Molecular Forms of Atrial Natriuretic Factor in Normal and Failing Human Myocardium

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Background. Atrial natriuretic factor (ANF) is produced by myocardial tissue, and the plasma ANF concentration is known to be elevated in congestive heart failure (CHF). Data from animal models indicate that myocardial concentrations of ANF are depleted in CHF, and this has given rise to the hypothesis that CHF is characterized by depletion of stored ANF. To date, the molecular forms of ANF and their concentrations in atrial and ventricular myocardium remain poorly characterized in the normal and the failing human heart.

Methods and Results. We measured ANF concentrations in fresh tissue from failing human hearts explanted at the time of cardiac transplantation and from organ donors whose normal hearts could not be used for transplantation. We determined total ANF and α, β, and γ ANF concentrations in the right and left atrial appendages, atrial free walls, and ventricles. In normal hearts, ANF concentration in the atrial appendages was 40-fold higher than ANF in the rest of the atrial free wall and in the ventricles. In the failing hearts, atrial appendage ANF concentrations increased 5- to 10-fold, and atrial free wall ANF concentrations increased 200-fold. Analysis of molecular forms of ANF demonstrated significant increases in the γ and β forms in the left atrial appendage of failing hearts. α, β, and γ ANF forms were also significantly increased in right and left atrial free wall tissue from failing hearts. In addition, failing hearts were characterized by absolute and relative increases in the precursor form γ ANF.

Conclusions. These data from fresh tissues suggest that cardiac ANF stores are not decreased in severe CHF in humans; rather, chronic CHF is characterized by marked increases in atrial ANF tissue concentrations, particularly the β and γ ANF forms. These findings are consistent with intracellular accumulation of precursor ANF forms in severe chronic human CHF. (Circulation 1993;88:364-371)

KEY WORDS • peptides • atrial natriuretic factor • heart failure • circulation

Atrial natriuretic factor (ANF) is a peptide hormone produced principally by the heart, which has natriuretic, diuretic, and vasodilatory properties.1-3 Because ANF has important volume-regulatory characteristics it is postulated to play a role in volume homeostasis under normal conditions and in pathophysiological states such as congestive heart failure (CHF).3 It is well recognized that plasma ANF concentrations are elevated in patients with CHF,4-7 and the extent of elevation has been shown to correlate positively with atrial pressure.5,6 Plasma ANF concentrations may change within minutes of alterations in atrial pressure,5,6,8-10 and the diuretic response to intravenous infusion of ANF occurs rapidly.7 These observations are consistent with a model in which ANF is stored in atrial tissue for rapid release and in which ANF functions as a promptly acting modulator of acute changes of intravascular volume. The role of ANF in chronic heart failure is less well understood. Because symptomatic CHF may ensue despite the increased plasma ANF concentrations, some investigators have suggested that cardiac production of ANF may become inadequate in severe heart failure, ie, that there is a “relative deficiency” of ANF. In the rat, acute hypertensive stress11,12 or myocardial infarction13,14 results in decreased atrial ANF tissue concentration. Chronic hemodynamic stress such as that in the spontaneously hypertensive rat15-17 or the cardiomyopathic hamster18-21 also results in decreased ANF tissue levels. In contrast to these data suggesting depletion of stored ANF in the atrium, other studies show heart failure to be characterized by stimulation of ANF atrial and ventricular synthesis.12,14,20,32 Nevertheless, integrated physiological studies in animals are consistent with the concept that the effectiveness of ANF in modulating intravascular volume in chronic heart failure may be diminished. Using the canine rapid ventricular pacing model of acute and chronic heart failure, Redfield and coworkers22 demonstrated that chronic heart failure animals stressed with volume expansion showed no further increase in plasma ANF levels. Volume loading studies in chronic human heart failure have shown conflicting results, showing no increase in plasma ANF or mixed responses.34,35 Taken together,
all of these findings support the hypothesis that a state of maximal ANF release may be achieved in heart failure. Progressive fluid retention in chronic heart failure could, therefore, be a consequence of ANF depletion, inadequate production of ANF, or altered ANF processing and release from the myocardium.36

