Coexpression of Endothelin-Converting Enzyme-1 and Endothelin-1 in Different Stages of Human Atherosclerosis

Christian Ihling, MD; Thomas Szombathy, MD; Bernd Bohrmann, PhD; Manfred Brockhaus, PhD; Hans E. Schaefer, MD; Bernd M. Loeffler, MD

Background—Endothelin-converting enzyme (ECE)-1 activates endothelin-1 (ET-1) and may thus contribute to the regulation of vascular tone and cell growth during atherosclerosis.

Methods and Results—To evaluate ECE-1 immunoreactivity concerning big ET-1/ET-1, we performed qualitative and quantitative immunohistochemistry in normal internal mammary arteries (n=10), in coronary arteries with adaptive intimal fibrosis (n=10), in aortic fatty streaks (n=10), and in distinct regions of advanced carotid plaques (n=15). Furthermore, we determined ECE-1 activity in the control specimens and in the inflammatory intimal regions of carotid plaques. Double immunolabeling showed that ECE-1 was present in endothelial cells, vascular smooth muscle cells, and macrophages. All ET-1+ cells were simultaneously ECE-1+. Most importantly, there were significantly more ET-1+ cells in the intima and media when atherosclerosis was in an inflammatory stage than when it was in a noninflammatory stage. Moreover, ECE-1 activity was upregulated in the intima of carotid plaques, although immunohistochemically, there were no significant differences between the number of ECE+ cells in the different compartments of the arterial wall.

Conclusion—Together with ET-1, ECE-1 is abundantly present in human arteries and at different stages of atherosclerotic plaque evolution. The upregulation of the ECE-1/ET-1 system is closely linked to the presence of chronic inflammation and is present in very early stages of plaque evolution. Therefore, enhanced production of active ET-1 may substantially contribute to cell growth and the regulation of vascular tone in advanced atherosclerotic lesions and in the very early stages of plaque evolution, when a plaque is still imperceptible clinically. (Circulation. 2001;104:864-869.)

Key Words: endothelin ■ atherosclerosis ■ inflammation

Endothelin (ET)-1, the most potent vasoconstrictive substance known today,1 exerts different biological activities in a large variety of noncardiovascular and cardiovascular diseases, including hypertension2 and atherosclerosis.4 Consequently, ET1 and ET2 receptors, which mediate the effects of ET-1, have been detected in numerous human tissues, including endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), as well as other cell types.3

The transcription of ET-1 messenger RNA is increased by different factors.1 Posttransitionally, the precursor of ET-1 is processed by furin5 to generate the functionally inactive big ET-1, which is activated through the proteolytic action of endothelin-converting enzyme (ECE).6 Recently, 2 homologous proteins, ECE-1 and ECE-2, with different tissue distributions, pH optima, and sensitivities to phosphoramidon that both efficiently catalyze the hydrolysis of big ET-1, have been isolated and the corresponding genes cloned.7,8 So far, 3 isoforms of ECE-1 have been identified in humans (ECE-1a, ECE-1b, and ECE-1c); they are encoded by the same gene and are produced by alternative splicing. The ECE-1 isoforms are structurally similar membrane-bound enzymes with an extracellular domain containing the catalytic active center. Although ECE-1a and ECE-1c are localized on the plasma membrane, ECE-1b seems to be predominantly localized in intracellular vesicles.9

ET-1 plays an important role in the maintenance of basal vascular tone10 and has been implicated in the pathophysiology of vasospastic reactions.11–13 In addition, ET-1 has mitogenic properties on VSMCs.14 Recent studies indicate that in apoE-deficient mice, ET-1 contributes to atheroma formation via the stimulation of the ET1 receptor and, hence, has an important impact on the progression of atherosclerosis.4 Furthermore, it has been shown that big ET-1/ET-1 is locally produced in the atherosclerotic intima by macrophages and that chronic inflammatory processes may be involved in the generation of big ET-1/ET-1.15,15 Moreover, there is evidence that ECE-1 is present in human atherosclerotic tissue and that its activity is upregulated.16,17 Because ECE-1 activates ET-1 and may thus also contribute to the regulation of vascular tone and cell growth during atheroscler-
Atherosclerotic lesions were graded as previously described. 18

Tissue Specimens
Histologically normal internal mammary arteries (IMAs; n=10) from patients undergoing aortocoronary bypass surgery were used as controls. Coronary arteries with adaptive intimal fibrosis (AIF; n=10) were derived from the explanted hearts of patients undergoing heart transplantation for idiopathic dilated cardiomyopathy.

