Ventricular Myocyte Caspases Are Directly Responsible for Endotoxin-Induced Cardiac Dysfunction

Steve Lancel, PhD; Olivier Joulin, MD; Raphael Favory, MD; Jean Francois Goossens, PhD; Jérome Kluza, PhD; Claude Chopin, MD; Pierre Formstecher, MD, PhD; Philippe Marchetti, MD, PhD*; Remi Neviere, MD, PhD*

Background—Although most of the deleterious effects of sepsis-induced apoptosis have been attributed to increased lymphocyte cell death, caspase activation may directly alter cell function of different organ systems. We postulated that left ventricular (LV) cardiomyocyte caspase activation is directly involved in sepsis-induced heart contractile dysfunction.

Methods and Results—LV cardiomyocytes isolated 4 hours after rat treatment with endotoxin injection (10 mg/kg) displayed major reductions in contractile reserve and myofilament response to Ca²⁺. Concomitantly, endotoxin also induced increases in LV cardiomyocyte caspase-3, -8, and -9-like activities, which were associated with sarcomeric structure destruction and cleavage of components of the cardiac myofilament. Interestingly, zVAD.fmk treatment of septic rats prevented LV cardiomyocyte contractile dysfunction, reductions in myofilament response to calcium, troponin T cleavage, and sarcomere destruction. Serum (10%) of endotoxin-treated rats induced contractile dysfunction, caspase-3–like activity increase, and troponin T cleavage of naive LV cardiomyocytes. The effects of septic serum were prevented in LV cardiomyocytes isolated from zVAD.fmk- or zDEVD.cmk-treated rats or LV cardiomyocytes preincubated with zVAD.fmk or zDEVD.cmk.

Conclusions—The results show an important relationship between endotoxin-induced caspase activation and reduced contractile reserve and sarcomere disarray at the level of single LV cardiomyocytes. (Circulation. 2005;111:2596-2604.)

Key Words: apoptosis ■ myocardial contraction ■ myocytes, cardiac ■ inflammation ■ shock

In the United States, >500 000 patients per year develop bacterial sepsis, with an estimated rising incidence of ≈1.5% per year.1,2 Sepsis remains the chief cause of death in intensive care units, with mortality rates between 30% and 70%.2,3 Impaired myocardial contractile function is a well-documented feature that greatly contributes to the mortality associated with this pathological condition.2,4 A large body of evidence4–6 suggests that exposure of animals and humans to endotoxin (lipopolysaccharide) mimics the deleterious effects of bacterial sepsis on myocardial function. Although sepsis is generally viewed as a disease aggravated by an inappropriate inflammatory response to endotoxin, implication of apoptosis processes in the pathogenesis of sepsis has been recently demonstrated.2,7

Most of the deleterious effects of endotoxin-induced apoptosis have been attributed to increased circulating and lymphoid tissue resident lymphocyte cell death.7,8 In these studies, protection induced by apoptosis inhibition involved prevention of bacterial growth, alteration in the immune response, and remote effects of apoptosis inhibitors on different cell types.7,8 Alternatively, it could be questioned whether endotoxin-induced activation of apoptosis pathways may directly induce myocardial dysfunction, which can be reduced by apoptosis inhibitors. Indeed, endotoxin may trigger end-stage apoptosis of myocardial cells, which is associated with heart multiple caspase activation and cytochrome c release from the mitochondria.9–12 Interestingly, in vivo caspase activity blockade by broad-spectrum and effector caspase (ie, caspase-3) inhibitors reduced endotoxin-induced heart caspase-3 activity and myocardial dysfunction.11,13 In these studies, however, indirect effects of caspase inhibitors on the extent of activation of immune cells cannot be ruled out.

The major goal of this study was to investigate the role of effector caspase activation in left ventricular (LV) cardiomyocyte dysfunction induced by endotoxin. First, we confirmed that caspase-3 activation and contractile dysfunction may be described at the level of LV cardiomyocytes isolated...
from septic rat hearts. Second, we developed an in vitro model to test whether caspase inhibition would prevent LV cardiomyocyte contractile dysfunction induced by septic serum, independently of the effects of caspase inhibitors on immunocompetent cells infiltrating the myocardium. Third, we proposed that endotoxin-induced effector caspase activation is centrally involved in LV cardiomyocyte contractile dysfunction and reduced myofilament response to calcium with major sarcomere disorganization and contractile protein cleavage.

