Gene Transfer of Connexin43 Mutants Attenuates Coupling in Cardiomyocytes

Eddy Kizana, Connie Y. Chang, Eugenio Cingolani, Genaro A. Ramirez-Correa, Rajesh B. Sekar, M. Roselle Abraham, Samantha L. Ginn, Leslie Tung, Ian E. Alexander, Eduardo Marban

Abstract—Modification of electrical conduction would be a useful principle to recruit in preventing or treating certain arrhythmias, notably ventricular tachycardia (VT). Here we pursue a novel gene transfer approach to modulate electrical conduction by reducing gap junctional intercellular communication (GJIC) and hence potentially modify the arrhythmia substrate. The ultimate goal is to develop a nondestructive approach to uncouple zones of slow conduction by focal gene transfer. Lentiviral vectors encoding connexin43 (Cx43) internal loop mutants were produced and studied in vitro. Transduction of neonatal rat ventricular myocytes (NRVMs) revealed the expected subcellular localization of the mutant gene product. Fluorescent dye transfer studies showed a significant reduction of GJIC in NRVMs that had been genetically modified. Additionally, adjacent mutant gene-modified NRVMs displayed delayed calcium transients, indicative of electrical uncoupling. Multi-site optical mapping of action potential (AP) propagation in gene-modified NRVM monolayers revealed a 3-fold slowing of conduction velocity (CV) relative to nontransduced NRVMs. In conclusion, lentiviral vector–mediated gene transfer of Cx43 mutants reduced GJIC in NRVMs. Electrical charge transfer was also reduced as evidenced by delayed calcium transients in adjacent NRVMs and reduced CV in NRVM monolayers. These data validate a molecular tool that opens the prospect for gene transfer targeting gap junctions as an approach to modulate cardiac conduction. (Circ Res. 2007;100:1597-1604.)

Key Words: gap junction ■ gene transfer ■ connexin43

Gap junctions are channels that permit intercellular communication. In mammalian tissues these channels are ubiquitously expressed and serve diverse biological functions.1 Within the heart, gap junctions mediate electrical impulse transmission which underlies its coordinated mechanical activity.2 Acquired heart disease and the ensuing gap junction remodeling can alter electrical conduction in a manner which begets arrhythmias. For example, this was found to be true of ventricular tachycardia (VT) occurring after chronic myocardial infarction (MI) where focal conduction slowing forms an essential part of the arrhythmogenic substrate.2 The clinical burden of this pathophysiology is evidenced by the fact that post-MI VT is a substantial cause of sudden cardiac death (SCD).3

Contemporary therapies for VT/SCD include antiarrhythmic drugs, implantable defibrillators, and catheter-based radiofrequency ablation. The first of these has met with limited efficacy in clinical trials largely because of increased proarrhythmic events.3 Defibrillators, although efficacious, are associated with a considerable cost and do not directly impact the underlying pathophysiology. Radiofrequency ablation is associated with modest success rates even when performed by highly-experienced operators and, furthermore, many patients are not amenable to this approach.3 Studies exploring new treatment approaches, such as gene therapy, are emerging with a recent example reporting inhibition of post-MI VT in a porcine model by targeting repolarization.4 With these issues in mind, a novel alternative approach based on gene transfer technology targeting gap junctional intercellular communication (GJIC) was pursued. This approach has several potential advantages including targeting of the mechanism causing the conduction disturbance, creation of a nondestructive therapy, and the potential to confer a permanent effect depending on the vector employed.

Here we explore the potential of a genetic inhibitor or loss-of-function approach directed at GJIC. Specifically, overexpression of internal loop mutants of the Cx43 gene are employed to uncouple cells connected by gap junctions.5 These mutants result in gap junctions that lack electrical communication when heterologously expressed in cell pairs.5

1597
Furthermore, they also confer a dominant-negative effect when expressed in cells with endogenous wild-type Cx43.9–11 We find that lentiviral vectors encoding Cx43 internal loop mutants are capable of significantly modulating electrical propagation by reducing GJIC in neonatal rat ventricular myocytes (NRVMs). Moreover, this effect is achieved without inducing toxicity in target cells. These functional effects are substantially diminished when Cx43 GFP C-terminal chimeras are used.

