Heat Shock Protein 90α–Dependent Translocation of Annexin II to the Surface of Endothelial Cells Modulates Plasmin Activity in the Diabetic Rat Aorta

Hetian Lei, Giulio Romeo, Andrius Kazlauskas

Abstract—The goals of this article were (1) to identify cell surface proteins whose expression was regulated by diabetes and (2) to assess their contribution to diabetic complications. We purified heat shock protein 90α (Hsp90α) from the membrane fraction of high glucose–treated endothelial cells (ECs) as a binding partner for a diabetes-specific phage. Further investigation revealed that high glucose elevated cell surface Hsp90α in cultured cells, and that diabetes increased the amount of Hsp90α on the luminal surface of the aorta. We also found that high glucose or diabetes promoted the association of Hsp90α with annexin II and increased the expression of annexin II on the surface of aortic ECs. Finally, plasmin activity was increased by high glucose or diabetes, and this change was partially reversed with an annexin II antibody. These findings reveal a novel glucose-regulated interaction between Hsp90α and annexin II, and raise the possibility that increased expression of annexin II, which promotes the generation of plasmin, is linked to clotting abnormalities associated with the diabetic state. (Circ Res. 2004;94:●●●●●●.)

Key Words: heat shock protein 90α ■ annexin II ■ endothelial cells ■ high glucose ■ diabetes

Diabetes is a chronic disease that affects a large segment of the world’s population. Diabetic complications compromise the function of numerous organs and seriously threaten an individual’s quality of life. For instance, diabetic individuals show an increased frequency of cardiovascular accidents, which are often related to accelerated thrombosis.1 Thrombogenesis is accompanied by an array of abnormalities, which are often related to accelerated thrombosis.2 Thrombogenesis is accompanied by an array of abnormalities, including enhanced platelet-mediated aggregation,2 altered endothelial-dependent vasodilation, and an imbalance between local pro- and anticoagulant factors. However, the events contributing to the diabetes-mediated thrombotic process and their interrelationships have not been fully elucidated in vivo, especially at the tissue level.

We developed a novel approach to identify proteins associated with diabetes. This two-step strategy involved first screening peptide phage display libraries over the surface of endothelial cells (ECs), and then purifying the binding partners for the peptides that were isolated in the screen. Using this approach, we recently learned that EC dysfunction could be induced by overexpressing profilin-1.3 These findings demonstrate the utility of our proteomics-based approach for identifying molecular changes contributing to diabetic complications.

Molecular chaperones are abundant cellular proteins that promote numerous aspects of protein stability.4 The two members of the 90-kDa molecular chaperone family are the 90-kDa heat shock protein (Hsp90) and the 94-kDa glucose-regulated protein (Grp94). Hsp90 has two isoforms, Hsp90α and Hsp90β, which are 76% identical.5 There are over 100 known Hsp90 client proteins including steroid receptors, transcription factors, tyrosine kinases, serine/threonine kinases, G-protein subunits, endothelial nitric oxide synthase, and telomerase.6 Outside of a report of autoantibodies to Hsp90 in the serum of diabetic patients,7 there is no known link between Hsp90 and diabetes mellitus (DM) or the associated vascular complications.

Annexin II (annexin A2, p36, calpactin I, or lipocortin II) is a member of the annexin family of calcium-dependent phospholipid binding proteins.8 Although it was originally thought to be cytoplasmic, annexin II was found on the surface of a wide variety of cell types including ECs, tumor cells, monocytes, macrophages, and neuronal cells.9 One of the functions of annexin II is to independently bind both tissue plasminogen activator (t-PA) and plasminogen, which enhances plasmin generation.9 A variety of cell types secrete plasminogen activator inhibitor (PAI-1),10,11 which may play a role in the delayed fibrinolysis associated with diabetes. In contrast to the extensive literature on coagulation defects and PAI-1 in diabetic patients, the role of annexin II in diabetes-mediated clotting defects has not been investigated.

Materials and Methods

Animals and Aorta Isolation

Sprague-Dawley male rats were made diabetic by injecting streptozotocin, and insulin was given as needed to achieve a slow weight...
gain while maintaining hyperglycemia and glycosuria. Thoracic aortas of 5-month diabetic or control rats were isolated as previously described. The protocol for the use of animals was approved by the Schepens Animal Care and Use Committee.

