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Dysfunction of annexin A2 contributes to hyperglycaemia-induced loss of human endothelial cell surface fibrinolytic activity

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Summary

Hyperglycaemia impairs fibrinolytic activity on the surface of endothelial cells, but the underlying mechanisms are not fully understood. In this study, we tested the hypothesis that hyperglycaemia causes dysfunction of the endothelial membrane protein annexin A2, thereby leading to an overall reduction of fibrinolytic activity. Hyperglycaemia for 7 days significantly reduced cell surface fibrinolytic activity in human brain microvascular endothelial cells (HBMEC). Hyperglycaemia also decreased tissue type plasminogen activator (t-PA), plasminogen, and annexin A2 mRNA and protein expression, while increasing plasminogen activator inhibitor-1 (PAI-1). No changes in p11 mRNA or protein expression were detected. Hyperglycaemia significantly increased AGE-modified forms of total cellular and membrane annexin A2. The hyperglycemia-associated reduction in fibrinolytic activity was fully restored upon incubation with recombinant annexin A2 (rA2), but not AGE-modified annexin A2 or exogenous t-PA. Hyperglycaemia decreased t-PA, upregulated PAI-1 and induced AGE-related disruption of annexin A2 function, all of which contributed to the overall reduction in endothelial cell surface fibrinolytic activity. Further investigations to elucidate the underlying molecular mechanisms and pathophysiological implications of A2 derivatisation might ultimately lead to a better understanding of mechanisms of impaired vascular fibrinolysis, and to development of new interventional strategies for the thrombotic vascular complications in diabetes.

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Keywords

Annexin A2; hyperglycaemia; fibrinolytic activity; advanced glycation end-product; human brain microvascular endothelial cells

Introduction

Diabetes is an established independent risk factor for atherothrombotic cardiovascular and cerebrovascular disease (1). Impaired fibrinolysis on the surface of endothelial cells has been identified as a key pathogenic factor that contributes to the thrombotic vascular complications of diabetes (2, 3). Diabetes and hyperglycaemia are known to decrease tissue type plasminogen activator (t-PA) and increase plasminogen activator inhibitor 1 (PAI-1) expression in endothelium (3, 4). However, a complete understanding of the molecular mechanisms underlying fibrinolytic dysfunction in diabetes remains to be elucidated.

Vascular endothelial cell luminal surfaces maintain their thrombo-resistance by dynamically balancing the haemostatic and fibrinolytic systems. The fibrinolytic characteristics of the vascular endothelium are modulated by the activity of t-PA, which facilitates conversion of plasminogen to active plasmin (5). Annexin A2, a member of the annexin superfamily of calcium-dependent phospholipid-binding proteins, has been identified as an endothelial cell co-receptor for t-PA and plasminogen, and functions to accelerate plasmin generation (6). Emerging reports have documented that recombinant annexin A2 enhances plasmin generation on vascular endothelium *in vitro* and reduces thrombus formation *in vivo* (7, 8). Furthermore, recombinant annexin A2 combined with low-dose t-PA improves thrombolytic reperfusion efficacy in a rat model of focal embolic stroke (9-11). Taken together, these experimental findings demonstrate the important role of annexin A2 in modulating fibrinolytic activity *in vivo*.

Interestingly annexin A2 was discovered as one of a few key endothelial plasmalemmal proteins that form early glucose adducts. Annexin A2 glycation has been postulated to impair plasmin generation on the endothelial cell surface under hyperglycaemic conditions (12). Elevated blood glucose levels foster early glycation of proteins, promoting formation of advanced glycation end-products (AGEs) and reactive oxygen species (13). The formation and accumulation of AGEs accelerated in diabetes, and may lead to vascular complications (4, 14). The possibility of fibrinolytic impairment as a result of annexin A2 dysfunction or annexinopathy, has not yet been well studied but is of great interest (7, 15).