**Methods**

**Animal Preparation**

Adult male Sprague-Dawley rats (weight, 250 to 300 g) were anesthetized with pentobarbital sodium (50 mg/kg IP). Treatments were administered intravenously via the dorsal penile vein. Four hours after treatment, rats were used for cardiomyocyte isolation and serum preparation, as previously described.14 The protocol was approved by the Animal and Use Committee of Lille, and the care and handling of the animals were conducted in accordance with our national institutional guidelines.

**LV Cardiomyocyte Shortening and [Ca2+]i Measurement**

LV cardiomyocytes (viability >85%) were plated (100 000 cells per milliliter) and stored at 37°C until use. For contraction amplitude (IonOptix), cells were placed in a flow chamber (30°C) on the stage of a microscope (E800, Nikon) and electrically stimulated at 1 Hz (voltage adjusted to maximize capture). The fluorescent Ca2+ indicator fluo-3 acetoxymethyl ester (6 μM) for 20 minutes, 37°C (Molecular Probes, Inc) was used to measure intracellular Ca2+ as previously described.14 Fluorescence brightness (excitation 465 to 495 nm; emission 515 to 555 nm) was recorded with the use of a CCD camera (C2400-08). Calibration of [Ca2+]i was performed by the use of ionomycin, butanedione monoxime, and MnCl2 solutions, as previously described.14

**Serum and Heart Tumor Necrosis Factor-α Determination**

Serum and heart homogenates were prepared for tumor necrosis factor-α (TNF-α) enzyme-linked immunosorbent assay (Quantikine Murine rat TNF, R&D Systems), as described previously.13

**Caspase-3, -8, -9–Like Activity Assay**

After incubation in assay buffer A (in mmol/L: HEPES 50, NaCl 100, EDTA 1, dithiothreitol 10, with CHAPS 0.1%, glycerol 10%; in μg/mL: aprotinin 10, leupetin 10, pepstatin 10; pH 7.32), cells were lysed with a Kontes glass. Next, 200 μg of proteins was diluted in 200 μL of assay buffer, then either Ac-DEVD-AMC, Ac-IETD-AMC, or Ac-LEHD-AMC (50 μmol/L; Biomol) was added. Sample fluorescence (excitation 380 nm; emission 437 nm) was measured at 2 hours (Spex Fluoromax, Is-Horiba).

**Immunohistochemistry for Active Caspase-3**

Frozen sections of heart tissue (8 μm) were used to detect caspase-3 by antibodies recognizing the cleaved active form of the enzyme (BD Pharmingen) according to the manufacturer’s instructions.

**Western Blot Studies**

After cell lysis (buffer A) electrophoresis, polyclonal anti-caspase-3 antibody (BD Pharmingen) and polyclonal anti-caspase-7 antibody (Cell Signaling Technology) were used. After cell fractioning into mitochondrial and cytosolic compartments and electrophoresis, monoclonal anti–cytochrome c antibody (BD Pharmingen) was used. After heart homogenate preparation, polyclonal anti–Bcl-2 and anti-Bax (Santa Cruz Biotechnology) antibodies were used. After cell lysis with specific lysis buffer for myofilament preparation13 and electrophoresis, monoclonal anti–α-actinin, anti–troponin T (Sigma), anti–troponin C (Novocastra Laboratories Ltd), and anti–troponin I (Advanced Immunotech) antibodies were used.

**Mitochondrial Membrane Potential**

Mitochondrial membrane potential was measured (FACS Calibur Analytic Flow Cytometer (BD Biosciences) in LV cardiomyocytes with the use of the fluorescent probe JC-1 (1 μmol/L, 30 minutes), which produces green fluorescence in the cytoplasm and red-orange fluorescence when concentrated in respiring mitochondria that have a negative internal potential. Data were analyzed with Windows Multiple Document Interface for Flow Cytometry 2.8 software.

