Tumor Necrosis Factor-α Upregulates Angiotensin II Type 1 Receptors on Cardiac Fibroblasts

Devorah Gurantz, Randy T. Cowling, Francisco J. Villarreal, Barry H. Greenberg

Abstract—Angiotensin II (Ang II) plays an important role in post–myocardial infarction (MI) remodeling. Most Ang II effects related to remodeling involve activation of the type 1 receptor (AT₁). Although the AT₁ receptor is upregulated on cardiac fibroblasts post-MI, little is known about the mechanisms involved in the process. Consequently, we tested whether growth factors known to be present in the remodeling heart increased AT₁ mRNA levels. Using quantitative competitive reverse transcription–polymerase chain reaction, we found that norepinephrine, endothelin, atrial natriuretic peptide, and bradykinin had no significant effect on AT₁ mRNA levels. Ang II, transforming growth factor-β, and basic fibroblast growth factor reduced AT₁ mRNA levels \( P < 0.02 \). Tumor necrosis factor-α (TNF-α), however, produced a marked increase in AT₁ mRNA. After 24 hours of TNF-α incubation, AT₁ mRNA increased by 5-fold above control levels \( P < 0.01 \). The EC₅₀ for the TNF-α effect was 4.6 ng/mL (0.2 nmol/L). Interleukin (IL)-1β caused a 2.4-fold increase, whereas IL-2 and IL-6 had no significant effect. Studies of TNF-α enhancement of AT₁ mRNA levels demonstrate that the increase was not due to a change in transcript stability. TNF-α treatment for 48 hours also resulted in a 3-fold increase in AT₁ surface receptor and a 2-fold increase in Ang II–induced production of inositol phosphates. The present findings provide evidence for TNF-α regulation of AT₁ receptor density on cardiac fibroblasts. Because TNF-α concentration and AT₁ receptor density increase in the myocardium after MI, these results raise the possibility that TNF-α modulates post-MI remodeling by enhancing Ang II effects on cardiac fibroblasts. (Circ Res. 1999;85:272-279.)

Key Words: AT₁ ■ cardiac fibroblast ■ tumor necrosis factor-α ■ post–myocardial infarction remodeling

There is considerable evidence that the renin-angiotensin system plays an important role in post–myocardial infarction (MI) cardiac remodeling. After MI, the renin-angiotensin system is activated both systemically and locally within the heart. Angiotensin II (Ang II) is a growth factor that can stimulate processes known to be associated with cardiac remodeling, such as myocyte growth (hypertrophy) and extracellular matrix (ECM) protein synthesis (fibrosis). Moreover, the administration of angiotensin-converting enzyme inhibitors and Ang II receptor blockers inhibits post-MI remodeling and reduces mortality in experimental animal models. Angiotensin-converting enzyme inhibitors also reduce mortality in MI survivors.

Cardiac fibroblasts are involved in post-MI remodeling through the generation of replacement scar tissue in the infarct zone and the production of fibrosis in noninfarcted segments of myocardium. Ang II stimulation of cardiac fibroblasts increases cell division and enhances production of ECM proteins such as fibronectin and collagens. Ang II also induces cardiac fibroblasts to secrete a paracrine growth factor(s) that stimulates hypertrophy of cardiac myocytes. These growth-promoting effects of Ang II are mediated through the Ang II type 1 receptor, AT₁. Stimulation of this G protein–coupled receptor results in the activation of phospholipase C, the production of inositol phosphates (IP), and a rise in intracellular calcium. There is evidence that AT₁ mRNA levels and receptor density are increased after MI and that these changes occur predominantly in cardiac fibroblasts. Although upregulation of the AT₁ receptor would be expected to modulate fibroblast activities involved in post-MI remodeling, little is known about the mechanism(s) responsible for this increase in receptor density.