We previously reported that elevated D-glucose concentrations decreased cell surface fibrinolytic activity of human umbilical vein endothelial cells (HUVEC), and that addition of recombinant annexin A2 (rA2) partially restored this activity (8). The underlying molecular mechanisms for this observation, however, remain unknown. In this study, we tested the hypothesis that hyperglycaemia-induced loss of fibrinolytic activity reflects dysfunction of the cell membrane fibrinolytic receptor annexin A2, leading to reduced plasmin production on cultured human brain microvascular endothelial cells (HBMEC).

Materials and methods

Reagents

D-Val-Leu-Lys-AMC was obtained from MP Biomedicals (Irvine, CA, USA). Lys-plasminogen was obtained from Haematologic Technologies, Inc (Essex Junction, VT, USA). Mouse anti-human AGE monoclonal antibody was obtained from Novus Biologicals (Littleton, CO, USA). Antibodies for sodium potassium ATPase (Na^+/K^+ ATPase), annexin A2, PAI-1, and t-PA were obtained from Abcam (Cambridge, MA, USA). Plasminogen antibody, S100A10 (p11) antibody and t-PA were obtained from Sigma (St. Louis, MO, USA). All other routine laboratory chemicals were purchased from Sigma, unless otherwise indicated. All reagents were dissolved in sterile purified water prior to dilution into culture medium.

Cell cultures

Primary human brain microvascular endothelial cells were purchased from Cell Systems Corporation (Kirkland, WA, USA) and cultured as previously described (16). Briefly, cells (passage 4 to 9) were grown in EBM-2 Basal Medium (Lonza, Baltimore, MD, USA) supplemented with Endothelial Cell Growth Medium-2 (Lonza). On the second day, cells were exposed to high D-glucose (25 mmol/l) or to an equivalent concentration of L-glucose (25 mmol/l). Control cells were cultured under euglycaemic (5 mmol/l D-glucose) conditions. After 7 days, cells were harvested for assessment of fibrinolytic activity, RT-PCR, immunoprecipitation, and immunoblot analysis.

Preparation of recombinant human annexin A2 and AGE-annexin A2

Histidine-tagged recombinant human annexin A2 (rA2) was produced in *Escherichia coli* from a bacterial expression vector containing full-length human annexin A2 cDNA according to the method described earlier (7). The purity of rA2 was confirmed by SDS-PAGE followed by Coomassie blue staining, and its identity was verified by immunoblot analysis (9). AGE-annexin A2 was produced according to the protocols previously described (17). Briefly, recombinant annexin A2 (2 mg/ml) was incubated with 33 mmol/l glycoaldehyde at 37°C for 7 days in phosphate-buffered saline (PBS; pH 7.4). Aliquots were obtained from each reaction mixture and dialysed against PBS.

Cell viability and cell counting measurements

Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl) 2, 5-diphenyl-tetrazolium bromide (MTT) reduction assay. After incubation with D-glucose or L-glucose, cells were placed in media with 0.4% MTT. After 3 hours (h) at 37°C, the media was removed and cells were dissolved in dimethyl sulfoxide. Formazan formation was measured by reading absorbance at 570 nm with a reference setting of 630 nm on a microplate reader (FL600, Bio-Tek Instruments Inc., Winooski, VT, USA). Cell numbers were counted by trypan blue dye exclusion analysis. Briefly, culture medium was replaced by PBS/0.4% trypan blue, which stains nonviable cells. Four photographs (10-20x) were taken for each well. The data represented 6-12 separate wells assayed per data point, with about 500-1,000 cells counted per well.