**Confocal Microscopy Studies**

Ventricular sarcomere organization and caspase-3–like activation were assessed on isolated LV cardiomyocytes and visualized by confocal microscopy (Leica TCS NT, Microsystemes). According to the manufacturer’s instructions, caspase-3–like activation was detected in living cells with the use of carboxyfluorescein-labeled fluoroethyl ketone peptide inhibitor of caspase-3 (FAM-DEVD-FMK) from CaspTag Caspase 3/7 in situ assay kit (Chemicon International).
International). Polymerized actin fibers, α-actinin, and troponin T were visualized by Texas red-phalloidin (Molecular Probes), mouse monoclonal anti–α-actinin, and anti–troponin T (Sigma), respectively. Images were quantified and processed with the use of Leica TCS NT software (PowerScan module).

**Experimental Design**

In the first series of experiments, LV cardiomyocytes of hearts of control (500 μL IV saline), endotoxin *Escherichia coli* serotype 055:B5 (Sigma)–treated (10 mg/kg IV in 500 μL saline) control, and zVAD.fmk-treated endotoxin-treated rats were isolated for physiological and biological evaluation. Caspase inhibitors were administered at the same time as endotoxin.

In the second series of experiments, the effects of control and septic serum were studied on naive LV cardiomyocytes incubated with caspase inhibitors (zVAD.fmk or zDEVD.cmk; 100 μmol/L). Alternatively, LV cardiomyocytes were isolated from rats treated with caspase inhibitors (zVAD.fmk or zDEVD.cmk; 3 mg/kg body wt, 4 hours).

In the third series of experiments, the effects of endotoxin 100 and 500 mg/mL were studied on LV cardiomyocyte survival, contractile function, caspase–3–like activity, and troponin T cleavage.

**Statistical Analysis**

Data represent mean±SEM and were analyzed by ANOVA procedures. When a significant difference was found, we identified specific differences between groups using a sequentially rejective Bonferroni procedure. After application of Bonferroni correction, significance was achieved with *P*<0.01 for comparisons with control.

**Results**

**Endotoxin Rat Treatment Induces Cardiomyocyte Caspase Activation, Contractile Dysfunction, and Sarcomeric Destruction**

Four hours after endotoxin treatment, rats presented with diarrhea, polypnea, and pilo-erection. Endotoxin treatment induced downstream caspase activation in LV cardiomyocytes, which was evidenced as caspase–3–like activity increases (Figure 1A), active caspase–3–like staining increases (Figure 2A), and proteolytic activation of caspase-3 (Figure 2B). Proteolytic caspase-7 activity was not detected in LV cardiomyocytes of control and endotoxin-treated rats (data not shown). Cell viability (trypan blue exclusion) and nuclear condensation (Hoechst 33342 staining, 1 μg/mL) of LV cardiomyocytes isolated from control and endotoxin-treated rats were quantified over time. Cell viability in control and septic LV cardiomyocytes remained up to 80% over time (4, 24, and 96 hours; data not shown). Few condensed nuclei (Hoechst staining) were detected (≤5%) in control and septic LV cardiomyocytes at 4 and 24 hours after isolation (data not shown). In LV cardiomyocytes of septic rats, caspase-3 activation was associated with heart and plasma TNF-α level (Figure 1B) and upstream caspase-8-like activity (Figure 1A) increases. Mitochondrial-related factors were investigated as a possible upstream pathway that would regulate caspase-3 activation. In LV cardiomyocytes of septic rats, we observed loss of mitochondrial membrane potential, reduced Bcl-2/Bax protein ratio, cytosolic cytochrome c release (Figure 1C), and caspase-9-like activity increases (Figure 1A).

To assess sarcomeric integrity, we evaluated sarcomeric unit structure, contractile protein cleavage, and caspase activities in the same population of LV cardiomyocytes. Septic LV cardiomyocytes, in which caspase–3–like activity was increased (Figure 2A), displayed hazy, destroyed, and disorganized sarcomeric unit structure (Figure 2A) and troponin T cleavage (32-kDa fragment) (Figure 2B). α-Actinin remained unchanged (Figure 2A, 2B). No significant proteolytic cleavages were detected for troponin I (32 kDa), troponin C (19 kDa), or actin (43kDa) (data not shown).

