Extracellular Hsp90α is a Potential Serum Predictor of Atherosclerosis in type 2 Diabetes

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Abstract

Background: Atherosclerosis is the main pathological change in diabetic angiopathy, and vascular inflammation plays an important role in early atherosclerosis. Heat shock protein 90, a cellular molecular chaperone, was recently determined to be secreted extracellularly, but the specific mechanism remains unclear. This study explored the relationship between Hsp90 and diabetic peripheral artery disease through serological analyses of different groups of diabetic patients and investigated the relationship between extracellular Hsp90α and vascular inflammation at the cellular level.

Methods: Seventy-seven selected patients were divided into three groups. The relationships among serum Hsp90, oxidative stress indexes and patient outcomes and the correlations among the indexes were analysed. An oxidative stress endothelial injury model was established under high glucose in vitro to explore the role of eHsp90 release in atherosclerosis progression.

Results: Serum Hsp90 and MDA levels tended to increase in different groups with peripheral vascular disease aggravation. Hsp90α was correlated with MDA to some extent and was predictive. In vitro, high glucose and low H_2O_2 treatment increased extracellular Hsp90 secretion, and endothelial cell conditioned medium and recombinant human Hsp90α increased monocyte migration ($P<0.05$).

Conclusions: Extracellular Hsp90α participates in endothelial cell injury in diabetic vascular disease and initiates the inflammatory response by promoting monocyte migration.

Trial registration: NCT04787770, ClinicalTrials.gov, Registered 9 March, 2021 - Prospective registered

Background

Diabetes mellitus (DM) has become a global public health problem. Approximately 4.2 million people die each year from DM or its complications. [1] Many studies have indicated that type 2 DM (T2DM) is closely associated with atherosclerotic vascular disease. Lower extremity arterial disease (LEAD) is the most common initial manifestation of cardiovascular disease in individuals with T2DM. [2] Individuals with DM are two to four times more likely to develop LEAD than those without DM. Up to 80% of people with T2DM die from cardiovascular complications, and the disease reduces the average life expectancy by approximately 10 years. [3] Therefore, the early prevention and diagnosis of LEAD is particularly important for diabetic patients.

The initial pathogenesis of atherosclerosis (AS) includes the formation of advanced glycosylation end products, excessive production of reactive oxygen species (ROS), activation of the PKC pathway, and other processes. [4] The different pathogenic mechanisms induced by hyperglycaemia all reflect a single upstream process, namely, overproduction of ROS, suggesting that ROS play a key role in atherosclerotic injury. [5] Ross proposed that AS is a pathological process characterized by a chronic inflammatory response. [6] When the levels of metabolic abnormalities are increased, superoxide can also activate and participate in the pathogenesis of various complications. However, there is no marker reflecting the

current states of the vascular endothelium and the inflammatory response that can be applied for early clinical detection, which makes early diagnosis difficult.

Heat shock protein 90 (Hsp90) is an important molecular chaperone that is widely involved in immune regulation. Lei et al. [7] proposed that Hsp90α is involved in the pathogenesis of diabetic angiopathy. In recent years, Hsp90 inhibitors have been found to have protective effects against diabetic angiopathy. For example, Lazaro et al. [8] reported that application of an Hsp90 inhibitor (17-DMAG) effectively reduced renal injury in a diabetic mouse model. Kim et al. [9] found that inhibiting Hsp90 can reduce the formation of AS by inhibiting the migration and proliferation of vascular smooth muscle cells (VSMCs). Madrigal-Matute et al. [10] found that Hsp90 is highly expressed in the thin fibrous cap area of human carotid atherosclerotic plaques. All the above findings suggest that Hsp90 may be a new target for AS therapy. Extracellular Hsp90 (eHsp90) has been reported to be involved in a variety of physiological and pathological processes. Our team found that eHsp90 can be released outside the cell through exosomes under stress conditions, which in turn affects cytoskeletal proteins to promote the migration of epithelial cells, participating in damage repair. [11–13] Although Hsp90 has been indicated to be correlated with diabetic vascular disease, previous studies have focused more on its role as a molecular chaperone. Whether eHsp90 is involved in vascular inflammation or fibrosis is still unclear.

In this study, in vivo serological studies and in vitro cell models were used to explore the direct relationship between serum Hsp90 concentrations and subclinical AS in patients with T2DM and the role of eHsp90α in diabetic vascular disease in an oxidative stress injury model. The findings will provide a new perspective for investigation into early therapeutic targets for diabetic vascular disease.

Methods

In vivo study

Study subjects

From August 30, 2019, to September 28, 2020, a total of 77 patients in the Endocrinology and Metabolism Department of the Nanfang Hospital of Southern Medical University were randomly enrolled in this study. The patients were divided into four groups: the DM group (12 patients), the DM+LEAD group (45 patients) and the Critical limb ischemia (CLI) group (20 patients). According to the central limit theorem, the sample size greater than 30, which can be considered as obeying normal distribution. The subjects were grouped according to their clinical presentations and the results of laboratory and imaging studies.

Inclusion criteria

a. DM group: T2DM was diagnosed according to the World Health Organization (WHO) 1999 criteria.

b. DM+LEAD group: Patients were diagnosed with T2DM according to the above DM diagnostic criteria and determined to have LEAD on the basis of the following:

(1) Symptoms and signs of AS (intermittent claudication, resting pain, decreased or absent pulse in the dorsal foot artery, etc.);

(2) An ankle-brachial arterial pressure index (ABI)<0.9, a toe-brachial arterial pressure index (TBI)<0.7, no three-phase foot pulse graph waveform, or a percutaneous oxygen partial pressure (TcPO2)<30 mmHg; or

(3) Evidence of uneven thickening, AS, atherosclerotic plaques, arterial stenosis or obstruction in the carotid and/or lower extremity arteries on vascular colour Doppler ultrasonography.

c. CLI group: CLI was diagnosed for patients who met the above diagnostic criteria for DM+LEAD and who had lower extremity ischemic infection, ulceration, and/or deep tissue destruction.

The patients and/or their families were informed and agreed to participate in the study. Patients were excluded if they met any of the following exclusion criteria before admission:

(1) History of diabetic ketoacidosis or hyperosmolar status within 30 days,

(2) History of diabetic coma within 3 months or any hypoglycaemic event within 1 month

(3) History of cancer, immune system disease, or any hypoglycaemic event within 1 month

(4) History of other types of DM

(5) Age less than 18 years old

The study was designed and implemented in accordance with the Declaration of Helsinki (2013), approved by the ethics committee of Nanfang Hospital (NFEC-2020-268), and registered on the Clinical Trials website (NCT04787770).

All patients participation in this survey were voluntary, with informed consent.

Sample collection

Blood samples (3 ml) were collected under aseptic conditions from the peripheral veins of the subjects. The collected samples were transported in coolers on ice within a temperature range of 0℃-4℃ to the laboratory designated for the study. Serum samples were obtained via cold centrifugation at 3000 rpm for 15 min. The clear supernatants were aspirated and stored at -80°C until they were used for biochemical analysis of Hsp90α, Hsp90β and MDA using a spectrophotometer.

Assessment of HSP90α and Hsp90β

The levels of serum HSP90α and Hsp90β were measured by using ELISA kits (JiangLanChun, China). All procedures were performed according to the manufacturer's instructions. The absorbance was measured at a wavelength of 450 nm. The concentrations of Hsp90α and Hsp90β protein in each sample were calculated according to a standard curve of optical density values.

