Circulating Heat Shock Protein 60 Is Associated With Early Cardiovascular Disease

A. Graham Pockley, Ruhia Wu, Carola Lemne, Rolf Kiessling, Ulf de Faire, Johan Frostegård

Abstract—The phylogenetically conserved nature of heat shock proteins (Hsp) has led to the proposition that they may provide a link between infection and the inflammatory component to vascular disease. Hypertension is associated with atherosclerosis. Here, we measured circulating heat shock protein and heat shock protein antibody levels in association with borderline hypertension. Seventy-two men with borderline hypertension patients and 75 normotensive control subjects (diastolic blood pressure 85 to 94 and <80 mm Hg, respectively) were selected from a population-screening program. The levels of Hsp60; Hsp70; and anti–human Hsp60, anti–human Hsp70, and anti–mycobacterial Hsp65 antibodies were determined with enzyme immunoassay. The presence of carotid atherosclerosis and the intima-media thickness values were determined with ultrasonography. A major novel observation in this report was the detection of circulating Hsp60, which was present at a significantly enhanced level in patients with borderline hypertension. Furthermore, serum Hsp60 was associated with intima-media thicknesses (P<0.01). Anti–Hsp65 antibody levels were higher in borderline hypertension (P<0.001), whereas Hsp70 and anti–Hsp70 antibody levels did not differ. In contrast to anti–Hsp65 antibody, anti–Hsp60 antibody levels were lower in borderline hypertension (P<0.03), although the difference was quantitatively small. None of the parameters evaluated were associated with atherosclerosis, metabolic factors, or smoking. We identified elevated Hsp60 levels in patients with borderline hypertension and an association between early atherosclerosis and Hsp60 levels. The physiological role of Hsp60 release has yet to be defined, but given the proinflammatory properties, these proteins could be involved in the induction/progression of both hypertension and atherosclerosis, as well as being markers for early cardiovascular disease. (Hypertension. 2000;36:303-307.)

Key Words: heat shock proteins ▪ hypertension, borderline ▪ atherosclerosis ▪ human

Evidence is accumulating that suggests the immunological component to the development of atherosclerosis may, at least in part, involve the expression of and reactivity to heat shock proteins. In addition to being constitutively expressed, heat shock proteins are induced in response to a number of biological and physicochemical agents. They are involved in repair processes and the intracellular assembly, folding, and translocation of oligomeric proteins. The highly conserved nature of heat shock proteins has led to the proposition that there is a link between immune responses to infection and the development of autoimmunity. Evidence for the involvement of heat shock proteins in vascular disease has arisen from studies that reported elevated levels of Hsp65 antibodies in patients with carotid atherosclerosis, coronary heart disease, or borderline hypertension (BHT).

Although heat shock proteins are typically regarded as being intracellular, they can be expressed on the surface of mononuclear cells and stressed aortic endothelial cells. They can also be released from cultured rat glial and human islet cells, and Hsp60 and Hsp70 have been identified in the serum of healthy individuals. Given the potential involvement of heat shock proteins in the pathogenesis of vascular disease and the association between hypertension and atherosclerosis, we determined the levels of circulating Hsp60; Hsp70; and anti–human Hsp60, anti–human Hsp70, and anti–mycobacterial 65-kDa protein antibodies in normotensive (NT) and BHT subjects. We make the novel observation that circulating levels of Hsp60 are increased in BHT subjects.

Methods

Study Groups

The study groups and their recruitment have been described previously. Samples from 72 men with BHT (defined as a diastolic blood pressure [DBP] of 85 to 94 mm Hg) and 75 age-matched NT control subjects from the original population were used in the present study. The BP of the control subjects was measured on 2 occasions a few weeks apart. For participation in the study, DBP had to be

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≤80 mm Hg on both occasions. BP measurements were made by the same nurse.

The study was approved by the local ethics committee of the Karolinska Hospital and was conducted in accordance with the Helsinki Declaration. All subjects gave informed consent.

**Study Program**

All subjects were investigated according to the same schedule. BHT patients and their NT counterparts were assessed simultaneously whenever possible or no more than 4 weeks apart. Blood samples were taken after the participant rested for 15 minutes in the supine position between 8:00 AM and 9:30 AM after a fast of 8 to 12 hours.