In contrast to animal models, few data are available on ANF concentration in human myocardium, and those that have been reported are inconsistent. Immunohistochemical staining of human tissue obtained by endocardial biopsy or necropsy indicates that atrial ANF is present in heart failure, and ANF staining in ventricular tissue increases during heart failure.20,27,31 The ANF content of failing human myocardium obtained at necropsy reflected no change in atrial concentration but an increase in left ventricular ANF concentrations.29 Other studies using a small number of human tissues obtained by surgery, necropsy, and endomyocardial biopsy have suggested that failing hearts had increased atrial and ventricular ANF concentrations.29 Surgical specimens of human atrial tissue from failing hearts have been reported to contain high ANF concentrations, but normal control tissues were either not available27 or were obtained from necropsy hearts.38

In addition to the conflicting reports on ANF tissue concentrations in CHF is the inadequate characterization of the molecular forms of ANF in CHF. ANF exists in three forms: the prohormone γ-ANF (amino acids 1-126), the circulating peptide α-ANF (amino acids 99-126), and β-ANF (a 56-amino acid antiparallel dimer of α-ANF).37-40 Membrane proteases cleave α-ANF from γ-ANF during release.41-43 In normal subjects without CHF, α-ANF is the principle circulating form, but small amounts of β- and γ-ANF may be found in plasma; the N-terminal 1-98 ANF fragment can also be detected.44,45 Although biological activity resides in the circulating α-ANF form under normal circumstances, β-ANF may be converted to α-ANF in plasma and has been shown to have biological effects when administered intravenously to normal human subjects.46 The biological activity of the β-ANF dimer is potentially important since, in chronic human heart failure, the plasma concentrations of α-, β-, and γ-ANF subspecies are increased.44,45,47,49 Analysis of right atrial tissue samples obtained from diseased hearts at surgery suggests that there may be increases in α- and β-ANF concentrations.20,37,38; data on right atrial γ-ANF concentrations are inconsistent.28,38

The current study was therefore designed to address the limitations and inconsistencies of previous reports of human myocardial concentrations of total ANF and its molecular forms in chronic heart failure. We characterized ANF in human myocardial tissue from multiple cardiac sites in patients with severe chronic CHF and from normal control subjects. Only fresh tissue was studied. Failing hearts were removed at the time of cardiac transplantation, and normal myocardium was obtained from organ donors whose normal hearts could not be used because of lack of an appropriate recipient. Tissues were analyzed for total ANF concentration and for the concentrations of γ-, β-, and α-ANF.

**Methods**

**Tissue Samples From Failing Hearts**

Myocardial samples were obtained from nine hearts explanted at the time of cardiac transplantation. All patients had long-standing severe (New York Heart Association class III to IV) heart failure. In six patients the etiology was dilated cardiomyopathy and in three it was severe coronary artery disease with a remote history of one or more myocardial infarctions. The patients ranged in age from 16 to 59 years (mean, 44.9±1.6). The mean weight of the failing hearts, which consisted principally of left and right ventricular tissue, was 537±41 g.

Samples were taken within 10 minutes of excision of the heart and were frozen at −80°C until the radioimmunoassay for ANF was performed. Samples were approximately 1.0 to 3.0 g in size. Samples were taken, when appropriate tissues were accessible on the heart, from the right atrial (RA) and left atrial (LA) free walls, from the right and left atrial appendages (RAA and LAA), and from the endocardial-midmyocardial wall levels of the right and left ventricles. (See Fig 1.) Atrial tissue was not available from each site in every patient because surgical technique in some cases left most of the atrial tissue in the recipient. Free wall atrial tissue (RA and LA) was obtained from the atrial tissue that remained on the explanted heart and was located near the atrioventricular junction, distant from the appendage. Ventricular samples were taken from tissues remote from areas of myocardial scar.