Aortic fatty streaks (n=10) were obtained from the proximal aortas of heart transplant specimens and autopsied individuals, and advanced carotid plaques (CPs; n=15) were taken from patients undergoing carotid endarterectomy. We performed a comparative examination of fixed CPs removed during surgery with CPs from autopsied patients whose death had occurred ≤12 hours before our examination. The results showed that neither the quality nor the quantity of ET-1 and ECE-1 staining was affected by the post mortem delay. The tissue was routinely fixed in 4% unbuffered formalin and then prepared according to standard methods. Serial sections were stained with hematoxylin and eosin and elastica-von Gieson’s stain and used for immunohistochemistry. To determine ECE-1 activity, the tissue was snap-frozen in liquid nitrogen.

Antibody Production
A soluble monomeric form of human ECE-1 was produced by fusing the ECE-1 cDNA encoding the extracellular part of ECE-1 (ECE-1e) with the human alkaline phosphatase signal sequence. Then, ECE-1e was expressed in HEK293-EBNA cells, and ECE-1e secreted into the culture medium was purified by affinity chromatography, including wheat-germ and ricinus communis agglutinin affinity and anion exchange chromatography to near homogeneity. Swiss Albino mice were immunized to human ECE-1 by subcutaneous injection of purified ECE-1e in complete Freund’s adjuvants and subsequently boosted with purified ECE-1e. The production of ECE-1e antibodies was tested in an ECE-1 capture assay by serial dilution of serum samples. Drained lymph node cells from anti-ECE-1e mice were fused with PAI myeloma cells according to standard procedures.

Briefly, the ECE-1 capture assay was performed by biotinylation of purified ECE-1e (bio-ECE-1e) with N-hydroxysuccinimide–biotin according to the manufacturer’s instructions. Diluted plasma samples of immunized mice or culture medium of fusion clones were reacted with bio-ECE-1e in anti-mouse IgG-coated 96-well plates. Plates were thoroughly washed, and bound bio-ECE-1e was detected with peroxidase-labeled streptavidin.

Cell clones derived after fusion were characterized for anti-ECE-1 antibody production by the ECE-1 capture assay for the anti-ECE-1 antibody. Culture medium of the 26 best cell clones was used to characterize the ability and specificity of the produced antibodies to detect human ECE-1 after Western blotting. The best clone (ECE 6) was recloned to yield a stable expressing cell line, and larger amounts of IgG were produced. The IgG was purified from the culture medium according to standard procedures.

Immunohistochemistry
We used polyclonal rabbit sera as primary antibodies against ET-1 (1:250, IHC 6901, Peninsula) and T-lymphocytes (1:100; CD3; DAKO), and monoclonal antibodies against leukocyte common antigen (LCA) (1:200; DAKO), α-actin (1:600; DAKO), macrophages (1:100; CD 68; KP1), DAKO) and ECE-1 (1:500). Immunohistochemistry was performed as previously described. 12,13,15 The specificity of the ECE-1 antibody was tested by preabsorbing the antibody with an excess of human ECE-1 before immunohistochemistry, which resulted in a complete loss of staining (Figure 1D). To confirm the absence of inflammatory cells in the specimens with AIF and in the media, we performed staining for LCA. For CD 3 staining, the sections were pretreated with proteinase K for 1 minute; for CD 68 and α-actin staining, antigens were unmasked by pressure cooking in 10 μmol of citric acid (pH 6) for 5 minutes.

Morphometric Analysis and Statistical Methods
Using morphometric software, 5 to 15 random, nonoverlapping, microscopic high-power fields (HPF) of histologically distinct regions were analyzed by scoring the total number of nuclei and cells stained for ET-1 and ECE-1. A cell was scored positive for ECE-1 only when the typical membranous staining pattern was present in...
by guest on July 26, 2017 http://circ.ahajournals.org/ Downloaded from

>50% of the cell circumference and only when the staining was undoubtedly associated with a cell nucleus.