From the same rat heart, cell shortening was measured. In contrast to control LV cardiomyocytes, fractional cell shortening was severely depressed in septic LV cardiomyocytes (Figure 2C).

**zVAD.fmk Prevents Endotoxin-Induced Cardiomyocyte Multiple Caspase Activation and Contractile Dysfunction**

zVAD.fmk treatment had no effects on clinical signs of sepsis and endotoxin-induced heart and plasma TNF-α level increases (data not shown). zVAD.fmk prevented endotoxin-induced LV cardiomyocyte caspase-3 activation (Figure 3A) and proteolytic procaspase-3 cleavage (data not shown), which were associated with reduced caspase–3–like, death receptor–dependent caspase-8, and mitochondrial-related caspase-9 activities (Figure 3B). zVAD.fmk inhibited sarcomere alterations induced by endotoxin (Figure 3C).

Fractional shortening and calcium transient were significantly decreased in LV cardiomyocytes isolated from endotoxin-treated rat hearts compared with control LV cardiomyocytes (Figure 4A, 4B). Reductions in calcium transient in LV cardiomyocytes isolated from endotoxin-treated rat hearts were mainly related to increases in diastolic [Ca2+]i (Figure 4C). Rat treatment with zVAD.fmk prevented changes in cell shortening, calcium transient, and diastolic [Ca2+]i, observed in septic LV cardiomyocytes.

**Figure 2.** Endotoxin treatment induces caspase–3–like activation, sarcomeric structure alterations, troponin T cleavage, and contractile dysfunction. Each experiment was performed on the same population of LV myocytes isolated from control (a to e) or endotoxin-treated (f to j) rats (n=6 in each group). A, top, Confocal visualization of active caspase–3–like stained by FAM-DEVD-FMK (a, f), polymerized actin fibers stained by Texas red-phalloidin (b, g), and colocalization of active caspase–3–like and polymerized actin fibers (c, h). N indicates nucleus localization. Magnification ×400. Bars=5 μm. Line graphs are semiquantitative analysis of actin fiber organization, which was appreciated as fluorescence pixel intensity along a longitudinal axis line of cells. Control cells exhibit regular oscillations between high- and low-fluorescence intensities, representative of well-organized sarcomeres, which were not present in cells isolated from endotoxin-treated rats. Arrow on graph represents space without actin fibers. Bar graphs represent percentage of cells displaying destroyed sarcomeres. One hundred fifty cells obtained from 6 rats in each group were analyzed. Black pattern represents altered sarcomeres; white pattern, well-organized sarcomeres. Bottom, Confocal visualization of α-actinin staining (d, i). Cells isolated from control and endotoxin-treated rats present the same well-organized pattern displayed on line graphs. Troponin T confocal visualization (e, j) is shown. Cells isolated from endotoxin-treated rats displayed a hazy structure associated with a low fluorescence intensity that is not observed in control cells. B, Western blotting of caspase-3, α-actinin, and troponin T. α-Actinin was not altered by rat treatment. Active caspase-3 (17 kDa) was detected on cells isolated from endotoxin-treated rats. Troponin T (41 kDa) was cleaved in a 32-kDa fragment in cells obtained from septic rats. C, Cells isolated from endotoxin-treated rats present a lower cell shortening than control cells. *P<0.01 vs control.
LV cardiomyocyte contractile reserve was further studied by increasing extracellular [Ca²⁺], from 0.5 to 3.0 mmol/L at a constant pacing frequency of 1 Hz. Diastolic length decreased slightly, but there were no differences among the groups (Figure 5A). In control LV cardiomyocytes, fractional cell shortening increased in response to the increased [Ca²⁺] (Figure 5B). In contrast to control LV cardiomyocytes, fractional cell shortening was severely depressed in septic LV cardiomyocytes, which was prevented by zVAD.fmktreatment (Figure 5B).

