Effect of Amiodarone on the Expression of Myosin Isoforms and Cellular Growth of Cardiac Muscle Cells in Culture

Asish C. Nag, Mei Li Lee, and Deborah Shepard

The effect of amiodarone on the expression of myosin isoforms and the growth of neonatal rat cardiac muscle cells in culture was studied by native gel electrophoresis, assays of DNA and protein synthesis, and electron microscopy. Cardiac myocytes exposed to amiodarone in the absence of triiodothyronine (T3) showed predominant V1. When cardiac myocytes were exposed to amiodarone in the presence of T3, they expressed prevalent isomyosin V3, or both V3 and V1 equally. Supraphysiological concentration of T3 counteracted the effect of amiodarone on myocytes, showing the expression of predominant isomyosin V1. Amiodarone has inhibitory effects on DNA synthesis and differentiation of cardiac myocytes. Myocytes treated with amiodarone showed maximum labeling index with 11% labeled cells after day 1. Subsequently, the labeling indexes declined and on the third day ceased, as opposed to the control culture, which attained a peak in labeling index with 60% labeled myocytes on the third day. The labeling indexes declined, showing 11% labeled myocytes at the terminal time point. Myocytes treated with amiodarone lost most of the well-organized myofibrils and other organelles, and instead contained sparse, scattered segments of myofibrils, free myofilaments, many mitochondria with disrupted cristae, and autophagic vacuoles. The results demonstrated that amiodarone has a direct influence on the expression of isomyosin by cardiac myocytes. Furthermore, this drug has inhibitory and degrading effects on the growth and differentiation of cardiac myocytes. (Circulation Research 1990;67:51-60)

Amiodarone, a benzofuran derivative and a potent antiarrhythmic drug, has been used extensively for the treatment of cardiac arrhythmias and angina1,2 in many countries and recently was introduced for use in the United States. Studies of amiodarone's effect on the heart have offered two types of findings by different groups of investigators. One has suggested that the chronic treatment with amiodarone may have effects that resemble those of hypothyroidism.1-9 Another finding has indicated that both hypo- and hyperthyroidism occurred in some patients treated with amiodarone for cardiac arrhythmias or angina.10,11 Ikeda et al3 reported that amiodarone caused prolongation of repolarization in cardiac tissue. This type of repolarization was found to be an effect produced by thyroidectomy in rabbits.4 It was observed that simultaneous administration of thyroxine (T4) inhibited amiodarone-induced lengthening of repolarization of atrial and ventricular action potentials.5 In addition, amiodarone has been found to cause a number of characteristic features of hypothyroidism such as bradycardia,6,7 prolonged systolic time intervals,8 and a decrease in Ca2+-ATPase activity of cardiac myosin.9 Lindenmeyer et al7 have been able to revise the bradycardia by administration of supraphysiological doses of triiodothyronine (T3). Bagchi et al9 reported that amiodarone treatment for 6 weeks in rats resulted in lower heart weight, decreased atrial production of 14C-CO2 from labeled glucose, decreased myocardial Ca2+-ATPase activity, and more synthesis of V3 isomyosin compared with that of the control. These effects were similar to those observed in hypothyroid rats but were lesser in magnitude. Although the effects of amiodarone treatment suggest hypothyroidism, serum thyroid-stimulating hormone, serum T4 is increased during amiodarone treatment, and serum T3 remains in the normal range.9,10 Martino et al12 reported the occurrence of hypo- and hyperthyroidism after chronic treatment with amiodarone. Hyperthyroidism showed elevation in serum T3 or free T3 concentrations, and hypothyroidism was best diagnosed by showing an elevated serum thyrotropin concentration. Both hypo- and hyperthyroidism have been noted to occur in some patients treated with amiodarone when the patients with hypothyroidism...
had subnormal T₄ levels, and the patients with hyperthyroidism had elevated T₃ levels.¹³

Past studies have been carried out in the in vivo system where various endogenous factors might control the drug actions in the body. The present study examines the effect of amiodarone on cardiac muscle cells that have been cultured and exposed to amiodarone in the presence or absence of exogenous thyroid hormone. The specific objective of this study is to investigate the influence of amiodarone on the expression of myosin isoforms and cellular growth of cardiac muscle cells in culture in the presence or absence of thyroid hormone. The rationale for the presence or absence of thyroid hormone in the culture is to examine whether there is interaction between the drug and thyroid hormone in the expression of isomyosins and the growth of cardiac myocytes.