**Tissue Samples From Normal Hearts**

Samples were obtained from 12 normal hearts which, in most cases, could not be used as transplant donor organs only because of the lack of a suitable recipient. In two cases, the heart was not used because of concern that the organ donor might have an active infection. In one case, the heart was not used because of suspicion of right ventricular contusion; subsequent histological assessment of tissue from this heart, however, showed normal myocardium. These patients did donate other organs (kidneys in all cases, with variable numbers of patients donating aortic homografts, livers, and corneas). These normal donors ranged in age from 2 to 42 years (mean, 24.3±0.9). Causes of death were motor vehicle accident (n=4), head trauma (n=2), aspiration pneumonia (n=1), circulatory collapse associated with acute alcohol intoxication (n=1), and uncertain cause of death (n=4). Conventional transplant practice is to harvest other organs first so that the heart maintains normal circulation to them. Tissue samples were obtained from these normal hearts within 10 minutes of the completion of the harvest of noncardiac organs and were frozen at −80°C for subsequent radioimmunoassay. Heart weights were not obtainable on these specimens because the hearts were not excised. Samples were obtained from all anatomic sites in 10 hearts and from the ventricles only in 2 hearts.

**Extraction of ANF and Radioimmunoassay**

Extraction of ANF and radioimmunoassay were performed as previously described.38,47,50 Approximately 100 mg of frozen myocardial tissue was treated with 1 mol/L acetic acid and 20 mmol/L HCL in a boiling water bath for 10 minutes. Tissues were then homogenized with a Polytron homogenizer (Brinkman Company, Westbury, NY), centrifuged at 15 000g for 60 minutes at 4°C, and the clear supernatant was stored at −30°C until subjected to radioimmunoassay or chroma-
FIG 1. Total tissue atrial natriuretic factor (ANF) concentrations are depicted for normal (n=10) and failing (n=9) hearts. Concentrations are expressed as nanograms per milligram of protein. Bold print signifies concentration for failing hearts. Probability values are for ANF concentration comparisons (normal vs failing myocardium) at each anatomic site. CHF, congestive heart failure; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

tography. For β-ANF and α-ANF, synthetic peptide standards were obtained from the Peptide Institute (Minou, Japan); for γ-ANF, cytochrome C was used as a molecular marker to identify γ-ANF on high-performance gel permeation chromatography (HP-GPC), the retention time for γ-ANF being in agreement with previous reports.51 Recovery of β-ANF and α-ANF using synthetic peptide standards was >90%. The intra-assay and interassay coefficient of variance for the three ANF radioimmunoassays was <5%. The cross-reactivities of the anti-α-human ANF antiserum with rat ANF and β-human ANF were 16% and 90% on a molar basis, respectively. The sensitivity of the radioimmunoassay was 2 pg per tube, with 50% displacement at 30 pg per tube. The cross-reactivity of the anti-ANF antiserum with brain natriuretic peptide was <0.001%.

High-Performance Gel Permeation Chromatography

ANF components of different molecular weight were measured by radioimmunoassay after separation with HP-GPC using a TSK-GEL G 2000 SW column (7.5×600 mm; Toyo Soda, Tokyo), as previously described.38 The column was eluted with 10 mmol/L trifluoroacetic acid containing 0.2 mol/L sodium chloride and 30% acetonitrile as a solvent at a flow rate of 0.3 mL/min.

Statistical Analysis

Data are expressed as mean±SEM. Because the size of these data sets makes it unlikely that the data were normally distributed, nonparametric tests were used. Groups were compared using the Wilcoxon signed rank test for paired data and the Mann-Whitney test for unpaired data. Differences were taken to be significant at the P≤.05 level (two tailed).

Results

Total ANF Concentrations in Cardiac Tissue

Normal hearts. The total ANF concentrations in normal tissues, expressed in nanograms per milligram of protein, are presented in Table 1 and Fig 1. Concentrations of ANF in the RA free wall and LA free wall were 4.3±1.1 and 2.6±0.5 ng/mg, respectively (difference, P=.03). Recovery of ANF using synthetic peptide standards was >90%. The intra-assay and interassay coefficient of variance for the three ANF radioimmunoassays was <5%. The cross-reactivities of the anti-α-human ANF antiserum with rat ANF and β-human ANF were 16% and 90% on a molar basis, respectively. The sensitivity of the radioimmunoassay was 2 pg per tube, with 50% displacement at 30 pg per tube. The cross-reactivity of the anti-ANF antiserum with brain natriuretic peptide was <0.001%.

