–mediated phosphatidylinositol hydrolysis. Finally, it has been reported that the binding of \(^{125}\)I-ET-1 to cultured vascular smooth muscle cells is attenuated by a nonhydrolyzable GTP analogue, guanosine 5′-O-(3-thiotriphosphate) (GTP\(_γ\)S).34 These findings provide evidence that ET receptors are coupled to a G protein. In contrast, it has been reported that GTP\(_γ\)S failed to alter \(^{125}\)I-ET-1 binding to rat renal papillary and glomerular membranes.22 In preliminary studies, we have also found that the binding of ET isoforms to rat cardiac membrane is not modified in the presence of 0.1 mM of GTP\(_γ\)S under the same assay condition as the present study (unpublished observation). Thus, we find it unlikely that the two binding sites shown in the present study are due to the changes in conformation of a single receptor coupled to a G protein.

Although we have discussed the role of ET receptors on cardiac muscle membranes, we cannot eliminate the possibility that the cell origin of the ET receptors detected here was some other cell type. When cardiac muscle is prepared for binding studies, many additional cell types are also present, such as neurons and smooth muscle cells. Many binding studies have indicated that numerous cell types contain ET receptors10 and that the receptor densities may be very different between these cell types. For example, the \(B_{\text{max}}\) of ET receptors in brain capillary endothelial cells is 100 times higher than that in vascular smooth muscle cells.35 Although cardiac sarcolemmal membranes contribute the greatest mass to the membrane preparation used in this study, the contamination of other cell types that potentially contain high densities of ET receptors may conceivably have a significant effect on the apparent \(K_d\) and \(B_{\text{max}}\) values obtained in this study.

If it is of great interest that the ET isoforms that we investigated produced large positive inotropic responses in ventricular preparations of the neonate but very weak or no responses in the adult ventricles. In contrast, these isoforms induced positive inotropic and chronotropic responses in both the neonatal and the adult atria. Therefore, the present studies indicate that the biological activity of ET isoforms changes most dramatically during development in the ventricles but that the responses of atria appear to be more stable. Our binding studies indicated that, corresponding to this change in biological activity with development, the characteristics of ET receptors changed much more dramatically in ventricles than in atria. Although the neonate ventricle exhibited two distinctly different binding sites for \(^{125}\)I-ET-3, we found evidence for only one site in the adult ventricle. Furthermore, there was a much greater reduction in the amount of binding sites for ET during development in ventricles than in atria. Thus, all these data taken together suggest that the decline of the positive inotropic effect of ETs in the ventricles during development is the result of changes in the characteristics of ET receptors.

**Acknowledgment**

We thank Dr. K.D. Keef, Department of Physiology, University of Nevada School of Medicine, Reno, Nev., for kindly reading the manuscript.

**References**


KEY WORDS • endothelin receptor • endothelin-3 • cardiac contractility • heart rate • development

Downloaded from http://circres.ahajournals.org/ by guest on October 22, 2017
Characteristics of binding of endothelin-1 and endothelin-3 to rat hearts. Developmental changes in mechanical responses and receptor subtypes.

T Ishikawa, L M Li, O Shinmi, S Kimura, M Yanagisawa, K Goto and T Masaki

Circ Res. 1991;69:918-926
doi: 10.1161/01.RES.69.4.918

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 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/69/4/918

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/