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Heat-shock protein 27 is a major methylglyoxal-modified protein in endothelial cells

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Abstract **In endothelial cells cultured under high glucose conditions, methylglyoxal is the major intracellular precursor in the formation of advanced glycation endproducts. We found that endothelial cells incubated with 30 mM D-glucose produced approximately 2-fold higher levels of methylglyoxal but not 3-deoxyglucosone and glyoxal, as compared to 5 mM D-glucose. Under hyperglycaemic conditions, the methylglyoxal-arginine adduct argpyrimidine as detected with a specific antibody, but not N^{e}-(carboxymethyl)lysine and N^{e}-(carboxyethyl)lysine, was significantly elevated. The glyoxylase I inhibitor HCCG and the PPARγ ligand troglitazone also increased argpyrimidine levels. Increased levels of argpyrimidine by glucose, HCCG and troglitazone are accompanied by a decrease in proliferation of endothelial cells. A 27 kDa protein was detected as a major argpyrimidine-modified protein. With in-gel digestion and mass spectrometric analysis, we identified this major protein as heat-shock protein 27 (Hsp27). This argpyrimidine modification of Hsp27 may contribute to changes in endothelial cell function associated to diabetes.**

Keywords: Endothelial cells; Methylglyoxal; Advanced glycation endproducts; Diabetes; Heat-shock protein 27

1. Introduction

Prolonged exposure to hyperglycaemia has detrimental effects on endothelial cell function and is the primary causal factor in the majority of diabetic complications [1,2]. A potential mechanism by which hyperglycaemia and its immediate biochemical sequelae induce damage in endothelial cells includes an increased formation of advanced glycation endproducts (AGEs) [3]. AGEs are formed from the reaction of glucose and other reducing sugars with amino groups of proteins. AGEs can be formed extracellularly, but it is now apparent that intracellular sugars and their derivatives can participate in glycation and AGE formation [4], as was initially demonstrated for growth factors [5]. In endothelial cells, the highly reactive dicarbonyl compound methylglyoxal has been identified as a major precursor in the formation of intracellular AGEs [6]. Methylglyoxal is formed non-enzymatically by dephosphorylation of triose phosphates and is efficiently catabolised to D-lactate by the glyoxalase pathway, consisting of glyoxalase I and glyoxalase II and the cofactor glutathione [7].

Methylglyoxal reacts with arginine residues to form the non-fluorescent products 5-hydro-5-methylimidazolone, tetrahydropyrimidine and the major fluorescent product argpyrimidine[8]. Plasma concentrations of methylglyoxal [9] as well as 5-hydro-5-methylimidazolone [10] and argpyrimidine [8] are increased in diabetic patients. Furthermore, the presence of argpyrimidine has been demonstrated in human lenses, in human atherosclerotic lesions and in the arterial wall of kidneys of diabetic patients [8,11]. Although it has been reported that methylglyoxal primarily reacts with arginine, the methylglyoxal-lysine adducts N^{e}-(carboxyethyl)lysine, imidazolysine and methylglyoxal-derived lysine dimer MOLD have also been identified [12,13].

Methylglyoxal is involved in a variety of detrimental processes such as cell growth arrest, dysfunction, apoptosis and necrosis, most probably mediated through modifications of proteins [7,14]. Since these events are closely related to the development and progression of diabetes complications, it is of importance to identify the proteins that are modified by methylglyoxal. In this study we demonstrated in human endothelial cells that hyperglycaemia produced higher levels of methylglyoxal. Under these conditions, argpyrimidine is significantly elevated. We identified heat-shock protein 27 (Hsp27) as the major argpyrimidine-modified protein.