Materials and Methods

Cell Culture

Ventricles of 4- to 5-day neonatal rats were used for isolation of cells. The isolation procedure was the same as those of our previous studies.¹⁴,¹⁵ Briefly, the ventricular tissue mince was dissociated into single-cell suspension by incubation in 0.15% trypsin, 0.025% collagenase, 4% chicken serum, and 96% Ca²⁺- and Mg²⁺-free Tyrode’s solution. The ventricular cells were incubated in a basic medium containing 99% Eagle’s basal medium with Earle’s salts, 1% bovine serum albumin, norepinephrine (10⁻⁵ M/ml), insulin/transferrin/selenium mixture (0.1/100 ml), ascorbic acid (0.02 mg/ml), epidermal growth factor (10 ng/ml), 2% calf serum, and 1% penicillin/streptomycin mixture. To grow cells in the presence of amiodarone, at least 2% serum is needed. The cells were grown in three experimental conditions: one set of culture was grown in the above medium with amiodarone (30–40 μg/ml) (amiodarone was a gift of Dr. A. Urdang, Sanofi, New York); the second set of culture was grown in the above medium with T₃ (10⁻⁵ M/ml) and amiodarone with the same concentrations as above. The growth medium of the third set was the same as in the second one with the exception of the T₃ dose, which was three times the second set. The maximum dose of this drug was determined on the basis of experimentation that showed cell death at a higher concentration than the doses used. Controls for three sets of cell culture were grown in their respective culture media without amiodarone. The cells were plated at a density of 1×10⁶ cells per 35-mm dish and cultured for 7 days in an incubator in a humidified atmosphere of 5% CO₂ in air. The cultures were terminated at intervals of 4 and 7 days of culture for biochemical studies. Eleven experiments were carried out for these studies.

Preparation of Myosin and Cells

The cultured cells were scraped out of the plates with a plastic scraper at selected intervals as mentioned above. Myosin extraction was carried out following our previous method¹⁴ with certain modifications. Briefly, the cells were homogenized by a Dounce homogenizer (Corning Glass Works, Parkridge, Ill.) in a buffer containing (mM) NaCl 40, EGTA 5, Na₂HPO₄ 3, and phenylmethylsulfonyl fluoride 1 (pH 7.2). The homogenate was centrifuged at 7,974g for 20 minutes, and the pellet was immersed and centrifuged at 139,238g for 3 hours in a modified extraction solution containing 100 mM Na₂P₂O₇, 15 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin (pH 8.8). The supernatant was collected and used for electrophoresis. Myosin from intact neonatal and adult ventricles was prepared by extracting tissue homogenate with 20 vol of the above extraction buffer. Protein concentration was determined by the method of Bio-Rad (Richmond, Calif.).

Electrophoresis

Pyrophosphate gels were prepared following essentially our previous method¹⁴ with minor modifications that included 3.88% acrylamide and 0.12% bisacrylamide in a buffer containing 20 mM Na₂P₂O₇ (pH 8.8), 2 mM cysteine, and 10% glycerol (vol/vol). A prerun of 1 hour was carried out under conditions identical with those of electrophoresis with a constant 78 V. Myosin samples (100 μl) in 50% glycerol were loaded directly on the top of the gels, and electrophoresis was run overnight (16 hours) with a constant 90 V. Staining and densitometer tracing of gels were essentially the same as those in our previous studies.¹⁴,¹⁷

Assay of [³H]Thymidine Incorporation Into Heart Cells

Cells were continuously exposed to 1 μCi/ml [³H]thymidine (TdR) for 24 hours before termination of cultures. The cultures were terminated at intervals of 1, 2, 3, and 5 days of incubation, and cells were scraped out of the plates after rinsing in cold 0.01 M phosphate buffer (pH 7.5) containing 0.01 M sodium pyrophosphate. Subsequently, the cells were pelleted by centrifugation, suspended in sodium pyrophosphate, and solubilized in 0.05N NaOH. The solution was then assayed for radioactivity in toluene-based scintillation fluid in a Packard Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.) as described previously.¹⁸

