Serum Heat Shock Protein 70 Levels Predict the Development of Atherosclerosis in Subjects With Established Hypertension

Alan G. Pockley, Anastasia Georgiades, Thomas Thulin, Ulf de Faire, Johan Frostegård

Abstract—Although heat shock proteins (Hsp’s) are present in the sera of healthy individuals and at elevated levels in subjects with early cardiovascular disease, their physiologic role in and value for predicting the development and/or progression of atherosclerosis have not been evaluated. Serum was obtained from 218 subjects with established hypertension (diastolic pressure >95 mm Hg) before their enrollment in the European Lacidipine Study on Atherosclerosis. Hsp60 and Hsp70, and anti-human Hsp60, anti-human Hsp70, and anti-mycobacterial Hsp65 antibody levels were measured by enzyme immunoassay. As an indicator of the presence/progression of atherosclerosis, the means of the maximum intima-media (I-M) thicknesses in the far walls of common carotid arteries and bifurcations (CBMmax) were determined by ultrasonography at the time of enrollment and 4 years afterward. Increases in I-M thicknesses at follow-up were less prevalent in subjects having high serum Hsp70 levels (75th percentile) at the time of enrollment (odds ratio, 0.42; 95% confidence interval [CI], 0.22 to 0.8, \( P=0.008 \)). Although a similar trend was observed for serum Hsp60 levels, this was not statistically significant (odds ratio, 0.6; 95% CI, 0.32 to 1.11, \( P=0.10 \)). There was no relation between anti-Hsp antibody levels and changes in I-M thicknesses. The relation between Hsp70 levels and changes in I-M thickness was independent of age, atenolol or lacidipine treatment, smoking habits, and blood lipid levels. These findings indicate that circulating Hsp70 levels predict the development of atherosclerosis in subjects with established hypertension, and an intriguing possibility is that Hsp70 protects against or modifies the progression of atherosclerosis in this subject group. (Hypertension. 2003;42:13672–13677.)

Key Words: atherosclerosis ■ heat shock proteins ■ hypertension, chronic ■ human ■ carotid arteries ■ ultrasonography

Evidence suggests that the inflammatory component of atherosclerosis might, at least in part, involve immune reactivity to heat shock proteins (Hsp’s).1,2 Although Hsp’s are typically regarded as intracellular proteins, Hsp60 and Hsp70 are present in the sera of clinically normal individuals,3,4 serum Hsp60 levels are associated with early atherosclerosis in clinically normal individuals,3,4 and Hsp70 levels are elevated in peripheral vascular disease.5 However, the physiologic role for circulating Hsp’s and their value for monitoring the pathogenesis and predicting the rate of progression of the disease have not been evaluated. This study reports that serum Hsp70 levels predict the progression of atherosclerosis in a 4-year follow-up study of 218 subjects with established hypertension enrolled in the European Lacidipine Study on Atherosclerosis (ELSA).6–8 The observation that increases in carotid intima-media (I-M) thicknesses at follow-up were significantly less prevalent in subjects having high serum Hsp70 levels at the time of enrollment indicates that Hsp70 is not only a specific and early marker for the cardiovascular disease process but also that it might protect against or modify the progression of atherosclerosis.

Methods

Study Group

Sera were obtained from 218 subjects with established hypertension (diastolic pressure >95 mm Hg) before their entry into the Swedish Study Group component of the ELSA.6–8 Samples were collected after a 4-week washout period with no medication, and cholesterol and triglyceride levels were determined as described.9 Blood pressure was measured with a mercury manometer with the subject in a sitting position on 3 occasions at 5-minute intervals after 15 minutes of rest.9 Measurements were taken at baseline, at each drug titration visit, and every 6 months thereafter. Subjects were subsequently assigned to treatment with the \( \beta \)-blocker atenolol (\( n=109 \)) or the calcium antagonist lacidipine (\( n=109 \)). The study was approved by the Karolinska Hospital Ethics Committee and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent.

Carotid Artery Ultrasound

Carotid artery ultrasound was performed and the data analyzed as described.8,10 In brief, the right and left carotid arteries were

Received February 28, 2003; first decision April 2, 2003; revision accepted July 3, 2003.