2. Materials and methods

2.1. Materials

M199, heat-inactivated newborn calf serum (NBCS), foetal calf serum (FCS) and glutamine were purchased from BioWhittaker (Verviers, Belgium). Human serum (HS) and pyrogen-free human serum albumin were obtained from Sanquin Research (Amsterdam, the Netherlands), penicillin/streptomycin from Gibco (Breda, the Netherlands), and hep-

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Abbreviations: AGEs, advanced glycation endproducts; Hsp27, heat-shock protein 27; HUVEC, human umbilical cord vein endothelial cells

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arine from Leo Pharmaceutics Products (Ballerup, Denmark). Gelatin was purchased from Merck (Darmstad, Germany). Crude endothelial cell growth factor was prepared from bovine brain [15]. Monoclonal mouse anti-argpyrimidine IgG was from Dr. K. Uchida and was characterised before. Monoclonal mouse-anti-Hsp27 IgG was purchased from Cell Signalling (Beverly, MA, USA) and polyclonal goat anti-Hsp27 IgG was from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture

Human umbilical cord vein endothelial cells (HUVECs) were isolated from umbilical cords according to Jaffe et al. [16]. The cells were seeded onto 1% gelatine coated dishes and were cultured in M199, supplemented with 10% heat-inactivated NBCS, 10% HS, 1% glutamine, penicillin (100 U/ml) streptomycin (10 μg/ml), heparin (5 U/ml) and endothelial cell growth factor (150 μg/ml) and were kept at a constant temperature of 37 °C in an incubator under 5% CO_2 and 20% O_2 atmosphere.

2.3. Cell proliferation assay

HUVEC were pre-cultured in 24-well plates (Greiner bio-one) with 5 or 30 mM D-glucose for 48 h. 18 h prior to harvesting, [^{3}H] thymidine (1 μCi/ml; Amersham Biosciences, Baie d'Urfe, Canada) was added and the incorporation into newly synthesised DNA was determined as described.

2.4. Preparation of anti-methylglyoxal antibodies

Methylglyoxal-keyhole limpet hemocyanin (KLH) was prepared by the reaction of methylglyoxal (10 mM) with KLH for 7 days at 37 °C and was used as antigen for the immunisation of mice. Methylglyoxal-KLH was emulsified in an equal volume of Freund's complete adjuvant and three mice were intradermally injected at multiple sites. The mice were boosted with the same amount of methylglyoxal-KLH emulsified in Freund's incomplete adjuvant 21 days later and antisera obtained 14 days after the booster were tested. The booster was repeated twice. Ten days after the final booster, antisera were tested with methylglyoxal-albumin and the mouse with the highest titer was used for fusion. We obtained 40 positive clones as tested with methylglyoxal-albumin and one of them was further characterised. This monoclonal antibody was from the IgG1 subclass, and has an at least 10-fold preference for tetrahydropyrimidine as compared to argpyrimidine or 5-hydro-5-methyl-4-imidazolone. Details of the characterisation of this antibody will be described elsewhere.

2.5. Western blotting

HUVECs were lysed in ice-cold lysis buffer (PBD, 1% Triton X-100, 12 mM sodiumdeoxycholate and 0.1% sodium dodecyl sulfate) with a cocktail of protease inhibitors (Roche) and subsequently sonicated. Equal amounts of protein, as determined using the Bradford protein assay (Bio-Rad, Munchen, Germany), from cells incubated under different conditions were used. The lysates were separated by 12% SDS–PAGE and then transferred electrophoretically to a PVDF membrane. The membrane was incubated for 1 h at room temperature with the primary antibody (anti-argpyrimidine, 1:200 000; anti-tetrahydropyrimidine, 1:2000, anti-Hsp27, 1:10 000). Then the membrane was washed three times and incubated for 1 h at room temperature with HRP-conjugated goat anti-mouse IgG (Dako, 1:2000). HRP was visualised by the ECL plus detection system (Amersham Biosciences).

2.6. Analysis of 1,2-dicarbonyl compounds and AGEs

HUVECs cultured in 25 cm^2 flasks were washed with ice-cold PBS and scraped in 200 μl acetic acid (0.1 M) and homogenised by sonication. The concentrations of methylglyoxal, 3-deoxyglucosone and glyoxal were determined by reversed-phase HPLC with gradient elution after derivatisation to their respective dimethoxyquinoxaline derivatives as recently described [17]. Concentrations of CML and CEL were measured by stable-isotope dilution tandem mass spectrometry (API 3000 from Sciex/Applied Biosystems) as recently described [18].