Determination of plasmin activity

The fibrinolytic activity was examined by plasmin activity assay as described previously (7). Briefly, endothelial cells were incubated with t-PA and/or annexin A2 for 60 minutes (min) at 4°C in PBS containing 0.2% bovine serum albumin (BSA). After washing three times in the same buffer, the cells were exposed to varying concentrations of plasminogen and the plasmin-specific fluorogenic peptide substrate D-Val-Leu-Lys-AMC (100 µmol/l). Plasmin generation in each well was measured at 5-min intervals with excitation set at 360 nm and emission at 460 nm on a fluorescence plate reader. Readings were expressed as relative fluorescence units/min² (RFU/min²). To remove endogenous plasminogen activators from cell surfaces, the cells were washed with acid glycine buffer (0.05 mol/l glycine, pH 3; 0.1 mol/l NaCl) for 3 min and then neutralised with 0.5 mol/l HEPES (pH 7.5) and 0.1 mol/l NaCl before adding t-PA and/or plasminogen (18). Thereafter the cells were incubated with varying concentrations of t-PA (0 to 1 µmol/l, 60 min, 4°C) and then washed. Plasmin generation was determined as described above at a plasminogen concentration of 0.2 µmol/l. Maximum rates of plasmin generation were calculated and plotted against t-PA concentration for estimation of the binding capability of t-PA to endothelial cells.

Quantitative real-time polymerase chain reaction analysis

The mRNA levels of annexin A2, p11 (S100A10), t-PA, PAI-1, and plasminogen in cultured HBMEC were measured by real-time reverse transcription-PCR analysis following a standard method with minor modification. In brief, total RNA extraction was accomplished using the RNeasy mini kit (Qiagen Sciences, German-town, MD, USA) according to the manufacturer's instructions. Reverse transcription was performed using Superscript II RNase H-Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) to obtain cDNA. Specific primers used for quantification were as follows: annexin A2 forward, 5'-ATGTCTACTGTTACGAAATC-3'; annexin A2 reverse, 5'-AATGAGAGAGTCCTCGTCGG-3'; t-PA forward, 5'-ATCTTGCGGAGAACATACCG-3'; t-PA reverse, 5'-TGCACTCTTCCCTCTCTGT-3'; PAI-1 forward, 5'-AGTCGAGGCCTCATGACAAC-3'; PAI-1 reverse, 5'-GTCTTGGTGCTGATGTGTGG-3'; Plasminogen forward, 5'-ACACCTCCACCATCTTCTGG-3'; Plasminogen reverse 5'-ATCCTTTCCTTCCCAAAC-3'; p11 forward, 5'-CCACAGCTGGAGGAAGAGAC-3'; p11 reverse, 5'-AGAGCCCAAACCATGTTC-3'. Real-time PCR was performed on an ABI prism 7000 sequence detection systems (Applied Biosystems, Foster City, CA, USA). Data were analysed according to the comparative threshold cycle method with glyceraldehyd-3-phosphate dehydrogenase expression for sample normalisation. Melting curves for each PCR reaction were generated to ensure the purity of the amplification products.

Immunoprecipitation and immunoblotting

Cells were lysed in lysis buffer (Cell Signaling Technology, Beverly, MA, USA), and protein concentration was determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Cell membrane proteins were separated from intracellular proteins using Mem-PER Mammalian Membrane Protein Extraction Reagent Kit (Pierce Biotechnology, Rockford,

IL, USA). Protein lysates (20 µg per lane) were separated in precast 4-12% Tris-glycine SDS-polyacrylamide gels (Invitrogen), and proteins were transferred to PVDF membranes (Invitrogen). After blocking with 0.2% I-block (Tropix, Bedford, MA, USA), PVDF membranes were incubated overnight at 4°C with the indicated primary antibodies, and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia, Piscataway, NJ, USA). Immune complexes were visualised by enhanced chemiluminescence (Amersham Pharmacia). For immunoprecipitation assays, a commercial Immunoprecipitation Kit (Invitrogen) was used. Briefly, 1.5 mg of Protein A Dynabeads were incubated with 10 µg of an annexin A2 specific antibody and incubated for 10 min at room temperature with rotation. Following several washes the bound bead/antibody complex was added to 100 µl of enriched cell lysates sample, mixed by pipetting, and then incubated for 30 min at room temperature with rotation. The captured bead/Protein A/antigen complex was washed several times and eluted for immunoblotting with anti-AGE antibody (Abcam, Cambridge, MA, USA), which recognises Nε-(carboxymethyl) lysine (CML), a major antigenic AGE structure (19).