**Analysis of Total Serum Immunoglobulin Levels**

Serum IgG, IgM, and IgA levels were determined with immunoturbidimetry (Hitachi 911 analyzer) with anti-IgG, anti-IgM, and anti-IgA reagents and calibrators (DAKO).

**Serum Hsp60 and Hsp70 levels**

Serum Hsp60 and Hsp70 levels were determined with immunoassay. Briefly, 96-well microtitre plates (Nunc Immulonplate Maxi-sorp; Life Technologies) were coated through overnight incubation at 4°C with murine monoclonal antibodies to human Hsp60 (clone LK.1.) or Hsp70 (clone C92F3A-5; StressGen) in carbonate buffer, pH 9.5 (2 μg/mL). Plates were washed with PBS containing 1% vol/vol Tween 20 (PBS/T) and blocked by incubation with 1% wt/vol bovine serum albumin in PBS/T.

Serum samples were added, and bound heat shock protein was detected by the addition of rabbit polyclonal anti-Hsp60 or anti-Hsp70 antibody (1:1000; SPA-804 and SPA-812; StressGen). Bound polyclonal antibody was detected with alkaline phosphatase-conjugated murine monoclonal antibody to rabbit immunoglobulins (Sigma Chemical Co), followed by p-nitrophenyl phosphate substrate (Sigma Chemical Co). The resultant absorbance was measured at 405 nm with a Titertek Multiscan MCC/340 plate reader. Standard dose-response curves were generated in parallel with recombinant human Hsp60 (StressGen), recombinant human Hsp70 (StressGen), and the concentrations of Hsp60 and Hsp70 were determined by comparison with these standard curves with ASSAYZAP software (Biosoft). The interassay variability of the Hsp60 and Hsp70 immunoassays was <10%.

**Serum Anti–Heat Shock Protein Antibody Levels**

Heat shock protein antibody levels were also determined as described previously. Microtiter plates were coated with recombinant human Hsp60 (StressGen), recombinant human Hsp70 (StressGen), or recombinant Mycobacterium bovis Hsp65 (100 μL, 2 μg/mL). M. bovis Hsp65 was provided by the UNDP/World Bank/WHO Special Program for Research and Training in Tropical Diseases (Braunschweig, Germany).

Plates were washed, and nonspecific binding sites were blocked. Serum samples (typically 1:100 in PBS/T) were added, and bound antibodies were detected with alkaline phosphatase conjugated polyclonal goat anti-human IgA/G/M (Sigma Chemical Co) followed by p-nitrophenyl phosphate substrate substrate. Antibody concentrations were determined by comparison with a standard curve that was generated with samples of predetermined high levels that had been assigned a concentration of 1000 arbitrary units/mL (AU/mL).

**Plasma Lipoprotein and Insulin Levels**

Serum VLDL-, LDL-, and HDL-cholesterol and triglyceride levels were determined as described previously. The major plasma lipoprotein fractions were determined with preparative ultracentrifugation and lipid analyses of the lipoprotein fractions. Venous blood samples were also taken for the determination of blood glucose (Kodak Ectachem) and plasma insulin (radioimmunassay; Kabi Pharmacia) levels.

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**Table 1. Basic Characteristics of the Study Groups**

<table>
<thead>
<tr>
<th>Variable</th>
<th>NT (n=75)</th>
<th>BHT (n=72)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>50.0±5.6</td>
<td>50.0±5.6</td>
<td>...</td>
</tr>
<tr>
<td>BP systolic, mm Hg</td>
<td>125±11.0</td>
<td>141±9.6</td>
<td>0.001</td>
</tr>
<tr>
<td>BP diastolic, mm Hg</td>
<td>75±3.9</td>
<td>89±2.4</td>
<td>0.001</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.6±3.2</td>
<td>25.9±3.2</td>
<td>0.009</td>
</tr>
<tr>
<td>Waist-hip ratio</td>
<td>0.90±0.5</td>
<td>0.92±0.5</td>
<td>0.022</td>
</tr>
<tr>
<td>Smokers, %</td>
<td>37</td>
<td>32</td>
<td>...</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>5.5±0.8</td>
<td>5.5±1.0</td>
<td>...</td>
</tr>
<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.27±0.3</td>
<td>1.16±0.3</td>
<td>0.016</td>
</tr>
<tr>
<td>LDL-cholesterol, mmol/L</td>
<td>3.78±0.89</td>
<td>3.85±0.71</td>
<td>0.011</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>1.34±0.8</td>
<td>1.57±0.7</td>
<td>0.015</td>
</tr>
<tr>
<td>Insulin, mU/L</td>
<td>14.2±4.3</td>
<td>17.4±5.6</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Values are mean±SD. Group differences were determined by Student’s t test (parametric data) or the Mann-Whitney U test (skewed variables).

