Increased Serum Levels of Heat Shock Protein 70 Are Associated With Low Risk of Coronary Artery Disease


Objective—Previous studies suggest that heat shock protein (HSP) 60 has a contributory role in atherosclerosis development. We examined whether circulating HSP70 protein and anti-HSP70 antibodies are associated with coronary artery disease (CAD).

Methods and Results—Blood samples from 421 patients (62% men, mean age 57 years) evaluated for CAD by coronary angiography were tested. Serum HSP70 was detectable in 67% of study subjects with levels ranging from 0.2 to 27.1 ng/mL (mean, 1.08; median, 0.5). HSP70 levels were higher in non-CAD patients than CAD patients (median, 0.72 versus 0.34; \( P = 0.0006 \)). Individuals with HSP70 levels above the median (0.5 ng/mL) had half the risk of CAD than individuals with levels below the median (adjusted odds ratio, 0.52; 95% confidence limit, 0.32 to 0.86). The association of high HSP70 levels with low CAD risk was independent of traditional CAD risk factors (\( P = 0.011 \)). Disease severity (number of diseased vessels) was also inversely associated with HSP70 protein levels (\( P = 0.010 \)). The adjusted odds ratio of having multivessel disease for patients with high HSP70 protein levels was 0.54 (95% confidence limit, 0.36 to 0.81). In contrast, no association between anti-HSP70 IgG seropositivity and the prevalence of CAD was found (\( P = 0.916 \)).

Conclusions—These data provide the first evidence that high levels of human HSP70 are associated with the low CAD risk, probably through its multiple protective effects on a cell’s response to stress. (Arterioscler Thromb Vasc Biol. 2003;23:1055-1059.)

Key Words: coronary artery disease \( \bullet \) heat shock protein \( \bullet \) antibodies \( \bullet \) protective effect

Heat shock proteins (HSPs) constitute a large family of proteins that aid in a cell’s response to acute stress. The importance of these proteins is evident by the fact that they are ubiquitous and highly conserved across species. Although the HSPs are mainly intracellular molecules, when overexpressed in response to stress, they can be transported to and reside in the cell membrane, where they may be recognized by the immune surveillance system and function as autoantigens. They can also be released from cells into the blood and have biological activity. Therefore, when overexpressed, HSPs may evoke responses other than those associated with their intracellular location, and these responses may have the potential for deleterious consequences. For example, serum levels of HSP60 were significantly elevated in subjects with prevalence/incident carotid atherosclerosis as well as in patients with borderline hypertension. Antibodies to HSP60 have been reported to be associated with both the presence and severity of clinically significant coronary artery disease (CAD).

Experimental evidence suggests a cardioprotective role of another member of this family, HSP70, in several examples of acute myocardial stress. For example, the hearts of transgenic mice overexpressing HSP70 exhibit enhanced resistance to ischemic injury. Because antibodies to HSP70 have been detected, as they have to HSP60, this raises the possibility that HSP70 could trigger autoimmune responses that might attenuate any intrinsic beneficial cellular effects or, like the response to HSP60, may even exacerbate the development of atherosclerosis. However, limited experimental or clinical data in regard to the effects of HSP70 on atherogenesis are available.

It was the purpose of the present investigation to determine whether associations exist between HSP70 expression and atherogenesis. Specifically, we determined whether circulating HSP70 protein and anti-HSP70 antibodies are associated with CAD.

Methods

Subjects

Four hundred twenty-one individuals, under a National Institutes of Health Institutional Review Board–approved protocol, entered the
Sera were obtained from study subjects before the time of coronary angiography. The samples were frozen at −80°C, and aliquots were thawed only when performing specific tests. Serum HSP70 protein levels were determined using commercially available ELISA kits (StressGen Biotechnologies Corp). The concentrations of HSP70 protein were determined by comparison with a standard curve according to manufacturer’s direction. The standard curve has a range of 0.78 to 50 ng/mL, and the sensitivity of the assay is 0.2 ng/mL.

IgG antibodies against human HSP70 were determined by ELISA. Ninety-six-well microtiter plates were coated with 5 μg/mL recombinant HSP70 (StressGen Biotechnologies Corp) in 100 μL carbonate/bicarbonate buffer (pH 9.6) per well at 4°C overnight. After washing wash buffer (Wampole) and blocking with 3% BSA in PBS at room temperature for 3 hours, plates were incubated with 100 μL serum samples diluted in Serum Diluent (Wampole) to 1 in 50 at room temperature for 1 hour. After an additional wash, the plates were incubated with horseradish-peroxidase–conjugated goat anti-human IgG diluted 1 in 10 000 with PBS. After washing, 100 μL chromogen/substrate solution containing tetramethylbenzidine (Wampole) was added to wells. Absorbance at 450 nm was measured after 10 minutes following addition of the stopping solution (Wampole). After correction for background absorbance, a serum sample was considered positive for antibodies against human HSP70 if the optical density exceeded a prospectively defined cutoff value of 0.60. This cutoff value is calculated from the negative and positive control absorbance values.