**Cell Culture**

The porcine aortic endothelial cell (PAEC) line was kindly provided by Dr Lena Claesson-Welsh, the primary bovine aortic endothelial cells (BAECs) were from P. D’Amore laboratory (Schepens Eye Research Institute), and rat aortic endothelial cells (RAECs) were isolated and cultured according to Doukas et al. All three of the cell types were cultured in a 1:1 mixture of Dulbecco’s modified eagle medium (low glucose): F12 nutrient mixture (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bio-Products). Human aortic endothelial cells (HAECS) and human coronary artery endothelial cells (HCAECs) (Clonetics) were cultured in EGM-MV Bulletkit (Clonetics). d-glucose was added to the medium to achieve “high glucose” (30 mmol/L), manitol was used as isoosmolar control, and normal glucose was 7.5 mmol/L.

**Phage and Peptides**

Phage 11 and phage 28 were obtained by screening a phage display peptide library. The peptide 11 and 28 sequences corresponding to peptide 11 and 28 were SVPPGFL and WCAVSRK, respectively. Synthetic peptides were flanked by COOH-GG, GS-NH$_2$-amide, and synthesized by the Tufts University Core Facility (Boston, Mass).

**Phage ELISA**

Enzyme-linked immunosorbent assay (ELISA) for phage 11 binding to HAECs, HCAECs, PAECs, RAECs, treated with normal or high glucose for 2 weeks was performed as described previously.14

**Immunofluorescence**

For competition experiments, synthetic peptide 11 or peptide 28 (0.01 μmol/L) was incubated with aortic sections (30 minutes at 37°C) before adding the cognate phage (6×10$^6$ pfu/mL). Immunofluorescence analysis of the binding of phage 11 and phage 28 to aortic sections was performed as described.3

**Immunofinity Chromatography, Silver Staining, and Microsequencing**

Peptide 11 (pI: 5.9) and peptide 28 (pI: 7.0) were coupled to Affi-Gel-15 matrix (BIO-RAD) according to the manufacturer’s instructions, and used in immunoaffinity chromatography experiments as previously described.3 For details regarding the isolation of binding partners for peptide 11, see the expanded Materials and Methods section in the online data supplement at http://circres.ahajournals.org.

**Retroviral Plasmids, Production, and Infection**

A pBluescript SK (-) plasmid containing the full-length human Hsp90α cDNA (2.95 kb) was generously provided by Dr Yahara. The Hsp90α cdNA was subcloned into the pLHDCX retroviral vector as SalI/Nosi fragment. pLHDCX’ or Hsp90α-pLHDCX was transfected into the packing 293GPG cell line.15 PAECs were infected using the resulting viruses and then selected in histidine-free medium (low glucose) and subjected to fluorescence-activated cell sorter (FACS) analysis as described in the online data supplement.

**FAC Analysis**

Cultured PAECs were detached with cell dissociation solution (Sigma) and subjected to fluorescence-activated cell sorter (FACS) analysis as described in the online data supplement.

**Fibrinolytic Activity Assay**

The fibrinolytic activity assay was based on a published protocol9,17 and was described in the online data supplement.

**Statistics**

Comparisons were made using unpaired and paired t test for flow cytometric experiments, using one-way ANOVA followed by unpaired t test for the fibrinolytic activity assay. A confidence level of 0.05 was considered statistically significant.

**Results**

Hsp90α Is a Binding Partner for Phage 11

We previously screened a peptide phage display library over the luminal surface of the aorta isolated from rats that had experienced 5 months of streptozotocin-induced diabetes. Some of the phage that was selected in this manner was individually tested for their ability to recognize ECs in aorta sections. For example, Figure 1A shows that phage 11 recognized many of the cells in the aortic section from diabetic rats, including the cells on the luminal surface (arrows). A much weaker signal was observed when phage 11 was used to stain aortic sections from nondiabetic rats (Figure 1B) or from retina or kidney sections of diabetic or control animals (data not shown). Phage 28 did not bind the diabetic sections (Figure 1C) and is representative of over 120 other phage that were individually tested on diabetic aorta sections.3 Preincubation of the diabetic sections with the synthetic peptide 11 competed the binding of phage 11 (Figure 1D), whereas peptide 28 was not effective (Figure 1E). These data indicate that phage 11 preferentially recognizes the diabetic aorta, and appears to recognize the endothelium of the aorta.