A variety of growth factors, including neurotransmitters, hormones, and cytokines, are increased systemically and/or locally in the heart after MI. Many of these agents are known to modulate fibroblast activities such as cell proliferation and ECM synthesis. Thus, we hypothesized that some of these factors may be involved in the post-MI regulation of the AT₁ receptor density on cardiac fibroblasts. In initial experiments, we assessed the effects of selected candidate agents on AT₁ mRNA levels in neonatal rat cardiac fibroblasts. Results derived from the testing of various humoral candidates indicate that tumor necrosis factor-α (TNF-α) has a unique capacity to substantially increase AT₁ mRNA levels.

TNF-α is a pleiotropic cytokine that plays an important role in the response to tissue injury and wound healing.
Increased amounts of this cytokine have been detected in regions of the infarcted heart23–26 where AT₁ upregulation is known to occur. Consequently, we proceeded to characterize TNF-α effects on AT₁ mRNA levels. In this study, we demonstrate that TNF-α enhancement of AT₁ mRNA levels is not due to a change in transcript stability. Increases in mRNA levels are associated with increases in receptor density and with the enhanced production of IP in response to Ang II treatment. Thus, these findings provide evidence that TNF-α increases the density of functional AT₁ receptors on cultured cardiac fibroblasts and suggest a potential important in vivo role for this cytokine in the setting of MI.

Materials and Methods

Cell Cultures

Neonatal rat cardiac fibroblasts were prepared from hearts of 1- to 2-day-old Sprague Dawley rats as described by Kim et al.5 For each experiment, cells were plated from frozen stock (passage 0) in medium (DMEM high glucose; Gibco-BRL) containing 10% FBS. At 90% confluency, medium was replaced with serum-free medium (DMEM high glucose; Gibco-BRL) containing 10% FBS. At 90% confluency, medium was replaced with serum-free medium for 24 hours, and cultures were treated according to the experimental design. Fibroblasts were exposed to norepinephrine (NE, Sigma), bradykinin (BK; Sigma), endothelin (ET; Peninsula Laboratories), atrial natriuretic peptide (ANP; Peninsula Laboratories), human transforming growth factor-β (TGF-β; R&D Systems), human recombinant interleukin (IL)-1β, human recombinant IL-6, and rat recombinant interleukin (IL)-2 (all from R&D Systems) in serum-free medium for 24 hours. (See Figure 2 for doses used.) Recombinant rat TNF-α (Biosource International) was used for all experiments and administered according to the experimental protocol. Actinomycin D was obtained from Calbiochem. Rabbit polyclonal anti-TNF-α antibody was purchased from Genzyme Diagnostics and Biosource International.

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>TNF&lt;sub&gt;α&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNA (ng)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Figure 1. Competitive quantitative RT-PCR for AT<sub>1</sub> mRNA levels in cardiac fibroblasts. A, Ethidium bromide-stained agarose gels of PCR products (Target) obtained from 3 different quantities of RNA isolated from untreated or TNF-α (10 ng/mL)-treated cells. RNA was amplified in the same reaction mixture with known amounts of deletion mutant AT<sub>1</sub> cRNA (Mutant). Competition between amplification of the target and the mutant RNAs can be observed (see Materials and Methods). B, Plot of the log ratio of intensity of the corresponding pairs of bands (○, TNF-α treated; □, untreated) is plotted against log RNA concentration. The amount of RNA containing the same number of AT<sub>1</sub> mRNA molecules as the mutant is denoted with an arrow and represents equal amplification.](http://circres.ahajournals.org/)}
Receptor Binding Assays

Binding of Ang II was performed on intact adherent cells plated in multwell plates, which had been treated with 100 ng/mL TNF-α for 48 hours. The procedure used was described by Villarreal et al., with modifications according to Widdowson et al. The modification included the use of varying concentrations of 0.1 to 10 nmol/L [*3H]Ang II (Amersham Life Science) for total binding, incubation of the cells with ligand for 2 hours at 4°C, and protein determination from an aliquot of each culture dish well. On the basis of specific activity, [*3H]Ang II counts were converted to fmol Ang II-bound and normalized per mg of protein. Nonspecific binding was determined in the presence of “cold” Ang II, and competition for binding was assessed in the presence of losartan and PD123319 (RBI). Maximal binding, Bmax, and the dissociation constant, KD, were derived in 2 ways. Specific binding was plotted against [*3H]Ang II concentration and fitted to a hyperbolic curve according to the equation

\[ B = \frac{B_{\text{max}} \times ([*3H]\text{Ang II})}{K_D + ([*3H]\text{Ang II})}, \]

where \( B \) is amount of [*3H]Ang II bound and \( B_{\text{max}} \) and \( K_D \) are derived (Figure 5) using the Prism program (GraphPad). \( B_{\text{max}} \) and \( K_D \) were also determined by means of Scatchard plot analysis.