From the Division of Clinical Sciences (North), University of Sheffield (A.G.P.), Sheffield, UK; the Division of Cardiovascular Epidemiology, Institute of Environmental Medicine, Karolinska Institute (A.G., U.d.F.), Stockholm; the Department of Cardiology, Karolinska Hospital (U.d.F.), Stockholm; the Department of Internal Medicine, Lund University Hospital (T.T.), Lund; and the Department of Medicine, Unit of Rheumatology and CMM, Karolinska Hospital (J.F.), Stockholm, Sweden.

Correspondence to Dr. A. Graham Pockley, Clinical Sciences Centre, Northern General Hospital, Herries Road, Sheffield, S5 7AU, UK. E-mail g.pockley@sheffield.ac.uk

© 2003 American Heart Association, Inc.

Hypertension is available at http://www.hypertensionaha.org

DOI: 10.1161/01.HYP.0000086522.13672.23
examined with a duplex scanner (Biosound 2000 IIA) equipped with an 8.0 MHz annular-array transducer. The I-M thickness was determined in the far wall as the distance between the leading edge of the lumen-intima echo and the leading edge of the media-adventitia echo. The change in mean maximum I-M thickness of the 4 far walls in the distal common carotid arteries and carotid bifurcations bilaterally (CBMmax) at the 4-year follow-up was used as a surrogate indicator for atherosclerosis. A comparable approach for monitoring the progression of atherosclerosis has been used in other intervention studies.\(^3,9\) Assays were performed blindly in the Division of Clinical Sciences (North), Sheffield, UK.

### Statistical Methods

Statistical analyses were performed with commercially available software (SAS version 8, SAS). Hsp and Hsp antibody levels were dichotomized at the 75th percentile into “high” and “low” groups. Cutoff levels were 560 ng/mL for Hsp60, 300 ng/mL for Hsp70, and 190 U/mL for anti-Hsp60 antibodies, 85 U/mL for anti-Hsp70 antibodies, and 300 U/mL for anti-mycobacterial Hsp65 antibodies. Associations between Hsp and Hsp antibody levels and the progression of atherosclerosis (increases in I-M thickness; yes/no) for a 4-year follow-up were determined by logistic regression analysis, and odds ratios (ORs) and 95% confidence intervals (CIs) were calculated. Adjustments for the confounding variables age, smoking habits, serum cholesterol, serum triglycerides, and mode of antihypertensive treatment (lacidipine, atenolol) were made. A 2-tailed probability value <0.05 was considered to represent a statistically significant relation.

### Results

The characteristics of the subjects at enrollment are given in Tables 1 and 2. Of the 218 subjects, carotid plaques were detected in 77 (35%) at enrollment and in 84 (38%) at the 4-year follow-up. Increases in I-M thicknesses at follow-up were not as prevalent in subjects having high serum Hsp70 levels (75th percentile) at enrollment (OR, 0.42; 95% CI, 0.22 to 0.80, \(P=0.008\); Table 3). Although a similar relation was observed for serum Hsp60 levels, this was not statistically significant (OR, 0.6; 95% CI, 0.32 to 1.11; \(P=0.10\); Table 3). There was no relation between anti-Hsp antibody levels and changes in I-M thicknesses. There were no significant differences in the proportion of males and females in any of the subgroups analyzed (Tables 1 and 2).

Baseline I-M thickness was significantly associated with the development of plaque; however, logistic regression analysis demonstrated a significant relation between Hsp70 levels (dichotomized into “high” and “low” groups) and changes in I-M thickness. Hsp70 continued to add to the prediction of change in I-M thickness, even when baseline

### Tables

#### Table 1. Prediction of Changes in I-M Thickness Over a 4-Year Period

<table>
<thead>
<tr>
<th>Parameter</th>
<th>I-M Thickness Increased (&lt;1.3 mm → &gt;1.3 mm)</th>
<th>Others</th>
<th>(P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>58±1.8</td>
<td>58±0.5</td>
<td>0.805</td>
</tr>
<tr>
<td>Sex, % male</td>
<td>50</td>
<td>48</td>
<td>0.500</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>150.8±3.00</td>
<td>149.5±0.93</td>
<td>0.669</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>91.9±1.73</td>
<td>91.6±0.84</td>
<td>0.891</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>236.6±9.2</td>
<td>231.9±2.6</td>
<td>0.580</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>58.1±8.4</td>
<td>55.3±1.9</td>
<td>0.664</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>146.9±9.0</td>
<td>149.8±2.7</td>
<td>0.742</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>130.4±12.2</td>
<td>131.8±4.1</td>
<td>0.917</td>
</tr>
<tr>
<td>I-M thickness, mm</td>
<td>1.19±0.03</td>
<td>1.22±0.02</td>
<td>0.384</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