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Results

Total ANF Concentrations in Cardiac Tissue

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Failing hearts. Total ANF concentrations in the tissues of nine failing hearts are presented in Table 2 and Fig 1. Total ANF concentrations in the right atrium and left atrium were 844.5±269.6 and 961.4±433.7 ng/mg protein, respectively; these concentrations were not different from each other (P=.32). In failing hearts, the RAA concentration was 693.2±390.2 and the LAA concentration was 973.2±207.3 ng/mg protein; these were not different from each other (P=.07). The ANF concentrations in the failing right ventricle was 4.5±1.3 and in the failing left ventricle was 12.7±6.7 ng/mg protein; these were not different from each other (P=.17).

Comparison of normal and failing hearts. The most striking difference between the total ANF concentrations in normal and failing myocardium was in the atria. In the LAA the ANF concentration increased from 188.4±50.2 ng/mg protein in the normal heart to 973.2±207.3 ng/mg protein in the failing heart (P<.001). In the LA free wall it increased from 2.6±0.5 to 961.4±433.7 ng/mg protein (P<.001). In the RAA, the concentration tended to increase from 72.6±12.9 in
normal hearts to 693.2±390.2 ng/mg protein (P=.16) in the failing hearts. In the RA free wall it increased from 4.3±1.1 to 844.5±269.6 ng/mg protein (P<.001). In the failing hearts, therefore, a marked increase in total ANF is observed in atrial tissues, with the greatest proportional increase being in the free wall of the atria. In the normal heart, the ANF concentration is highest in the atrial appendages and much lower in the rest of the atrium. In the failing heart, the ANF concentration increases 5- to 10-fold in the atrial appendages and 200- to 370-fold in free wall atrial tissue.

By contrast, tissue ANF concentrations remained low in the failing right ventricle (4.5±1.3 ng/mg protein) and were not different from concentrations in the normal RV (3.2±0.4 ng/mg protein) (P=.8). In the left ventricle, the ANF concentration, 12.7±6.7, was not significantly greater than that in the normal left ventricle, 3.7±0.5 ng/mg protein (P=.9). However, as can be observed in Table 2, there was variability in RV and LV concentrations, with several individual failing hearts showing substantially greater ventricular concentrations of ANF (ie, patients 4, 5, and 9).

Molecular Forms of ANF in Cardiac Tissue

Normal hearts. The concentrations of γ-, β-, and α-ANF in normal cardiac tissues are presented in Table 3 and Fig 2. In the normal RAA and LAA the γ-ANF storage form predominates, and β-ANF and α-ANF are also found in lesser but comparable quantities. In the RA, LA, RV, and LV tissues, all three molecular forms are also detectable, although in much smaller quantities.

Failing hearts. As described above, the total ANF concentrations in the atrial appendages increase markedly in the failing hearts. Although there was a tendency for all of the absolute concentrations of the molecular forms of ANF to increase in the RAA and LAA (Fig 2

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Concentrations are expressed as nanograms per milliliter of protein. RAA, right atrial appendage; LAA, left atrial appendage; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; NA, not available.

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Concentrations are expressed as nanograms per milligram of protein. Dx, diagnosis; RAA, right atrial appendage; LAA, left atrial appendage; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; DCM, dilated cardiomyopathy; CAD, coronary artery disease; NA, not available.
and Table 3), there were also shifts in the proportion of the forms in the failing hearts. While there was a modest increment in α-ANF in the RAA and LAA, there were greater increases in γ- and β-ANF forms, particularly in the LAA ($P<.01$) (Fig 2). The relative increase in γ-ANF is further emphasized in Table 4, which depicts the increase in the ratio of γ-ANF/α-ANF in the failing RAA and LAA tissues.

Similar changes were noted in the right atrium and left atrium. (Fig 2 and Tables 3 and 4). The absolute concentrations of all ANF molecular forms increased significantly in the failing atria ($P \leq .01$ for each molecular subtype at each atrial anatomic site). All three molecular forms are present, and the β- and γ-ANF forms predominate in the RA and LA tissues. There is a marked accumulation of γ-ANF in the right atrium and left atrium, as shown in Table 4, where the failing atrial free walls and left atrial appendage have a significantly higher ratio of γ-ANF/α-ANF.