2.7. Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was carried out. In brief, endothelial cells were solubilised in lysis buffer for 30 min (9 M urea, 2% CHAPS, 20 mM Tris, pH 7.5, 0.5% dithiothreitol, and 0.5% IPG buffer 3–10) and then centrifuged. 370 μl of the supernatant was used for the rehydration and simultaneously loading of the proteins to the IPG strip (Immobiline 18 cm DryStrip 3–10 NL, Amersham Biosciences), at 30 V for 12 h. The voltage was increased to 8000 V and focused for a total of 65 000 V/h. Immediately after being focused, IPG strips were wrapped in plastic foil and stored at −80 °C. Prior to SDS–PAGE, IPG strips were equilibrated in 6 M urea/2% SDS/1% dithiothreitol/50 mM Tris, pH 8.8/30% glycerol for 15 min, followed by equilibration in 6 M urea/2% SDS/2.5% iodoacetamide/50 mM Tris, pH 8.8/30% glycerol for 15 min. The second dimension separation was run overnight using the Isodalt System (Amersham Biosciences) in 1.5-mm 11% gels (Duracryl, Genomic Solutions) at 25 mA per gel at 15 °C. After electrophoresis, a gel was fixed and stained using silver. The gel was washed once with water and stored at room temperature in a plastic sealing until tryptic digestion. A gel, which was run in parallel, was subjected to Western-blotting and immunostained with anti-argpyrimidine.

2.8. Digestion of proteins from two-dimensional gels

The corresponding immunopositive argpyrimidine spot from the silver-stained gel was manually excised with a round bottom dermal slicer of 3-mm diameter. The gel pieces were destained in 60% acetonitrile in 25 mM ammonium bicarbonate buffer, pH 8.5, and then dehydrated with 100% acetonitrile. The shrunken gel pieces were re-swelled in 25 mM ammonium bicarbonate buffer, dehydrated again in 100% acetonitrile, and dried in a speedvac. The gel pieces were rehydrated in 8 μl of trypsin solution (20 μg/ml) for 1 h, followed by addition of 50 μl of 25 mM ammonium bicarbonate buffer to completely immerse the gel pieces. After incubation overnight at room temperature, the supernatant was loaded into a self-packed Poros R2 (PerSeptive Biosystems) micro-tip, and eluted in 5 μl 50% acetronitrile/1% formic acid directly into a spraying electrode and analysed by an electrospray Q-TOF (Micromass) mass spectrometer.

2.9. Immunoprecipitation

HUVECs were lysed in lysis buffer. The lysate was incubated with agarose conjugated with goat anti-Hsp27 antibody as prepared with the Immunolink Plus Immobilization Kit (Pierce, Rockford, USA). Western blot analysis of the lysate before and after immunoprecipitation was performed with mouse anti-Hsp27 and anti-argpyrimidine as described above.

2.10. Data presentation and statistics

Data are expressed as mean ± S.D. The n values represent number of independent experiments with three determinations within one experiment. Statistical differences were determined using paired 2-tailed Student's t test. A $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Methylglyoxal is significantly elevated in endothelial cells cultured under hyperglycaemic conditions

Human endothelial cells cultured under hyperglycaemic conditions (30 mM D-glucose, 96 h) induced an increase in the concentrations of methylglyoxal as compared to cells cultured under standard conditions (5 mM D-glucose) (Fig. 1). Under the same experimental conditions we did not find a significant increase of 3-deoxyglucosone and glyoxal (under standard condition the concentrations were 0.012 and 0.085 nmol/mg protein, respectively) and also not of the AGEs CML and CEL (under standard conditions 0.07 and 0.01 μmol/mmol lysine, respectively). Simultaneously, the proliferation of endothelial cells was reduced (Fig. 2). To evaluate whether an intracellular formation of methylglyoxal is associated with a reduction in cell proliferation, we treated endothelial cells with a direct inhibitor of glyoxalase I, the HCCG-diester, or the PPARγ ligand troglitazone, which has been reported to be a potential inhibitor of glyoxalase I synthesis inside cells [19].