From the IMAs, 5 HPF of the media were evaluated; from the specimens with AIF and from the fatty streaks, 5 HPF from the intima and the adjacent media were investigated. Furthermore, from the CPs, 5 random HPF from areas showing severe chronic inflammation (defined by the presence of numerous CD68+ macrophages, CD3+ T-lymphocytes, and newly formed microvessels), 5 HPF from cell-depleted, fibrotic plaque regions (defined by the absence of inflammatory infiltrates, the presence of α-actin+ VSMCs, and extracellular matrix), and 5 HPF from the media subjacent to the plaque tissue were examined. All data are reported as mean±SD. The Bonferroni-Dunn test was applied to compare data from different plaque types and plaque regions and data from the controls.

ECE Activity Assay
To measure ECE activity, we prepared 10-μm-thick serial frozen sections (n=22) of IMAs (n=6) and CPs (n=6) and stored 20 sections from each specimen in Eppendorf tubes at -80°C. The first and last sections of a series were mounted on glass slides and stained with hematoxylin and eosin to confirm the presence of severe chronic inflammation in the CPs and the absence of atherosclerotic lesions in the IMAs. Thereafter, we performed planimetry and calculated the volume of the remaining 20 sections (n=10 of 10 cases in 100% of the ECs and in 47.2% of the medial VSMCs, mainly in the subendothelial compartment of the media (10 of 10 cases; 3.7±3.2%). ECE-1/ET-1 double immunoreactivity was present in 10 of 10 specimens and was localized to ECs (100%). Cell counting revealed that 16.8±16.8% of the intimal VSMCs and 12.5±11.9% of the medial VSMCs were ET-1+. As shown by ECE-1/ET-1 double immunolabeling, cellular ET-1 staining never occurred without ECE-1 staining (10 of 10 cases; Figure 2B).

Results
IMA With a Normal Histology
In the controls, ECE-1 immunoreactivity was present in 10 of 10 cases in 100% of the ECs and in 47.2±19.6% of the medial VSMCs (α-actin+; Figures 1A through 1C). In contrast to the widespread occurrence of ECE-1 immunoreactivity, ET-1 immunoreactivity was weak and localized predominantly to a subset of ECs (30% of the ECs were ET-1+) and scattered medial VSMCs, mainly in the subendothelial compartment of the media (10 of 10 cases; 3.7±3.2%). ECE-1/ET-1 double immunolabeling confirmed that all ET-1+ cells were also ECE-1+ (Figure 2A).

Coronary Arteries With AIF
In the vessels with AIF, ECE-1 immunoreactivity was present in 10 of 10 specimens and was localized to ECs (100%), intimal VSMCs (α-actin+; 44.0±19.8%), and medial VSMCs (α-actin+; 59.3±22.4%). ET-1 immunoreactivity was present in most of the ECs (70%). In the intima and the media, ET-1 immunoreactivity had a patchy distribution: it was focally extracellular with varying intensity (10 of 10 specimens). Cell counting revealed that 16.2±16.8% of the intimal VSMCs and 12.5±11.9% of the medial VSMCs were ET-1+. As shown by ECE-1/ET-1 double immunolabeling, cellular ET-1 staining never occurred without ECE-1 staining (10 of 10 cases; Figure 2B).

Atherosclerosis
Aortic Fatty Streaks
In the fatty streaks, ECE-1 immunoreactivity was present in 10 of 10 cases in ECs (100%), intimal macrophages (CD68+), intimal VSMCs (α-actin+; 46.1±20.5%), and medial VSMCs (α-actin+; 42.3±21.9%) of the intimal cells). Double immunolabeling with ECE-1 and CD3 revealed that intimal T-lymphocytes were never ECE-1+. Whenever present, ECs above fatty streaks showed a strong ET-1 reaction. Quantitative evaluation revealed that 52.7±20.3% of the intimal cells and 20.1±9.5% of the medial VSMCs were ET-1+. In the intima and media, small foci of diffuse extracellular ET-1 immunoreactivity were present. As confirmed by double immunolabeling (ECE-1/ET-1), ECs (100%), intimal foam cells (CD68+ and α-actin+), and medial VSMCs coexpressed ET-1 and ECE-1 (Figures 2C and 2D).