**Carotid Ultrasonography**

The right and left carotid arteries were examined with a duplex scanner (Acuson 128XP/5) with a 7.0-MHz linear-array transducer. Subjects were investigated in the supine position with the head turned slightly away from the sonographer. The intima-media (I/M) thickness was determined as described previously. Plaque was defined as localized I/M thickening, with a thickness of ≥1 mm and a 100% increase in thickness compared with normal, adjacent wall segments; it was scored as being present or absent. The common, internal, and external carotid arteries on both sides were screened.

**Statistical Methods**

Variables were tested for skewness and groups with skewed variables were compared by the nonparametric Mann-Whitney U test. The Student’s t test was used for the comparison of normally distributed data. Categorical variables were compared by the χ² test. The Spearman rank correlation was used to assess interrelationships among heat shock proteins, heat shock protein antibodies, metabolic variables, and BP levels. Nonnormally distributed variables were logarithmically transformed. Data are presented as mean±SEM for normally distributed data and as median and first and third quartile (Q1 and Q3) values for nonnormally distributed data. The level of statistical significance was set at a value of P<0.05.

**Results**

**Characteristics of Patients and Control Subjects**

The characteristics of the 2 study groups are presented in Table 1. The mean BP level for the NT group was 125/75 (±11/5) mm Hg compared with 141/89 (±10/2) mm Hg for the BHT group. The BHT group also had a significantly higher body mass index and waist/hip ratio (Table 1).

BHT men had a significantly altered metabolic profile with fasting hyperinsulinemia and dyslipoproteinemia (Table 1), as has been reported previously. In the BHT group, 26% of the subjects had detectable carotid plaques on 1 or both sides, whereas the incidence in the NT group was 16% (19 versus 10 subjects, NS).

**Heat Shock Protein and Heat Shock Protein Antibody Levels**

Hsp60 levels were markedly higher in the BHT group (P=0.001), whereas there was no significant difference in Hsp70 levels (Table 2). Hsp60 was present in all of the...
patients with BHT (100%) and 73 of the 75 control subjects (97%). Circulating Hsp70 was detectable in 36 of the 65 individuals with BHT (55%) and 37 of the 75 control subjects (49%). Anti–human Hsp60, anti–human Hsp70, and anti–mycobacterial Hsp65 antibodies were detected in all of the BHT samples analyzed. Anti-Hsp60 antibodies were detected in all 75 of the control samples, but there were 10 individuals who were negative for anti–mycobacterial Hsp65 antibodies and 2 individuals who were negative for anti-Hsp70 antibodies. The anti–mycobacterial Hsp65 antibody levels were significantly higher in the BHT group (P<0.001; Table 2), which confirms previous findings in the same patient group. In contrast to anti-Hsp65 antibody levels, anti–human Hsp60 antibodies were significantly lower in the BHT group, although the differences were quantitatively small (P<0.03; Table 2). Anti-Hsp70 antibody levels in the BHT group were not different than those in NT subjects (Table 2).

### Relationships Among Hsp, Anti-Hsp Antibodies, I/M Thickness, BP Levels, and Metabolic Variables

There were significant associations between Hsp60 and DBP (r=0.34, P<0.0001) and 24-hour systolic BP (SBP, r=0.179, P=0.037) in the complete study group. There also was a trend toward an association between Hsp60 and SBP and 24-hour DBP, but this was not of statistical significance. Furthermore, anti-Hsp65 antibody levels were associated with DBP (r=0.187, P=0.028) but not with SBP (r=0.146, P=0.087) or 24-hour DBP (r=0.159, P=0.07). There was no relationship between Hsp70 and anti-Hsp70 antibody levels with BP.