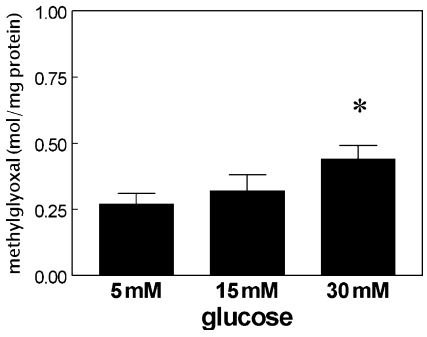

Fig. 1. Methylglyoxal concentrations in HUVECs cultured under normo- and high glucose conditions. HUVECs were cultured under standard conditions (5 mM D-glucose), 15 mM D-glucose and 30 mM D-glucose for 4 days. Cells were scraped in acetic acid (0.1 M) and the methylglyoxal concentrations in cell extracts were measured by HPLC, as described in Section 2. Data are the means ± S.D. of four independent experiments $^{*}P < 0.01$.

These potential glyoxalase I inhibitors also significantly reduced the proliferation by approximately 80%. In agreement with the data on [3H]-thymidine incorporation, cell counting as estimated by phase contrast microscopy also revealed comparable lower cell numbers after incubation with high glucose (30 mM) concentrations, but also with HCCG and troglitazone. Under the above mentioned conditions, we did not observe morphological differences of the cells, or an effect of cell adhesion.

3.2. MGO-adduct argpyrimidine in endothelial cells

In order to study the formation of specific methylglyoxal-adducts, we have used two monoclonal antibodies. HUVECs cultured under hyperglycaemic conditions show elevated levels of argpyrimidine at a protein of a molecular weight of ~25 kDa as compared to cells cultured under normoglycaemic conditions (Fig. 3B). In contrast, an antibody with a preference for tetrahydropyrimidine above argpyrimidine and 5-hydro-5-methylimidazole did not show such a difference in endothelial cells cultured under identical conditions (Fig. 3A). Cells treated with troglitazone, or HCCG-diester also showed significantly higher levels of argpyrimidine (Fig. 3B). Under these conditions, we did not find changes in the free methylglyoxal concentration (data not shown).

Cells cultured under hyperglycaemic conditions have significantly higher levels of argpyrimidine compared to cells cultured under normoglycaemic conditions (Figs. 3B and 4), but in time, the argpyrimidine levels inside cells decreased (Fig. 4), whereas the absolute difference between normoglycaemia and hyperglycaemia remained the same. The decrease of argpyrimidine inside endothelial cells, as estimated in four independent experiments, was approximately 15% per day.

3.3. Identification of argpyrimidine-positive protein and Hsp27 in endothelial cells

To identify the major argpyrimidine-containing 25 kDa protein, we subjected cell lysates from HUVECs to two-dimensional electrophoresis. One gel was used for Western blotting and stained with an antibody against argpyrimidine. Two pro-

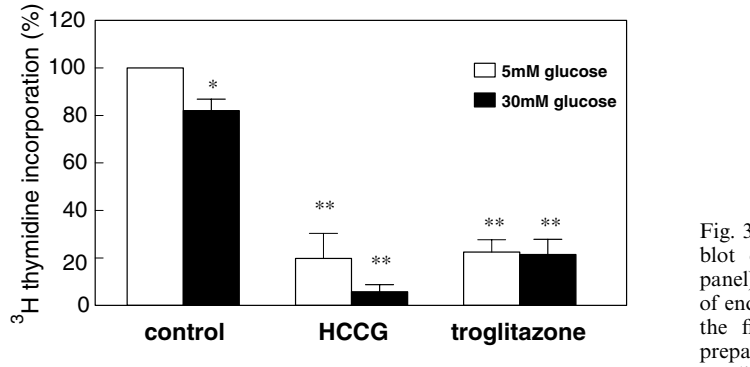

Fig. 2. The effect of high glucose concentrations, HCCG and troglitazone on HUVEC proliferation. HUVECs were cultured under 5 mM D-glucose or 30 mM D-glucose for 4 days, without or with the glyoxalase I inhibitor (HCCG-diester, 48 h, 5 μM) or troglitazone (24 h, 0.1 mM). The [^{3}H] thymidine incorporation into newly synthesized DNA of endothelial cells was evaluated. Means ± S.D. of four independent experiments are given; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (5 mM D-glucose).