The preferential staining of the cells at the luminal surface of the diabetic aorta (Figure 1A) suggested that the ECs express the binding partner(s) for phage 11 and that this interaction was enhanced by diabetes. We reasoned that the isolation of phage 11 binding partner(s) might provide new insight into the pathogenesis of diabetic vascular disease. To this end, we used phage 11 as an affinity matrix to isolate binding partners from lysates of cells from the luminal aorta. Unfortunately, this approach was not successful. As an alternative, we coupled a synthetic peptide corresponding to peptide 11 to a matrix and used it to purify proteins from the membrane fraction of PAECs exposed to high glucose. The reason for choosing PAECs is that PAECs showed the greatest glucose-stimulated binding of phage 11 in the five types of endothelial cells tested using ELISA (data not shown). As shown in the silver-stained gel in Figure 2A, a subset of proteins (indicated by dots) were recovered from the high glucose–treated samples using peptide 11 but not peptide 28. The recovery of these proteins was lower from the membrane fraction of PAECs treated with normal glucose. We concluded that these proteins were specific for both high glucose treatment and for peptide 11. Consequently, the bands indicated with dots were subjected to mass spectromet-
ric analysis, and 40 of the 84 peptides for the second dotted band from the top matched Hsp90/H9251. The peptides for the third, fourth, and fifth bands were not instructive, whereas the top band will be the subject of a separate report.

To test if Hsp90/H9251 directly associated with phage 11, we mixed purified Hsp90/H9251 with phage 11 and assayed for coimmunoprecipitation of Hsp90/H9251 with phage. As shown in Figure 2B, Hsp90/H9251 coprecipitated with phage 11, but not with phage 28. Furthermore, excess peptide 11 competed (73.4±3.6%; P<0.05) with phage 11 for association with Hsp90a. These data indicate that Hsp90α directly interacts with phage 11 and support the idea that Hsp90α is a binding partner for phage 11.

High Glucose and Diabetes Increase the Surface Expression of Hsp90α in Aortic ECs

The observations that phage 11 bound to the luminal surface of a diabetic aorta (Figure 1) and high glucose increased the recovery of Hsp90α from the membrane fraction of PAECs (Figure 2A) suggested that glucose or diabetes increased Hsp90α on the surface of cells. To investigate this idea, we used three approaches. First, FACS analysis revealed that high glucose elevated the cell surface expression of Hsp90α by 90±12% (P<0.05) when compared with normal glucose cultured cells, whereas mannitol had no effect (Figure 3A and online Figure 1 in the online data supplement). Second, biotinylation of cell surface proteins (Figure 3B), and third, immunofluorescence of Hsp90α in nonpermeabilized PAECs (Figure 3C) confirmed these results. In contrast to the events at the cell surface, the total level of Hsp90α did not change (Figures 3D and 3E and online Figure 2). Taken together, these data indicate that exposure to high glucose increased the Hsp90α expression on the surface of PAECs.

We next took two approaches to compare the level of Hsp90α at the luminal surface of the aorta in diabetic and control rats. Immunofluorescence with anti-Hsp90α antibodies showed enhanced staining of the luminal surface of aortic sections from diabetic rats as compared with control (Figures 4A and 4B), which was consistent with phage 11 binding aortic sections (Figure 1). The endothelial cell–specific marker PECAM-1 colocalized with Hsp90α, indicating that the enhanced signal was within the ECs. As a second approach, we biotinylated the luminal surface of the aortas, purified the biotinylated proteins and subjected them to Western blot. As shown in Figure 4D, diabetes increased the amount of Hsp90α on the aortic luminal surface by 120% as compared with the control aorta. A cytosolic protein, p44/p42 MAP kinase (Erk), was not detectable under these conditions (Figure 4D). In contrast to the high glucose data with PAECs (Figure 3D), diabetes elevated Hsp90α by 78% in total lysates prepared from the luminal surface of the aorta.

The results of the data shown in Figures 1 through 4 provide evidence that Hsp90α is a binding partner for phage 11. Furthermore, diabetes elevates Hsp90α in the aortic endothelium, and this change includes increased expression of Hsp90α on the luminal surface of the aorta. Finally, the translocation of Hsp90α to the cell surface can be mimicked in vitro by exposing PAECs to high glucose.