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical analysis was performed by one-way ANOVA followed by the Newman-Keuls test for multiple comparison tests (using SPSS 16.0 software; SPSS Inc., Chicago, IL, USA). Statistical significance was set at $p < 0.05$.

Results

Hyperglycaemia model in cultured HBMEC

We exposed cultured HBMEC to a series of D- and L-glucose concentrations over a range of incubation periods. Our data showed that exposure of HBMEC to 25 mmol/l D-glucose for 7 days had no significant effect on either cell viability or cell number. A slight impairment of mitochondrial function (as assessed by the MTT test) and reduction in cell counts (trypan blue exclusion analysis) were observed at 9 days, and reached statistical significance at 11 days when compared to euglycaemic controls (5 mmol/l D-glucose) (•Figure 1A, B). High L-glucose (25 mmol/l), an osmotic control, had no effect on cell viability or cell number, even at 11 days (•Figure 1A, B). In contrast, more severe hyperglycaemia at 50 mmol/l D-glucose for 7 days induced a concentration-dependent and significant reduction in cell viability and cell number (•Figure 1C, D). Thus, 25 mmol/l D-glucose for 7 days was chosen as the basic hyperglycaemia model in our subsequent *in vitro* experiments.

Hyperglycaemia reduced plasmin generating activity on the surface of cultured HBMEC

Hyperglycaemia (25 mmol/l D-glucose) decreased cell surface plasmin generating activity of cultured HBMEC progressively over time, as compared to the normal control group (5 mmol/l D-glucose). A significant reduction in plasmin activity was detected on days 5 and 7. However, the corresponding 25 mmol/l L-glucose osmotic control had no effect on cell surface plasmin activity (•Figure 2).

Exogenous t-PA failed to fully restore hyperglycaemia-induced loss of cell surface plasmin generating activity

When HBMEC were treated with varying concentrations of t-PA, there was a t-PA concentration-dependent increase in plasmin generation under both euglycaemic and hyperglycaemic conditions. However, hyperglycaemia significantly reduced (60%) the maximum level to which t-PA restored cell surface plasmin activity when compared to euglycaemic controls (•Figure 3). This result suggested that hyperglycaemia might impair the functional interaction of t-PA to its cell surface binding site(s) on the surface of HBMEC.

Hyperglycaemia impaired t-PA and plasminogen binding to the surface of cultured HBMEC

After cells were cultured in the presence of 5 or 25 mmol/l D-glucose for 7 days, addition of exogenous t-PA failed to restore the hyperglycaemia-induced reduction in plasmin generating activity; this result suggested an impairment in the ability of t-PA to convert plasminogen to plasmin (•Figure 4A). After removing endogenous membrane-bound t-PA with an acid-glycine buffer, and then providing exogenous t-PA followed by three washes, we found that the reduced plasmin activity was only partially restored in hyperglycaemia-treated cells (•Figure 4B); this result indicated a reduced ability of t-PA to bind to the cell membrane. Likewise, provision of exogenous lys-plasminogen failed to restore the hyperglycaemia-induced loss of plasmin generating activity, further indicating impairment of the ability of t-PA to convert plasminogen to plasmin in the setting of hyperglycaemia (•Figure 4C). Finally, after removing endogenous membrane-bound t-PA with acid-glycine buffer, there was no detectable plasmin generating activity following incubation with exogenous plasminogen (•Figure 4D); this result suggested that membrane binding of t-PA was essential for t-PA-mediated conversion of plasminogen to plasmin at the cell surface. Taken together, these data suggested significantly reduced cell surface binding capacity for t-PA and plasminogen leading to reduced plasmin generation in the setting of hyperglycaemia.