Assay of Cellular Protein

The cells were collected from the culture dishes by gently scraping them out with a rubber policeman. The cells were then pelleted by centrifugation and subsequently suspended in phosphate-buffered saline followed by solubilization in 0.05N NaOH. Aliquots were taken for protein estimation using the Bio-Rad Coomassie blue assay. Data, expressed as micrograms of protein, are the means of determinations made on six or more individual tissue culture dishes.
**Autoradiography and Periodic Acid-Schiff Technique**

Cells were labeled with the same dose and length of exposure to $[^{3}H]Tdr$ as described above. The cells were fixed in cold formaldehyde:alcohol (1:9) for 24 hours at 4°C and subsequently processed for periodic acid-Schiff (PAS) technique and autoradiography as described previously. Cardiac muscle cells were identified by PAS technique that stains cardiac muscle cells for their glycogen content. For scoring labeling indexes, at least 200 cells were counted under a light microscope. Triplicate plates were used for each time point, and pooled results of five experiments were recorded as mean values for labeled or unlabeled cells with their standard deviation.

**Electron Microscopy**

The cells in monolayer were processed for electron microscopy after 1, 3, 5, and 7 days of culture, following our previous methods. Essentially, the methodology involves rinsing cultured cells in Tyrode’s solution several times and fixing them in half-strength Karnovsky’s fixative for 1.5 hours at room temperature. The cells then were rinsed in cacodylate buffer (pH 7.4) several times and post-fixed in 1% osmium tetroxide for 1.5 hours at 4°C. The fixed cells were embedded in situ in Epon 812 after dehydration. The embedding consisted of two stages. First, polymerization of Epon was carried out at 55°C overnight, and subsequently the embedded culture was peeled off the flask. Second, the peeled embedded culture was polymerized further at 60°C for 24 hours for proper hardening. Thin sections were prepared and stained with uranyl acetate and lead citrate. Sections were examined and photographed in a Philips Electronic Instruments 410 LS electron microscope (Mahwah, N.J.) operated at an accelerating voltage of 60 kV.

**Results**

Myosin from neonatal rat cardiac myocytes in culture was examined by pyrophosphate gel electrophoresis. The culture was exposed to amiodarone under three experimental conditions as follows: 1) myocyte culture was exposed to amiodarone (30–40 $\mu$g/ml) in the absence of $T_3$; 2) myocyte culture was exposed to amiodarone (30–40 $\mu$g/ml) in the presence of $T_3$ ($10^{-5}$ M); 3) myocyte culture was exposed to amiodarone (30–40 $\mu$g/ml) in the presence of three times the amount of $T_3$ in experiment 2. The optimum concentrations of amiodarone for cell culture were determined by experimentation. The cells did not survive in concentrations higher than 40 $\mu$g/ml.

Controls basically included two cultures: 1) myocytes grown in the absence of amiodarone and $T_3$ treatments; and 2) myocytes grown in the presence of $T_3$ treatment and the absence of amiodarone. In addition, myosin from cultured neonatal rat cardiac myocytes was compared with myosin of neonatal and adult rat cardiac myocytes in vivo.

**Myosin Isoform Profiles**

The cultured myocytes with treatment of the drug and without $T_3$ contained predominant isoform $V_1$ (Figure 1). The $V_2$ band was not as clear as those of isoforms $V_1$ and $V_3$. Although the expression of myosin isoform profiles in response to different doses of amiodarone was alike (Figure 1), the amount of myosin isoforms in cultured cardiac myocytes exposed to differential doses (30, 35, 40 $\mu$g/ml) of

**FIGURE 1.** Native gel electrophoresis patterns of myosin from a) intact 5-day neonatal rat ventricle, and b) 7-day control culture of neonatal rat cardiac myocytes, growth without amiodarone in the culture medium. Cells were isolated from the same source as above; c) 7-day culture with 30 $\mu$g/ml amiodarone culture medium, d) 7-day culture with 35 $\mu$g/ml amiodarone culture medium, and e) 7-day culture with 40 $\mu$g/ml amiodarone medium.