Annexin II Is an Hsp90α Target In Vitro and In Vivo

Because Hsp90α is a chaperone and functions to bind proteins, we hypothesized that the diabetes-induced move-
Hsp90α and annexin II interacted in a glucose-regulated fashion, we performed a series of coimmunoprecipitation experiments on PAECs that had been treated with normal or high glucose. The results show that annexin II coprecipitated with Hsp90α from total cell lysates, and that the recovery of annexin II was increased by 108±16% (P<0.05) in response to high glucose treatment (Figures 5A and 5B). The reciprocal experiment (in which annexin II immunoprecipitates were probed with anti-Hsp90α antibodies) confirmed these observations (Figures 5A and 5B).

To determine whether Hsp90α interacted with annexin II in intact tissue, and whether the interaction was affected by diabetes, lysates from the aortic luminal surface of diabetic or control rats (n=8) were pooled, and Hsp90α was immunoprecipitated. Western blot analysis showed that there was 390% more annexin II coimmunoprecipitated from the diabetic versus control setting (Figure 5C). Neither of these proteins was recovered with a nonimmune IgG (Figure 5C). These data demonstrate that Hsp90α exists in a complex with annexin II, and that their interaction is enhanced by diabetes.

The finding that high glucose or diabetes increased the amount of Hsp90α on the cell surface and its association with annexin II suggested that annexin II might be translocated to the cell surface as well. To investigate this idea, we tested if exposing cells to high glucose altered the cell surface level of annexin II. FACS analysis revealed that exposure of PAECs to high glucose for 2 weeks resulted in a 86±12% (P<0.05) increase in the level of annexin II (Figure 6A). Geldanamycin (GA), an inhibitor of Hsp90,18 reversed this increase (Figure 6A and online Figure 3), but the total level of annexin II in PAECs was not altered (Figure 6B), suggesting that Hsp90α was mediating this change. This idea was strongly supported by the observation that overexpression of Hsp90α elevated the cell surface level of both Hsp90α and annexin II (Figure 6D). Moreover, GA treatment eliminated the rise in annexin II observed in cells overexpressing Hsp90α (Figure 6D and online Figure 4). In contrast to the change in the level of annexin II on the cell surface, overexpression of Hsp90α or GA treatment (data not shown) did not alter the total level of annexin II in PAECs (Figure 6C). We conclude that (1) high glucose elevates annexin II on the surface of cells, (2) this event is dependent on Hsp90α activity, and (3) a rise in Hsp90α is sufficient to elevate annexin II.

**Diabetes Increases Annexin II on the Luminal Surface of the Aorta**

To relate our annexin II findings in cultured cells to the in vivo setting, we assessed annexin II expression at aortic ECs of diabetic and control rats. As shown in Figure 7A, more annexin II (61%) was recovered from luminal surface of diabetic rat aortas than from control aortas; in contrast, the total level of annexin II in the rat aortic ECs did not change in response to diabetes (Figure 7B). We used an immunofluorescence approach to test the luminal surface of the aorta for the presence of annexin II, and found that there was a modest increase in the staining with anti-annexin II antibodies in the diabetic samples, as compared with controls (online Figure 5). Taken together, these two approaches indicate that...
diabetes increases the level of annexin II on the luminal surface of the aorta.

Plasmin Generation in Response to High Glucose In Vitro and Diabetes In Vivo

A number of investigators have found that elevating annexin II increased plasminogen activation.\(^9,17\) As an independent measure of the presence of annexin II, we tested plasmin generation in cells that were exposed to high glucose, or in isolated aorta specimens from diabetic and control rats. As shown in Figure 8A, an 86.5±2.1\% (\(P<0.05\)) increase in the amount of plasmin generation was observed in response to exposing PAECs to high glucose. This increase was partially reversed (54±2.2\%, \(P<0.05\)) by preincubating cells with an anti-annexin II antibody, which had been shown by other groups to partially attenuate annexin II–dependent plasminogen to plasmin generation.\(^9,19\) The antibody inhibited plasmin activity in control cells by 30.1±2.2\% (\(P<0.05\)) (Figure 8A). Although elevation of Hsp90\(\alpha\) was not the only change induced by high glucose, overexpressing Hsp90\(\alpha\) was sufficient to increase plasmin generation by 35±2.6\% (\(P<0.05\)) (Figure 8B).