Cell Counts

Cells were plated and treated in a fashion identical to that for binding studies. Medium was removed and saved. After trypsinization, cell suspension in a known volume was pooled with the original cell medium. Cells were counted in the presence of trypan blue to identify nonviable cells.

Production of Inositol Phosphates

For the determination of Ang II–induced IP production, fibroblasts were plated and treated for 48 hours with TNF-α (100 ng/mL) in inositol-free medium. During the last 24 hours of TNF-α treatment, cells were loaded with [*3H]myo-inositol (NEN). Isolation of IP was performed according to a previously described procedure. IP production in response to Ang II exposure over the range of 10^-9 to 10^-6 mol/L were measured at 45 minutes in the presence of LiCl (10 mmol/L). To determine the effect of Ang II receptor antagonists, cells were incubated with either losartan (AT1 antagonist) or PD123319 (AT2 antagonist). Because cell counts indicated minimal variations in cell number among wells, data were expressed as counts per well.

Data Analysis

Data are presented as mean±SEM. Significant differences were determined by t test or ANOVA. Curve fits were generated using the Prism computer program (GraphPad). A P value <0.05 was considered statistically significant.

Results

Modulation of AT1 mRNA Levels

The possibility that growth factors present in the remodeling heart might be playing a role in the regulation of AT1 receptor density was initially assessed by measuring the effect of these agents on AT1 mRNA levels. Control AT1 mRNA levels in the cultures of untreated neonatal cardiac fibroblasts ranged from 3000 to 8000 molecules per nanogram RNA with an average of 5667±536 (n=14). The effects of the various agents on AT1 mRNA levels were expressed as change from control levels within each experiment (Figure 2). NE, ET, ANP, and BK did not significantly affect AT1 mRNA levels. Ang II, TGF-β, and bFGF each significantly reduced AT1 mRNA levels to 38%, 24%, and 44% of control levels, respectively (all \( P<0.02 \)). Exposure to TNF-α, however, increased AT1 mRNA nearly 5-fold above control levels (\( P<0.01 \)). IL-1β increased AT1 mRNA levels by 2.4-fold (\( P<0.04 \)), whereas IL-6 and IL-2 had no significant effects on AT1 mRNA levels.

TNF-α Effects on AT1 mRNA Are Time and Dose Dependent

To characterize the time dependence of the TNF-α effect, total RNA was extracted from cardiac fibroblasts at 2, 6, 12,
24, and 48 hours after exposure to serum-free medium containing TNF-α (10 ng/mL, 0.57 nmol/L) and from control fibroblasts exposed to serum-free medium in the absence of TNF-α (Figure 3A). TNF-α treatment resulted in an increase in AT1 mRNA as early as 6 hours after exposure. AT1 mRNA levels reached a 5-fold (*P<0.01) increase above control levels by 24 hours, and this effect was maintained at 48 hours.

Dose dependency of TNF-α on AT1 mRNA levels was determined at 24 hours of exposure. TNF-α concentrations used for the dose response determination ranged from 0.1 to 500 ng/mL (6 pmol/L to 3 nmol/L, Figure 3B). The TNF-α effective dose for 50% AT1 mRNA upregulation was found to be 4.6 ng/mL (0.26 nmol/L).

In the above experiments, cardiac fibroblasts were exposed to a single application of TNF-α for up to 48 hours. The effects of prolonged exposure to TNF-α were determined by applying 100 ng/mL TNF-α to fibroblasts every 48 hours for up to 6 days. The results showed that AT1 mRNA levels increased to 8.4-fold of basal levels after 4 days and were 7-fold higher after 6 days. Removal of TNF-α after 48 hours of exposure resulted in the return of AT1 mRNA to basal levels within 3 days.