Histopathology and immunohistochemistry

Blood vessels were obtained from amputated tissue discarded during surgery in three CLI patients, immersed in paraformaldehyde for at least 24 h for fixation, paraffin-embedded, and cut into 2-μm sections. The samples were stained with haematoxylin and eosin (HE) for histopathological assessment. Immunohistochemistry for Hsp90α was performed.

Patients participation in this research were with informed consent.

In vitro study

Cell culture

Human umbilical vein endothelial cells (HUVECs) and the human monocyte line THP-1 were obtained from the China Center for Type Culture Collection. HUVECs were cultured in DMEM/F12 (Gibco, USA), and THP-1 cells were grown in complete RPMI-1640 medium supplemented with 10% FBS (ExCell Bio, China) and 1% penicillin/streptomycin in a 37°C incubator with a humidified atmosphere and 5% CO_2.

Antibodies and reagents

The primary antibodies included Hsp90α (07-2174, Merck, USA), Hsp90β (SPC-177, StressMarq Biosciences, Canada), LRP1 (BS9805M, Bioworld Technology, USA), p-Akt (66444-Ig, Proteintech, USA), Akt (#2920S, CST, USA), and β-actin (RM2001, RayBiotech, China).

The primary reagents included recombinant human Hsp90 alpha protein and recombinant human Hsp90 beta protein (SPR-101C and SPR-102C, StressMarq, Canada) and 17AAG (HY10211, MCE, USA). A non-commercial anti-secreted Hsp90α monoclonal antibody (mAb), 1G6-D7, was kindly donated by the University of Southern California Keck School of Medicine (USA).

Cell viability assay

Cell viability was evaluated with a Cell Counting Kit-8 (CCK-8) assay, which was carried out following a standard procedure in 96-well plates. Briefly, cells were seeded into a 96-well plate at a density of 5×10^3 cells/well in 100 μl of medium and grown to 80% confluence. After treatments, the medium was replaced with fresh medium containing 10% CCK-8 reagent. The absorbance at 450 nm was measured after a 2-h incubation at 37°C. Triplicate wells were included for each group.

Detection of MDA content and SOD activity

Following treatment, supernatants were collected, and the levels of lipid peroxidation were determined with a Micro-MDA Assay Reagent Kit (KGT003, Keygen Biotech, China). SOD activity was detected by a SOD assay kit (KGT00150, Keygen Biotech, China) according to the manufacturer's instructions. MDA and SOD concentrations were determined based on the constructed standard curve and are expressed in nmol/(mg total protein).

Measurement of ROS

Intracellular changes in ROS generation were measured with an ROS assay kit (S0033, Beyotime, China). According to the manufacturer's instructions, cells were incubated with 10 μM DCFH-DA in a cell incubator with 5% CO_2 at 37°C for 20 min. Then, the fluorescence intensity of the cells was detected by using a flow cytometer.

Protein extraction and protein expression analysis

HUVECs were plated in 10-cm dishes. When the cells grew to 80% confluence, the medium was replaced with fresh medium without FBS. After 24 h, conditioned medium (CM) from the serum-free cultures was collected, centrifuged and filtered through a Millipore Amicon Ultra-4 (50K) column. The total cell lysates were centrifuged at 13,000 × g for 15 min at 4°C, and the total protein concentrations were determined using a BCA Protein Assay Kit (Keygen, China). CM and cell extract samples were electrophoresed through 10% SDS polyacrylamide gels under denaturing conditions and transferred to PVDF membranes (EMD Millipore, USA). The membranes were blocked in 5% non-fat milk that was dissolved in 1× TBST and then incubated with the corresponding primary antibodies at 4°C overnight. The membranes were subsequently washed in 1× TBST and incubated with secondary antibodies for 2 h at room temperature. Specific antigen-antibody interactions were detected with enhanced chemiluminescence.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 10 min at room temperature. Permeation was performed by incubating the pre-treated cells with ice-cold methanol at -20°C for 15 min. The cells were incubated with primary antibodies (1:100) at 4°C overnight. Then, the cells were washed with cold PBS and

incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Abcam, USA) (1:200) at room temperature for 2 h. After the nuclei were stained with DAPI, images were captured using an Olympus FV1000 confocal laser scanning microscope (Tokyo, Japan).

mRNA expression analysis

Total cell RNA was isolated with RNAiso (Takara, China), and 1000 ng of total RNA was used for reverse transcription with a PrimeScript RT Reagent Kit. Real-time quantitative PCR was performed using SYBR Premix Ex Taq and Premix Taq.

The following primers were used: Hsp90AA1, F: AGGAGGTTGAGACGTTCGC, R: AGAGTTCGATCTTGTTTGTTCGG; Hsp90AB1, F: GGATGACAGCGGTAAGGATAAG, R: GAGCCCGACGAGGAATAAATAG; and β-actin, F: GCCATGTACGTTGCTA TCCA, R: CCTCGTAGATGGGCACAGT.

Cell migration assay

A Transwell migration assay was applied to study the transmigration behaviour of THP-1 monocytes. Twenty-four transwell inserts with pore sizes of 3 μm (Corning, USA) were employed. A total of 10^6 THP-1 monocytes in 200 μl of serum-free RPMI 1640 medium were loaded into the upper chamber of each Transwell insert. Then, 600 μl of endothelial cell CM was treated with H_2O_2 and 17AAG, or RPMI medium was added to the lower chamber. The cells were then allowed to migrate for 8 h. The cells that migrated across the membrane to the lower chamber were counted under an inverted microscope (Nikon Eclipse TS100, Japan).

Statistical analysis

All experiments were repeated three times. SPSS 22.0 was used for statistical processing of the data. Normally distributed continuous variables are described as the mean ± standard deviation (x±s). Continuous variables with a skewed distribution are expressed as the median (interquartile range) (Q1, Q3). Categorical variables are expressed as the number of cases (%). The differences between groups were analysed by one-way ANOVA, Dunnett's t-test, Student's t-test, K-W test and chi-square test according to the characteristics of the data. Spearman correlation analysis was used to analyse the correlations of related indexes. A receiver operating characteristic (ROC) curve was drawn to evaluate the predictive value for LEAD in diabetic patients. The significance level was $p < 0.05$.

Results

In vivo study

Hospital-based cross-sectional study

Characteristics of the study subjects

The baseline demographic and clinical information of the 77 patients (45 males [58.4%] and 32 females [41.6%]) included in this study is displayed in Table 1. All patients were considered to have T2DM as defined by the WHO 1999 criteria. Forty-five of these 77 patients were considered to have LEAD, and 20 others had CLI. Univariate analysis showed that among the clinical features and risk factors studied, age, course, past medical history, CRP, HDL, WBC count and Hb were significantly associated with the patient outcomes ($p < 0.05$).

