The lost correlation between heat shock protein 70 (HSPA1A) and plasminogen activator inhibitor-1 in patients with type 2 diabetes and albuminuria

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Received: 4 August 2015 / Accepted: 17 August 2015 / Published online: 4 December 2015
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Abstract We aimed to study the relation between plasma levels of stress-induced heat shock protein 70 (HSPA1A) with plasminogen activator inhibitor-1 (PAI-1) and high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (Apo-A1), and HDL-C/Apo-A1 ratio. In a matched case-control study on patients with diabetes (40 patients with albuminuria and 40 without albuminuria matched for age, sex, and body mass index), we observed that plasma levels of HSPA1A and PAI-1 are increased in patients with albuminuria (0.55 ± 0.02 vs. 0.77 ± 0.04 ng/ml, p value < 0.001 for HSPA1A; 25.9 ± 2 vs. 31.8 ± 2.4 ng/ml, p value < 0.05 for PAI-1). There was a significant correlation between HSPA1A and PAI-1 in diabetic patients without albuminuria ($r = 0.28$; p value $= 0.04$), but not in those with albuminuria ($r = 0.07$; p value $= 0.63$). No association was found between HSPA1A and HDL-C, between HSPA1A and Apo-A1, or between HSPA1A and HDL-C/Apo-A1 ratio. We concluded that there is a direct correlation be-

tween plasma HSPA1A and PAI-1 levels in patients with diabetes, which is lost when they develop albuminuria.

Keywords Heat shock protein 70 · Plasminogen activator inhibitor-1 · High-density lipoprotein · Albuminuria · Diabetes

Introduction

Heat shock proteins (HSPs) are a family of molecular chaperones that exert housekeeping functions under physiologic conditions and protect the cell against stressful stimuli (Beckmann et al. 1990; Hartl and Hayer-Hartl 2002). Inducible HSPs are highly upregulated by heat shock factors (HSF) under stressful conditions to serve their protective roles. Extracellular levels of HSPs are increased in several diseases (Abe et al. 2004; Najafizadeh et al. 2015; Son et al. 2015), including diabetic

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nephropathy (Morteza et al. 2013b). Diabetic patients with albuminuria are at considerably higher risk of developing cardiovascular events (Matsushita et al. 2015). As HSPs are widely involved in the pathogene of cardiovascular diseases (Rizzo et al. 2011), they may partially explain the association between albuminuria and increased cardiovascular risk.

Heat shock protein 70 (HSP70) is the most conserved member of HSP family and has been the subject of extensive researches. The stress-inducible HSP70 (HSPA1A) exerts cytoprotective functions as an intracellular chaperone but may induce immunological responses in extracellular space (Mansilla et al. 2012). Atherosclerosis is an inflammatory disease and is not merely the accumulation of lipids within the artery wall (Ross 1999). Increased plasma level of plasminogen activator inhibitor-1 (PAI-1) (Hirano et al. 1997; Stehouwer et al. 2002) and dysfunctional high-density lipoprotein (HDL) molecules (Navab et al. 2009) are contributing factors and may be the results of this localized inflammation.

This study aimed to examine the association between plasma levels of HSPA1A and PAI-1, as well as HDL-cholesterol (HDL-C) and apolipoprotein-A1 (Apo-A1, the main constituent Apo-protein of HDL molecule) in patients with diabetes-induced albuminuria.

Methods

This was a 1:1 matched case-control study on patients with type 2 diabetes, diagnosed at an outpatient diabetes clinic in Vali-Asr Hospital affiliated with Tehran University of Medical Sciences (TUMS). Diabetes was diagnosed according to the American Diabetes Association criteria (Diagnosis and classification of diabetes mellitus 2009). Normoalbuminuria was defined as urinary albumin excretion rate (UAER) <15 mg/12 h, and albuminuria was defined as UAER between 100 and 400 mg/12 h. Cases and controls were matched for age, sex, and body mass index (BMI). For each case with albuminuria, a diabetic patient without albuminuria of the same gender, with age ± 3 years, and BMI ± 1 kg/m² was selected. Exclusion criteria were type 1 diabetes, acute or chronic renal failure (estimated glomerular filtration rate (eGFR) <60 ml/min), past medical history of glomerulonephritis or hematuria, RBC >5 per high power field or RBC cast in urine analysis, congestive heart failure, acute infections, pregnancy, diabetic ketoacidosis, nonketonic hyperosmolar diabetes, thyroid disorders, autoimmune diseases, hormone replacement therapy or use of oral contraceptive pills, and hospital admission in recent 3 months.

