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CIRCULATING ANTI-HSP70 LEVELS IN NASCENT METABOLIC SYNDROME: THE CASALE MONFERRATO STUDY

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ABSTRACT

The metabolic syndrome (MetS) confers an increased risk of both type 2 diabetes and cardiovascular diseases (CVD). Heat shock protein 70 (Hsp70), an intracellular polypeptide, can be exposed on the plasma-membrane and/or released into the circulation, eliciting both native and immune responses that may contribute to vascular damage. Our aim was to assess if serum anti-Hsp70 antibody levels were cross-sectionally associated with uncomplicated MetS. A cross-sectional case-control study from the non-diabetic cohort of the Casale Monferrato Study was performed. Subjects with established CVD and/or abnormal renal function were excluded. Case subjects (n= 180) were defined as those fulfilling the criteria for the diagnosis of MetS. Control subjects (n= 136) were completely free of any component of the MetS. Serum anti-Hsp70 levels were measured by immunoenzymatic assay. We found that anti-Hsp70 antibody levels were significantly higher in cases than in control subjects [122.6 (89.5-155.6) vs 107.1 (77.3-152.4) μg/ml, p=0.04], even after age- and sex-adjustment. In logistic regression analysis, higher levels of log-anti-Hsp70 conferred greater odds ratio (OR) for MetS, independently of age and sex. There was a statistically significant trend of ORs across quartiles of anti-Hsp70 and values greater than 108.0 μg/ml conferred a 77% increased OR of MetS as compared with values in the lower quartiles. The strength of the association slightly decreased after further adjustment for apolipoprotein B, smoke, and albumin excretion rate. In conclusion, our results shows that serum anti-Hsp70 antibody levels are independently associated with nascent MetS.

Keywords: metabolic syndrome, heat shock proteins, anti-heat shock protein 70 antibodies, cardiovascular disease.

Introduction

The metabolic syndrome (MetS), which confers an increased risk for both type 2 diabetes and cardiovascular disease (CVD), comprises a cluster of cardio-metabolic abnormalities with adiposity, insulin resistance, low-grade inflammation, and oxidative stress as its central pathophysiological features (Eckel et al., 2005).

Heat shock protein 70 (Hsp70), an ubiquitous, intracellular, highly conserved polypeptide, is important for cytoprotection and cell survival. Hsp70 is induced by oxidative stress in various cell types (Hendrick et al., 1995), and by insulin in cardiomyocytes (Li et al., 2006). In type 2 diabetes there is a deficiency in both skeletal muscles and liver Hsp70 expression that is believed to contribute to the pathogenesis and/or progression of insulin-resistance. Indeed, Hsp70 induction by various means enhances insulin sensitivity in both rodents and humans likely through inhibition of c-jun terminal kinase activation (Hendrick et al., 1995; Chung et al., 2008).

In stress conditions, including inflammation, oxidative stress, and endothelial injury, Hsp70 can also be exposed on the plasma-membrane and/or released into the circulation. The underlying mechanisms are still unclear; however, both passive and active mechanisms have been proposed (Joly et al., 2010). In contrast with intracellular Hsp70, extracellular Hsp70 has potent pro-inflammatory effects, secondary to Hsp70 interaction with toll like receptors resulting in inflammatory cytokine production (De Maio, 2011; Zhang et al., 2010, Joly et al., 2010).

Extracellular Hsp70 can also elicit an autoimmune response with production of anti-Hsp70 antibodies and this has been implicated in atherosclerosis both progression and severity in humans (Pockley et al., 2003). Altered levels of circulating antibodies against Hsp70 have been associated with components of MetS, type 2 diabetes, and diabetic micro/macrovascular complications (Wu et al., 2001; Ghayour-Mobarhan et al., 2005 and 2007; Gruden et al., 2009). However, there are no data on circulating anti-Hsp70 levels in large series of patients with MetS prior to the development of CVD and type 2 diabetes. Therefore, the aim of the present study was to investigate if serum anti-Hsp70 antibody levels are associated with MetS, uncomplicated by diabetes and CVD.