Hyperglycaemia decreased annexin A2 expression in cultured HBMEC

Hyperglycaemia (25 mmol/l D-glucose; 7 days) significantly reduced mRNA levels for plasminogen (49.3% reduction), t-PA (73.3% reduction), and annexin A2 (62.6% reduction), while significantly increases PAI-1 (931.7% increase) mRNA; p11 mRNA levels remained unchanged (•Figure 5A). Consistent with these results, protein expression was significantly decreased for annexin A2 (23.6% reduction), t-PA (31.7% reduction), and plasminogen (29.8% reduction), while PAI-1 protein levels were significantly increased (128.2%); no change in p11 protein expression was detected (•Figure 5B, C). These results suggested that, in addition to the perturbed ratio of t-PA (and plasminogen) to PAI-1 induced by hyperglycaemia, decreased annexin A2 protein expression may also contribute to the loss of plasmin generating activity at the HBMEC cell surface.

Hyperglycaemia induced AGE-modified forms of annexin A2 in cultured HBMEC

After exposure to elevated D-glucose for 7 days, total cell and cell membrane proteins were extracted from HBMEC. Annexin A2 and AGE-annexin A2 protein expression were

determined by immunoblot and immunoprecipitation (►Figure 6A, C). Optical density quantitation showed that 7 days of hyperglycaemia significantly increased total cell and membrane AGE-modified forms of annexin A2 by 3 and 4.4-fold, respectively (► Figure 6B, D).

Recombinant A2, but not AGE-annexin A2, rescued hyperglycaemia-induced loss of plasmin generating activity

The hyperglycaemia-induced loss of plasmin generating activity was restored by adding rA2 in a dose-dependent manner (► Figure 7A), suggesting central role for annexin A2 in mediating cell surface plasmin generation. Importantly, the hyperglycaemia-induced decrease in plasmin activity was fully restored by rA2, but not by AGE-rA2 (► Figure 7B), confirming the dysfunctional nature of AGE-modified annexin A2, a possible contributor to hyperglycaemia-induced fibrinolytic impairment. Because lipoprotein(a) [Lp(a)] can block the binding of plasminogen to annexin A2 resulting in inhibition of annexin A2-mediated cell surface plasmin generation (5, 20), we pretreated cells with rA2 in the presence of Lp(a) and noted significant inhibition of the ability of rA2 to restore hyperglycaemia-impaired plasmin activity (► Figure 7C). This observation further supported impaired fibrinolytic assembly of the t-PA-annexin A2/p11-plasminogen complex under hyperglycaemic conditions (21).

Discussion

On the vascular endothelial cell surface, the fibrinolytic system is comprised of an inactive proenzyme, plasminogen, and t-PA, which can convert plasminogen to the active enzyme, plasmin; plasmin degrades fibrin, releasing soluble fibrin-degradation products (22). Impairment of the fibrinolytic cascade is associated with a wide range of atherothrombotic conditions and risk factors (20, 23, 24). Atherogenesis is a complex process involving vascular inflammation, injury, and lipid accumulation, as well as platelet and fibrin deposition (25). A link between deficient fibrinolytic activity and the development of the atherosclerotic plaque has been suggested by findings of increased fibrin deposition and decreased fibrinolytic activity in the walls of atherosclerotic arteries (26-28). Metabolic syndrome is a well-studied process that links impaired fibrinolysis and atherosclerosis (25, 29). Metabolic syndrome has been strongly associated with both development of type 2 diabetes and atherothrombosis (25). However, the exact role of impaired vascular fibrinolysis in the initiation or progression of atherosclerosis is not yet clear (24). Hyperglycaemia is one of several potential contributors to hypofibrinolysis in diabetes (30). Dysfunction of the endothelial cell fibrinolytic receptor annexin A2 has been hypothesised to account for hyperglycaemia-induced hypofibrinolysis, but still remains largely unexplored (8, 12, 15).