To assess myofilament response to Ca²⁺ in single LV cardiomyocytes, a repetitive electric tetanization technique (10 Hz; 10 to 20 s) was used in thapsigargin (2 μmol/L)-treated cells, as previously described. In the setting of sarcoplasmic reticulum Ca²⁺-ATPase inhibition by thapsigargin, this procedure results in reproducible maintained elevation of [Ca²⁺], during tetanic shortening, thus allowing assessment of a steady state relation between shortening and [Ca²⁺], in intact cells. Compared with control LV cardiomyocytes, the relationship between tetanic elevation of [Ca²⁺], and fractional shortening was shifted rightward in septic LV cardiomyocytes, which suggests reductions in myofilament response to Ca²⁺. Reductions in myofilament response to Ca²⁺ were largely prevented in septic LV cardiomyocytes isolated from rats treated with zVAD.fmktreatment (Figure 5C).

**In Vitro Effects of Serum From Endotoxin-Treated Rats on Cardiomyocyte Contractile Function, Caspase-3–Like Activity, and Troponin T**

In vitro serum treatment had no effects on LV cardiomyocyte cell death (trypan blue exclusion and Hoechst 33342 staining; data not shown). Exposure of control LV cardiomyocytes with serum of endotoxin-treated rats induced sustained depression of fractional shortening (Figure 6A) and calcium...
transient (data not shown) starting at 20 minutes, whereas control serum had no effect. Septic serum induced progressive increases in LV cardiomyocyte caspase-3–like activity (Figure 6B) and troponin T cleavage (Figure 6C), whereas control serum had no effect.

Serum from endotoxin-treated rats induced reductions in LV cardiomyocyte shortening (Figure 7A), calcium transient (data not shown), and increases in caspase-3–like activity (data not shown), and troponin T cleavage (Figure 7B). Serum from endotoxin plus zVAD.fmк or zDEVD.cmк had similar effects (data not shown). In contrast, these abnormalities were prevented in LV cardiomyocytes incubated (15 minutes) with caspase inhibitor (zVAD.fmк or zDEVD.cmк; 100 μmol/L) or

Figure 4. Effects of rat treatment on cardiomyocyte function. In each experiment, LV cardiomyocytes were isolated from control, zVAD.fmк-, endotoxin-, or endotoxin+zVAD.fmк-treated rats (n=6 in each group). Results are mean±SEM of 72 cells; *P<0.01 vs control. A, Fractional shortening; B, calcium transient, and C, diastolic [Ca^{2+}] of LV cardiomyocytes.

Figure 5. Effects of rat treatment on Ca^{2+}-dependent contractile reserve. In each experiment, LV cardiomyocytes were isolated from control, zVAD.fmк-, endotoxin-, or endotoxin+zVAD.fmк-treated rats. Results are mean±SEM of 50 cells; n=6 rats in each group. Influence of extracellular calcium concentration [Ca^{2+}] on diastolic cell length (A) and on cell shortening (B) is shown. C, Relationship between intracellular calcium concentration [Ca^{2+}] and tetanic cell shortening.
isolated from caspase inhibitor (zVAD.fmk or zDEVD.cmk; 3 mg/kg body wt)–treated rats (Figure 7A, 7B).

Exposure (60 minutes) of LV cardiomyocytes to 100 and 500 ng/mL endotoxin had no effects on cell viability, fractional cell shortening, caspase-3–like activity, and troponin T (Table).

**Discussion**

Myocardial dysfunction encountered in septic shock has been attributed to either the cardiodepressive properties of circulating cytokines or the impairment of cardiac regional perfusion, and endotoxin may act as a causative agent in both settings. The present study suggests the new finding that
caspase-3 activation plays an important role in endotoxin-induced cardiomyocyte dysfunction, which may be related to changes in calcium myofilament response, contractile protein cleavage, and sarcomere disorganization. Caspase activation has been implicated in contractile dysfunction of various models of myocardium injury.15,17,18 In these models, contractile dysfunction was not associated with elevated LV cardiomyocyte end-stage nuclear apoptosis.18 Consistently, we observed that in vivo endotoxin treatment induced LV cardiomyocyte caspase-3 activation, without major terminal apoptosis.13 Both death receptor– and mitochondrial-related factors represent possible upstream pathways that would regulate effector caspase activation. These results are consistent with previous studies showing activation of whole heart multiple caspases in response to sepsis.9,11–13 Along with cardiomyocyte caspase-3 activation, endotoxin treatment induced major reduction of contractile function with troponin T cleavage and sarcomere disarray. Endotoxin also produced alterations in myofilament Ca2+ response,16,19 which may participate in the septic myocardial dysfunction. Importantly, functional and morphological alterations of septic cardiomyocytes were prevented by caspase inhibitor zVAD.fmk, suggesting that caspase-3 activation may underlie, at least part, cardiomyocyte contractile dysfunction and calcium homeostasis perturbations. Beneficial effects of caspase inhibition could not be related to changes in TNF-α production because zVAD.fmk did not prevented endotoxin-induced increases in serum and heart TNF-α levels. These results are, however, consistent with studies indicating that caspase activation may directly alter myofilamentary structure and calcium pumps and channels.20,21