Advanced Atherosclerotic CPs
In the CPs, ECE-1 immunoreactivity was present in 15 of 15 cases in plaque inflammatory regions (34.2±26.9% of the cells), plaque fibrotic regions (40.9±28.4% of the cells), and the media beneath the plaque (33.9±19.8% of the cells). Luminal ECs and microvascular ECs showed an inconstant ECE-1 immunoreactivity (15% of luminal ECs were ECE-1+). Double immunolabeling confirmed that macrophages (CD68+), intimal VSMCs, and medial VSMCs (α-actin+) were ECE-1+, whereas T-lymphocytes (CD3+) were ECE-1- (Figures 3A through 3D). ET-1 immunoreactivity localized mainly to plaque regions with signs of chronic inflammation.
(55.2±21.2% of the cells) and occurred both intracellularly and extracellularly. Double immunolabeling (ET-1/ECE-1) revealed that, in those regions, extracellular ET-1 immunoreactivity colocalized with extracellular ECE-1 immunoreactivity (Figure 3D). In plaque fibrotic regions, ET-1 staining was focal, weak, localized to the cytoplasm of 5.2±4.3% of the VSMCs (α-actin+), and always found together with ECE-1 immunoreactivity. In the media beneath the plaque, ET-1 immunoreactivity was observed both intracellularly and extracellularly in a patchy distribution in 13.3±9.4% of VSMCs (α-actin+). Double immunolabeling (ET-1/ECE-1) revealed that cellular ET-1 immunoreactivity never occurred without ECE-1 immunoreactivity (Figure 3D).

### Statistical Analysis of ECE-1 and ET-1 Immunostaining

Table 1 summarizes the mean ECE-1 and ET-1 staining grades in the intima and media.

#### ECE in Intima

In the intima, we found no significant differences between the number of ECE-1+ cells in different compartments of inflammatory and noninflammatory atherosclerotic lesions (Table 2). Thus, chronic inflammation does not influence the extent of ECE-1 immunoreactivity in the intima of atherosclerotic lesions.

#### ECE in Media

In most cases, the number of ECE-1+ cells in the media beneath the different plaque types were not significantly different (Table 3). However, in the media beneath fatty streaks and CPs, ECE-1 staining was significantly lower compared with the media beneath AIF.

#### ET-1 in the Intima

There were significantly more ET-1+ intimal cells in specimens with inflammatory atherosclerosis than in those without it (Table 4). Correspondingly, in plaque fibrotic regions, there were significantly fewer ET-1+ cells than in plaque inflammatory regions and fatty streaks. Thus, ET-1 is upregulated as

### Table 1. Quantitative and Statistical Results

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Staining, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endothelium</td>
</tr>
<tr>
<td>IMA</td>
<td></td>
</tr>
<tr>
<td>ECE</td>
<td>100</td>
</tr>
<tr>
<td>ET</td>
<td>30</td>
</tr>
<tr>
<td>AIF</td>
<td></td>
</tr>
<tr>
<td>ECE</td>
<td>100</td>
</tr>
<tr>
<td>ET</td>
<td>70</td>
</tr>
<tr>
<td>FS</td>
<td></td>
</tr>
<tr>
<td>ECE</td>
<td>100</td>
</tr>
<tr>
<td>ET</td>
<td>100</td>
</tr>
<tr>
<td>CP</td>
<td></td>
</tr>
<tr>
<td>ECE</td>
<td>85</td>
</tr>
<tr>
<td>PI</td>
<td>34.2±26.9</td>
</tr>
<tr>
<td>PF</td>
<td>40.9±28.4</td>
</tr>
<tr>
<td>ET</td>
<td>100</td>
</tr>
<tr>
<td>PI</td>
<td>55.2±21.2</td>
</tr>
<tr>
<td>PF</td>
<td>5.2±4.3*</td>
</tr>
</tbody>
</table>

Values are mean±SD. FS indicates fatty streak; PI, plaque region with inflammation; and PF, cell-depleted fibrotic plaque.

*P<0.001 vs ECE; †P<0.01 vs ECE.
atherosclerosis progresses and reaches its peak values in fatty streaks; its occurrence is closely linked to the presence of chronic inflammation.