Materials and Methods

**Molecular Cloning**

The cDNAs for rat Cx43 (Eric Beyer, University of Chicago, Ill, USA), rat Cx43GFP fusion (Dale Laird, University of Western Ontario, London, Canada), and cat Cx43Δ130 to 166 deletion mutant (Vladimir Krutovskikh, International Agency for Research on Cancer, Lyon, France) were sub-cloned into the lentiviral vector plasmid, pRRLsin18.cPPT.CMV.GFP.Wpre (pPPT.CMV.GFP, Inder Verma, The Salk Institute for Biological Studies, Calif, USA) after removal of GFP. The resulting plasmids were designated pPPT.CMV.Cx43, pPPT.CMV.Cx43GFP, and pPPT.CMV.Cx43Δ166, respectively. Additional vector plasmids encoding Cx43Δ130 to 166 and Cx43Δ130 to 170GFP were produced by PCR-based site-directed mutagenesis using pPPT.CMV.Cx43 and pPPT.CMV.Cx43GFP as the respective templates. Column-purified mutagenesis primers 5'-GGAGTTTGCAAGACGACGGCAAGTGGAATATGAG-3' (upper, phosphorylated) and 5'-CGCTTCAAGGTGTCATCACCAGTTG-3' (lower) were designed to result in a 24-bp deletion in the Cx43 coding region. The mutagenesis reaction was based on the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene). The deletion was confirmed by sequencing and the resulting plasmids designated pPPT.CMV.Cx43Δ8 and pPPT.CMV.Cx43Δ8GFP.

**Lentiviral Vector Production**

A third-generation lentiviral vector system was used for gene transfer. Vector was produced by calcium-phosphate coprecipitation transfer. Vector was produced by calcium-phosphate coprecipitation of the 4 lentiviral vector plasmids into 293T cells by the Polybrene method. Vector was applied overnight and media changed the following morning.

**Cell Culture**

293T and HeLa cells (ATCC) were maintained in DMEM culture medium (Gibco) supplemented with 10% fetal bovine serum, (FBS), Hyclone) and penicillin/streptomycin (Gibco). NRVMs were enzymatically dissociated from the ventricles of 2- to 3-day-old Sprague-Dawley rats (Harlan, Indianapolis, Ind) with the use of trypsin (US Biochemicals) and collagenase (Worthington) as previously described.12 For optical mapping experiments, 106 cells were plated on multichamber slides (Becton-Dickinson) coated with fibronectin (25 μg/mL, Micro multidimensional dissociation kit (Vladimir Krutovskikh, International Agency for Research on Cancer, Lyon, France) were sub-cloned into the lentiviral vector plasmid, pRRLsin18.cPPT.CMV.GFP.Wpre (pPPT.CMV.GFP, Inder Verma, The Salk Institute for Biological Studies, Calif, USA) after removal of GFP. The resulting plasmids were designated pPPT.CMV.Cx43, pPPT.CMV.Cx43GFP, and pPPT.CMV.Cx43Δ166, respectively. Additional vector plasmids encoding Cx43Δ130 to 166 and Cx43Δ130 to 170GFP were produced by PCR-based site-directed mutagenesis using pPPT.CMV.Cx43 and pPPT.CMV.Cx43GFP as the respective templates. Column-purified mutagenesis primers 5'-GGAGTTTGCAAGACGACGGCAAGTGGAATATGAG-3' (upper, phosphorylated) and 5'-CGCTTCAAGGTGTCATCACCAGTTG-3' (lower) were designed to result in a 24-bp deletion in the Cx43 coding region. The mutagenesis reaction was based on the ExSite PCR-Based Site-Directed Mutagenesis Kit (Stratagene). The deletion was confirmed by sequencing and the resulting plasmids designated pPPT.CMV.Cx43Δ8 and pPPT.CMV.Cx43Δ8GFP.