**FIGURE 2.** Electrophoretic profiles of myosin components in adult rat ventricle in vivo and neonatal cardiac myocytes in vitro. a) Intact adult rat ventricle, b) 7-day control culture grown in the presence of $10^{-3}$ M triiodothyronine ($T_3$) without amiodarone, c) 7-day culture grown in the presence of $10^{-5}$ M $T_3$ and 40 $\mu$g/ml amiodarone medium, and d) 7-day culture grown in the presence of $3 \times 10^{-3} M$ $T_3$ and 40 $\mu$g/ml amiodarone medium.
amiodarone was not alike. With the increased dose of the drug, there was an increase in \( V_1 \) content in cells grown without \( T_3 \) (Table 1). The intact ventricles from 5-day neonatal rats without the treatment of amiodarone expressed predominant myosin isoform \( V_1 \), whereas the cultured myocytes without treatment of the drug and \( T_3 \) (control culture 1) showed predominant isoform \( V_3 \) as observed in our previous studies.\(^{14} \)

When cardiac myocytes in culture were exposed to amiodarone in the presence of added \( T_3 \), the expression of myosin isoform \( V_3 \) was prevalent (Figure 2) or as strong as \( V_1 \) (Figure 3), unlike those of control myocytes grown in the absence of the drug and presence of added \( T_3 \) (control culture 2). The control expressed predominant myosin isoform \( V_1 \). This observation conformed with those of Bagchi et al.,\(^9 \) who reported preferential synthesis of \( V_3 \) isomyosin over the control using an in vivo system in which endogenous thyroid hormones were present. In this experiment, the isomyosin content was also found to be drug dose dependent, showing an increase in the isomyosin \( V_3 \) content with increased dose. Cardiac myocytes exposed to amiodarone (30–40 \( \mu \text{g/ml} \)) in the presence of triple the usual dose of \( T_3 \) expressed predominant isomyosin \( V_1 \) (Figure 2).

**DNA Synthesis in Heart Cells**

Because there is no significant difference in DNA synthesis between \( T_3 \) treated and untreated myocytes, the results discussed do not include \( T_3 \) treatment. DNA synthesis as determined by autoradiography of cardiac muscle cells grown in the presence or absence of amiodarone is presented in Figure 4. Initially, control cardiac myocytes grown in the absence of amiodarone showed approximately 15% labeled myocytes, whereas experimental myocytes grown in the presence of amiodarone exhibited 11% labeled myocytes after 24 hours of culture. With the continuation of the culture, however, the control cardiac myocytes exhibited a gradual rise in the number of labeled cells. The labeling index of control myocytes peaked on the third day, showing approximately 60% labeled myocytes. The labeling index declined to approximately 11% on the fifth day of culture (Figure 4). Cardiac myocytes exposed to amiodarone showed a sharp decline in labeling indexes after showing 11% labeled myocytes 24 hours after culture (Figure 4). The labeling of experimental myocytes ceased on the third day of culture, unlike that of the control culture, which showed labeled cells until termination of culture on the fifth day.

The profile of incorporation of \(^{[3]H}\text{TdR}\) by heart cells as determined by the scintillation counter is presented in Figure 5. The incorporation of \(^{[3]H}\text{TdR}\) into DNA of heart cells, which included cardiac muscle and nonmuscle cells without exposure to the drug, was significantly higher \((p>0.001)\) than that of the cells exposed to the drug. The results indi-
cated that the incorporation of $[^3]$H]Tdr per unit protein in the experimental cells declined sharply after 24 hours of incubation, showing minimal incorporation value on the terminal time point of the culture (Figure 5).

The cellular protein content at different time points of the culture is presented in Figure 6. The protein content in the control culture continued to increase throughout the culture period, whereas that of the experimental culture initially showed a slightly lower value than that of the control culture. Subsequently, the values for protein content in the drug-treated culture decreased significantly ($p > 0.001$) compared with those of the control culture.