In this study, our experimental results showed that (i) endothelial cell surface fibrinolytic activity (plasmin activity) was significantly decreased following 7 days of hyperglycaemia (► Figure 2); (ii) decreased cell surface plasmin activity was not fully restored by exogenous t-PA or plasminogen (►Figure 3 and ►Figure 4); (iii) hyperglycaemia-induced a decrease in annexin A2 expression, and an increase in AGE-modified annexin A2 (►Figure 5 and ►Figure

6), and (iv) hyperglycaemia-related hypofibrinolysis was completely rescued by adding recombinant annexin A2, but not AGE-modified annexin A2 (•Figure 7). These results suggest that hyperglycaemia resulted in AGE-derivatisation of annexin A2 that impaired the binding of endogenous t-PA and plasminogen, resulting in a reduction in endothelial cell surface fibrinolytic activity.

Cell membrane annexin A2 functions as a co-receptor for t-PA and plasminogen in endothelial cell surface plasmin generation (31, 32). By RT-PCR and immunoblot analyses, we found that hyperglycaemia significantly decreased annexin A2 mRNA (62.6% reduction) and protein expression (23.6% reduction) (•Figure 5). These data indicated that down-regulation of annexin A2 might contribute to the hyperglycaemia-induced fibrinolytic deficiency.

It is worth noting that hyperglycaemia and/or diabetes may decrease t-PA expression and increase PAI-1 *in vitro* and *in vivo* (3). The t-PA/PAI-1 imbalance has been thought to be an important mechanism, but it explains only a portion of the observed reduction in fibrinolysis (33-35). In the present study, consistent with previously published work by others (35), we confirm that hyperglycaemia decreased plasminogen and t-PA expression while increasing PAI-1 expression. However, we also found a complete rescue of hyperglycaemia-induced hypofibrinolysis upon addition of rA2, but not t-PA (•Figure 3 and •Figure 4). This suggested that a mechanism separate from the reduced t-PA/PAI-1 ratio might be involved. The annexin A2 heterotetramer is comprised of two p11 (S100A10) subunits and two annexin A2 subunits on the endothelial cell membrane that regulate t-PA dependent cell surface fibrinolytic activity (6, 36). Although p11 is stabilised by annexin A2, we found that the expression of p11 was not significantly altered by hyperglycaemia (•Figure 5).

Although annexin A2 is among the three cell membrane proteins that are most vulnerable to glycation in type 1 diabetic rats, the fundamental mechanisms behind this pathology have not been delineated (12). In this study, we found that hyperglycaemia significantly increased total and cell membrane AGE-modified forms of annexin A2 (AGE-annexin A2) by about 3- and 4.4-fold, respectively (•Figure 6). Moreover, rA2, but not AGE-rA2, successfully restored hyperglycaemia-impaired fibrinolytic activity. Taken together, these findings clearly demonstrate that hyperglycaemia induces glycation of annexin A2, which appears to contribute to impaired endothelial cell surface fibrinolytic activity. Furthermore, assembly of the t-PA-annexin A2/p11-plasminogen complex appears to be crucial for efficient t-PA-mediated conversion of plasminogen to plasmin at the cell surface (38). Although we did not dissect the specific alteration in fibrinolytic assembly in this study, our experimental findings showed that adding AGE-rA2, t-PA, or plasminogen after removal of endogenous t-PA, or blocking plasminogen binding to annexin A2 by Lp(a) failed to rescue hyperglycaemia-induced loss of fibrinolytic activity on the cell surface (•Figure 7). These data raise the possibility of impaired fibrinolytic assembly under conditions of hyperglycaemia.

There are, however, other mechanisms that may be involved. For example, both t-PA and plasminogen have multiple protein binding sites on the endothelial cell membrane (39), and possible decreases in non-annexin A2 binding sites might also impair fibrinolytic assembly.