**Calcium Transient Analysis**

For calcium transient studies, 2×10^6 NRVMs were plated in 35-mm glass-bottom dishes (MatTek Cultureware), transduced, and analyzed 72 hours later. Cells were loaded with Rhod2-AM (2 μmol/L) (Molecular Probes) for 18 minutes, then washed once with PBS and subsequently placed in normal Tyrode solution with 2 mmol/L calcium. Calcium transients were recorded at 37°C from randomly-selected PPT.CMV.GFP, PPT.CMV.Cx43Δ7, and PPT.CMV.Cx43Δ8GFP transduced cells. Images were acquired on an inverted confocal laser-scanning microscope (Perkin Elmer/Nikon). Offline analysis was performed on randomly selected adjacent cells. Calcium transients were plotted and analyzed to determine the temporal relationship between the peaks of calcium transients. After studying...
control GFP-transduced cells, a >0.10 seconds difference between peaks was considered to be delayed and indicative of electrical uncoupling.

**Transmitochondrial Membrane Potential (ΔΨ_m) Assay**

To determine the effect of lentiviral transduction and Cx43-mutant overexpression, ΔΨ_m was assessed by tetramethylrhodamine ethyl ester (TMRE; Molecular Probes) labeling using a flow cytometer as previously described.11 Four days after transduction, 1 × 10^6 NRVMs were loaded with 100 nmol/L TMRE for 20 minutes in the dark at 37°C. Labeled NRVMs were washed with PBS, trypsinized, and subjected to flow cytometry (FACScan, Becton Dickenson).

**Optical Mapping of Action Potential Propagation**

Coverslips with cultured NRVMs were inspected, and those with defects or nonbeating cultures were rejected before transduction. Cells were transduced with vector 3 to 4 days after culture and optical mapping performed 3 to 4 days after transduction. Coverslips were placed in a custom-designed chamber, stained with 5 μmol/L di-4-ANEPPS (Molecular Probes) for 5 minutes, and continuously superfused with warm (36.5°C) oxygenated tyrode solution consisting of (in mmol/L) 135 NaCl, 5.4 KCl, 1.8 CaCl_2, 1 MgCl_2, 0.33 NaH_2PO_4, 5 HEPES, and 5 Glucose. Cells were stimulated with monophasic 10-ms pulses at 2 Hz delivered by a bipolar line electrode at twice diastolic threshold. The electrode was positioned in the vertical orientation, 3 to 4 mm from the edge of the coverslip. A 3000-ms recording was taken after a 10-beat drive train. Three recordings were made for each coverslip. Action potentials were recorded from 253 sites using a custom-built contact fluorescence imaging system as previously described.12 The relative activation times at each recording point were used to calculate CV. To avoid electrode-associated artifacts, CV was determined over paths from mid-field to 1 mm from the periphery of the mapped area in the direction of AP propagation. CV was determined perpendicular to isochrones and averaged from 9 to 10 paths for each coverslip.

**Statistical Analysis**

Data were analyzed for mean, standard deviation, and standard error of the mean (SEM) using Microsoft Excel XP. The quantitative figures in this work represent the mean ± SEM of the measurement. Analysis of the statistical significance of data sets was performed using 2-sample t test assuming equal variances or using a 2-sample χ^2 test for determining the significance of the difference of 2 independent proportions. A value of P < 0.05 was considered significant.

**Results**

**Functional Analysis of Vectors Encoding Cx43 Mutants**

The expression of PPT.CMV.Cx43Δ8GFP was studied in NRVMs (Figure 1C and 1D). Trafficking defects have previously been reported with Cx43 internal loop mutants.8 These, however, could be rescued by coexpression of wild-type Cx43. Consistent with these observations, fluorescent microscopy of the GFP-tagged Cx43 mutant revealed the expected subcellular localization with predominance of GFP fluorescence in dense linear clusters at the cell membrane, with additional signal seen in the cytoplasm of approximately 10% of cells studied (Figure 1C). Western analysis revealed substantial overexpression of the fused molecule relative to endogenous Cx43 in this cell type (Figure 1D).
NRVMs Are Efficiently Transduced by Lentiviral Vectors Without Excess Apoptosis

To determine the permissiveness for lentiviral vectors, NRVMs were transduced with PPT.CMV.GFP at varying MOIs and GFP expression assessed by flow cytometry. NRVMs were efficiently transduced by PPT.CMV.GFP with 95% of target cells expressing GFP after transduction at an MOI of 20 (Figure 2). This MOI was used unless stated otherwise.