Experimental procedures

The Casale Monferrato study is an ongoing population-based study started in 1988 in the town of Casale Monferrato North-West of Italy. A non-diabetic cohort (n=2,211) was recruited in 2005 from an age- and sex-stratified sample of 3,700 individuals, aged 45-74 years, randomly identified through the files of the resident population, as previously detailed (Bruno et al., 2009). Subjects were examined at the diabetes clinic and blood samples collected after overnight fasting, and stored at -80° C. Ethics committee approval was obtained and all subjects provided written informed consent. A cross-sectional case-control study was designed from the non-diabetic cohort of the Casale Monferrato study, after exclusion of subjects (n=659) with CVD, serum creatinine levels ≥2 mg/dl, and/or high sensitive C reactive protein (hs-CRP) levels ≥ 3mg/l. Cases were those who fulfilled the criteria of the updated National Cholesterol Education Program's Adult Treatment Panel III report for the diagnosis of MetS. Control subjects were selected to be completely free of any component of the MetS. Applying these criteria, this yielded 180 cases and 136 controls with full data on complications and samples available for analysis. The sample size provides a power of 82% (α=0.05) to detect a difference in log-anti-Hsp70 within the cohort of at least one-third of standard deviation (SD).

Smoking status was classified as current smoker, never smoker, ex-smoker (smoking cessation at least a month prior to the visit). Hypertension was defined as systolic blood pressure >140 mmHg and/or diastolic blood pressure >90 mmHg or treatment with antihypertensive drugs. CVD was defined as a positive medical history of a cardiovascular event, including myocardial infarction, angina pectoris, coronary artery bypass graft and stroke, and/or ischemic changes on a resting 12-lead electrocardiogram, classified according to the Minnesota Code. The WHO Rose questionnaire was also administered and people with symptoms suggestive of CVD underwent further investigations to confirm the diagnosis (Bruno et al., 2009).

Fasting blood samples were taken in the morning visit from all recruited subjects and plasma glucose levels measured using the glucose-oxidase method. Triglycerides, total-cholesterol, HDL-cholesterol, apolipoprotein B, serum creatinine and insulin were measured by standard techniques,

hs-CRP by immunoturbidimetry (Roche-Diagnostic), and albumin excretion rate (AER) by nephelometry on single overnight urine collections. Anti-Hsp70 antibody levels were measured in serum samples (diluted 1:1000) by ELISA (EKS-750-Enzo Life Sciences) (Ghayour-Mobarhan et al., 2005). Assay sensitivity and range were 6.79 ng/ml (6.79-1000 ng/ml) and the intra- and inter-assay coefficients of variation (CV) were below 10%. Serum IgG levels were determined by immunonephelometry (Siemens BN II Analyzer) with anti-IgG reagents and calibrators (Siemens). The CV for both intra- and inter-assay was < 4%.

Data were expressed as mean (SD) or geometric means (interquartile range). Logistic regression analysis was used to estimate the odds ratios (ORs) of serum anti-Hsp70 antibody levels for MetS, independently of potential confounders and cardiovascular risk factors (age, sex, apoB, smoke, log-AER). Variables were retained in the final model if they added significantly to the likelihood of models or to the estimated coefficients of predictors. To assess pattern of ORs across increasing serum anti-Hsp70 antibody levels, they were categorized by the quartile distribution in controls.

We tested for linear trends across quartiles by entering a single ordinal term into the models. As ORs in the first and second anti-Hsp70 quartiles were similar, they were aggregated as the reference category in the final analyses and compared with the lowest quartiles. P value of less than 0.05 was considered to indicate statistical significance. Analyses were performed with Stata (Stata Release 10.0, Stata Corporation, College Station, Texas).

Results

The study population (n=316) had a mean age of 58.2 years (SD 8.3). As shown in Table 1, cases were older than controls and had a greater proportion of men. As expected, mean waist circumference, BMI, systolic and diastolic blood pressure, blood glucose, insulin and triglyceride levels were greater, while HDL-cholesterol levels lower in cases than in controls. Cases also had a more adverse cardiovascular risk profile with significantly higher values of LDL-cholesterol, apoB, hs-CRP, and AER.

Anti-Hsp70 antibodies were measurable in all the 316 samples with right skewed distribution of values. Anti-Hsp70 antibody levels were significantly higher in cases than in control subjects

(Table 1), even after age- and sex-adjustment (118.2 vs 106.1 p=0.02). However, the difference was no longer significant after further adjustment for BMI (p=0.09). Mean IgG levels were similar between groups.

Logistic regression analyses was performed to assess if anti-Hsp70 antibody levels were associated with MetS, independently of potential confounders and cardiovascular risk factors. Models showed that higher levels of log-anti-Hsp70 conferred greater ORs for MetS (Table 2). This association remained statistically significant after adjustment for age and sex (Table 2 - Model 2). A statistically significant trend of ORs across quartiles of anti-Hsp70 was observed (p=0.04). Anti-Hsp70 values in the two upper quartiles (> 108.0 μg/ml) conferred a 77% increased OR of MetS as compared with values in the lower quartiles. The strength of the association between anti-Hsp70 and MetS slightly decreased after further adjustment for apoB, smoke, and AER.