Table 1
Baseline characteristics of the study subjects

Parameter	Patients			$F(\chi^2)$	p
	DM	DM + LEAD	CLI		
N (%)	12(15.6)	45(58.4)	20(26.0)		
Male/female (n)	7/5	27/18	11/9	0.143	0.931
Age (years)	46.17±11.91	58.31±11.32	63.50±11.52	8.698	<0.001
BMI (kg/m^2)	22.28±2.61	23.33±3.61	23.56±3.07	0.583	0.561
Course (%)				12.720	0.013
< 5years	9(75.0)	16(35.6)	4(20.0)		
5-10years	2(16.7)	11(24.4)	3(15.0)		
≥10years	1(8.3)	18(40.0)	13(65.0)		
Smoking (%)	6(50.0)	16(35.6)	4(20.0)	3.173	0.205
Hypertension (%)	5(41.7)	20(44.4)	12(60.0)	1.574	0.455
Hyperlipidaemia (%)	3(25.0)	20(44.4)	8(42.1)	1.501	0.472
Family history (%)	4(33.3)	17(37.8)	5(20.0)	1.012	0.603
Past medical history (%)	0	3(6.7)	8(40.0)	14.933	0.001
2hPBG (mmol/L)	11.59±6.72	11.82±4.47	9.31±4.58	1.591	0.212
GLU (mmol/L)	6.39 (4.37–13.70)	7.79(5.19–12.25)	9.73(6.86–16.43)	1.681	0.193
HbA1c (mmol/mol)	8.09±2.38	9.50±2.63	8.88±2.48	0.457	0.635
CRP (mg/L)	1.12(0.87–12.78)	2.57(0.83–8.56)	46.15(5.96–92.40)	7.144	0.002
TG (mmol/L)	1.51(1.05–2.57)	1.72(0.98–2.43)	1.47(1.02–2.72)	0.526	0.593
CHOL (mmol/L)	4.38(3.61–5.51)	4.19(3.26–5.22)	3.88(2.99–4.83)	0.307	0.737
HDL-C (mmol/L)	1.01(0.80–1.44)	0.92(0.79–1.17)	0.75(0.61–0.90)	3.171	0.048

BMI: Body mass index; 2hPBG: 2 hour postprandial blood glucose; GLU: glucose; CRP: C-reactive protein; TG: triglyceride; CHOL: cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol WBC: white blood cell; Hb: haemoglobin; Data are the mean ± SD or median (interquartile range)

Parameter	Patients			$F(\chi^2)$	p
	DM	DM + LEAD	CLI		
LDL-C (mmol/L)	2.91±0.87	2.62± 0.92	2.43± 1.26	0.793	0.456
WBC ($\cdot 10^9$/L)	6.51(5.40-10.15)	7.31(6.01–9.44)	10.16(7.82–14.36)	6.808	0.002
Hb (g/L)	130.75±24.32	120.64±25.48	102.63±23.89	5.426	0.006

BMI: Body mass index; 2hPBG: 2 hour postprandial blood glucose; GLU: glucose; CRP: C-reactive protein; TG: triglyceride; CHOL: cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol WBC: white blood cell; Hb: haemoglobin; Data are the mean ± SD or median (interquartile range)

Comparison and correlation analyses of serum levels of Hsp90α, Hsp90β and MDA in the study subjects

The serum levels of Hsp90α, Hsp90β and MDA in the different groups of patients and controls are shown in Fig. 1A-C. In pairwise comparisons, serum HSP90α levels were significantly higher in DM + LEAD or CLI patients than in DM patients ($p < 0.01$, respectively). The results showed that the serum Hsp90α and Hsp90β levels gradually increased with the progression of the disease, and T2DM patients were characterized by a state of continuous and chronic oxidative stress. The relationships among Hsp90α, Hsp90β and MDA levels in the study subjects are shown in Fig. 1D and E. Pearson's correlational analysis showed there were no significant correlations between Hsp90α and MDA levels ($r = 0.3648$, $p = 0.1366$) and no correlation between Hsp90β and MDA levels ($r=-0.0992$, $p = 0.7251$). The results suggest that the serum levels of Hsp90α can reflect, to some extent, the degree of oxidative stress in vivo.

Diagnostic efficacy of HSP90α and Hsp90β for DM + LEAD

ROC curve analysis was conducted to assess the diagnostic efficacy of Hsp90α and Hsp90β for DM + LEAD, and the results are shown in Fig. 1F. The analysis was performed after dividing the patients into two groups: the DM group and the DM + LEAD group. Hsp90α showed better diagnostic performance in the DM + LEAD group (AUC = 0.882) than in the DM group ($p = 0.002$). The diagnostic efficacy of Hsp90β was not statistically significant (AUC = 0.630, $p = 0.283$). These results demonstrate that serum Hsp90α shows better performance for initial diagnosis of early DM in patients with atherosclerotic changes than in those without such changes.

T-test based on clinical characteristics of HSP90α levels

A T-test based on the clinical characteristics of the serum Hsp90α levels is depicted in Table 2. Except for BMI and past medical history, serum Hsp90α was not associated with other clinical characteristics of the study population. The results indicated that weight control and previous history examination are more

important for patients with diabetes. However the elevated levels of Hsp90α indicated an abnormal baseline level in patients.

Table 2
T-test based on clinical characteristics of HSP90α levels

Variable	t	*p*
Sex	0.058	0.954
Age (cutpoint, 60)	1.600	0.118
BMI (24)	2.394	0.022
Smoking	0.216	0.830
Hypertension	-1.079	0.287
Hyperlipidaemia	0.663	0.511
Family history	-0.419	0.677
Past medical history	-2.144	0.038
2hPGB (7.8)	0.474	0.638
GLU (5.9)	1.038	0.306
HbA1c (6.0)	-0.358	0.723
CRP (15)	-1.427	0.162
TG (1.7)	0.752	0.457
CHOL (5.2)	-0.992	0.328
HDL (1.55)	-0.468	0.643
LDL (3.36)	-1.238	0.224
WBC (9.5)	-1.510	0.139
Hb (115)	-0.865	0.393

Histopathological sections

Both inflammatory cell infiltration and elevated Hsp90α expression were present. Tissue sections prepared from human atherosclerotic lesions were subjected to HE staining (Fig. 2A, C and E) and immunohistochemistry (Fig. 2B, D and F). We found thickening of the vascular endothelium in CLI patients. Inflammatory cells infiltrated the intima heavily, and Hsp90α was abundantly expressed in the endothelium and smooth muscle. In addition, the expression area of Hsp90α was consistent with the area of inflammatory infiltration.

In vitro study

Construction of a HUVEC model of oxidative injury induced by H_2O_2

The results of H_2O_2 concentration screening (Fig. 3A) indicated that the survival rate of HUVECs gradually decreased with increasing H_2O_2 concentrations and H_2O_2 treatment times. After treatment with 300 μM H_2O_2 for 24 h, the cell survival rate was nearly 80%. After treatment for 24 h, cells exposed to H_2O_2 at 200 μM and 300 μM exhibited significantly different MDA contents (Fig. 3B) and SOD activities (Fig. 3C) than control cells. ROS detection (Fig. 3D) indicated that the ROS levels of HUVECs treated with 300 μM H_2O_2 for 24 h were increased.

These results suggest that 300μM H_2O_2 treatment for 24 hours can successfully establish the oxidative stress injury model of endothelial cells

Increased eHsp90 secretion in the vascular endothelial injury model

HUVECs were treated with H_2O_2 at a range of doses. eHsp90α and eHsp90β secretion increased after treatment, but the total amount of intracellular Hsp90 did not change significantly (Fig. 4A). After H_2O_2 treatment, the total amount of eHsp90α and eHsp90β from HUVECs was the highest at 24 h, but the change in intracellular Hsp90 was not obvious (Fig. 4B). Treatment with H_2O_2 and 17AAG alone or in combination significantly increased the secretion of Hsp90 by endothelial cells. In addition, the expression of Hsp90 mRNA was increased in the group treated with 17AAG (Fig. 4C and D). H_2O_2 had little effect on the expression of Hsp90AA1 and Hsp90AB1. The immunofluorescence results (Fig. 4E) showed that Hsp90α localization shifted with treatment; specifically, the fluorescence at the cell edges increased, suggesting that the expression of Hsp90α in the membrane and periphery may have increased.