Serum C-Reactive Protein Levels

Serum C-reactive protein (CRP) was measured by fluorescence polarization immunoassay (FPIA) technology (TDxFLEx analyzer, Abbott Laboratory). Using this assay, 95% of healthy individuals (n=202) had a CRP level of ≤0.5 mg/dL and 98% had levels ≤1.0 mg/dL, respectively, in their sera. The between-run coefficient of variation of this assay (n=31) was 4.5% and 2.2% at mean levels of 1.10 and 2.94 mg/dL, respectively.

Statistical Analysis

Data distributed normally are presented as the mean and standard deviation (SD), whereas data distributed nonnormally are presented as the median and interquartile range (IQR). Comparisons between end points were made using t test parametric distributions and the Mann-Whitney U test for nonparametric distributions. Categorical data were analyzed by the χ2 test or Fisher’s exact test for small samples. All tests were 2-sided. The dichotomous variables indicating the presence and severity of CAD were modeled as a function of other factors using multiple logistic regression. The odds ratio (OR) was used as a measure of the presence and severity of CAD in patients with a given risk factor compared with those without that factor or as a multiplicative factor for each unit increase in age or HSP70 levels. The covariates considered were age, male sex, smoking, diabetes, hypercholesterolemia, hypertension (traditional CAD risk factors), serum CRP, HSP70 protein, and anti-HSP70 antibody levels. All covariates were examined as predictors of the presence and severity of CAD in univariate analyses and as a group in one multivariate model.

Results

Characteristics of Patients

A total of 421 subjects were studied; 62% were male and 72% were white, ranging in age from 30 to 82 years (mean, 57.3; median, 57.0 years). There were 258 (61%) with angiographic evidence of CAD (≥50% stenosis of at least one major coronary artery by coronary angiography). With the exceptions of hypertension, traditional CAD risk factors (age, male sex, diabetes, and hypercholesterolemia) were significantly associated with the risk of CAD by both univariate and multivariate analyses. Smoking was significantly associated with CAD by univariate analysis but not multivariate analysis (Table 1).

Relation of HSP70 Protein and HSP70 Antibodies to CAD Risk

Serum HSP70 protein was detectable in 283 of 421 (67%) study subjects, with levels ranging from 0.2 to 27.1 ng/mL (mean, 1.08; median, 0.5 ng/mL). HSP70 protein levels were higher in non-CAD patients than CAD patients (median, 0.72 and IQR, 1.42 versus median, 0.34 and IQR, 1.21 ng/mL; P=0.001). In addition, the high HSP70 level group (above the median value >0.5 ng/mL) contained 51% CAD patients, whereas the low HSP70 group had 71% CAD patients (P<0.001). Multivariate logistical regression analysis revealed that individuals with elevated levels of HSP70 had half the risk of CAD than individuals with low levels (adjusted OR, 0.52; 95% confidence limit [CL], 0.32 to 0.86). The association of high HSP70 level with low CAD risk was independent of traditional CAD risk factors (P=0.011).

Figure 1 shows the effects of HSP70 protein on CAD risk in each HSP70 protein concentration group (displayed by HSP70 quartile). Individuals in the highest quartile of HSP70 levels (>75th) had 59% less CAD risk compared with those in the lowest quartile (25th). The ORs with 95% CL of CAD risk associated with HSP70 protein concentration equal to or greater than the 25th, 50th, 75th, and 90th percentiles of the control distribution were 0.42 (0.26 to 0.66), 0.46 (0.28 to 0.75), 0.44 (0.24 to 0.79), and 0.38 (0.17 to 0.85), respectively. Adjustment for traditional CAD risk factors had no impact on the risk reduction. There was nonlinearity of the relation between HSP70 protein concentration and CAD risk reduction. In contrast to HSP70 protein, anti-human HSP70

### TABLE 1. Association of Presence of CAD With Traditional Risk Factors

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Univariate OR (95% CL)</th>
<th>Multivariate† OR (95% CL)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (10 y)*</td>
<td>&lt;0.001</td>
<td>2.5 (1.9 to 3.2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Male sex</td>
<td>&lt;0.001</td>
<td>3.9 (2.3 to 6.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.006</td>
<td>1.4 (0.8 to 1.4)</td>
<td>0.343</td>
</tr>
<tr>
<td>Diabetes</td>
<td>&lt;0.001</td>
<td>3.3 (1.7 to 6.8)</td>
<td>0.001</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>&lt;0.001</td>
<td>2.2 (1.3 to 3.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.148</td>
<td>0.9 (0.4 to 2.2)</td>
<td>0.316</td>
</tr>
</tbody>
</table>

*In 10-year increments of age.
†Adjusted covariates: age, male sex, smoking, diabetes, hypercholesterolemia, and hypertension.
antibodies were detected in only 34% of study subjects. No difference in the prevalence of anti-HSP70 seropositivity between CAD and non-CAD patients was found (34.2% versus 33.7%, respectively; \( P = 0.916 \)).