#### Table 2. Demographics of the “Low” and “High” Hsp70 Groups at Enrollment

<table>
<thead>
<tr>
<th>Parameter</th>
<th>All Subjects</th>
<th>“Low” Hsp70 (&lt;300 ng/mL, (n=169))</th>
<th>“High” Hsp70 (&gt;300 ng/mL, (n=49))</th>
<th>(P), “Low” vs “High”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>58±0.5</td>
<td>58±0.6</td>
<td>58±1.1</td>
<td>0.657</td>
</tr>
<tr>
<td>Sex, % male</td>
<td>50</td>
<td>52</td>
<td>44</td>
<td>0.220</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>149.6±1.00</td>
<td>149.9±1.12</td>
<td>148.7±1.84</td>
<td>0.657</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>91.7±0.60</td>
<td>91.9±0.72</td>
<td>91.2±1.15</td>
<td>0.593</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>231.9±2.55</td>
<td>229.0±2.96</td>
<td>241.7±4.71</td>
<td>0.036</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>55.3±2.00</td>
<td>53.1±1.99</td>
<td>62.9±5.24</td>
<td>0.037</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>149.0±2.66</td>
<td>147.7±3.08</td>
<td>153.5±5.22</td>
<td>0.365</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>132.3±4.00</td>
<td>132.2±4.59</td>
<td>132.4±7.96</td>
<td>0.985</td>
</tr>
<tr>
<td>I-M thickness, mm</td>
<td>1.21±0.019</td>
<td>1.19±0.02</td>
<td>1.28±0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Evidence of carotid plaque</td>
<td>35</td>
<td>20</td>
<td>30</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Values are mean±SEM.
I-M thickness and age were forced into the model. Age, smoking habits, blood lipids, or treatment with atenolol or lacidipine did not add significantly to the prediction of I-M change, the latter being true despite the observation that lacidipine has been shown to slow the progression of asymptomatic carotid atherosclerosis.8

The I-M thickness was >1.3 mm in 73 subjects at the 4-year follow-up, 20% of whom had exhibited high Hsp70 levels at the time of enrollment. In addition, only 2 of the 22 subjects (9%) in whom plaque had developed during the 4-year period (ie, the I-M thickness was <1.3 mm at enrollment but >1.3 mm at follow-up) exhibited high levels of Hsp70 at baseline. This suggests that Hsp70 might attenuate plaque development.

Discussion

Hsp’s are typically regarded as intracellular molecules with important chaperone and cytoprotective functions.14 However, observations that they can be released from a range of cell types have stimulated interest in the intercellular signaling capacity of extracellular Hsp’s and their role as regulators of physiologic and immune responses.15

This study reports that the progression of carotid atherosclerosis in subjects with established hypertension is not as prevalent in subjects having high serum Hsp70 levels. Although we have previously reported elevated levels of Hsp70 in the peripheral circulation of patients with peripheral and renal vascular disease,5 we have not observed any relation between Hsp70 levels and I-M thickness in these subjects with established hypertension.9 nor have we observed any relation between Hsp70 levels and I-M thickness in subjects with borderline hypertension.5 The elevated levels of Hsp70 in peripheral and renal vascular disease might result from the inflammatory response that is associated with established atherosclerotic disease, and this notion is supported by the observation that although higher than those in controls, Hsp70 levels in patients with localized renal vascular disease were significantly lower than those in patients with more disseminated peripheral vascular disease. It is difficult to draw parallels between the events leading to elevated Hsp70 levels in overt and clinically established symptomatic vascular disease with those involved in the more subtle changes associated with increases in I-M thickness.

Given other studies that have shown anti-mHsp65 antibody levels to predict cardiovascular events,16,17 our observation that anti-Hsp antibody levels did not predict increases in I-M thickness suggests that Hsp antibody levels might be more predictive of unstable plaque and acute events rather than stable plaque progression.

The source of serum Hsp’s and the precise relation between circulating Hsp70 and the progression of atherosclerosis are unknown. However, our observations indicate that Hsp70 is a specific and early marker for cardiovascular disease process. Our finding also suggests that Hsp70 might protect against or modify the progression of atherosclerosis, although it is possible that it reflects an underlying, yet undisclosed, association, further clarification of which is required.