Endothelial cell CM and hrHsp90α induce THP-1 migration

To study the effect of chronic oxidative stress on the development and progression of AS, we next examined the effect of endothelial cell CM on THP-1 cells. Compared with the control medium, H_2O_2-treated CM induced THP-1 migration. CM from endothelial cells treated with 17AAG alone or H_2O_2 in combination exhibited a significantly decreased ability to induce THP-1 migration (Fig. 5A). We observed that hrHsp90α exposure increased the number of THP-1 cells that migrated to the lower chamber. The chemotactic effect of hrHsp90β on THP-1 monocytes was not obvious. We used 1G6-D7, a newly generated mAb that selectively targets the dual lysine region in secreted Hsp90α, to identify the specificity of Hsp90α for chemotactic THP-1 monocytes. RPMI 1640 medium pre-treated with 17AAG and 1G6-D7 for 30 min and containing hrHsp90α or hrHsp90β was added to the lower chamber. The results showed that hrHsp90α promoted the migration of monocytes to a certain extent, while the effect of recombinant

Hsp90β was not obvious. The chemotactic ability of monocytes was weakened by the addition of 17AAG or 1G6-D7 (Fig. 5B).

eHsp90α activates THP-1, LRP1 and Akt kinase

Next, we treated THP-1 cells with hrHsp90α, and Western blotting showed that the LRP1 protein content was significantly elevated in the treated cells. To confirm the involvement of the eHsp90α-Akt pathway, we determined that stimulation with eHSP90α induced time-dependent activation of p-Akt (Fig. 5C).

Discussion

In our study, we clarified the relationships of oxidative stress with serum Hsp90α and Hsp90β levels in diabetic patients. We propose that serum Hsp90α has reference value for the early diagnosis of DM + LEAD, and our findings indicate that low-dose and long-term ROS exposure can increase eHsp90 secretion from endothelial cells. While blocking the effect of Hsp90, we used a previously developed mAb specific for eHSp90 to further verify the role of eHsp90. eHsp90α acts as a chemokine to induce THP-1 cell migration, which may result from a compensatory response of early inflammation and oxidative stress in AS, initiating the atherosclerotic process (Fig. 6).

The incidence and mortality rates of atherosclerotic cardiovascular disease (ASCVD) are significantly higher in diabetic patients than in nondiabetic patients. [14] Fifty percent of patients with T2DM had complications at the time of diagnosis. [15] However, fewer than 10% of patients have typical clinical symptoms. In most patients, the degree of vascular stenosis exceeds 50% at initial diagnosis, and the lack of effective treatments affects the patients' prognoses. [16] Recent studies have reported several potential serum markers for the screening of diabetic vascular complications, such as CD36, YKL-40, [17] hepatocyte growth factor, [18] serum fibroblast growth factor 23, [19] serum sclerostin, [20] serum growth differentiation factor 15, [21] and circulating Hsp27. [22] In addition, heat shock proteins (HSPs) can be transported to the plasma membrane and released extracellularly, resulting in detectable levels of HSPs in the blood. This finding has prompted clinical studies exploring the potential use of HSPs and anti-HSP antibodies as serum biomarkers for DM complications. [23] Available data suggest that circulating Hsp27, [24] Hsp60, [25] Hsp70[26] and anti-HSP levels may be used as biomarkers for diabetic vascular disease. In T1DM, elevated serum Hsp90 levels have been found to be related to cellular autoimmunity in children. However, differences in Hsp90 levels do not predict whether individuals with positive autoantibodies will develop T1DM. [27] New findings confirmed the role of Hsp90 as a putative autoantigen triggering inflammation within human carotid atherosclerotic plaques. [28] However, there have been no studies on the relationships between Hsp90 and T2DM macrovascular complications.

Based on the current standardized management of clinical DM, we explored whether there were any controllable factors among the groups of patients. Clinical data of 77 patients were collected in this study. The results showed that disease severity gradually increased with age and that DF patients had ulcer infections. Low levels of HDL are also associated with an increased incidence of coronary heart

disease in diabetic patients. Abnormal Hb levels may be related to renal anaemia and renal dysfunction. An elevated WBC count indicates the persistence of a chronic inflammatory response. Therefore, we sought to investigate whether there is an indicator that can be included in primary prevention strategies that has reference value for early prediction. Numerous studies have reported that patients with DM have chronic inflammation, but now many inflammation indicators are closely related to acute infection, such as CRP and PCT; [29] thus, these indicators may not truly reflect chronic inflammation. Serological studies have found that the degree of oxidative stress in diabetic patients is cumulative. Therefore, we detected differences in the levels of Hsp90α and Hsp90β in serum samples of each group (Fig. 1). Correlation analysis revealed that there was a certain correlation between HSP90α and MDA, but there was no statistical significance, suggesting that circulating Hsp90α may reflect a patient's oxidative stress state to a certain extent. Predictive analysis indicated that serum Hsp90α shows better performance for the initial diagnosis of LEAD in early DM patients than in diabetic patients without atherosclerotic changes. A T-test further revealed that serum Hsp90α levels in patients with abnormal BMI were significantly higher than those in the normal group. The serum Hsp90α levels in patients with a past medical history were also higher than those in the control group, suggesting that weight control and previous history examination are required for the prevention of DM + LEAD. Due to the small number of samples at present, confounding factors could not be well controlled, and multivariate analysis could not be carried out. In further studies, we will include more samples to make the results more representative.

AS is also related to apoptosis of macrophages, smooth muscle cells and endothelial cells. During cell activation and apoptosis, endothelial cells can release many types of extracellular vesicles (EVs), and new evidence in the field shows that endothelial cell-derived EVs participate in the development of AS. During this process, NO and oxidative stress can induce endothelial cell apoptosis and ROS generation before atherosclerotic plaques form, and ROS-induced apoptosis of endothelial cells has the potential to initiate AS. [30] It is generally believed that abnormal glucose metabolism damages the arterial endothelial barrier while increasing platelet and inflammatory cell aggregation, which increases local oxidative stress levels. Destruction of the barrier function of vascular endothelial cells is a crucial initial step in this process. [31] Oxidative stress is a potential mechanism for the development of endothelial cell dysfunction that is common to all risk factors. [32] Where does Hsp90 in the serum of diabetic patients come from? Previous studies have focused mostly on Hsp90 in cells and on cell membranes, and our initial findings suggested that Hsp90α is a predictive molecule. However, the cells that release Hsp90 into the serum under conditions of high glucose and low H_2O_2 remain unclear. Endothelial cells are the direct targets of stimulation by blood glucose and lipids in vessels and can be damaged by these components. Thus far, no reports have suggested that endothelial cells can secrete Hsp90. Next, we verified cytologically that oxidative stress upregulates the expression of eHSp90 in HUVECs and that this process is not accompanied by an increase in Hsp90 gene expression or a decrease in intracellular protein expression. These findings are in contrast to those of a study by Profumo et al. [33] in which oxidative stress upregulates Hsp90 expression on the surfaces of endothelial cells and reduces Hsp90 secretion. There may be a link between the two findings. The expression of eHsp90 may be related to the duration of oxidative stress in cells. After a short (2 h or 4 h) treatment, no change in the amount of eHSp90 was

observed in the cell culture medium. Previous studies have rarely used indicators to simultaneously measure oxidative stress at both the population and cell levels or used such indicators as references. In our study, we used MDA to measure the oxidative stress state of the population and found that MDA levels were lower in cells after H2O2 treatment than in patient serum. These findings suggest that endothelial cells under low-concentration H_2O_2 treatment exhibit increased expression of eHsp90, which can simulate oxidative stress in humans.