Ultrastructural Organization of Cardiac Myocytes

Cardiac myocytes in culture exposed to amiodarone were examined with the electron microscope to determine differentiation of their myofibrils and other cellular organelles during different time periods of culture. Myocytes after 24 hours of culture in the presence of amiodarone exhibited abundant myofibrils, mitochondria, free ribosomes, polysomes, glycogen, and sarcoplasmic reticulum similar to those of controls grown in the absence of amiodarone. Unlike the controls, myocytes contained scattered autophagic vacuoles and whorls of phospholipid. Mitochondria of the experimental myocytes often contained small, hollow, dense circular profiles absent in the controls (Figure 7). As the culture continued, well-organized myofibril content of the experimental myocytes decreased significantly, showing scattered segments of myofibrils and free myofilaments in the sarcoplasm of the cells after 3 days of culture (Figure 8). In addition, these myocytes contained abundant free ribosomes and polysomes, autophagic vacuoles, and whorls of phospholipid. Many mitochondria exhibited disrupted cristae. The whorls of phospholipid content increased in number after 6 days of culture, showing amorphous matrix in the central region of the whorl (Figure 9). The myofibril content and other cellular organelles of cardiac myocytes at this terminal point of culture did not differ significantly from those of 3-day-old culture myocytes. In contrast to the experimental myocytes, the control myocytes after 7 days of culture contained abundant organized myofibrils, mitochondria, ribosomes, glycogen, and sarcoplasmic reticulum (Figure 10).

**Figure 4.** Quantitation of labeled cardiac myocytes grown in the presence or absence of amiodarone; 30 µg/ml amiodarone culture medium was used. The dose of $[^3]$H]thymidine and length of cell exposure to the radioactive isotope are described in "Materials and Methods." The standard deviation in different determinations did not exceed 10% of the mean.

**Figure 5.** The incorporation of $[^3]$H]thymidine (Tdr) into neonatal rat heart cells exposed to amiodarone-free or amiodarone-containing media; 30 µg/ml amiodarone medium was used. The dose of radioactive isotope and the length of exposure of cells to the isotope are discussed in "Materials and Methods." SD bars are included.
Discussion

The present study shows that treatment with amiodarone produced two main types of results concerning the expression of myosin isoforms. When cardiac myocytes were exposed to amiodarone in the absence of added T₃ in the medium, myocytes expressed predominantly myosin isoform V₁, whereas addition of T₃ in the medium containing amiodarone showed

![Figure 6](image1.png)

**FIGURE 6.** Quantitation of cellular protein in neonatal heart cells grown in the presence or absence of amiodarone. The concentration of amiodarone was 30 μg/ml medium. Values are mean ± SD of six determinations at each time point.

![Figure 7](image2.png)

**FIGURE 7.** Electron micrograph of a portion of cardiac muscle cell after 24 hours of culture in 30 μg amiodarone. The micrograph shows specifically abundant myofibrils and mitochondria along with dense circular profiles (Cp) in the mitochondria and a whorl of phospholipid (P) in the sarcoplasm. Mf, myofibril; Sr, sarcoplasmic reticulum; Rb, ribosomes. Magnification, ×21,420.
the preferential expression of isomyosin V₁. It appears that amiodarone probably competes with T₃ in T₃-receptor sites and thus counteracts the influence of T₃ on the expression of myosin isoform V₁ during the treatment of cardiac myocytes with amiodarone in the presence of T₃. This interpretation is in agreement with the idea that the intracellular inhibition of the conversion of T₄ to T₃ is not the ultimate mode of the action of the amiodarone effect on heart rate. It is thought that amiodarone interacts with T₃ at its receptor or somewhere later along the pathway from the T₃-receptor interaction to the final effect of T₃ on the heart rate. Furthermore, the present interpretations agree with the previous findings⁶ that patients receiving amiodarone had a slowing heart rate, which gradually increased after withdrawal of the drug. This previous report⁶ conforms with the interpretation of the present data that the drug probably competes with T₃ for receptor sites and thus inhibits the function of T₃ in the myocardium. After withdrawal of the drug, the receptor sites become available only to T₃, and the heart rate returns to its original state. Because amiodarone and T₃ apparently are competing for the common receptor sites, the sites probably are distributed between these two extracellular agents, resulting in the formation of two pools of receptor-agent complexes, such as receptor-drug complex and receptor-hormone complex. It is possible that, as a result of the generation of two heterogeneous pools of receptor-agent complexes, there has been an alteration in the receptor function, which did not cause induction of the expression of isoform V₁ although each of these agents (amiodarone and T₃) independently can cause the expres-
FIGURE 9. Electron micrograph of a portion of experimental cardiac myocyte after 6 days of culture showing specifically a large number of whorls of phospholipid (P), scanty myofibrils (Mf), autophagic vacuole (Av), and damaged mitochondria (Dm). Magnification, ×15,030.