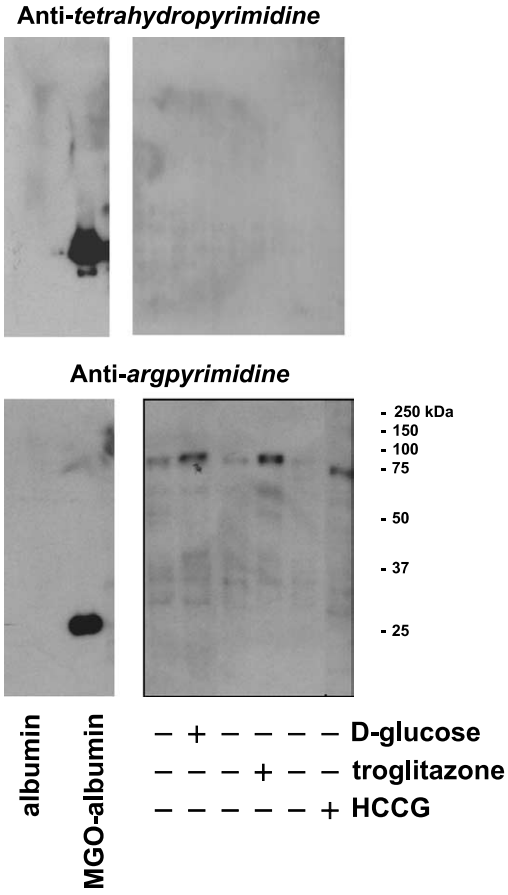

Fig. 3. Methylglyoxal-modified proteins in endothelial cells. Western-blot detection of tetrahydropyrimidine-containing proteins (upper panel) and argpyrimidine-containing proteins (lower panel) in lysates of endothelial cells, cultured under different conditions as indicated in the figure. Left panels: standard albumin and a MGO-albumin preparation. Right panels, lanes 1 and 2: cells cultured under standard conditions of 5 mM glucose (lane 1) or high glucose (30 mM D-glucose, lane 2) for 6 days. Lanes 3 and 4: cells cultured in medium with 20% FCS (lane 3), and 100 μM troglitazone (lane 4) for 24 h. Lanes 5 and 6: cells cultured under standard conditions without (lane 5) and with 5 μM HCCG for 54 h (lane 6). The molecular weights of the protein standards in kilodaltons are indicated. MGO-albumin (0.1 μg) is used as the positive control and migrated at a molecular weight of ~69 kDa. For all conditions, 10 μg of cellular protein are used.

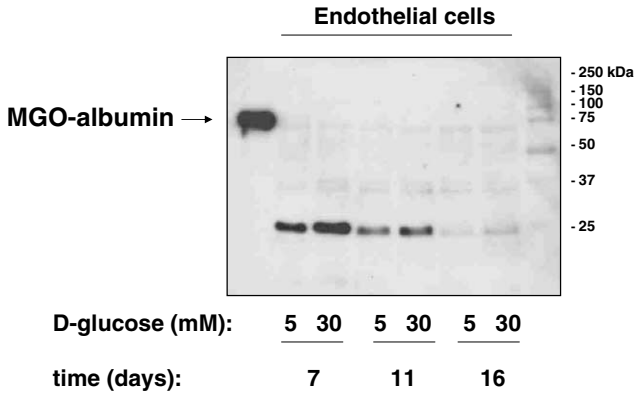

Fig. 4. Western blot of Argpyrimidine containing proteins in HUVECs. HUVECs were cultured under standard conditions (5 mM D-glucose) or at a high glucose concentration (30 mM D-glucose) for 7, 11 and 16 days. Cell lysates were detected for the presence of argpyrimidine by Western blotting. For all conditions, 10 μg of cellular protein is used. The molecular weights of the protein standards in kilodaltons are indicated. MGO-albumin (0.1 μg) is used as the positive control and migrated at a molecular weight of ~69 kDa. This is a representative experiment out of series of four experiments with identical results.