Demographic and anthropometric data including age, sex, height, weight in light clothing, and waist circumference, as well as duration of diabetes, were recorded. Smoking status, family history of diabetes, and drug history were determined through interview. Blood pressure was measured twice after 5 min with mercury sphygmomanometer in a valid method

(Nargesi et al. 2014). The BMI (kg/m²) was calculated according to the Quetelet formula. eGFR was calculated using the Modification of Diet in Renal Disease (MDRD) formula. All participants gave written informed consent before participation. Ethics committee of Endocrinology and Metabolism Research Center of TUMS approved the study protocol. This study complied with the principles of the Declaration of Helsinki.

Urine and blood samples

Patients were instructed in 24-h timed urine collection for the measurement of UAER. Urinary albumin was determined by calorimetric methods using commercial kits (ZiestChem Diagnostics, Tehran, Iran). Blood samples were collected after 12 h of overnight fasting, and biochemical analysis was made subsequently. Glucose measurements were made using glucose oxidase method (intra-assay coefficient of variants (CV)=2.1 %, inter-assay CV=2.6 %). Creatinine was measured using calibrated Jaffe method (Parsazmoon, Karaj, Iran). Total cholesterol, HDL-C, low-density lipoprotein cholesterol (LDL-C), and triglycerides were determined using direct enzymatic methods (Parsazmoon, Karaj, Iran). HbA1c was estimated by high-pressure liquid chromatography. HSPA1A was measured using a quantitative sandwich ELISA immunoassay (EKS-715, Stressgen). The intra- and inter-assay CVs were 4.5 and 7 %, respectively. Apo-A1 was measured (Cobas INTEGRA Tinaquant Apolipoprotein A-1 ver.2) following the principles of antigen-antibody reaction using immunoturbidometric method (intra-assay CV=0.8 %, inter-assay CV=1.7 %). PAI-1 was measured by sandwich ELISA (Human PAI-1 PicoKine ELISA kit, EK0859, Boster Bio, CA, USA; intra-assay CV=5–6.5 %, inter-assay CV=6.4–8.2 %).

Statistical analysis

Data is presented as mean $\pm$ standard error of mean (SEM) or number (percent). For variables with non-Gaussian distributions, log-transformed values were used in analysis and geometric means $\pm$ antilog(SEM) are presented. Paired-samples *t* test and chi-squared test were used for group comparisons, as appropriate. Pearson *r* correlation and linear regression models were used for data modeling. STATA (version 11.2, StataCorp, Texas, USA) was used for statistical analysis. Two-sided *p* values <0.05 were considered statistically significant.

Results

Study population consisted of 40 patients with diabetes and albuminuria (cases) and 40 patients with diabetes and without albuminuria (controls). Baseline characteristics of study population are presented in Table 1. Groups were matched for age, sex, and BMI. There was no significant difference between