To exclude the possibility that impurities in the TNF-α or the release of autocrine factors from fibroblasts were responsible for the upregulation of the AT1, experiments were performed in the presence of neutralizing antibodies to TNF-α. The use of TNF-α antibodies either concomitantly with TNF-α (n=2) or with conditioned medium from fibroblasts pretreated with TNF-α (n=1) suppressed the induction of AT1 mRNA.

TNF-α Does Not Increase AT1 mRNA Levels by Enhancing Message Stability
Previous studies had demonstrated that selected humoral agents can increase AT1 receptor density by enhancing transcript stability (e.g., insulin treatment of rat vascular smooth muscle cells32). Thus, the effect of TNF-α on AT1 mRNA stability was investigated. After induction of cardiac fibroblast AT1 mRNA levels by TNF-α (50 ng/mL) for 24 hours, cultures were treated with the transcription inhibitor actinomycin D (5 μg/mL) in the continuing presence of TNF-α. Total RNA was then extracted from fibroblasts at various defined intervals. AT1 mRNA levels were quantified by RT-PCR as described above. Figure 4 depicts the fraction of AT1 mRNA levels relative to the levels at the beginning of actinomycin D treatment (time=0) for untreated and TNF-α–treated cultures (mean±SEM, n=3). Although TNF-α enhanced AT1 mRNA levels in a manner depicted in Figure 3, it did not alter the rate of mRNA degradation in the presence of actinomycin D, which indicates that it did not increase the stability of the AT1 transcripts.

TNF-α Enhances Density of AT1 Cell Surface Receptor
Binding studies were performed to determine whether TNF-α increased cell surface AT1 receptor density. Figure 5A illustrates that total binding levels for [3H]Ang II (10 nmol/L) were approximately doubled at 24 hours and further increased to 3.8 above control levels by 48 hours, whereas nonspecific binding in the presence of excess unlabeled Ang II (10-6 mol/L) remained essentially unchanged. Addition of the selective AT1 antagonist losartan (10-5 mol/L) resulted in nearly complete displacement of bound radioligand, whereas addition of the AT2 antagonist PD123319 (10-5 mol/L) had no significant effect, which demonstrates that an increase in AT1 receptor density was the primary cause for the increase in Ang II binding to cultured fibroblasts.
Saturation binding experiments were performed to obtain the dissociation constant ($K_d$) and receptor density ($B_{max}$) for control and TNF-α–treated cardiac fibroblasts. Although the dissociation constant for binding was not significantly different in control fibroblasts (4.12 ± 0.58 nmol/L, n = 3) and TNF-α–treated fibroblasts (4.53 ± 0.47 nmol/L, n = 3), $B_{max}$ in treated cells (1313 ± 124 fmol/mg protein) was 2.8-fold higher ($P < 0.01$, n = 3) than $B_{max}$ in control cells (466 ± 70 fmol/mg protein). Data from a representative experiment are illustrated in Figure 5B and 5C.

The effect of TNF-α on cell proliferation was assessed to determine whether increases observed in surface receptor density were due to an increase in cell number. No significant differences in the number of viable or dead cells between control and TNF-α–treated fibroblasts was noted (25 wells counted per each cell group in 4 individual experiments). On the basis of the number of cells per well, total protein level per well, and level of Ang II binding, the number of AT$_1$ receptors was estimated to be $\approx 10^5$ per cell.