Overexpression of Cx43 in differentiating skeletal myoblasts has been reported to result in extensive cell death. Implicating the Cx43 transgene, this event occurred independently of the 3 different vector systems used for gene transfer. Importantly, in the current study transduction-associated cell toxicity was not visually evident in NRVM monolayers. There was no excess of monolayer defects or detached cells in the cell culture supernatant (Figure 3A and 3B). Additionally, optical signals from action potentials were of equal quality between nontransduced and Cx43-gene modified NRVMs (Figure 3C and 3D). Furthermore, loss of the mitochondrial transmembrane potential, an early apoptotic event, did not occur at an increased frequency in cells transduced with Cx43Δ7 mutants, despite the use of a moderate MOI of 20 (Figure 3E to 3H). It has been previously demonstrated that maintenance of mitochondrial membrane potential (ΔΨm) is a critical determinant of myocyte survival.

Overexpression of Cx43 Mutants Attenuates Dye Transfer in NRVMs

To measure the effect of mutant Cx43-encoding vectors on GJIC, a fluorescent dye transfer system with quantification using flow cytometry was used. In this system, NRVMs
selected cocultures. In addition, nontransduced and mutant channels.

formation from the effects on the diffusive properties of mutants on changes in the rate of new gap junction channel
ever, that this method does not separate the effects of Cx43 dependent manner (Figure 4D). It should be noted, how-
coding the mutant Cx43 reduced GJIC in a vector dose-
transduced NRVMs served as positive and vector controls,
analysis, were obtained from the vast majority of available
olds between 12 to 18 V. Electrical signals, suitable for
throughout the experimental protocol without deterioration in
ning of AP propagation was performed on gene-modified

(receptor cells) were loaded with a gap junction permeable dye, calcein, and added to potential recipient NRVMs grown in confluent monolayer (Figure 4A to 4C). After a defined period of coculture, cells were harvested and the proportion of fluorescent cells determined by flow cytometry. Regions within the resulting flow cytometry fluorescence histogram were defined to include donor cells, noncalcein labeled cells, and cells undergoing dye transfer during the period of culture. These same regions were applied to all experimental samples. As a negative control, the nonspecific gap junction inhibitor α-glycyrrhetinic acid (AGA) was added to the media of selected cocultures. In addition, nontransduced and LacZ-transduced NRVMs served as positive and vector controls, respectively. These experiments revealed that vectors encoding the mutant Cx43 reduced GJIC in a vector dose-dependent manner (Figure 4D). It should be noted, however, that this method does not separate the effects of Cx43 mutants on changes in the rate of new gap junction channel formation from the effects on the diffusive properties of mutant channels.

Slowing of AP Propagation in Cx43 Mutant Gene-Modified NRVM Monolayers
To assess the effect of mutant Cx43 overexpression on electrical coupling in networked excitable cells, optical mapping of AP propagation was performed on gene-modified NRVM monolayers. Cultures maintained a robust condition throughout the experimental protocol without deterioration in AP signals. Stimulation was readily performed with thresholds between 12 to 18 V. Electrical signals, suitable for analysis, were obtained from the vast majority of available channels. Planar (or semi-planar) waves of AP propagation were observed in most cultures because of the use of a bipolar line electrode (Figure 5A to 5C). In cultures gene-modified with either PPT.CMV.Cx43Δ7 or PPT.CMV.Cx43Δ8 there was a greater than 3-fold slowing in CV relative to nontransduced cells (Figure 5D). As a control for the effects of transduction, CV was also assessed after genetic modification with PPT.CMV.GFP. This caused a small but significant slowing in CV. Notably, transduction of cells with PPT.CMV.Cx43Δ8GFP did not significantly impact CV (Figure 5D).

Delayed Calcium Transients Between Adjacent NRVMs Transduced With Cx43 Mutants
Transfection of NRVMs with a plasmid encoding Cx43Δ8GFP causes a 12% rate of desynchronization between adjacent NRVMs compared with 2% in controls.7 To extend these observations, NRVM monolayers were transduced with PPT.CMV.GFP, PPT.CMV.Cx43Δ7, or PPT.CMV.Cx43Δ8GFP, loaded with a calcium indicator and spontaneous calcium transients assessed in adjacent cells. We were unable to find truly desynchronized transients and therefore defined them as either nondelayed or delayed as defined by the temporal separation of transient peaks (Figure 6A and 6B). A low rate of delayed transients was found in control cells (Figure 6C). Consistent with the AP propagation studies,
Lentiviral vector–mediated gene transfer of Cx43 mutants substantially reduced GJIC in target NRVMs. Gene transfer at varying MOIs and CMV.Cx43