Table 1 Baseline characteristics of study population

	Patients without albuminuria (n=40)	Patients with albuminuria (n=40)	p value
Female n (%)	18 (45)	18 (45)	–
Age (years)	58.5±1.5	56.8±1.8	0.49
Weight (kg)	70.7±1.8	70.9±2	0.94
BMI (kg/m ²)	26.5±0.7	27.1±0.7	0.56
Waist circumference (cm)	92±1.5	93±1.5	0.63
Duration of DM (years)	9±1	9.3±1.3	0.83
SBP (mmHg)	122.2±3.6	123.1±2.9	0.86
DBP (mmHg)	72.4±1.6	72±1.4	0.86
FBS (mg/dl)	183±13.3	196±13.8	0.49
HbA1C (mg/dl)	8.4±0.3	8.9±0.4	0.43
Total cholesterol (mg/dl)	181±8.2	185±7.4	0.71
Triglycerides (mg/dl)	161±18	186±20	0.35
LDL-C (mg/dl)	102±6.7	99±4.9	0.71
HDL-C (mg/dl)	40.8±1.7	38.7±1.3	0.31
Apo-A1 (mg/dl)	146.8±4.8	139.3±4	0.22
HDL/Apo-A1	3.67±0.11	3.62±0.06	0.80
HSPA1A (ng/ml)	0.55±0.02	0.77±0.04	<0.001
Quantity for PAI-1 (ng/ml)	25.9±2	31.8±2.4	<0.05
eGFR (ml/min)	69±4.4	66±2.7	0.56
UAER (mg/12 h)	5.1±0.3	159±18.2	<0.001
Smoking n (%)	3 (7)	1 (2)	0.30
Family history of DM n (%)	27 (67)	22 (55)	0.25
Lipid-lowering drugs n (%)			<0.05
Statin	21 (52)	12 (30)	
Anti-hyperglycemic drugs n (%)			0.28
Glyburide	3 (7)	8 (20)	
Metformin	6 (15)	8 (20)	
Insulin	7 (17)	3 (7)	
Metformin+glyburide	7 (17)	4 (10)	
Metformin+insulin	17 (42)	17 (42)	

Data is presented as mean±SEM or number (percent). Group comparisons were made using paired-samples *t* test and chi-squared, as appropriate. As the distributions of HSP70, PAI-1, HDL-C, Apo-A1, and UAER were non-Gaussian, log-transformed values were analyzed and geometric mean±SEM were reported

BMI body mass index, *DM* diabetes mellitus, *SBP* systolic blood pressure, *DBP* diastolic blood pressure, *FBS* fasting blood sugar, *HbA1C* hemoglobin A1C, *LDL* low-density lipoprotein cholesterol, *HDL-C* high-density lipoprotein cholesterol, *Apo-A1* apolipoprotein A1, *HSPA1A* heat shock protein A1A, *PAI-1* plasminogen activator inhibitor-1, *eGFR* estimated glomerular filtration rate, *UAER* urinary albumin excretion rate

groups, except for serum HSPA1A and PAI-1, which were higher in cases, and for anti-lipid medications, which were used more frequently in controls. HDL-C, Apo-A1, and HDL-C/Apo-A1 ratio were not different between cases and controls.

We observed that HSPA1A had a significant correlation with PAI-1 in controls ($r=0.28$, p value=0.04), but not in

patients with albuminuria ($r=0.07$, p value=0.63; +ure-1). Neither in cases nor in controls, no significant association was found between HSPA1A and HDL-C ($r=0.18$, p value=0.26 in controls; $r=0.03$, p value=0.82 in cases), HSPA1A and Apo-A1 ($r=0.05$, p value=0.72 in controls; $r=0.06$, p value=0.70 in cases), or HSPA1A and HDL-C/Apo-A1 ratio ($r=-0.17$, p value=0.28 in controls; $r=0.02$, p value=0.89 in cases).

With median values of HSPA1A=0.64 ng/ml and PAI-1=29 ng/ml, study population was stratified into high-HSP (HSPA1A >0.64) vs. low-HSP (HSPA1A <0.64) and high-PAI1 (PAI-1 >29) vs. low-PAI1 (PAI-1 <29) subgroups. In a 2×2 table, we observed that clustering of HSPA1A and PAI-1 into high and low subgroups was associated (chi-square=4.05, d.f=1, p value=0.04).

Discussion

PAI-1 is an acute phase reactant and a marker of inflammation and endothelial cell dysfunction (Alessi and Juhan-Vague 2004). Elevated plasma level of PAI-1 is considered as a non-traditional risk factor for cardiovascular diseases (Brodsky et al. 2002). Rucker et al. demonstrated that heat shock stress increases the expression of PAI-1 in vascular tissues of rat muscle (Rucker et al. 2006). It was also reported that glycated LDL increased the expression of HSF1 (a physiologic transcription factor of HSP gene) and HSPA1A in endothelial cells in vitro (Zhao and Shen 2007). HSF1 bind to PAI-1 gene promoter and directly upregulates its expression. This effect can be inhibited by HSF1 small interference RNAs, suggesting a direct regulatory effect for HSF1 on PAI-1 gene expression (Zhao and Shen 2007). Plasma level of HSPA1A is increased in patients with albuminuria (Morteza et al. 2013b). In accordance, increased plasma level of PAI-1 was reported both in patients with diabetes (Hirano et al. 1997) and patients with albuminuria (Stehouwer et al. 2002). These observations imply a mechanistic link between HSPA1A and PAI-1 in patients with diabetic nephropathy.