To compare plasmin activity associated with the aorta, we isolated the aorta from diabetic or control rats, washed the luminal surface 10 times with cold PBS, and assayed plasmin activity as done with the PAECs. There was 41±2.5\% (\(P<0.05\)) greater activity in the diabetic samples (Figure 8C). Preincubation of the diabetic aorta with an anti-annexin II antibody inhibited the DM-induced increase in plasmin generation by 83±2.9\% (\(P<0.05\)) (Figure 8C). These data are consistent with the observation that there is more annexin II present in diabetic aortas (Figure 7A and online Figure 5).

Taken together, the data in Figures 7 and 8 show that (1) high glucose and diabetes mediated an Hsp90\(\alpha\)-dependent increase in annexin II on the surface of aortic ECs, and (2) the
elevation of annexin-II is, at least in part, responsible for the increased plasmin activity detected both in vivo and in vitro.

Discussion

Hsp90 is an abundant molecular chaperone that influences the activity and stability of many client proteins.20 The association between molecular chaperones such as Hsp90 and client proteins is stimulated by the partial unfolding of the client protein.4 More recently, an agonist-induced association of Hsp90 with client proteins has been reported.21 Our finding that high glucose promotes the interaction between Hsp90 and annexin II reveals an additional regulatory mechanism by which Hsp90 chooses a client protein.

There are several possible explanations for how high glucose treatment of PAECs increases complex formation between Hsp90 and annexin II. Although high glucose treatment elevates the amount of Hsp90 on the surface of ECs, this does not appear to be sufficient to explain increased association of Hsp90 and annexin II. The amount of annexin II that coprecipitates with Hsp90 is greater in high glucose-treated samples even after normalization for the amount of Hsp90. Annexin II is readily glycated in response to diabetes,22 and this type of posttranslational modification may be contributing to its enhanced association with Hsp90. Finally, Hsp90 may promote the heterotetramerization of annexin II with the p11 protein; this heterotetrameric form of annexin II is associated with the plasma membrane.5 Monomeric annexin II is cytoplasmic, whereas heterodimeric annexin II (which is composed of annexin II and 3-phosphoglycerate kinase) is nuclear.23

Whereas Hsp90 is largely cytoplasmic, numerous labs have reported expression of Hsp90 on the cell surface.5,24 For instance, the cell surface expression of Hsp family members (including Hsp90) can be induced by oxidative damage.25 The
extracellular expression of Hsp family members has been implicated in the pathology of Graves’ disease, which may relate to the ability of Hsp90α to participate in antigen presentation.5,24 Our work extends these observations by showing that high glucose treatment of cultured ECs increases the cell surface expression of Hsp90α/Hsp9251 and that there is enhanced expression of Hsp90α on the luminal surface of aortic ECs in diabetic rats.

Hsp90α does not have a classical signal peptide for secretion, and the mechanism by which it attains the extracellular localization remains unknown. Glucose-induced translocation of Hsp90α to the cell surface is blocked by geldanamycin (unpublished observations, 2004). This observation suggests that transporting of Hsp90α to the cell surface involves association of Hsp90α with a shuttle protein via the geldanamycin-binding domain of Hsp90α. We are currently investigating the possibility.

What is the physiological relevance of the novel observation that Hsp90α promotes the increased expression of...
annexin II on the surface of ECs? Given that these events are stimulated by high glucose in vitro and diabetes in vivo, a likely connection is to one or more of the diabetic complications. For instance, annexin II functions to promote the interaction between plasminogen and t-PA, resulting in the generation of plasmin. One of the substrates of plasmin is fibrin, and generation of plasmin is essential to fibrinolysis. Elevated annexin II was partially responsible for the increased plasmin activity in diabetes and high glucose–cultured ECs, which, in turn, would predict enhanced fibrinolysis. Yet, diabetes brings on reduced fibrinolysis. This apparent discrepancy may reflect an attempt to counteract the prothrombotic milieu associated with diabetes via upregulation/translocation of annexin-II. Importantly, the so-far-unappreciated role of annexin II in diabetic vascular disease and its regulation by Hsp90 deserve further investigation.