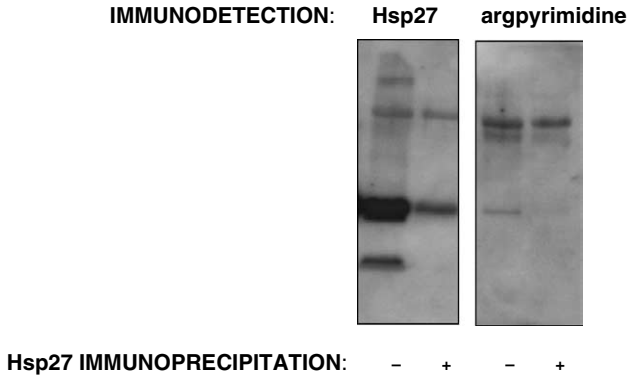

Fig. 6. Identification of Hsp27 as an argpyrimidine-containing protein in HUVEC A lysate of endothelial cells was immunoprecipitated with antibodies against Hsp27 that was covalently coupled to agarose. Western blot analysis was performed on the supernatant before and after Hsp27 immunoprecipitation followed by immunodetection with antibodies against Hsp27 (left panel) and argpyrimidine (right panel).

tein spots appeared around 25 kDa, most probably the phosphorylated and the unphosphorylated form of the protein (Fig. 5B). Subsequently, the other gel was silver-stained and one of the corresponding protein spots were removed from the gel and subjected to MALDITOF (Fig. 5A). The protein was identified as Hsp27. Western-blotting and immunodetection confirmed this finding (Fig. 5C). To prove that Hsp27 is the major argpyrimidine-containing protein, we performed immunoprecipitation experiments. A lysate of the endothelial cells was used for immunoprecipitation with agarose-coupled anti-Hsp27. Western blot analysis showed that argpyrimidine completely co-immunoprecipitated with Hsp27 (Fig 6), identifying the argpyrimidine-containing protein as Hsp27.

4. Discussion

Vascular endothelial cells exposed to high glucose concentrations might causes endothelial cell activation and, as a consequence, diabetic vascular complications. Studies in endothelial cells under high glucose conditions have exemplified the key biochemical pathways leading to endothelial dysfunction, including the accumulation of methylglyoxal-mediated formation of AGEs [3]. In this study, we demonstrated in human endothelial cells that high concentrations of D-glucose induced higher levels of methylglyoxal, but not glyoxal and 3-deoxyglucosone. With the use of a specific monoclonal antibody, we demonstrated that under these conditions the methylglyoxal-arginine adduct argpyrimidine was significantly increased, but not the methylglyoxal-lysine adduct CEL and CML, the latter in accordance with previous results [6]. In agreement with our result, it has been demonstrated that high concentrations of glucose also increased the concentration of methylglyoxal in transformed fetal bovine aortic endothelial cells [6] and induced synthesis of argpyrimidine in rat retinal endothelial cells [20] and rat measangial cells [23]. In addition to high glucose concentrations, we demonstrated that the glyoxalase I inhibitor HCCG also increased the levels of argpyrimidine in endothelial cells. Furthermore, the PPARγ agonist troglitazone, which has been demonstrated to down-regulate glyoxalase I mRNA in human astroglioma cells, rat cardiac myocytes and rat hepatocytes [19] also increased argpyrimidine production.