**Relation of HSP70 Protein and HSP70 Antibodies to CAD Severity**

A similar association of HSP70 protein levels falling below versus above the median (>0.5 ng/mL) was also observed with disease severity, as assessed by number of diseased vessels (Figure 2). HSP70 protein levels above the median were related to less CAD severity (\( P \) for trend=0.01). The adjusted OR of having multivessel disease for patients with high HSP70 levels was 0.54 (95% CL, 0.36 to 0.81; \( P = 0.003 \)). However, there was no association between anti-HSP70 antibodies and CAD severity (\( P = 0.264 \)).

**Relation of HSP70 Protein and HSP70 Antibodies to Traditional CAD Risk Factors**

The association of HSP70 protein to CAD risk factors is presented in Table 2. Elevated levels of HSP70 protein were not associated with male sex, smoking, diabetes, hypercholesterolemia, or hypertension on both univariate and multivariate analysis (all \( P > 0.05 \)). Although HSP70 protein was significantly associated with age, the low HSP70 protein level in patients with CAD was independent of age. No association between the presence of HSP70 antibodies and CAD risk factors was found (all \( P > 0.05 \), data not shown).

**Relation of HSP70 Protein and HSP70 Antibodies to Inflammation**

The mean level of CRP was 0.84±0.04 in individuals with high level of HSP70 protein and 0.90±0.05 mg/dL±SE in the low HSP70 protein group (\( P = 0.361 \)). The mean level of CRP was 0.88±0.05 in individuals with anti-HSP70 seropositivity and 0.87±0.04 mg/dL±SE in anti-HSP70 seronegativity (\( P = 0.905 \)). No correlation between CRP levels and either HSP70 protein levels or the presence of HSP70 antibodies was observed (both \( P > 0.05 \)).

**Discussion**

HSPs are abundant intracellular proteins, subdivided into different families according to their molecular weight. They are found in both prokaryotic and eukaryotic organisms and are highly conserved, and their main function seems to be as chaperones, involved in protein folding and transport.\(^1\)\(^2\)

HSP70 is one of the more extensively studied HSPs. With stress, HSP70 translocates to the nucleus and associates with...
TABLE 2. Association of HSP70 Levels With Traditional CAD Risk Factors

<table>
<thead>
<tr>
<th>Risk Factors*</th>
<th>HSP70 Protein Level‡</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>≤0.5 ng/mL</td>
</tr>
<tr>
<td>Age, y</td>
<td>59.2±11.1</td>
</tr>
<tr>
<td>Male sex</td>
<td>62.9</td>
</tr>
<tr>
<td>Smoking</td>
<td>50.7</td>
</tr>
<tr>
<td>Diabetes</td>
<td>24.6</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>53.8</td>
</tr>
<tr>
<td>Hypertension</td>
<td>55.6</td>
</tr>
</tbody>
</table>

*Data are mean±SD or percentage of patients.
†The median level of HSP70 protein is 0.5 ng/mL.
‡Univariate analysis.
§Adjusted covariates: age, male sex, smoking, diabetes, hypercholesterolemia, and hypertension.

nucleoli. It shares many of HSP class functions and, in addition, seems to protect against ischemic injury. Studies have demonstrated that exposure of isolated animal hearts to thermal or ischemic stress induces HSP70 expression, and subsequent studies showed that prior whole-body exposure to heat, which results in increased levels of HSP70, improves recovery of animal hearts from ischemia-induced injury. More convincing evidence of a protective role of HSP70 in ischemia-induced injury was found in genetically based studies. These have demonstrated that overexpression of HSP70 in cultured primary cardiac cells protects these cells against thermal or ischemic stress, whereas overexpression of HSP56 and HSP60 has no such protective effect. Studies also found that the hearts of transgenic mice overexpressing HSP70 are more resistant to ischemic injury. Because of the known protective effects of the HSPs but the possibility that HSPs can lead over the long term, through the development of autoantibodies, to chronic disease (as demonstrated for HSP60), the present investigation was undertaken. We sought to determine whether circulating HSP70 protein and anti-HSP70 antibodies are associated with CAD.

In our study, serum HSP70 was detectable in nearly 70% of study subjects. Most interestingly, it was significantly higher in non-CAD patients than in CAD patients, an association independent of traditional risk factors. Furthermore, disease severity, as assessed by number of diseased vessels, was also inversely associated with HSP70 levels. In contrast, antibodies to HSP70 were present in only one third of study subjects, and there was no association between antibodies to HSP70 and CAD.