In addition, we cannot exclude the possibility that the dissociation of the annexin A2/p11 tetramer and/or diminished translocation of annexin A2 to the cell surface could also contribute to impaired plasmin generation under hyperglycaemia (40). Further investigation is needed to address these questions, and these studies should be extended to diabetic animals and diabetic human subjects.

Although clinical information is largely lacking, knowledge obtained from initial experimental investigations indicate that impaired fibrinolytic activity may play an important role in arterial thrombus formation, which implicated in most episodes of acute myocardial infarction or brain infarction. (26, 41). The hypofibrinolytic status of the vessel wall may be caused by vascular injury, hyperglycaemia/hyperinsulinaemia, inflammation or atherosclerosis, and may lead to adhesion and accumulation of platelets, initiation of coagulation cascades, enhanced thrombin generation, and resistance to thrombolytic treatments (3, 7). Therefore, modulation of thrombogenesis and fibrinolysis in the vascular wall has been a major focus of research related to atherosclerosis and thrombotic vascular disease. Further investigation is needed to address the potential causal relationship between impaired fibrinolytic activity and other risk factors. A better understanding of the pathogenesis of vascular fibrinolytic dysfunction would elucidate further the efficacy and safety of atherothrombotic preventive and lytic therapy, and could enable the development of new therapeutic strategies to increase vascular fibrinolysis (24, 29).

In summary, our findings demonstrate that hyperglycaemia-induced loss of endothelial cell surface fibrinolytic activity is mediated in part by an increase in AGE-modified annexin A2 that impairs t-PA and plasminogen binding and subsequent plasmin generation. Further investigations to elucidate underlying molecular mechanisms may ultimately lead to a better understanding of vascular fibrinolysis and the development of new interventional strategies for diabetic vascular disease.

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What is known about this topic?

- Impaired fibrinolysis on the surface of endothelial cells has been identified as a key pathogenic factor that contributes to the thrombotic vascular complications in diabetes.
- Annexin A2 is an endothelial cell co-receptor for t-PA and plasminogen, and functions to accelerate plasmin generation. Annexin A2 was found as one of a few key endothelial plasmalemmal proteins that form early glucose adducts in diabetes rats.
- The possibility of hyperglycaemia-induced fibrinolytic impairment as a result of annexin A2 dysfunction or annexinopathy, has not yet been well studied, but is of great interest.

What does this paper add?

- Hyperglycaemia significantly decreased annexin A2 mRNA and protein expressions, and significantly increased AGE-modified annexin A2 (AGE-annexin A2).
- Hyperglycaemic reduction in fibrinolytic activity was fully restored by incubation with recombinant annexin A2 (rA2) but not by AGE-annexin A2 or exogenous t-PA.
- Our findings demonstrate that hyperglycaemia-induced loss of endothelial cell surface fibrinolytic activity is mediated in part by an increase in AGE-modified annexin A2 that impairs t-PA and plasminogen binding and subsequent plasmin generation.

Figure 1. Effect of hyperglycaemia on cell viability and cell number in cultured human brain microvascular endothelial cells (HBMEC)

Cells were exposed to high D-glucose (25 mmol/l) for various times or were exposed to various concentrations of D-glucose for 7 days. A) Cell viability at days 1-11 was assayed using the MTT reduction assay. B) Cell counts on days 1-11 were assessed by trypan blue exclusion. C) Cell viability on day 7 was assayed by the MTT assay. D) Cell counts on day 1-11 were assessed by trypan blue exclusion. The data are expressed as mean $\pm$ SD, n=5 per group. *p< 0.05 compared to control group (5 mmol/l D-glucose).

Figure 2. Effect of hyperglycaemia on fibrinolytic (plasmin) activity on the surface of cultured HBMEC

Cells were exposed to high D-glucose (25 mmol/l) for up to 7 days. Relative fluorescence units (RFU/min²) were used to represent plasmin activity. The data are expressed as mean $\pm$ SD (% of control), n=5 per group. *p<0.05 compared to each control group (5 mmol/l D-glucose).