AS is a lipid-driven inflammatory disease of the arterial intima. The balance of proinflammatory and anti-inflammatory mechanisms determines the final clinical outcome, which is characterized by gradual accumulation of lipids and inflammatory cells. [34] Two chemotactic processes are involved in AS: monocyte chemotaxis and smooth muscle fibroblast chemotaxis. Circulating leukocytes adhere to and migrate through the endothelial wall to the vascular smooth muscle layer of the intimal membrane. [35] The migration of monocytes is an early disease indicator. Ambade et al. [36] found that the EVs of mice with alcoholic liver disease can induce macrophage activation through Hsp90. Inhibitor treatment can reduce the formation of plaques to a certain extent by inhibiting the migration of VSMCs. [9] In addition, colocalization of inflammatory cells and Hsp90α was found in patient tissue samples, so we hypothesized that there may be a connection between Hsp90α and inflammatory cells. The results showed that eHsp90a could promote monocyte migration and initiate the inflammatory process of atherosclerosis. However, there have been no reports on the effects of eHsp90 on the LRP1 receptor and Akt kinase in monocytes. When we used recombinant Hsp90α to treat monocytes, we observed increased expression of LRP1 in a short period of time, which activated p-Akt, thereby promoting monocyte migration. Our study provides in vitro evidence of the key pathological role of eHsp90α in the control of diabetic vascular complications. This study did not go deep into the cell signalling mechanisms. In the future, we will study the pathway mechanism by knocking down or overexpressing genes, using pathway inhibitors and other means.

Conclusion

The current study shows that eHsp90α participates in the inflammatory process of AS to a certain extent and further aggravates adverse reactions at the beginning of the disease course. eHsp90α is expected to become a promising new target for disease prediction and for treatment to slow the progression of AS.

Abbreviations

DM: Diabetes mellitus; T2DM: Type 2 diabetes mellitus; PAD: Peripheral artery disease; DF: Diabetic foot disease; AS: Atherosclerosis; ROS: Reactive oxygen species; HUVECs: Human umbilical vein endothelial cells; THP-1: Human leukemia monocytic cell line; Hsp90: Heat shock protein 90; eHso90: Extracellular heat shock protein 90; 17-AAG: Tanespimycin; CM: Conditioned medium; BMI: Body mass index; 2hPBG: 2 hour postprandial blood glucose; GLU: glucose; CRP: C-reactive protein; TG: triglyceride; CHOL: cholesterol; HDL-C: high density lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol WBC: white blood cell; Hb: haemoglobin

Declarations

Ethics approval and consent to participate

The study was designed and implemented in accordance with the Declaration of Helsinki (2013), approved by the ethics committee of Nanfang Hospital (NFEC-2020-268), and registered on the Clinical Trials website (NCT04787770). All patients participation in this survey were voluntary, with informed consent.

Consent for publication

If the manuscript is accepted, we approve it for publication in Cardiovascular Diabetology.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xinyi Ding, Chuzhen Meng, and Hangming Dong performed the experiments, analysed the data and wrote the manuscript. Shili Zhang, Hui Zhou and Wenchong Tan analysed data. Lei Huang and Aiping He collected specimens. Jieyou Li and Jiali Huang contributed to the discussion. Wei Li, Fei Zou and Mengchen Zou designed the experiment and reviewed the manuscript.

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Figures

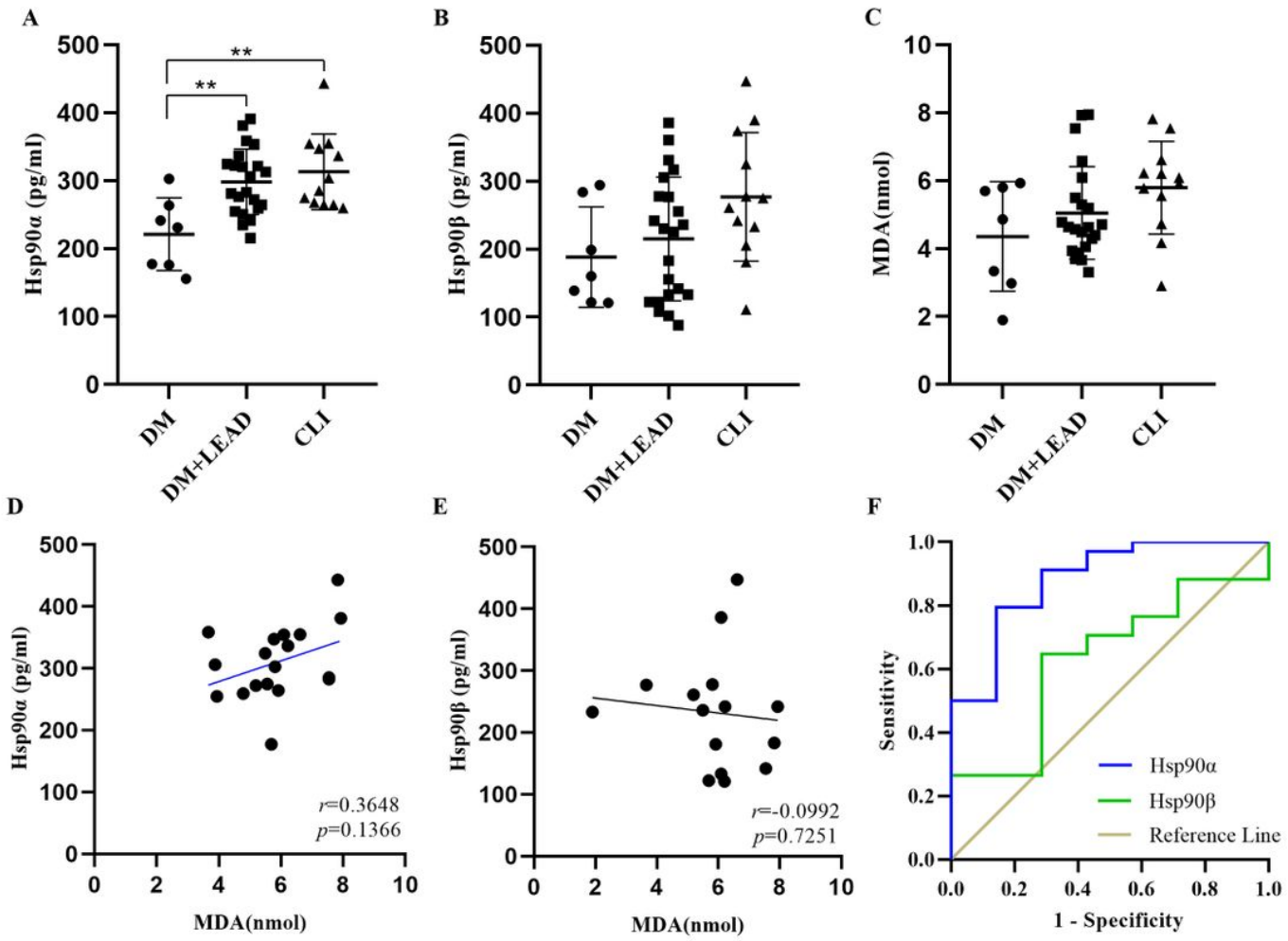

Figure 1

Hospital-based cross-sectional study. Levels of serum Hsp90α (A), Hsp90β (B) and MDA (C) in the different groups. Correlation between Hsp90α (D), Hsp90β (E) and MDA levels in serum samples. Pearson coefficient tests were conducted to calculate statistical significance. The ROC curve analysis of the diagnostic efficiency of Hsp90α and Hsp90β for DM and DM+LEAD. (F) The diagnostic ability to distinguish DM+LEAD patients from DM patients.