In the failing ventricles all three forms are detectable. Compared with normal hearts, there were no significant increases in the absolute concentrations of the ANF forms in failing hearts (Fig 2; note change in scale for ventricular versus atrial and atrial appendage concentrations). However, as noted in Table 4, there was a trend toward accumulation of the γ-ANF form in failing ventricles.

**Discussion**

The current investigation reports for the first time atrial and ventricular myocardial concentrations of the molecular forms of ANF in fresh normal and failing hearts. Chronic severe heart failure is characterized by marked alterations: the total ANF concentration in the atrial appendages increases 5- to 10-fold; ANF in the atrial free wall increases 200-fold or more, reaching levels comparable to those in atrial appendages. Furthermore, in heart failure, the atrial appendages manifest a marked increase in the absolute concentration of the γ- and β-ANF forms. In the atrial free wall, the absolute concentrations of all three ANF forms are increased in heart failure, with the most marked increases again being in γ- and β-ANF. It is of note that the relative proportion of γ-ANF increases in failing atrial tissues, resulting in an increase in the ratio of γ-ANF/α-ANF. In the ventricles, all three forms are detectable in normal hearts, and the concentrations of ANF forms are not altered in chronic heart failure in most patients.

Data from rodent models have suggested that atrial tissue ANF concentrations are decreased in both subacute and chronic circulatory failure as well as during acute, subacute, and chronic hypertensive stress. These findings have supported the hypothesis that CHF leads to a depletion of tissue ANF stores and, despite increased plasma ANF concentrations, to a condition in which demand exceeds the production capacity. Attempts to characterize ANF tissue activity in the human heart have been hampered by difficulties in obtaining optimal tissue specimens.

Previous reports derived from diseased human hearts are inconsistent. Tsuchimochi and coworkers also ex-

![FIG 2. Tissue concentration of the γ-, β-, and α-atrial natriuretic factor (ANF) molecular forms in the normal (NI) and failing hearts (CHF, congestive heart failure), expressed in nanograms per milligram of protein. Tissue sites are right atrial appendage (RAA), left atrial appendage (LAA), right atrium (RA), left atrium (LA), right ventricle (RV), and left ventricle (LV). Statistical comparisons are for normal vs failing tissue for each molecular subtype at each anatomic site. Note the change in scale for right ventricular and left ventricular concentrations compared with atrial concentrations. These data are also depicted in Table 3.](image-url)
TABLE 4. Ratio of γ-ANF/α-ANF Tissue Concentrations in Normal and Failing Hearts

<table>
<thead>
<tr>
<th>Normal</th>
<th>Failing</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>1.26±0.51</td>
<td>12.40±2.55</td>
</tr>
<tr>
<td>LA</td>
<td>1.22±0.51</td>
<td>14.43±3.35</td>
</tr>
<tr>
<td>RAA</td>
<td>4.12±0.89</td>
<td>8.20±2.43</td>
</tr>
<tr>
<td>LAA</td>
<td>6.01±1.37</td>
<td>20.11±4.22</td>
</tr>
<tr>
<td>RV</td>
<td>2.99±1.94</td>
<td>3.54±0.66</td>
</tr>
<tr>
<td>LV</td>
<td>1.23±0.36</td>
<td>2.74±0.81</td>
</tr>
</tbody>
</table>

ANF, atrial natriuretic factor (ng/ml protein); RA, right atrium; LA, left atrium; RAA, right atrial appendage; LAA, left atrial appendage; RV, right ventricle; LV, left ventricle.