Figure 3. Effect of exogenous t-PA on hyperglycaemia-induced loss of plasmin activity on cultured HBMEC

Cells were cultured with 5 or 25 mmol/l D-glucose for 7 days and then incubated with different concentrations of t-PA. Relative fluorescence units (RFU/min²) were used to represent plasmin activity. The data are expressed as mean $\pm$ SD, n=5 per group. *p< 0.05 compared to control group (5 mmol/l D-glucose).

Figure 4. Effect of exogenous t-PA or plasminogen on hyperglycaemia-induced reduction in cell surface plasmin activity

Cells were cultured with 5 or 25 mmol/l D-glucose for 7 days. A) Effect of exogenous t-PA (0.2 μ mol/l) on hyperglycaemia-induced reduction in plasmin activity. B) Effect of exogenous t-PA (0.2 μ mol/l) on hyperglycaemia-induced reduction in plasmin activity after acid-glycine buffer treatment. C) Effect of exogenous lys-plasminogen (0.2 μ mol/l) on hyperglycaemia-induced reduction in plasmin activity. D) Effect of exogenous lys-plasminogen (0.2 μ mol/l) on hyperglycaemia-induced reduction in plasmin activity after acid-glycine buffer treatment. The data are expressed as mean $\pm$ SD, n=5 per group. *p<0.05 compared to control group (5 mmol/l D-glucose).

Figure 5. Effect of hyperglycaemia on expression of proteins related to endothelial cell surface plasmin generation

Cells were cultured with 5 or 25 mmol/l D-glucose for 7 days. A) Real-time PCR for mRNA encoding plasminogen (PLG), t-PA, PAI-1, p11, and annexin A2 (ANNX). B) Representative immunoblots for total cellular protein expression of PLG, t-PA, PAI-1, p11 and annexin A2. C) Optical density quantification of immunoblot analysis. The data are expressed as mean $\pm$ SD (% of control), n=3 per group. *p<0.05 compared to control group (5 mmol/l D-glucose).

Figure 6. Effect of hyperglycaemia on production of AGE-modified annexin A2 in cultured HBMEC

Cells were cultured with 5 or 25 mmol/l D-glucose for 7 days. Protein expression was examined by immunoprecipitation (IP) followed by immunoblot analysis. A) Representative immunoblots for total cellular AGE-modified annexin A2 (AGE-ANNX) levels. β -actin served as the loading control. B) Optical density quantification of immunoblot analysis for total cellular AGE-ANNX levels. C) Representative immunoblots for cell membrane AGE-modified annexin A2 (AGE-ANNX) levels. Na^+/K^+ ATPase served as the loading control. D) Optical density quantification of immunoblot analysis for cell membrane proteins. The data are expressed as mean $\pm$ SD (fold increases), $n=3$ per group. * $p<0.05$ compared to control group (5 mmol/l D-glucose).

Figure 7. Effect of recombinant annexin A2/ or AGE-annexin A2 and lipoprotein(a) on hyperglycaemia-induced reduction in plasmin activity on the surface of cultured HBMEC Cells were cultured with 5 or 25 mmol/l D-glucose for 7 days. Plasmin activity on the cell surface was examined. A) Effect of recombinant annexin A2 on hyperglycaemia-induced reduction in plasmin activity. B) Effect of AGE-annexin A2 on hyperglycaemia-induced reduction in plasmin activity. C) Effect of recombinant annexin A2 in combination with Lp(a) pretreatment on hyperglycaemia-induced reduction in plasmin activity. The data are expressed as mean $\pm$ SD (% of control), n=5 per group. *p<0.05 compared to control group (5 mmol/l D-glucose); #p<0.05 compared to high D-glucose group (25 mmol/l) and &p<0.05 compared to annexin A2 group.