Cx43/H9004

7 or PPT.CMV.Cx43

nexin protein. These cells were transduced with either PPT.

express Cx43GFP (HeLaCx43GFP) as the exclusive con-

We hypothesized that the GFP-fused mutant protein was not efficient at inhibiting GJIC as its nonfused counterparts. To confirm this, a high rate of delayed NRVM transients was found after transduction with Cx43Δ7. Interestingly, NRVMs transduced with Cx43Δ8GFP displayed a significantly reduced rate of delayed transients, a finding supportive of the lack of conduction slowing observed in the AP mapping studies.

Cx43 Mutants Do Not Inhibit Dye Transfer in HeLa Cells Expressing Cx43GFP

We hypothesized that the GFP-fused mutant protein was not as efficient at inhibiting GJIC as its nonfused counterparts. To further assess this, a clone of HeLa cell-line was engineered to express Cx43GFP (HeLaCx43GFP) as the exclusive connexin protein. These cells were transduced with either PPT.CMV.Cx43Δ7 or PPT.CMV.Cx43Δ8 at varying MOIs and subjected to GJIC assessment by dye transfer and flow cytometry. In contrast to the results seen in the NRVM dye transfer studies, GJIC in HeLaCx43GFP was not inhibited by either vector even at high MOIs (Figure 7A to 7F). To confirm that Cx43 mutants could inhibit GJIC in the context of HeLa cells, these cells were gene-modified to stably express wild-type Cx43. After this, cells were further transduced with PPT.CMV.Cx43Δ8 and subjected to GJIC assessment. This revealed a dose-dependent reduction in dye transfer as assessed by flow cytometry (Figure 7G). These experiments support the notion that the Cx43 internal-loop mutant-induced GJIC inhibition is reduced when either the wild-type or the loop-mutant protein is fused to GFP.

Discussion

Lentiviral vector–mediated gene transfer of Cx43 mutants substantially reduced GJIC in target NRVMs. Gene transfer occurred with high efficiency resulting in widespread genetic modification of target cells and robust transgene over-expression at the expected subcellular localization. Vector or transgene associated toxicity, which could confound measures of cell coupling, was negligible. Dye transfer and calcium transient studies revealed a significant reduction in the extent of cell coupling. Similarly, AP propagation studies showed marked conduction slowing following transduction with the mutant constructs.

Gap junctions are essential for electrical impulse conduction in the mammalian heart. In this context, 2 important properties of gap junctions need to be borne in mind when targeting GJIC for manipulation of conduction. Firstly, GJIC is mediated by gap junctions whose constituent connexins vary according to the regional function subserved. Ventricular gap junctions, for example, are mostly composed of Cx43 whereas gap junctions of the conducting pathways of the His-Purkinje system (HPS) are abundant in Cx40. The conductance of gap junctions made of Cx40 is greater than that of channels made of Cx43, a property that underpins the increased impulse conduction velocity in the HPS compared with ventricular myocardium. Secondly, the heart exhibits marked redundancy with regard to gap junctions. Cx43 heterozygote knockout mice, for example, with 50% Cx43 levels, do not display reduced impulse conduction velocity when measured using sensitive methods. Even cultured myocytes from the homozygote mice displayed residual, albeit very slow, conduction. A molecular approach to inhibiting GJIC will, therefore, ideally require a capacity for targeting multiple connexin subtypes and effect at least a 50% functional reduction in target gap junctions to confer an observable phenotype in terms of impulse conduction.