**Upregulation of AT$_1$ Receptor by TNF-α Results in Enhancement of Ang II–Induced IP Production**

Figure 6 illustrates levels of Ang II ($10^{-9}$ to $10^{-6}$ mol/L)–induced IP production in untreated fibroblasts or fibroblasts pretreated for 2 days with TNF-α (100 ng/mL). Control levels used for normalization of the data in each experiment were derived from fibroblasts treated with neither TNF-α nor Ang II (Figure 6, control [0 mol/L Ang II]). In untreated fibroblasts, Ang II stimulated similar levels of IP production throughout a concentration range of $10^{-9}$ to $10^{-6}$ mol/L. Pretreatment of the cells with TNF-α, however, altered the profile of IP production. Basal levels (0 mol/L Ang II) were reduced to one third of the corresponding levels of untreated cells ($P < 0.001$, n = 3). Increasing concentration of Ang II progressively increased IP production in TNF-α–treated cells, reaching maximal levels at $10^{-7}$ to $10^{-6}$ mol/L. Ang II, with a significant 2-fold increase observed at $10^{-7}$ mol/L. Ang II relative to untreated cells (Figure 6, $P < 0.003$, n = 3). PD123319 (10$^{-5}$ mol/L), the AT$_2$ antagonist, did not significantly affect IP production, whereas losartan (10$^{-5}$ mol/L), the AT$_1$ antagonist, produced essentially complete blockade when used in 100-fold excess.

**Discussion**

Ang II activation of cardiac fibroblasts plays an important role in post-MI cardiac remodeling. Most of the known effects of Ang II on cardiac fibroblasts are mediated through the AT$_1$ receptor, and there is evidence that this receptor is upregulated after MI. The present study shows that TNF-α increases AT$_1$ mRNA levels in neonatal rat cardiac fibroblasts. The increase in AT$_1$ mRNA levels is associated with an increase in membrane receptor density and enhanced
Figure 6. Ang II–induced AT₁-mediated IP production is enhanced by TNF-α treatment (M indicates mol/L). Production of IP induced by Ang II (10⁻⁸ to 10⁻⁷ mol/L) is shown for untreated (open bars) and TNF-α-pretreated (closed bars, 100 ng/mL for 48 hours) fibroblasts. Values ( ³H]IP counts per well) were normalized in each experiment to the IP levels of fibroblasts treated with neither TNF-α nor Ang II (open bars, 0 mol/L Ang II). Results are presented as mean±SEM of 3 individual experiments. TNF-α tended to depress basal IP production in fibroblasts. Ang II dose-dependent enhancement of IP synthesis is observed for fibroblasts pretreated with TNF-α with a significant (P<0.003) 2-fold higher IP production than that of corresponding controls at 10⁻⁶ mol/L Ang II.

TNF-α is involved in the regulation of the AT₁ receptor, cultured neonatal fibroblasts were exposed to a group of preselected candidate neurotransmitters, growth factors, and cytokines. In vitro experiments are advantageous for this purpose in that a selected agent can be examined in the absence of systemic effects. The results demonstrate that TNF-α markedly increased AT₁ mRNA levels. IL-1β produced a smaller increase. A previous study done using cultured rat vascular smooth muscle cells showed that IL-1β produced an increase in AT₁ mRNA of magnitude similar to that observed in cardiac fibroblasts.33 Interestingly, in that study, TNF-α had no significant effect on AT₁ mRNA levels, which suggests that the upregulation seen in cardiac fibroblasts in the present study is cell or tissue specific.

The reduction by Ang II and lack of any significant effect with ET seen in these experiments are consistent with reported observations in cardiac fibroblasts.34 However, our results with NE treatment contrast with those previously reported, which noted a modest 60% upregulation of AT₁ mRNA levels after a 24-hour treatment of neonatal cardiac fibroblasts with NE.34 TGF-β₁, the secretion of which from cultured cardiac fibroblasts is stimulated by Ang II,21 decreased AT₁ mRNA to 24% of control levels. A similar reduction in AT₁ mRNA was seen with bFGF. A decrease in AT₁ mRNA levels induced by bFGF has been seen in vascular smooth muscle cells and has been attributed to a decrease in AT₁ gene transcription rate and destabilization of the AT₁ message.35