We have used an unbiased proteomics-based approach to identify proteins that are associated with diabetic complications. These efforts have led to three novel discoveries: (1) that high glucose induces a preferential association of certain proteins with Hsp90α, including annexin II; (2) that high glucose and diabetes mediate an increased expression of Hsp90α and annexin II on the endothelial surface; and (3) that the elevated annexin-II is, at least in part, responsible for the increased plasmin activity observed in both diabetic aorta and high glucose–cultured ECs.

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References


Figure 7. Diabetes increases annexin II on the luminal surface of aorta. A, The same set of biotinylated proteins used for the experiment shown in Figure 4D was reprobed using an anti-annexin II antibody. Quantitative analysis of bands indicated that diabetes increased the amount of annexin II on the luminal surface by 61%. B, Lysates were prepared from the luminal surface of aortas of six 5-month diabetic (DM) or control (C) rats (pool of 3 aortas per lane) and analyzed by Western blotting using anti-annexin II and anti-RasGAP antibodies. Quantitative analysis of bands indicated that diabetes had no effect on the total level of annexin II.

Figure 8. Increasing annexin II accelerates plasmin generation. A, PAECs treated with normal (N) or high glucose (H) for 2 weeks were preincubated for 2 hours at room temperature with an anti-annexin II antibody (N2, H2, 30 μg/ml) or nonimmune mouse IgG (N1, H1, 30 μg/ml) or 1% BSA in PBS (N, H), followed by Lys-plasminogen (0.15 μmol/L), t-PA (50 nmol/L), and a fluorogenic plasmin substrate, D-Val-Leu-Lys-AMC (100 μmol/L). Plasmin generation in each well was measured at 15-minute intervals at room temperature, with excitation set at 360 nm and emission at 460 nm, using a fluoroscent plate reader, and expressed as relative fluorescence units/minute (RFU/min). B, Fibrinolytic activity of PAECs expressing an empty vector (V) or the Hsp90α cDNA (O) was measured as in A. C, Fibrinolytic activity of aortic pieces isolated from 5-month diabetic (DM) or control rats (C) was assayed as described in A. Anti-annexin II antibodies were added to aortic luminal surface (C2, DM2) and normal IgG also was added as control (C1, DM1). In all 3 panels of this figure, the data are the mean ± SD of 3 independent experiments; *P<0.05.
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Additional Data

Material and Methods

Antibodies

The following antibodies were used: anti-M13 (Amersham Pharmacia Biotech, NJ), anti-Hsp90α (Stressgen, CA), anti-annexin II and anti-transferrin receptor (Zymed, CA), anti-rat PECAM-1 (BD Biosciences), anti-Erk (Cell Signaling, MA), anti-SM-actin (Dako, Denmark), anti-hsp90α, anti-annexin II, and non-immune IgG (Santa Cruz, CA), and Cy3-conjugated, FITC-conjugated antibodies (Jackson ImmunoResearch, PA). Anti-RasGAP and anti-VEGFR-2 were made at this laboratory.1,2

Immunoaffinity Chromatography, Silver Staining and Microsequencing

Immunoaffinity chromatography was done according to the instructions provided with the Affi-Gel matrix (BIO-RAD). Briefly, an affinity matrix was prepared by coupling peptide 11 or the negative control peptide 28 (0.5 mg in 1.5 ml DMSO) to 1.0 ml Affi-Gel 15 (BIO-RAD). Membrane fractions3 (1 mg) from PAECs treated with normal or high glucose for two weeks were applied to the affinity columns. After washing with 8 ml of 10 mM Tris HCl (pH 7.4) for three times, the bound proteins were eluted with 1 ml 100 mM glycine (pH 2.0). The eluates were collected in two 500 µl aliquots, which were buffered immediately with 200 mM sodium phosphate (pH 7.4), and concentrated in a 10,000 NMWL filter (Millipore) by centrifugation for 30 min at 4°C, 3,500 ×g. The concentrated samples were resolved by 10% SDS-PAGE, and the resulting gel was silver stained using Silver Quest (Invitrogen). The bands that were specific for peptide 11 and were more abundant in the high glucose samples were excised and destained. A piece of blank gel of the same size was processed in parallel
and served as a control to identify systemic contamination. Protein microsequencing was performed at the Harvard Microchemistry facility by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (µLC/MS/MS) on a finnigan LCQ DECA XP quadrupole ion trap mass spectrometer. The resulting peptide data were analyzed by local alignment search tool (BLAST) homology search.