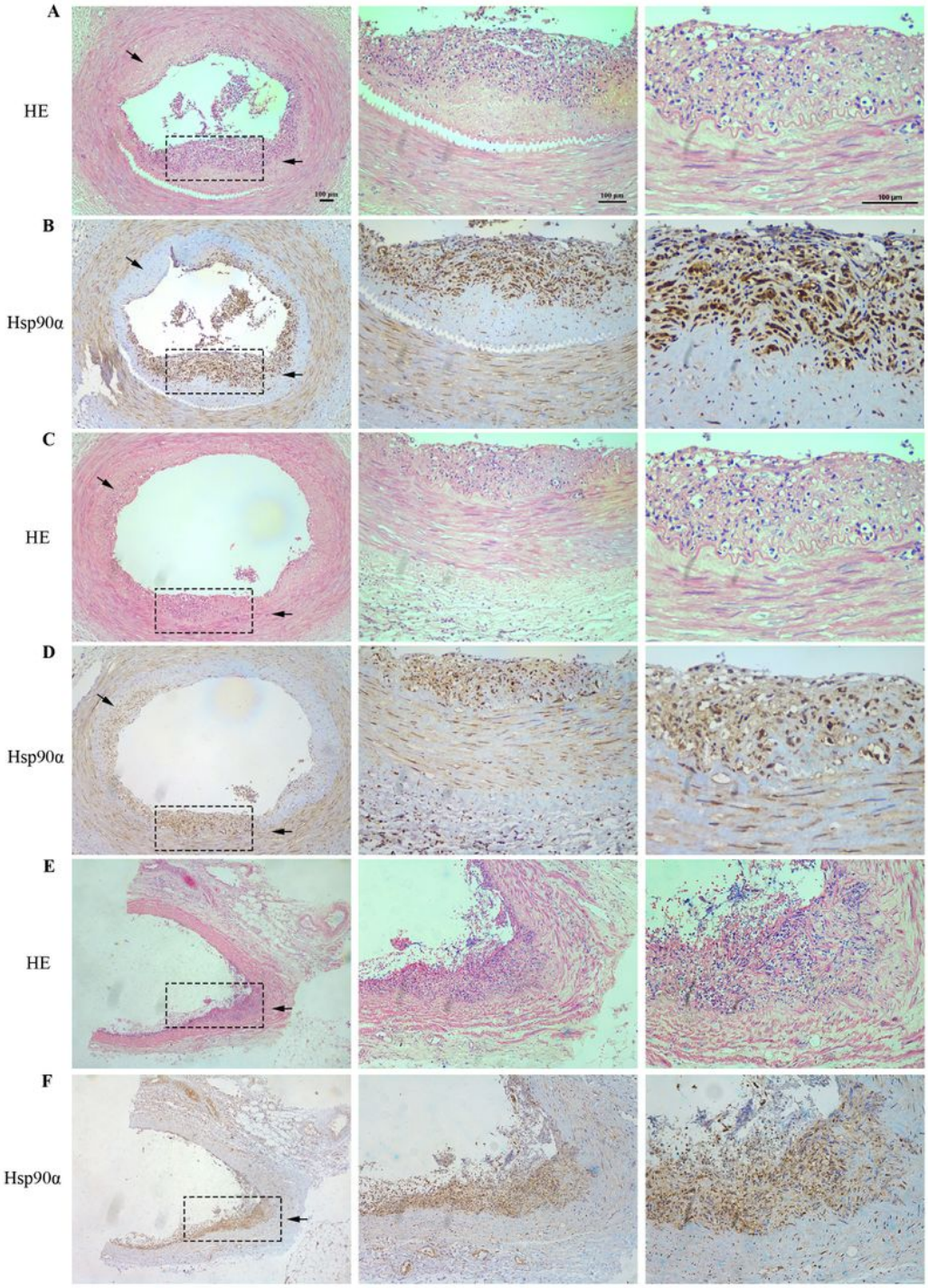

Figure 2

Histopathological changes in blood vessels. HE staining (A, C, E) and immunohistochemistry (B, D, F) (40×, 100×, 200×) of Hsp90α in the blood vessels of patients with CLI undergoing partial amputation. (n=3)