Three lines of evidence support the concept that circulating Hsp70 might protect against or modify the progression of cardiovascular disease. First, exogenous Hsp70 protects stressed aortic cells in vitro by a mechanism that does not require internalization of the protein.18 Second, intracellular stress responses are anti-inflammatory, in that the induction of Hsp70 in vascular endothelial cells prevents leukocyte-endothelial adhesion after administration of an inflammatory stimulus.19 Serum Hsp70 levels possibly reflect tissue expression, and elevated levels might therefore reflect the presence of an antiatherogenic state in the vasculature. Third, immune system reactivity to Hsp70 can be anti-inflammatory, as the induction of T-cell reactivity to self-Hsp70 epitopes down-regulates inflammatory disease by a mechanism that involves the induction of Th2 cells that produce the regulatory cytokine interleukin-10.20–22 The Th2 phenotype of self-Hsp70 reactivity is particularly relevant, given that downregulation of proinflammatory, Th1-type immune responses reduces atherogenesis in the apolipoprotein E–knockout mouse model of atherosclerosis.23 In addition, deviation of Hsp65/60 immune system reactivity toward a Th2 phenotype by mucosal vaccination with mycobacterial Hsp65 decreases atherosclerosis in LDL receptor–deficient mice.24,25 From these findings, it is becoming clear that Hsp’s and other antigens that are proposed as protagonists in atherogenesis can, under certain circumstances, protect against the disease.26–29

Perspectives

This study has demonstrated that circulating Hsp70 levels predict the rate of progression of atherosclerosis in subjects with established hypertension. Further studies are required to elucidate the relation between circulating Hsp’s and atherosclerosis and to provide more insight into the potential prognostic value of serum Hsp measurements and their therapeutic potential in these and other patient groups. However, our data highlight the intriguing possibility that Hsp’s, particularly Hsp70, might have therapeutic potential for preventing the progression of atherosclerosis, at least in individuals with established hypertension.

Acknowledgments

This study was supported by Boehringer-Ingelheim, Sweden, the Swedish Heart Lung Foundation, the King Gustav V 80th Birthday Fund, the Swedish Society of Medicine, the Swedish Rheumatism Association, the Söderberg Foundation, and the Swedish Science Fund. The UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases provided the recombinant myco-

| Table 3. Prediction of Changes in I-M Thickness Over a 4-Year Period and Hsp and Hsp Antibody Levels |
|-------------------------------|-----------------|-----------------|
| Variable                     | Odds Ratio (95% CI) | P               |
| Hsp60                        | 0.6 (0.32–1.11)   | 0.10            |
| Hsp70                        | 0.42 (0.22–0.80)  | 0.008           |
| Anti-human Hsp60             | 1.19 (0.63–2.24)  | 0.60            |
| Anti-human Hsp70             | 1.08 (0.58–2.02)  | 0.81            |
| Anti-mHsp65                  | 1.2 (0.61–2.20)   | 0.64            |

m indicates mycobacterial. OR for the 75th percentile and 95% CI were derived by logistic regression analysis in subjects for whom I-M thickness was increased (yes/no) at the 4-year follow-up examination.
bacterial Hsp65. J.F. is a Career Investigator of the Karolinska Institutet. We acknowledge the study coordinator of the ELSA for permission to use the Swedish patient data from Lund and Stockholm for these analyses. We are also grateful to Linda Nilsson, Lena Averfalk-Ohlsson, and Karin Falk for performing the ultrasound measurements.

References
Serum Heat Shock Protein 70 Levels Predict the Development of Atherosclerosis in Subjects With Established Hypertension

Alan G. Pockley, Anastasia Georgiades, Thomas Thulin, Ulf de Faire and Johan Frostegård

*Hypertension.* published online August 4, 2003;

*Hypertension* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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

Print ISSN: 0194-911X. Online ISSN: 1524-4563

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

http://hyper.ahajournals.org/content/early/2003/08/04/01.HYP.0000086522.13672.23.citation

**Permissions:** Requests for permissions to reproduce figures, tables, or portions of articles originally published in *Hypertension* 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 *Hypertension* is online at:

http://hyper.ahajournals.org/subscriptions/