Next, we evaluated the effects of caspase inhibitors on naive cardiomyocytes exposed to septic serum to test potential changes in host immune response and noncardiomyocyte cells associated with caspase inhibitor treatments.7,8 Consistent with studies in which similar in vitro models were used,4,6 we observed that septic serum induced cardiomyocyte dysfunction in the absence of detectable endotoxin levels. Any direct endotoxin effects on LV myocyte structures and functions were further excluded in our in vitro series of experiments. Septic sera induced cardiomyocyte caspase-3 activation, troponin T cleavage, and contractile dysfunction, which were prevented by the use, both in vivo and in vitro, of zVAD.fmk, a pan-caspase inhibitor, and zDEVD.cmk, a specific caspase-3–like inhibitor.22,23

In contrast, no beneficial effects on LV myocyte structures and functions were observed when serum obtained from endotoxin- and zVAD.fmk-treated rats was used. This is consistent with either high cell permeability and serum short half-life24,25 or absence of effects on immune response of small peptide caspase inhibitors. Caspase-3–like activation can be directly responsible for septic serum–induced cardiomyocyte contractile dysfunction. This contention is supported by the novel findings that caspase-3 may have a role in regulating cardiac contractility and that its inhibition may be associated with reversible states of depressed contractility.26 In addition, similar results emerged from studies showing that contractile performance and sarcomere disarray of failing ventricular myocytes can be corrected via caspase inhibition.17,18 Many putative caspase cleavage sites in cardiac contractile and structural proteins may be identified through databank research with caspase cleavage motifs. Additionally, exposure of myofibrillar proteins to activated caspase-3 results in α-actin, α-actinin, troponin T,15 and myosin light chain27 cleavage. In our model of sepsis, cardiomyocytes isolated from endotoxin-treated rats displayed troponin T cleavage and sarcomere disorganization, which were prevented by the broad-spectrum caspase inhibitor zVAD.fmk. Although these observations do not demonstrate a causal link, breakdowns of cardiac myofilament components and sarcomere disorganization induced by caspase activation suggest an important interrelationship between caspase activation and functional reserve in cardiomyocytes.

In summary, the results show an important relationship between effector caspase-3 activation and functional contractile reserve in cardiomyocytes in a rat endotoxin model of sepsis. Targeting caspases in cardiomyocytes may be beneficial not only for cardiomyocyte function but also for myocardial depression of sepsis.

Acknowledgments

This work was supported by grants from Fondation de l’Avenir pour la recherche médicale appliquée (ET1-306, 2001) and INSERM (Biologie et pathologie des régulations cellulaires). U459 and EA 2689 belong to IFR 114 IMPRT (CHU, COL, INSERM, IRCL, Université Lille 2). S. Lancel is supported by grants from Ministère de l’éducation nationale, de la recherche, et de la technologie MENRT 3857-2002.

References

3. Parker MM, Shellhammer JH, Bacharach SL, Green MV, Natanson C, Frederick TM, Damske BA, Parrillo JE. Profound but reversible myo-
Ventricular Myocyte Caspases Are Directly Responsible for Endotoxin-Induced Cardiac Dysfunction

Steve Lancel, Olivier Joulin, Raphael Favory, Jean Francois Goossens, Jérome Kluza, Claude Chopin, Pierre Formstecher, Philippe Marchetti and Remi Neviere

_Circulation_. published online May 16, 2005; Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2005/05/16/CIRCULATIONAHA.104.490979.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ 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_ is online at:
http://circ.ahajournals.org//subscriptions/