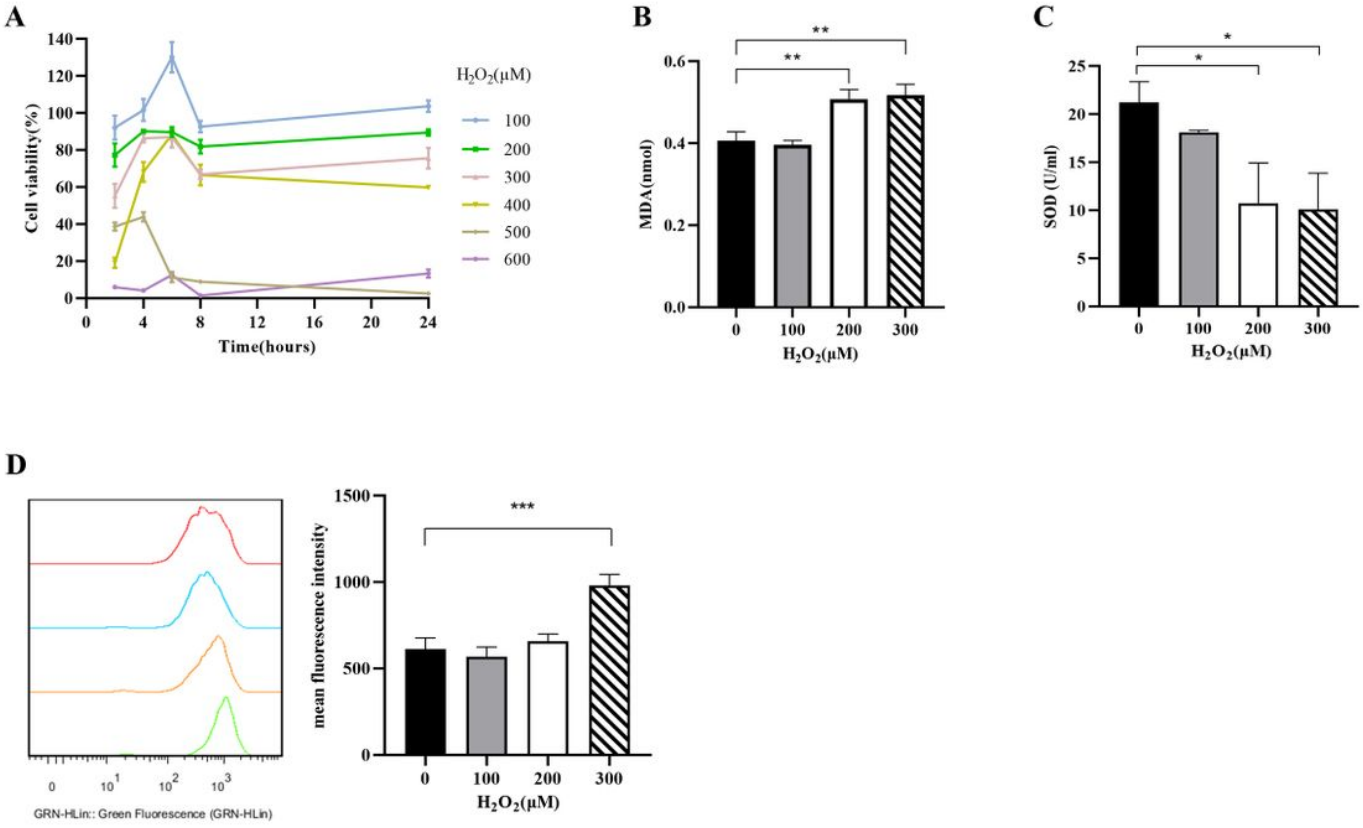

Figure 3

Construction of a HUVEC model of oxidative injury. (A) A CCK-8 assay was used to detect cell viability in HUVECs treated with different concentrations of H2O2 for 2, 4, 6, 8 and 24 h. (n=3) The levels of MDA (B) and SOD (C) were measured with assay kits. (n=3) HUVECs were treated with H2O2 (100, 200 and 300 μM) for 24 h, and then the intracellular levels of ROS (D) were detected. (n=3) All data represent the mean ±SD of three independent biological replicates. The differences between groups were analyzed by One-Way ANOVA, Dunnett, according to data feature. *p < 0.05, **p < 0.01, ***p <0.001 vs. control

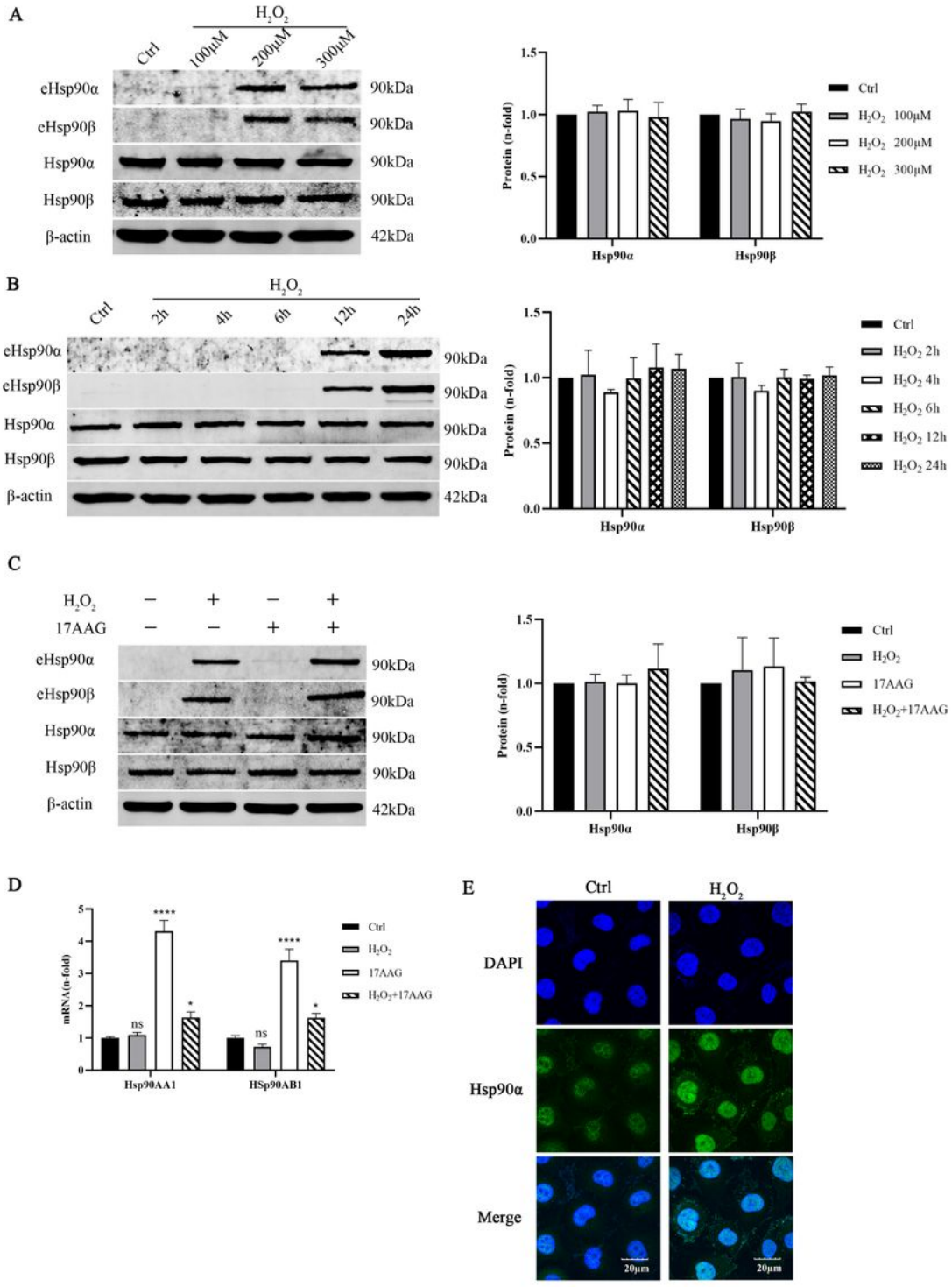

Figure 4

Increased eHsp90 secretion in a vascular endothelial injury model. (A) Western blot analysis concentrated medium and total cell extracts from HUVECs cultured with the indicated concentration of H2O2. ImageJ software was used to determine the relative intensities of extracellular and intracellular Hsp90α and Hsp90β bands.(n=3) (B) Extracellular and intracellular Hsp90α and Hsp90β expression was measured after H2O2 stimulation for different durations.(n=3) (C) Western blot analysis was performed to assess

the expression of related proteins in HUVECs pre-treated with 17AAG and then exposed to H2O2.(n=3) (D) qPCR was employed to observe the respective Hsp90α and Hsp90β mRNA levels after HUVECs were stimulated with H2O2 and/or 17AAG for 24 h.(n=3) (E) Representative confocal images of Hsp90α subunit localization in endothelial cells after 24 h of H2O2 stimulation are shown.(n=3) All data represent the mean ±SD of three biological replicates. The differences between groups were analyzed by One-Way ANOVA, Dunnett, according to data feature. *p < 0.05, ****p < 0.0001 vs. control.