**Biotinylation, and Western Blot**

Aortas of six 5-month diabetic or control rats were isolated as previously described, washed 10 times with 1 ml ice-cold PBS each time. PAECs treated with normal or high glucose were washed three times with ice-cold PBS. Aortas or cells were then incubated for 30 min in 0.5 mg/ml Sulfo-NHS-SS-biotin (Pierce)/PBS on ice. The samples were then washed 4 times with cold PBS to remove the unreacted biotin. EB (extraction buffer) was added (1 ml/plate or 80 µl/aorta), the cells were gently scrapped, the liquid was moved to an eppendorph tube, and centrifuged at 13,000 × g for 15 min at 4°C to remove insoluble debris. The resulting clarified supernatant was incubated with 30 µl neutravidin beads (Pierce) per sample for 4 hours. The neutravidin beads were washed 4 times with 1ml EB each time, and resuspended in 1 × SDS sample buffer. The samples were applied to 10% SDS-PAGE and immunoblotting using standard procedures as previously described. The Western blot signals were detected by chemiluminescence using the ECL plus kit (Amersham Pharmacia). Data were analyzed by densitomery using Quantity One (Bio-Rad).

**FACS Analysis**

The cells that were subjected to FACS included PAECs treated with normal, high glucose, or mannitol, PAECs infected with retrovirus harboring an empty vector (pLHDCX3) or the full length human Hsp90α cDNA in the pLHDCX3 vector. In all cases the cells were
cultured in 100 × 20 mm Falcon tissue culture dish (Becton), then washed two times with PBS, and detached with cell dissociation solution (Sigma). The cells were incubated with one of the following antibodies for 1 hr on ice: anti-Hsp90α, anti-Annexin II, non-immune IgG (Santa Cruz), and anti-transferrin receptor (Zymed). After two washes with ice-cold PBS, the cells were incubated with corresponding FITC-conjugated secondary antibodies (Jackson ImmunoResearch) for 30 min on ice. Following two ice-cold PBS washes, the cells were stained with propidium iodide according to the instructions provided with the apoptosis kit (Clontech). The cells were finally analyzed by flow cytometry in Coulter Beckman XL (Coulter C.).

**Fibrinolytic Activity Assay**

The fibrinolytic activity assay was based on the protocol published in Ishii et al.\(^5\) Briefly, PAECs that had been exposed for 2 weeks to normal or high glucose, or had been infected with retroviruses containing pLHDCX\(^3\) or pLHDCX\(^3\)-hsp90α, were plated in a 96-well culture plate (2×10\(^4\) cells/well) and incubated for 2 hours at 37 °C. The cells were washed three times with PBS (200 µL PBS/well), then were incubated for two hours at room temperature in 1% BSA/PBS with 30 µg/ml anti-annexin II IgG (Zymed) or non-immune mouse IgG (Sigma) or no antibody. After washing 3 times with PBS, Lys-plasminogen (Haematologic Technologies Inc.) (0.15 µM in PBS) was incubated with the cells for 1 hr at 37 °C. Following four additional washes, a substrate mixture consisting of PBS containing t-PA (tissue plasminogen activator, Sigma) (50 nM) and a fluorogenic plasmin substrate, D-Val-Leu-Lys-AMC (Enzyme Systems Products, 100 µM) was added to each well (100 µl/well). Plasmin generation was measured at 15-minute intervals at room temperature, with
excitation set at 360 nm and emission at 460 nm, using a fluorescent plate reader (Bio-Tek FL600), and expressed as relative fluorescent units/min² (RFU/min²).

The thoracic aorta (from 6-month diabetic or control rats) were washed 10 times with 1 ml ice-cold PBS each time, cut into 10 mm × 8 mm segments, and placed into individual well of a 24 well culture plate with the luminal surface facing up. The plasmin activity assay was performed as described above. After recording the fluorescence, the pieces of aorta were dried and weighed. The plasmin activity was corrected for the weight of the aorta in each sample.