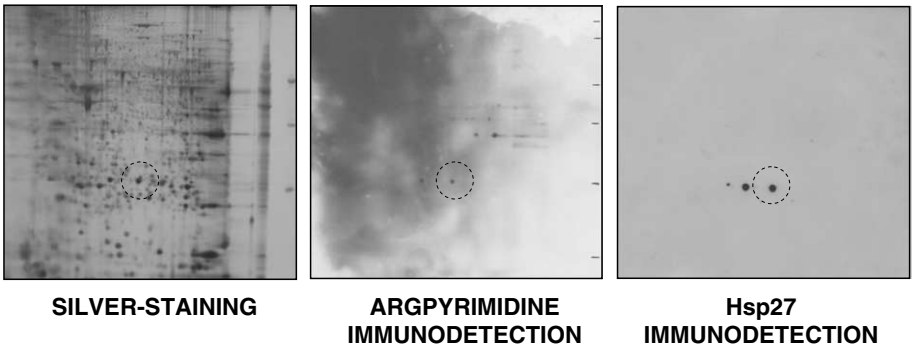

Fig. 5. Identification of argpyrimidine-positive protein and co-localisation with Hsp27. A cell lysate of 300 μg of total protein from HUVEC was used for two-dimensional electrophoresis. A gel was silver-stained and a second gel was subjected to Western blotting and stained with anti-argpyrimidine. After detection, the same immunoblot was stripped and stained with an antibody against Hsp27. The corresponding immunopositive protein (marked) was subjected to MALDITOF.

In contrast to the effect of glucose on methylglyoxal levels, we did not find changes in the free methylglyoxal concentration upon treatment of endothelial cells with HCCG and troglitazone. These experiments are in agreement with experiments in endothelial cells with glyoxalase 1 overexpression in which the levels of AGEs, but not free methylglyoxal, are decreased [6]. It might be that HCCG and troglitazone increases free methylglyoxal, which is immediately quenched by an interaction with proteins (such as to Hsp27) and therefore not found in its free form.

Since an increase of argpyrimidine levels upon treatment of endothelial cells with high D-glucose concentrations, HCCG or troglitazone are accompanied by impaired proliferation of these cells, these data suggest that methylglyoxal and/or methylglyoxal-adducts play a critical role herein. However, it should be emphasised that other pathways may also contribute to proliferation such as the activation of protein kinase C [21], which may act in concert with the MGO-modification of Hsp27. Brownlee and co-workers previously demonstrated that hyperglycaemia rapidly increases intracellular AGE formation in bovine aortic endothelial cells and they identified basic fibroblast growth factor (bFGF) as a protein modified by glycation [5]. Cytosolic AGE-bFGF was increased 6-fold accompanied by a marked decrease in mitogenic activity [5]. Although these data are consistent with the hypothesis that non-enzymatic glycation of intracellular protein alters vascular cell function, so far neither the identification of the glycation of bFGF nor the identification of other intracellular proteins modified by glycation are identified. With immunoprecipitation and immunoblotting we did not find bFGF as an argpyrimidine-modified protein (data not shown), indicating that other AGE-modifications on bFGF may be present.

In this study we identified with the use of specific antibodies and MALDITOF, that Hsp27 in human endothelial cells is a major argpyrimidine-modified protein. Although to a lesser extent, other proteins are modified as well (Figs. 3 and 4). Identification of these proteins was not successful, most probably due to the detection limit of the techniques. It should be emphasised, however, that other proteins may also be targets for methylglyoxal binding with the formation of other methylglyoxal-arginine adducts such as 5-hydro-5-methylimidazolone.

Recently, argpyrimidine formation in Hsp27 has also been identified in human carcinoma cells [22] and rat kidney mesangial cells [23], indicating that Hsp27 is a protein highly susceptible for methylglyoxal modification. Hsp27 is expressed throughout the body and plays a role in important cellular functions such as apoptosis [24] and actin polymerization [25]. In stressed cells, increased levels of Hsp27 facilitate the repair or destruction of damaged proteins, thus promoting cell recovery. Because methylglyoxal modifications of Hsp27 enhances the chaperone function of Hsp27, like that of α-crystallin [22,26] it has been speculated that methylglyoxal could protect cells against hyperglycaemia-induced damage in diabetes. Additionally, it has also been shown that Hsp27 can inhibit caspase-3 activity by binding to cytochrome c. A study in human carcinoma cells indicated that the methylglyoxal-induced modification Hsp27 is essential for oligomerisation of Hsp27, binding of Hsp 27 to cytochrome c and, subsequently, the protection of caspase-3 dependent cell death [22].

Taken together, we identified in human endothelial cell Hsp27 as a major argpyrimidine-modified protein. Although we do not know yet the role of this argpyrimidine modification of Hsp27 in human endothelial cells, this major AGE adduct may be associated with vascular complications in diseases such as in diabetes.

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