Discussion

In this cross-sectional population-based sample of non-diabetic subjects without clinical evidence of CVD, we have provided the first evidence of an independent association between anti-Hsp70 antibody levels and uncomplicated MetS.

Mean anti-Hsp70 antibody levels were significantly higher in cases than in controls. Excess body weight was likely a major determinant of this rise in anti-Hsp70 antibody levels as the difference between cases and controls was no longer significant after adjustment for BMI. In logistic regression analysis, serum anti-Hsp70 antibody levels greater than 108 μg/ml were associated with an almost 80% higher likelihood of MetS with respect to lower values, independently of age and sex. Although smoke (Newkirk et al., 2012), hypercholesterolemia (Guisasola et al., 2009), and microalbuminuria (Bianchi et al., 2008) have been associated with increased circulating anti-Hsp70 levels and cases had greater prevalence/levels of these risk factors, the strength of the association was only slightly reduced by further adjustment for apoB, smoke, and AER.

Previous studies have shown an association between circulating anti-Hsp70 antibody levels and single parameters of the MetS, such as hypertension, obesity, and dyslipidemia (Wu et al., 2001;

Ghayour-Mobarhan et al., 2005 and 2007); however, these clinically-based studies also included patients with type 2 diabetes and established CVD, making detangling analysis open to imprecision. Indeed, anti-Hsp70 antibody levels are often reduced in patients with CVD, likely because of immunocomplex formation (Dulin et al., 2010) and diabetic macro/microvascular complications have been associated with lower anti-Hsp70 levels (Gruden et al., 2009). Therefore, in the present study we have purposely selected patients with nascent MetS, uncomplicated by diabetes and CVD.

The underlying cellular mechanisms of anti-Hsp70 antibody rise in patients with nascent MetS remains elusive. However, it is likely to reflect a relatively greater exposure, either in the past or in the present, to extracellular Hsp70, possibly triggered by MetS-associated oxidative stress, which is a known inducer of extracellular Hsp70 release and/or membrane-bound Hsp70 exposure (Zhang et al., 2010). This is not in disagreement with recent studies in type 2 diabetes showing a reduced Hsp70 expression in insulin-sensitive tissues (i.e. skeletal muscles and liver) and linking this downregulation to the pathogenesis of insulin-resistance (Hendrick et al., 1995; Chung et al., 2008). Indeed, circulating Hsp70 levels also mirror expression in insulin-independent tissues, where Hsp70 expression is often enhanced (Yabunaka et al., 1998; Kavanagh et al., 2009). In addition, the dual role of intra- and extra-cellular Hsp70 is well recognized and differential mechanisms may regulate cytosolic and membrane-bound Hsp70 expression (Joly et al., 2010). In this regard it is noteworthy that insulin, whose levels are enhanced in insulin-resistant states, induces Hsp70 expression specifically on cardiomyocyte plasma-membranes (Li et al., 2006).

The rise in anti-Hsp70 antibody levels may play a role in the enhanced CV risk of patients with MetS. Indeed, anti-Hsp70 antibodies have been associated with atherosclerosis both progression and severity in humans (Pockley et al., 2003). Furthermore, in experimental animals anti-Hsp70 binding to endothelial Hsp70 triggers an inflammatory response that accelerates atherosclerosis (Zhang et al., 2010). On the other hand, the increase in anti-Hsp70 antibody levels may also represent a compensatory and protective response because anti-Hsp70 antibodies can prevent the deleterious effects of extracellular Hsp70 by clearing circulating Hsp70 and blocking membrane-bound Hsp70. Indeed, in contrast to cytoprotective intracellular Hsp70, extracellular Hsp70 act as

danger signals, eliciting both immune and inflammatory responses, and has deleterious inflammatory and pro-atherogenic activity (Zhang et al., 2010). In line with this hypothesis, a recent study, performed in an experimental model of hypertension-induced cardiac hypertrophy, has shown that hypertension induces both Hsp70 release and enhanced membrane-bound Hsp70 expression and that anti-Hsp70 antibodies can abolish cardiac fibrosis by suppressing Hsp70 conjugation with toll-like receptor 4 and hence to expression of pro-inflammatory and pro-sclerotic cytokines (Cai et al., 2010).