The previous in vivo studies concerning antiarrhythmic activity of amiodarone suggested that amiodarone inhibits the peripheral conversion of T4 to T3 and may block the metabolic action of thyroid hormone in bringing about the reduced heart rate.6,23,24 It is also reported that the effect of amiodarone was observed despite normal serum T3 and thyroid-stimulating hormone and elevated T4 concentrations, which most likely are due to inhibition of peripheral 5'-deiodinase rather than the effect of iodine released from the drug.2,25 The present studies show that amiodarone has a direct effect on the expression of myosin isoforms of cardiac muscle cells in culture. The preferential expression of myosin isoform V3 by cardiac myocytes exposed to amiodarone in the presence of physiological concentration of T3 results in reduced contractility owing to its lower ATPase activity. This effect probably includes the reduction
in oxygen demand on cardiac muscle cells and thus may cause the antianginal effect of the drug. Although a dose-dependent response of amiodarone was observed, the dose higher than 40 µg/ml killed the myocytes, showing its toxic effect on the cells. T3 above the physiological concentration reversed the expression of myosin isoform caused by amiodarone, showing an expression of predominant isoform V1. This observation agrees with that of in vivo studies and indicates that amiodarone causes a hypothyroid-like state in the myocardium. In the past, therapeutic induction of hypothyroidism helped the treatment of intractable angina.26 However, it is not known whether antithyroid activity of amiodarone causes the antiarrhythmic effect of the drug.

Amiodarone has significant influences on DNA synthesis, cellular protein content, differentiation, and survival of cardiac myocytes in culture. DNA synthesis in cardiac myocytes is inhibited and subsequently stopped by amiodarone after 3 days of culture in contrast to the control, which attained a peak in labeling index with approximately 60% labeled cardiac myocytes after 3 days of culture. The labeling index of the control cardiac myocytes gradually declined, showing 11% labeled myocytes after the terminal time point of culture on the fifth day. The data on cellular protein content in drug-treated cultures showed significant lower values compared with those of control cultures, which showed continuous increase in protein concentration throughout the culture period. These observations show that this drug has a strong inhibitory effect on cardiac cell proliferation and protein synthesis, and thereby the growth of the myocardium. The lower protein content in the experimental culture appears to be a reflection of the lower cell proliferation rate, of reduced protein synthesis, and, to a certain extent, of cell degradation. The effect of amiodarone on the differentiation of myofibrils and cellular organelles as observed by electron microscopy showed that this drug has retarding and degrading effects on these structures. Initially, cardiac myocytes contained...
abundant, well-differentiated myofibrils and cellular organelles, but as the culture continued, myofibrils were disassembled, showing scattered irregular segments of myofibrils and free myofilaments in the sarcoplasm. The myocytes contained many degraded mitochondria and autophagic vacuoles containing degraded cellular materials. A considerable number of degraded cardiac cell bodies were observed in late culture. These cell bodies were indicative of cell deterioration and death. The overall effect of this drug on the ultrastructure of cardiac cells is not conducive to the maintenance of cellular structures needed for proper functioning.

These studies have demonstrated that amiodarone has a direct influence on the cardiac myocytes for expression of myosin isoforms. Moreover, this drug has an inhibitory effect on the growth and differentiation of cardiac myocytes. The ultrastructural studies suggest that prolonged use of this drug can damage the myocardium.

Acknowledgments

The authors gratefully acknowledge the gift of amiodarone from Dr. A. Urdang, Sanofi, New York.

References


Key Words • amiodarone • cardiac myocytes • myosin isoenzymes • DNA synthesis • ultrastructure
Effect of amiodarone on the expression of myosin isoforms and cellular growth of cardiac muscle cells in culture.

A C Nag, M L Lee and D Shepard

* Circ Res. 1990;67:51-60
doi: 10.1161/01.RES.67.1.51

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1990 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/67/1/51

**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/