Given that DBP in subjects >60 years old plateaus and that there is an increasing relevance of SBP in subjects in that age group, data were also analyzed according to SBP of >140 mm Hg (n=39) or <140 mm Hg (n=106). The pattern was comparable to that observed when DBP was used. There was a trend for Hsp60 (P=0.072) and anti-Hsp65 (P=0.098) levels to be higher and anti-Hsp60 levels to be lower (P=0.023). Hsp70 and anti-Hsp70 levels did not differ significantly between patients and control subjects.

There was no significant association between heat shock proteins or heat shock protein antibody levels and BP parameters when the BHT and NT groups were analyzed separately.

I/M thickness was associated with Hsp60 levels in the entire group (I/M sin r=0.236, P=0.009; I/M dex r=0.255, P=0.004). This association was present in the BHT group alone (I/M sin r=0.236, P=0.009; I/M dex r=0.255, P=0.004) but not in the NT group. Hsp60 levels were higher in those individuals with carotid atherosclerosis than in those without (n=29; 435±1885 versus 1885±702 ng/mL, mean±SEM), although this difference was not of statistical significance. However, there was a trend toward Hsp60 levels and atherosclerosis being associated in the BHT group (P=0.06).

Serum Hsp60 levels were associated with VLDL (P=0.017) and triglyceride (P=0.05) levels, but there was no association between Hsp70 or anti-heat shock protein antibody with blood lipids or metabolic factors, nor was there a significant associations between LDL and any of the heat shock protein–related parameters. There was no correlation between smoking status and any of the heat shock protein parameters measured (data not shown).

There was no difference in immunoglobulin levels between the BHT group and controls (IgG 9.71±1.9 versus 9.76±2.3 mg/mL, IgM 2.25±0.8 versus 2.1±0.9 mg/mL, respectively), thus excluding the possibility that differences in antibody levels simply reflected altered total antibody levels.

### Discussion

In the present study, we identified enhanced levels of Hsp60 in the circulation of individuals with BHT. Hsp60 levels were associated with I/M thickness. Anti–human Hsp65 antibody

<table>
<thead>
<tr>
<th>Group</th>
<th>Hsp60, ng/mL</th>
<th>Hsp70, ng/mL</th>
<th>Anti-hHsp60, AU/mL</th>
<th>Anti-hHsp70, AU/mL</th>
<th>Anti-mHsp65, AU/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>Mean</td>
<td>Median</td>
<td>Interquartile range</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1197</td>
<td>192</td>
<td>68–672</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1358</td>
<td>40</td>
<td>40–400</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td></td>
<td>152</td>
<td>129</td>
<td>129–175</td>
<td>75</td>
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<td></td>
<td>35</td>
<td>29</td>
<td>29–44</td>
<td>75</td>
<td></td>
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<tr>
<td></td>
<td>54</td>
<td>38</td>
<td>38–76</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>BHT</td>
<td>Mean</td>
<td>Median</td>
<td>Interquartile range</td>
<td>n</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3640</td>
<td>662*</td>
<td>662–1739</td>
<td>66</td>
<td></td>
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<tr>
<td></td>
<td>1493</td>
<td>0†</td>
<td>0–300</td>
<td>65</td>
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</tr>
<tr>
<td></td>
<td>118</td>
<td>100†</td>
<td>100–151</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>32†</td>
<td>32–42</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td></td>
<td>83</td>
<td>68*</td>
<td>68–98</td>
<td>69</td>
<td></td>
</tr>
</tbody>
</table>

Antibody levels were determined from standard dose-response curves that were generated with sera with high levels of the respective antibody and are expressed as arbitrary units/mL (AU/mL). Heat shock protein and heat shock protein antibody levels demonstrated a lognormal distribution, and data are presented as median and first and third quartiles (Q25 and Q75) values.

*P<0.001 vs control; †P<0.05 vs control; ‡NS (P>0.05) vs control (Mann-Whitney U test).