References


Figure Legends

Supplemental Figure 1

PAECs treated with normal (N), high (H) glucose, or mannitol (M) were detached with cell dissociation solution. After two washes with ice-cold PBS, cells were incubated with anti-Hsp90α, anti-transferrin receptor, or non-immune IgG (5 µg/ml) for 1 hour on ice. The cells were washed twice with ice-cold PBS, then incubated with a FITC-conjugated secondary antibody (1:100 dilution) for 30 minutes on ice. After two washes with ice-cold PBS, cells were stained with propidium iodide (PI) for 10 minutes at room temperature in the dark. Cells were analyzed by flow cytometry (System II Vision 3.0 Software) in Coulter Epics XL. The results show that high glucose increased the fraction of Hsp90α-positive cells from 9.4% to 17.8%. The data are representative of 3 independent experiments.

Supplemental Figure 2

Same as Supplemental Figure 1, except that 1) cells were fixed with 3.7% formaldehyde in 0.1% Triton X-100/PBS for 10 minutes at room temperature immediately after detaching them from the dishes; 2) the cells were not stained with propidium iodide (PI). The detergent in the fixation buffer permeabilized the cells and thereby permitted access to both intracellular and extracellular compartments. The data are representative of 3 independent experiments.

Supplemental Figure 3

Same as supplemental Figure 1 except two: 1) in some cases, the cells were treated with 1 µmol/L geldanamycin (+GA) for 12 hours; 2) the primary antibody was anti-annexin II, or non-immune IgG (5 µg/ml) for 1 hour on ice. High glucose increased cell surface annexin II from 5.16% to 9.02%. The data were representative of 3 independent experiments.
Supplemental Figure 4

An empty vector (V) or the Hsp90α cDNA (O) was introduced into PAECs. The resulting cells were stained and analyzed by FACS as in Supplemental Figure 1. Overexpressing Hsp90α increased both Hsp90α (from 10.03% to 14.43%) and annexin II (from 5.48% to 7.45%) on the surface. The increase in annexin II staining was inhibited by treating the cells with 1 µmol/L geldanamycin (+GA) for 12 hours. The data were representative of three independent experiments.

Supplemental Figure 5

The thoracic aortas were isolated from sacrificed rats, which had endured 5 months of diabetes, or were age- and sex-matched controls. Before sacrifice, the rats were anesthetized, and then perfused with pre-warmed PBS through the left ventricle. The thoracic aorta was harvested, freed of periadventitial fat, dissected longitudinally and then the aorta pieces (about 6 × 8 mm) were placed luminal side up onto slides. The unfixed samples were blocked for 1 hour using 5% non-fat dry milk/PBS. The samples were first stained using an anti-annexin II antibody or non-immune IgG (5 µg/ml) for 1 hour at room temperature, and then a Cy3-conjugated secondary antibody (1:400 dilution) for 1 hour at room temperature. The samples were mounted with mounting medium, and examined and photographed with a Nicon Eclipse E-800 epifluorescent microscope. The images were representative of three independent experiment. Arrows represent annexin II binding to the luminal surface of aortas and the scale bar indicates 20 µm.
Supplemental Figure 1 (primary FACS data)

Normal goat IgG (H)  Hs90α (N)  Hs90α (M)

Normal mouse IgG (H)  Transferrin receptor (N)  Transferrin receptor (H)
Supplemental Figure 2 (permeabilized PAECs of primary FACS data)

- Normal goat IgG (H)
- Hs90α (N)
- Hs90α (H)
- Normal mouse IgG (H)
- Transferrin receptor (N)
- Transferrin receptor (H)

Percentage values: 0.80%, 99.3%, 98.9%, 1.21%, 99.7%, 99.9%
Supplemental Figure 3 (primary FACS data)

- Normal goat IgG(H): 0.80%
- Annexin II (N): 5.16%
- Annexin II (H): 9.02%
- Annexin II (M): 5.12%
- Annexin II (N+GA): 4.10%
- Annexin II (H+GA): 4.26%
Supplemental Figure 4 (primary FACS data)

IgG1 (O)  
Hsp90α (V)  
Hsp90α (O)  

Annexin II (V)  
Annexin II (O)  
Annexin II (V+GA)  
Annexin II (O+GA)  

IgG2 (V)  
Transferrin receptor (V)  
Transferrin receptor (O)  

[Graphs showing the percentage of cells in different categories]
Supplemental Figure 5
(immunofluorescence for annexin II on the luminal surface of the rat aorta)

annexin II/control

annexin II/diabetic aorta

Annexin II, DAPI

Scale bar: 20 µm

IgG/diabetic aorta