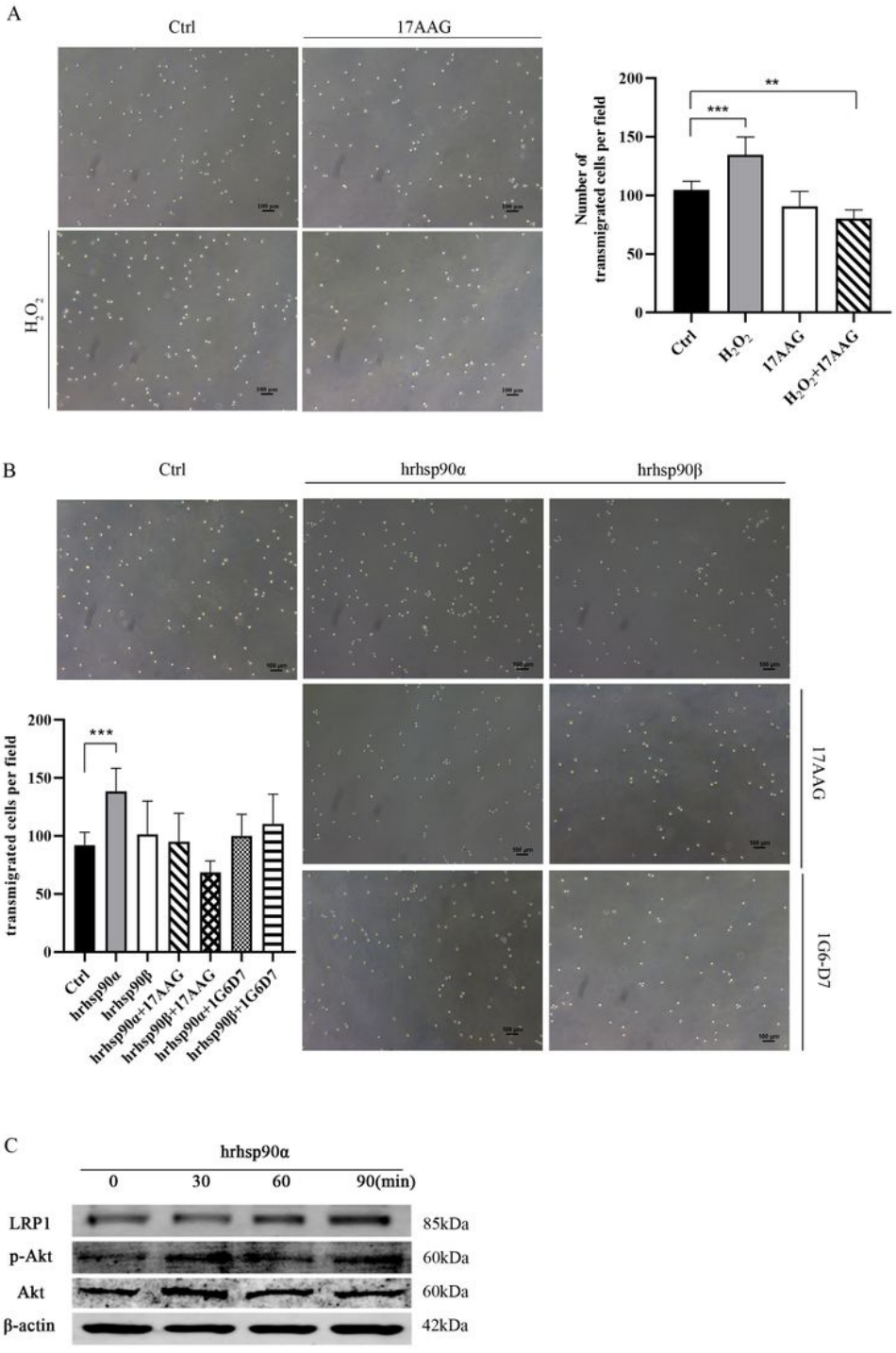

Figure 5

eHsp90 promotes monocyte migration, and recombinant Hsp90α activates LRP1 receptor expression. Representative images with quantification of the results for a THP-1 cells transwell migration assay in the presence of H2O2- and/or 17AAG-treated endothelial cell CM (A) and different reagents (B). (n=5) ∗represents significant difference compared with untreated CM or control; (C) LRP1 and p-Akt expression was measured after hrHsp90α stimulation for different durations. (n=3) All data represent the mean ±SD of three biological replicates. The differences between groups were analyzed by One-Way ANOVA, Dunnett, according to data feature. *p < 0.05, **p < 0.01, ***p <0.001.

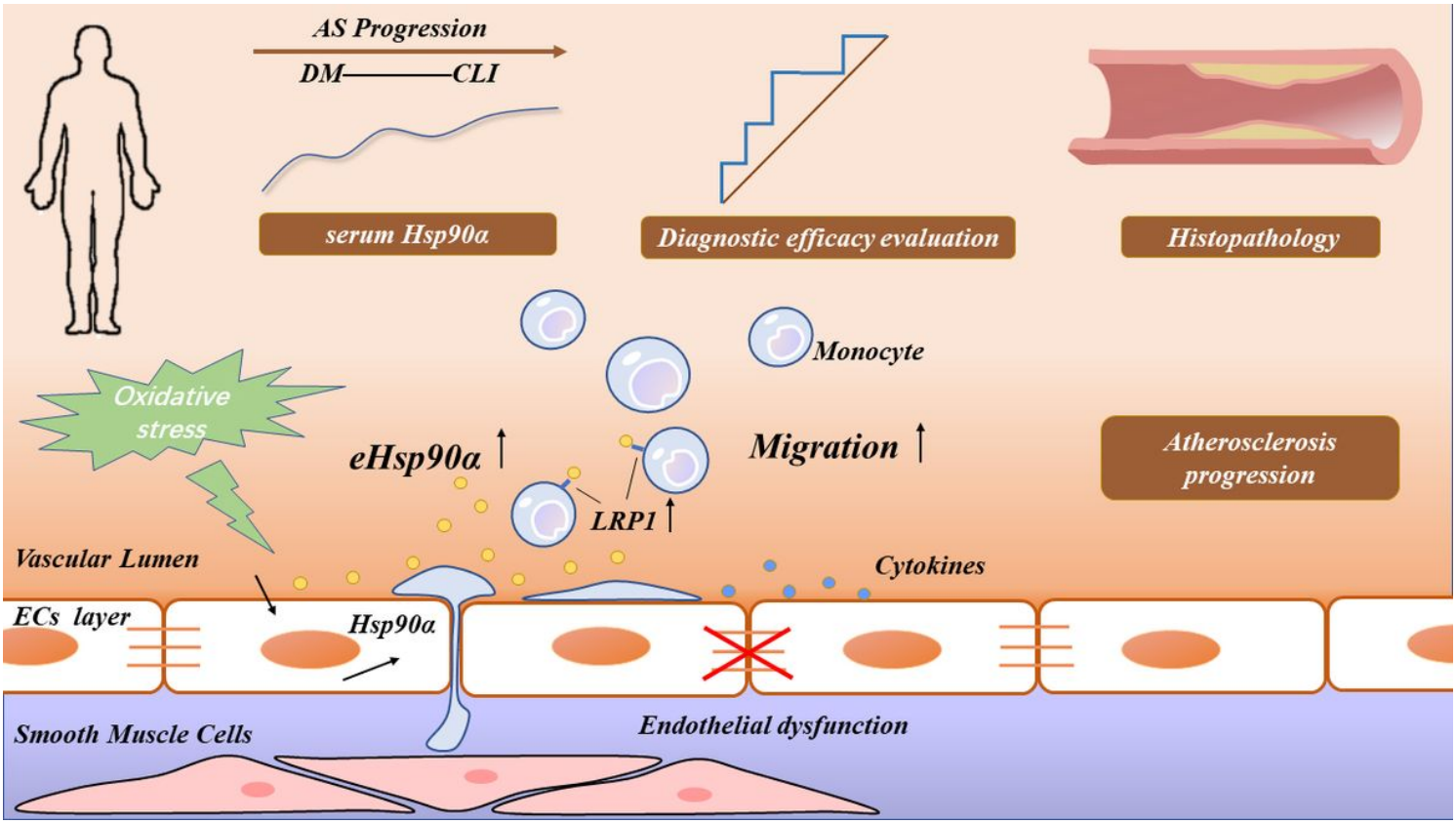

Figure 6

Model of extracellular Hsp90α participation in atherosclerosis.