Induction of AT₁ mRNA Levels

The increase in AT₁ mRNA with TNF-α is seen as early as 6 hours after exposure to the cytokine and peaks at 24 hours after treatment. Maximal effect of a single dose led to a 5-fold increase in mRNA levels, whereas continued application of TNF-α over 6 days resulted in a progressive increase in mRNA levels. Removal of the cytokine was associated with a return to basal levels within 3 days. These observations may have implications relevant to the in vivo setting after MI. AT₁ is upregulated after MI at the peri-infarction zone19,21 predominantly on cardiac fibroblasts.20 Investigators studying the post-MI heart have reported that macrophages infiltrating the necrotic region and its border zones, including cells surrounding the vasculature, appear to be involved in production of TNF-α.21,26 This production of TNF-α is sustained over an extended period in both the border zone and remote segments of the myocardium.26 Thus, the continued presence of high levels of TNF-α in the border zone and noninfarcted regions of the myocardium is consistent with the possibility that TNF-α may be responsible for the regulation of AT₁ seen in these regions.19,20

Increased AT₁ mRNA stability and/or enhancement of rate of AT₁ gene transcription could account for enhancement of AT₁ mRNA levels. However, the degradation rate of AT₁ mRNA was unaffected by TNF-α treatment, which indicates a lack of effect on message stability. Future work should address the possibility of enhancement of transcription and the identification of gene enhancer sequences that are responsive to TNF-α. The promoter for AT₁A has been isolated, and putative response elements have been identified on the basis of sequence analysis.36 Although the presence of putative response elements to TNF-α, such as activator protein-1 and nuclear factor-κB,37,38 have been identified on the AT₁ gene promoter, a systematic analysis of their activity in the cardiac fibroblasts is required.

Induction of Density of Functional AT₁ Receptors

The increase in mRNA was followed by an increase in AT₁ surface receptor density without change in receptor affinity. Our results also showed that upregulation of the AT₁ mRNA and receptor density occurs on individual cells rather than as a result of TNF-α–induced cell proliferation. Ang II–induced synthesis of IP has been previously described and is associated with AT₁ activation.32 Our data demonstrate that increased AT₁ receptor density in response to TNF-α results in the enhanced production of second-messenger IP by Ang II.

Potential Role of Fibroblast AT₁ Receptors in Cardiac Remodeling

This study provides evidence that TNF-α is involved in the upregulation of AT₁ receptor density on cardiac fibroblasts in the post-MI rat heart. The significance of this observation is related to the role that Ang II activation of the AT₁ receptor on cardiac fibroblasts plays in post-MI cardiac remodeling. Previous studies...
have shown that AT1 receptors are substantially more abundant on cardiac fibroblasts than on cardiac myocytes. Exposure of cardiac fibroblasts to Ang II leads to an increase in cell number. Ang II also stimulates production of the ECM proteins and III and fibronectin and other substances, such as TGF-β, which are related to the deposition of interstitial matrix and scar formation. Ang II also induces cardiac fibroblasts to produce a paracrine factor(s) that stimulates myocyte hypertrophy. All of these effects of Ang II on cardiac fibroblasts are mediated through the AT1 receptor. Increased density of AT1 receptors on cardiac fibroblast after MI would be expected to enhance Ang II–mediated effects on the remodeling process. The Ang II type 2 receptor, AT2, has been reported to mediate antigrrowth and antiproliferation functions. However, we found no evidence of AT2 upregulation in binding studies.

In summary, the results of this study demonstrate that TNF-α increases the density of functional AT1 receptors on cardiac fibroblasts. These findings identify a previously unrecognized association that could link the effects of disparate systems that are believed to be involved in post-MI remodeling.

Acknowledgments

This study was supported in part by the National Heart, Lung, and Blood Institute (Grant NL-03160, to F.J.V.). We thank Dr W. H. Dillmann for his helpful suggestions and Juan Alvergue for technical assistance.

References

Tumor Necrosis Factor-α Upregulates Angiotensin II Type 1 Receptors on Cardiac Fibroblasts
Devorah Gurantz, Randy T. Cowling, Francisco J. Villarreal and Barry H. Greenberg

_Circ Res._ 1999;85:272-279
doi: 10.1161/01.RES.85.3.272

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 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/85/3/272

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/