**ET-1 in the Media**

In the media, we generally found significantly higher ET-1 staining grades as atherosclerosis progressed in the intima (Table 5). Staining had already reached its peak in the media subjacent to fatty streaks. Interestingly, there were significantly fewer ET-1 cells in the media beneath CPs than beneath fatty streaks, and there was no statistically significant difference between the number of ET-1 VSMCs in the media beneath AIF and CPs.

**Relation of ECE-1-Staining to ET-1 Staining in the Intima**

In noninflammatory lesions (AIF) and noninflamed, fibrotic regions of CPs, the number of ECE-1 cells was significantly higher than the number of ET-1 cells (Table 1). In plaque regions with inflammation and in the intima of fatty streaks, the number of ET-1 cells was slightly higher than the number of ECE-1 cells, although this difference was not significant (Table 1).

**Relation of ECE-1-Staining to ET-1 Staining in the Media**

In the media beneath noninflammatory lesions, the number of ECE-1 cells exceeded the number of ET-1 cells significantly (Table 1), whereas in the media beneath inflammatory lesions, the number of ECE-1 cells was slightly higher than the number of ET-1 cells, although this difference was not statistically significant (Table 1).

**ECE Activity**

ECE-1 activity was significantly higher (42.9%, P<0.01 versus IMA) in the atherosclerotic tissue from CPs (119.6±12.6 ng/mm² in 24 hours) than in that from the IMA (83.7±7.9 ng/mm² in 24 hours). Thus, despite quantitative differences in the number of ECE-1 cells in these 2 compartments (IMA versus plaque regions with inflammation: P<0.0394), there is a functional upregulation of ECE-1 enzymatic activity in the atherosclerotic tissue with chronic inflammation.

**Discussion**

The results of the present study reveal that ECE-1 is abundantly present in different human arteries in ECs and intimal and medial VSMCs, and it colocalizes with big ET-1/ET-1. Therefore, big ET-1 may be locally converted into the biological active ET-1 to exert its pleiotropic effects. In atherosclerotic plaques with chronic inflammation, ECE-1 and ET-1 are present together in macrophages, VSMCs, and ECs. Quantitative analysis and functional studies revealed that ECE-1 activity is upregulated in the atherosclerotic tissue, although, on the immunohistochemical level, there were no significant differences between the number of ECE-1 cells in the different compartments of the arterial wall.

Our previous semiquantitative analysis of ET-1 immunoreactivity in coronary atherosclerotic plaques was confirmed;12,13 we found that ET-1 was upregulated as atherosclerosis progressed, mainly in regions with signs of chronic inflammation. Importantly, the results of this study extend our previous observations by demonstrating that the media subjacent to atherosclerotic plaques is also characterized by increasing numbers of ET-1 VSMCs and that the enzymatic activity of ECE-1 is upregulated with the progression of atherosclerosis. Taken together, these data raise the possibility that big ET-1 present in the media may be completely converted into its biologically active form. Thus, the availability of mature ET-1 may increase during the progression of atherosclerosis from an noninflammatory to an inflammatory state in the intima and in the media subjacent to the atherosclerotic plaque. Because vascular tone depends on the contraction of the media, ET-1 may therefore substantially contribute to the regulation of the vasoconstrictor tone of atherosclerotic arteries. Indeed, it has recently been shown that endogenous ET-1 exerts a vasoconstrictor effect in the epicardial arteries of patients with coronary artery disease, as evidenced by the vasodilation through the ET-1 receptor antagonist bosentan.20 Interestingly, in media subjacent to a CP, the number of ET-1 VSMCs is significantly lower than in media beneath a fatty streak lesion, and there is no significant difference between the number of ET-1 cells in media adjacent to AIF and media subjacent to a CP. This may be due to the medial atrophy with a reduction of the number of VSMCs, which is a common finding subjacent to advanced atherosclerotic plaques.