Despite the putative relation between HSPA1A and PAI-1 at DNA/mRNA level, the correlation between their extracellular levels has not been adequately explored, especially in patients with albuminuria. We found a significant (though weak) association between plasma levels of HSPA1A and PAI-1 in patients without albuminuria, while this relation was lost in those with albuminuria (Fig. 1). We have already observed this pattern of appearance and disappearance of associations for several oxidative and inflammatory markers in diabetes (Morteza et al. 2013a, b; Nakhjavani et al. 2012; Nakhjavani et al. 2015). Considering previous reports on the direct involvement of HSF1 in the regulation of PAI-1 gene expression (Zhao and Shen 2007), a direct correlation between

Fig. 1 Correlation between plasma HSP70 and PAI-1 levels in diabetic patients with and without albuminuria

plasma levels of these two factors was expected, as the same observed in our study. However, the observed divergence between their extracellular levels in patients with diabetic complications may represent altered gene regulation in this group. It is known that biochemical pathways and epigenetic patterns are altered in target cells under diabetic conditions (Villeneuve and Natarajan 2010). Our observations imply that HSF-PAI-1 pathway may also be differently modulated in patients with diabetic complications, compared with those with uncomplicated diabetes.

But, is HSPA1A a pro-inflammatory trigger in diabetic nephropathy or a cytoprotective response against inflammation? While intracellular levels of HSPA1A are decreased in T2DM and correlated with insulin resistance, extracellular HSPA1A levels are increased and correlated with oxidative damage and stress (Rodrigues-Krause et al. 2012). Serum HSPA1A concentrations are positively correlated with markers of inflammation, such as C-reactive proteins, monocyte count, and TNF- α (Mayer and Bukau 2005; Njemini et al. 2004). In fact, extracellular HSPA1A may act as a danger signal and trigger immunological responses (Mansilla et al. 2012). Interstitial fibrosis and glomerulosclerosis are two main pathological features of diabetic nephropathy, and inflammation plays a key role in the development of both (Qian et al. 2008). Inflammation may initially exert a protective response against hyperglycemia, but it turns into a detrimental condition if chronically persisted (Lawrence and Gilroy 2007). Unresolved renal inflammation promotes progressive fibrosis of glomerulus and tubulointerstitial structures (Meng et al. 2014). Extracellular HSPs may be a link between hyperglycemia and its resultant oxidative stress on one side, and the inflammation and the resultant fibrotic changes on the other side. Anti-hyperglycemic agents are the mainstay of diabetes treatment. However, diabetic

complications (especially macrovascular complications) still affect these patients. If HSPs are proved to be causally involved in the pathogenesis of inflammation in diabetic patients, they may be candidates for preventive and therapeutic interventions in these patients.

We found no difference in HDL-associated measures (HDL-C, Apo-A1, and HDL-C/Apo-A1 ratio) between diabetic patients with and without albuminuria. There was also no association between these indices and plasma HSPA1A level. Altogether, we found no evidence for a similar inter-talk between HSPA1A and reverse cholesterol transport mediators.

The cross-sectional design, lack of follow-up, and small sample size are some limitations of this study. However, recruitment of participants from a large population of diabetic patients, as well as its matched design, resulted in a large degree of homogeneity between study groups. Groups were comparable in respect to their baseline characteristics, except for higher usage of lipid-lowering drugs in those without albuminuria. This was a clinical study aimed to examine the relation between extracellular levels of HSPA1A and PAI-1. The study of their interaction at DNA/RNA level is surely needed to complement the initial findings.

In conclusion, we observed that extracellular levels of HSPA1A and PAI-1 are correlated in diabetic patients without albuminuria, while this correlation is lost in those who develop albuminuria. The interaction between HSPA1A and PAI-1 in diabetic complications may be different from those with uncomplicated diabetes.

Conflict of interest The authors declare that they have no competing interests.

Source of funding This study received no funding.

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