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weeks in rodents).12,14,19,21,23-26 In addition, humans were under medical treatment for heart failure, which may further extend the chronicity of their heart failure and modify its intensity compared with untreated heart failure in animal models. Sampling technique may also affect ventricular ANF measurements, since there is evidence for a decreasing gradient of ventricular ANF tissue concentration from the endocardium to the epicardium in the hamster and human heart.26,28,29 Studies using subendocardial biopsy samples from humans20,27,29 have shown ventricular recruitment, the magnitude of which could have been influenced by sampling a site particularly rich in ANF; the samples obtained in the present study contained endocardium but also included 2 to 5 mm of subendocardial tissue, which may have a lower ANF concentration. Studies that have reported increased tissue ANF from human left ventricular aneurysms may reflect a different disease condition, since the myocardium adjacent to aneurysm has been shown to be relatively rich in ANF, perhaps because of local stretch stimulation.33,54 Different ANF measurement methodology could also produce variance in assessment of ventricular ANF. Immunohistochemical techniques20,27 are less quantitative and difficult to compare with radioimmunoassay of tissue homogenates. Detection of increased ventricular tissue ANF mRNA has provided further evidence for ventricular recruitment of ANF production in heart failure.14,21,23-26 Measurement of ANF mRNA could be increased out of proportion to tissue ANF content if ventricular ANF is constitutively released, ie, released immediately after production and not stored in the prohormone γ-ANF form.55 The lower levels of ventricular ANF that we found do not necessarily indicate that the ventricle does not produce ANF but could be accounted for if such a constitutive release mechanism is operative in chronic heart failure.

ANF is generated from the prohormone γ-ANF (1-126) and is stored in this form in granules in atrial myocytes.39 The principle circulating form is α-ANF (99-126), and its dimer, β-ANF, may be detectable in small quantities in plasma of some normal subjects.47 All three forms are known to be elevated in plasma in human heart failure, and increases in plasma β-ANF have been noted in particular.48,49 Our findings show that all three ANF molecular forms are detectable in the normal atrial appendage, atrial free wall, and ventricle. Furthermore, our data demonstrate that human heart failure produces not only increases in total atrial ANF concentrations but also increases in the atrial concentrations of the γ-ANF storage form, the β-ANF form, and the α-ANF form. These data in fresh tissues are compatible with those of Naruse and coworkers,38 who compared failing atrial myocardium obtained at cardiac surgery with normal necropsy tissues. Of particular interest is the marked increase in the prohormone γ-ANF in severe CHF. The significance of the increase in tissue γ-ANF has yet to be elucidated, but such accumulations raise the possibility that chronic CHF may be characterized by impaired conversion of γ-ANF to α-ANF. It is also noted that the ratio of α-ANF to β-ANF in myocardium is lower than the reported ratio of circulating α-ANF to β-ANF; this may reflect conversion from the β to the α form on release into the circulation.49

The extent to which the myocardium is able to augment ANF production, combined with the well-
known increases in plasma ANF in heart failure, makes it unlikely that the clinical syndrome of human chronic CHF results from an exhaustion of cardiac ANF production capacity. Rather, other factors such as impaired processing of γ-ANF to α-ANF, myocyte loss, and diminished renal responsiveness to ANF may all contribute to sodium and water retention in chronic heart failure. The ultimate role of ANF in chronic heart failure will also need to be evaluated in the context of our understanding of other natriuretic peptides that are currently being described.

Potential problems in data interpretation could arise from the fact that failing hearts came from patients significantly older than the donor hearts. No data are available on the effect of age on myocardial tissue ANF concentration. Failing tissues were obtained from hearts after surgical excision, and failing hearts were removed after patients were placed on cardiopulmonary bypass; it is not known if these maneuvers affect tissue ANF concentrations. Both groups of hearts also experienced a period of several minutes of lack of coronary perfusion, and the effect of this on ANF production and release is unknown.

In summary, the current report of fresh human myocardial tissue indicates that in severe chronic heart failure there is a significant increase in ANF concentration in the appendages and a marked increase in ANF concentration in the atrial free wall. Increases are observed in all ANF forms but particularly in the γ-ANF precursor form. Thus, heart failure is characterized by increased rather than depleted cardiac ANF concentration.

**Acknowledgments**

These studies were supported by National Institutes of Health grants HL-14192 and HL-35323. The authors would like to thank Yuko Kato and Chikako Monzen for their able technical assistance and Candy Cope- man, DeAnn Rowe, and Sheila Ferguson for excellent preparation of the manuscript. We would like to thank the Coordinators of the Tennessee Donor Services for their assistance in obtaining normal tissue specimens.

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Circulation. 1993;88:364-371
doi: 10.1161/01.CIR.88.2.364

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