In line with recent in vivo findings in atherosclerotic rabbits,21 we observed the maximum upregulation of the ET-1 already in fatty streaks in the intima and in the media. Consequently, enhanced production of the active ET-1 may

---

**TABLE 3. P Values Between the ECE-1 Staining Grades in the Media**

<table>
<thead>
<tr>
<th></th>
<th>AIFM</th>
<th>FSM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMA</td>
<td>0.022</td>
<td>0.35</td>
<td>0.0086</td>
</tr>
<tr>
<td>AIFM</td>
<td>...</td>
<td>0.0002*</td>
<td>0.0001*</td>
</tr>
<tr>
<td>FSM</td>
<td>...</td>
<td>...</td>
<td>0.044</td>
</tr>
</tbody>
</table>

*P values are not significant unless P<0.0083. *Significant.

**TABLE 4. P Values for ET-1 Staining Grades in the Intima**

<table>
<thead>
<tr>
<th></th>
<th>FS</th>
<th>PI</th>
<th>PF</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIF</td>
<td>0.0001*</td>
<td>0.0001*</td>
<td>0.0027*</td>
</tr>
<tr>
<td>FS</td>
<td>...</td>
<td>0.5</td>
<td>0.0001*</td>
</tr>
<tr>
<td>PI</td>
<td>...</td>
<td>...</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

*P values are not significant unless P<0.0167. *Significant.

**TABLE 5. P Values for ET-1 Staining Grades in the Media**

<table>
<thead>
<tr>
<th></th>
<th>AIF</th>
<th>FSM</th>
<th>PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMA</td>
<td>0.0003*</td>
<td>0.0001*</td>
<td>0.0001*</td>
</tr>
<tr>
<td>AIF</td>
<td>...</td>
<td>0.0003*</td>
<td>0.6767</td>
</tr>
<tr>
<td>FSM</td>
<td>...</td>
<td>...</td>
<td>0.0005*</td>
</tr>
</tbody>
</table>

*P values are not significant unless P<0.0083. *Significant.

Abbreviations as in Table 3.
substantially influence local cell growth and the regulation of vascular tone in very early stages of plaque evolution. In addition, these data show that once the chronic inflammation in the intima is established, ET-1 is fully upregulated in the media.

Generally, as atherosclerosis progresses, the number of ET-1+ cells increases considerably, mainly in the intima; the difference between ECE-1+ cells and ET-1+ cells diminishes; and finally, in fatty streaks and plaque regions with inflammation, the number of ET-1+ cells exceeds the number of ECE-1+ cells slightly. However, because the enzymatic activity of ECE-1 is upregulated in atherosclerotic tissue with chronic inflammation, one may assume that despite the quantitatively lower expression of ECE-1 in fatty streaks and plaque regions with inflammation, big ET-1 may be completely activated. Thus, there is evidence that the ECE-1/ET-1 system is fully activated in fatty streaks and inflammatory regions of CPs and that great amounts of mature ET-1 may be produced in these inflammatory foci.

Interestingly, as shown by ligand-binding studies, maximal densities of the growth-promoting and vasoconstriction-mediating ETα receptor are present on medial VSMCs, whereas the ETα receptor is lacking on intimal VSMCs of advanced human coronary atherosclerotic plaques. Because ET-1 acts in a paracrine fashion, this raises the possibility that the ET-1 produced in advanced plaques may exert its constrictor effects in the media, especially if a plaque is located immediately adjacent to the media. Thus, ET-1 produced in the atherosclerotic intima may, in fact, be involved in the triggering of vasospastic reactions, which are often observed in the region of culprit lesions during coronary atherosclerosis.

In conclusion, together with ET-1, ECE-1 is abundantly present in different human arteries and different stages of atherosclerotic plaque evolution. Importantly, the upregulation of the ECE-1/ET-1 system is closely linked to the presence of chronic inflammation and is present in very early stages of plaque evolution. Therefore, enhanced production of active ET-1 may substantially contribute to cell growth and the regulation of vascular tone in advanced atherosclerotic lesions and in the very early stages of plaque evolution, when a plaque is clinically still imperceptible.

Acknowledgments
We thank Brigitte Plessow-Fueudenberg and Alfred Einhaus for excellent technical assistance.

References
Coexpression of Endothelin-Converting Enzyme-1 and Endothelin-1 in Different Stages of Human Atherosclerosis
Christian Ihling, Thomas Szombathy, Bernd Bohrmann, Manfred Brockhaus, Hans E. Schaefer and Bernd M. Loeffler

_Circulation_. 2001;104:864-869
doi: 10.1161/hc3301.094742
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2001 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/104/8/864