The Cx43 internal-loop mutants were exploited for their ability to form nonfunctional gap junctions when expressed in connexin-null cells. Furthermore, mutants of this type have been reported to confer either a partial or complete dominant-negative effect when coexpressed with wild-type Cx43. Moreover, Cx43 internal loop mutants are also capable of inhibiting GJIC when heterologously coexpressed with several wild-type connexins including Cx37 and Cx45. By forming heterotypic channels, mutant Cx43 can coassemble with endogenous Cx45, the other major ventricular cardiac connexin, and form dysfunctional channels. We found a substantial yet incomplete dominant-negative effect both in the dye transfer and AP propagation studies. For example, pharmacological uncoupling with AGA exceeded vector in inhibiting dye transfer, and no mutant-transduced monolayers failed to propagate APs. These observations can be explained by incomplete heteromultimerization of wild-type and mutant protein, resulting in some gap junctions composed entirely of wild-type protein. Alternatively, heteromultimerization may be an efficient process but result in gap junctions with residual low-level conductance. These possibilities could be explored in dual patch-clamp experiments performed in NRVM pairs, gene-modified to express Cx43 mutants. Another possible explanation is the presence of Cx40-based gap junctions in the NRVMs which, unlike Cx45, are not subject to inhibition by the Cx43 internal loop mutants.
Connexin-GFP fusion proteins have been widely used to study the biology of these molecules. It was surprising to observe normal AP propagation in NRVM monolayers transduced with Cx43/H9004GFP. This occurred in contrast to the marked slowing of AP conduction in NRVMs transduced with Cx43/H9004, a construct lacking the GFP tag but otherwise identical to the former. Similar results were also seen in the calcium transient studies in which the nonchimeric mutant conferred a significantly higher rate of transient delay. We hypothesized that this tag impaired the inhibitory effect of the mutant protein and was further explored in experiments with GFP fused to wild-type Cx43 and coexpressed with Cx43 mutants. These results revealed a lack of effect on GJIC of these vectors as evident by identical overlay of test and black plots in the M2 region even at high MOIs of 100 (C and F). In control experiments, HeLa cells stably expressing wild-type Cx43 displayed a dose-dependent reduction in GJIC after transduction with Cx43/H9004 (G); n=4 each; *P<0.05 compared NT.

An additional intriguing observation was the prolonged (>100ms) in-phase delay of calcium transients in adjacent mutant-transduced cells. A prior study in adult ventricular cell-pairs revealed the maximum delay in AP propagation to be 24 ms before complete uncoupling. To a first approximation, similar delays would be expected between calcium transients in cell pairs; the fact that we measured longer delays merits reflection. It is possible that cells in the monolayer are stretch-activated by their contracting neighbors; that is, they are mechanically rather than electrically coupled. Another possible contribution to differences in propagation arises from the obvious geometrical differences between cell pairs, where excitation proceeds only from one cell to its neighbor, versus monolayers, in which all surrounding cells may contribute to activation, possibly extending the safety margin. This important finding affects the interpretation of the published data on the persistence of mechanical activity in Cx43 knockout animals and on synchronization of calcium transients between host hearts and transplanted stem cells lacking gap junctions.

There are a number of potential translational applications for genetic GJIC inhibitors. By targeting focal areas of the heart, high local concentrations of vector can be delivered to the region of interest, thus transducing cells with multiple...
gene copies and placing a greater than 50% reduction in gap junctions within reach. The most clinically obvious application is the focal interruption of macro reentrant arrhythmias such as atrial flutter or more ambiguously, VT. Another possible application is controlling the ventricular response rate during atrial fibrillation by slowing conduction through the AV node. In this situation focal gene delivery to the AV node has the potential to result in the desired physiological effect. The importance of the development of this technology lies in the unsatisfactory contemporary approaches to the management of arrhythmias such as VT or subsets of patients with arrhythmias refractory to conventional treatment.

Sources of Funding
This work was supported by the Donald W. Reynolds Foundation. E.M. holds the Michel Mirowski, MD Professorship of Cardiology of the Johns Hopkins University. E.K. is funded by the Michel Mirowski, MD Fellowship of the Heart Rhythm Society. S.L.G. is supported by the Noel Dowling Research Fellowship.

Disclosures
None.

References
Gene Transfer of Connexin43 Mutants Attenuates Coupling in Cardiomyocytes: Novel Basis for Modulation of Cardiac Conduction by Gene Therapy
Eddy Kizana, Connie Y. Chang, Eugenio Cingolani, Genaro A. Ramirez-Correa, Rajesh B. Sekar, M. Roselle Abraham, Samantha L. Ginn, Leslie Tung, Ian E. Alexander and Eduardo Marbán

Circ Res. 2007;100:1597-1604; originally published online May 10, 2007;
doi: 10.1161/CIRCRESAHA.106.144956
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 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/100/11/1597

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/