There are certain limitations to our study. First, this is a cross-sectional study and this restricts our ability to assess temporal relationships between anti-Hsp70 antibody levels and MetS and to identify causal biological mechanisms underlying this association. However, no data on anti-Hsp70 antibodies and uncomplicated MetS exist; therefore, this study may serve as a reasonable starting point to explore this issue. Second, although serum samples were adequately stored, the possibility of protein degradation cannot be excluded; however, random misclassification would have biased downward our estimates, without affecting significant associations. Third, the presence of CVD was assessed based on clinical data and resting ECG; therefore, we cannot exclude the possibility that some patients with CVD and normal ECG were erroneously included; however, the WHO Rose questionnaire was administered and people with symptoms suggestive of CVD underwent further investigations. Last, lack of serum Hsp70 measurement limits interpretation of results; however, serum Hsp70 levels only partially reflect actual Hsp70 antigen exposure as extracellular Hsp70 is found not only free into the circulation, but also exposed on the cellular plasma-membrane.

In conclusion, this is the first study providing evidence that serum anti-Hsp70 antibody levels are independently associated with MetS, uncomplicated by diabetes and CVD. Further studies are required to determine causal relationships and elucidate underlying mechanisms of this association.

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Table 1. Physical and Clinical Characteristic of the 316 recruited subjects.

		Case subjects	Control subjects	P
N		180	136	
Age (years)		60.9 ± 8.6	54.6 ± 6.5	<0.0001
Males (%)		58.9%	23.5%	<0.0001
BMI[a] (kg/m^2)		29.3 ± 4.6	22.4 ± 2.8	<0.0001
Waist circumference (cm)		100.6 ± 9.8	77.2 ± 9.3	<0.0001
Systolic blood pressure (mmHg)		154.0 ± 18.7	118.4 ± 10.1	<0.0001
Diastolic blood pressure (mmHg)		94.9 ± 10.6	77.0 ± 6.6	<0.0001
Hypertension (%)		91.1%	1.5%	<0.0001
Total cholesterol (mmol/l)		222.6 ± 32.5	210.1 ± 32.3	0.003
LDL-cholesterol (mmol/l)		129.2 ± 35.4	117.8 ± 26.6	0.002
HDL-cholesterol (mmol/l)		58.1 ± 13.4	76.6 ± 16.7	<0.0001
Triglycerides (mmol/l)		170 (127-216)	76 (61-89)	<0.0001
ApoB (mg/dl)		106.7 ± 26.1	87.4 ± 18.5	<0.0001
Glucose (mg/dl)		102.0 ± 10.9	85.8 ± 7.8	<0.0001
Insulin (ng/ml)		13.5 (10.1-19.2)	6.0 (4.7-8.2)	<0.0001
AER μg/min		40.0 (25.6-72.9)	30.2 (18.7-47.9)	<0.0001
CRP[b] (mg/l)		1.4 (0.9-2.1)	0.7 (0.5-1.2)	<0.0001
Creatinine (mg/dl)		0.86 (0.71-0.97)	0.73 (0.68-0.84)	<0.0001
Smokers	**no**	101 (55.8%)	73 (54.1%)	0.22
	ex	49 (27.1%)	29 (21.5%)	
	yes	31 (17.1%)	33 (24.4%)	
Anti-Hsp70 (μg/ml)		122.6 (89.5-155.6)	107.1 (77.3-152.4)	0.04
IgG		11.1 ± 2.5	10.7 ± 2.2	0.19

[a]BMI: body mass index; [b]CRP: C reactive protein

Table 2: Odds ratios for metabolic syndrome by anti-HSP70 values in the case-control study within the Casale Monferrato Study.

		Model 1	Model 2	Model 3
		OR (95% CI)	**OR (95% CI)**	**OR (95% CI)**
Log-Anti-HSP70		1.67 (1.02-2.73)	2.02 (1.12-3.66)	1.84 (0.97-3.50)
Anti-HSP70 (μg/ml)	**<77.4**	1	1	1
	77.4-108.0	1.15 (0.59-2.24)	1.11 (0.51-2.41)	1.06 (0.46-2.47)
	108.1-152.4	1.82 (0.96-3.44)	1.81 (0.87-3.78)	1.81 (0.82-4.02)
	>152.4	1.48 (0.78-2.84)	1.92 (0.90-4.10)	1.62 (0.70-3.73)
P for trend		***0.12***	***0.04***	***0.13***
Anti-HSP70 (μg/ml)	**≤108.0**	1	1	1
	>108.0	1.53 (0.98-2.41)	1.77 (1.05-2.99)	1.67 (0.94-2.96)

Model 1: unadjusted. Model 2: adjusted for age and sex. Model 3: adjusted for age, sex, smoking, apolipoprotein B, albumin excretion rate.

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