The mechanisms responsible for the inverse relation between HSP70 serum levels and CAD are, at this time, conjectural. The elevated serum levels themselves could exert important biological effects. For example, exogenously administered HSP70 triggers cell surface–mediated proinflammatory signaling cascades that lead to the expression of multiple inflammatory cytokines. However, this activity would be expected to exert proatherosclerotic rather than antiatherosclerotic effects. Of relevance, the extracellular concentrations of HSP70 needed to exert signaling effects is approximately 2 orders of magnitude higher than the serum concentrations we measured in this investigation. Therefore, we believe the inverse associations we found between serum levels of HSP70 and CAD most likely result from the molecule’s intracellular effects, with the serum levels we measured probably reflecting, in a rough way, intracellular levels.

The most obvious intracellular protective mechanisms derive from the primary class action cell-chaperone effects of the HSPs, which have broad effects on intracellular proteins. Other actions could either derive from this function or could represent independent activities of HSP70. For example, Suzuki et al demonstrated that HSP72 (a major protein in HSP70 family) enhances manganese superoxide dismutase activity. This enzyme preserves mitochondrial function and limits mitochondrial-related apoptosis during myocardial ischemia-reperfusion injury. Ethridge et al found an inverse relationship between intracellular levels of HSP70 and the activity of COX-2, a major proinflammatory enzyme. Although it has been demonstrated that this relationship derives from COX-2 inhibiting HSP70 expression, it has also been suggested that this reciprocal relation is part of a negative feedback loop. If so, then this represents an important anti-inflammatory effect of HSP70. Importantly, Shimizu et al demonstrated in rats that HSP70 forms complexes with inhibitory Kβ levels and attenuates nuclear factor-κB activation. Because nuclear factor-κβ is in a key transcription factor modulating the expression of proinflammatory genes, this is another key anti-inflammatory activity of HSP70.

The disparate findings relating to the association to CAD of HSP60 versus HSP70 are interesting to consider. Anti-HSP60 antibodies are common in the population, and they have been demonstrated to be associated with the development of atherosclerosis. Elevated serum levels of HSP60 protein were also found to be related to atherosclerosis. In contrast, elevated anti-HSP70 antibodies are relatively uncommon. At this time it is unknown why one HSP has the capacity to elicit an autoimmune response and predispose to CAD whereas another does not.

It should also be pointed out that Pockley et al reported, in contrast to our findings, that 20 patients with peripheral vascular disease and 13 with renal vascular disease had elevated levels of circulating HSP70 compared with their age- and sex-matched controls. In these studies, there was a very large range of HSP70 concentrations (0 to 74 550 ng/mL in patients with peripheral vascular disease and 0 to 19600 ng/mL in controls). We studied 421 patients and found that the association between higher levels of HSP70 and lower prevalence of both CAD and CAD severity persisted (adjusted OR, 0.52; 95% CL, 0.32 to 0.86) after adjustment for traditional CAD risk factors (age, male sex, smoking, diabetes, hypercholesterolemia, and hypertension). Thus, the disparate results between our study and that of Pockley et al may relate to the type of patient studied, the assay used to quantitate HSP70 levels, and data adjustments included in each of the studies. Additionally, Schett et al found that myocardial injury leads to a release of HSP60 and a suppression of the anti-HSP65 immune response. Such results suggest that differences in levels of circulating HSP70 or...
HSP70 antibodies in non-CAD and CAD patients could be attributable to a consequence of immune complex formation. Such a possible mechanism would be a very worthy subject of future investigation.

In summary, this investigation provides the first evidence that high levels of human HSP70, as reflected by elevated serum levels, are associated with the low risk of CAD, presumably through its multiple intracellular protective effects on a cell’s response to stress. The results of this study suggest that the serum level of HSP70 protein is a potent marker for lowered CAD susceptibility and may be helpful, along with other currently recognized risk factors, in more accurately conveying the overall risk of an individual for CAD.

References

Increased Serum Levels of Heat Shock Protein 70 Are Associated With Low Risk of Coronary Artery Disease

Jianhui Zhu, Arshed A. Quyyumi, Hongsheng Wu, Gyorgy Csako, David Rott, Alexandra Zalles-Ganley, Jibike Ogunmakina, Julian Halcox and Stephen E. Epstein

*Arterioscler Thromb Vasc Biol.* 2003;23:1055-1059; originally published online May 1, 2003; doi: 10.1161/01.ATV.0000074899.60898.FD

*Arteriosclerosis, Thrombosis, and Vascular Biology* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Copyright © 2003 American Heart Association, Inc. All rights reserved.

Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://atvb.ahajournals.org/content/23/6/1055