**TABLE 2. Serum Heat Shock Protein and Heat Shock Protein Antibody Levels in NT and BHT Subjects**

There was no difference in immunoglobulin levels between the BHT group and controls (IgG 9.71±1.9 versus 9.76±2.3 mg/mL, IgM 2.25±0.8 versus 2.1±0.9 mg/mL, respectively), thus excluding the possibility that differences in antibody levels simply reflected altered total antibody levels.
levels were enhanced in BHT patients, whereas anti–human Hsp60 levels were slightly but significantly decreased.

The evidence for a heat shock protein–mediated component to atherosclerosis has arisen from a number of studies. The intensity of heat shock protein expression positively correlates with the severity of atherosclerosis, there is a localized enrichment of γδ T cells in the lesion, and immunization with recombinant mycobacterial Hsp65 can induce atherosclerotic lesions in normocholesterolemic rabbits. Elevated levels of circulating antibody to the mycobacterial 65-kDa heat shock protein have been reported in carotid atherosclerosis and coronary heart disease. The in vivo physiological significance of heat shock protein antibodies in the pathogenesis of vascular disease has yet to be clearly established. However, they have been shown to mediate endothelial cell cytotoxicity, and the observation that anti-Hsp65/60 antibodies in individuals with atherosclerosis recognize 3 distinct, conserved sequences may implicate them in the initiation of atherosclerosis via an autoimmune type of mechanism.

BHT is a condition that exhibits only minor cardiovascular changes, and the presence of circulating Hsp60 and anti–mycobacterial Hsp65 antibodies may reflect early vascular events. Raised BP has direct effects on the vasculature, and vessels subjected to greater mechanical and shear stress express heat shock proteins and are more prone to the development of atherosclerosis. In addition, Hsp70 mRNA levels are enhanced in hypertensive animals, and there is a greater accumulation of heat stress–induced Hsp70 mRNA in peripheral blood lymphocytes from hypertensive humans compared with that in NT control subjects. Although these findings demonstrate a stress response to hypertension, a definitive causative link between heat shock protein expression/release and the development of vascular disease has yet to be established.

It may be that the heat shock protein expression in the vasculature serves to protect against the effects of physiological and pathological insults such as mechanical stress, shear stress, hypercholesterolemia, free radicals, cytokines, and infections. The proposition has been that heat shock protein release is part of a response to protect neighboring cells, and this is supported by the observation that Hsp70 enhances the survival of stressed cultured arterial smooth muscle cells. However, it is possible that heat shock proteins potentiate later stages of atherogenesis by inducing heat shock protein antibodies, or by their expression on the endothelial cell surface.

The putative involvement of heat shock proteins in the establishment and progression of the atherosclerotic lesion is undoubtedly complex. Heat shock protein expression may be secondary to the inflammatory process, in that the expression of cytokines by both the vascular endothelium and infiltrating leukocyte populations drives the expression or release of heat shock proteins from the vessel wall. Human atherosclerotic plaques express a spectrum of inflammatory cytokines, including interferon-γ, interleukin-1α and -1β, and tumor necrosis factor-α, all of which have been shown to increase heat shock protein expression in a range of cell types. Alternatively, the localized expression of heat shock proteins may promote the production of cytokines, the enhanced expression of adhesion molecules, and the establishment and propagation of the inflammatory response. A number of studies lend support to this proposition. Bacterial and mycobacterial heat shock proteins induce proinflammatory cytokine expression, and bacterial heat shock proteins (DNAk and GroEL) induce intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on human vascular endothelial cells. Chlamydia and human Hsp60 activate human vascular endothelial cells to express E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 and activate vascular endothelial cells, smooth muscle cells, and macrophages to secrete interleukin-6. Heat shock proteins may also be expressed by lipid-laden “foam” cells in the atherosclerotic plaque, in that in vitro exposure to oxidized LDL induces Hsp60 expression by monocyctic cell lines and Hsp70 expression by human endothelial cells.

This is the first report of increased circulating heat shock protein levels in BHT. Their nature and origin in normal and diseased states have yet to be identified, as have the mechanisms that underlie their release and the influence that released proteins exert on physiological processes. The relationship between circulating heat shock proteins and anti–heat shock protein antibodies is undoubtedly complex, and additional studies with an aim of characterizing the circulating protein, defining its relationship with serum antibody, and elucidating its physiological importance are clearly warranted.

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References


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