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The defence against free radicals protects endothelial cells from hyperglycaemia-induced plasminogen activator inhibitor 1 over-production

A. Ceriello, F. Curcio, P. dello Russo, I. Pegoraro, G. Stel, P. Amstad and P. Cerutti

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It has been shown that hyperglycaemia may stimulate plasminogen activator inhibitor 1 (PAI-1) over-production in human endothelial cells in culture. At the same time, it has been shown that glucose may enolize, producing free radicals. In this study, the possibility that hyperglycaemia stimulates PAI-1 over-production in human endothelial cells in culture by generating free radicals has been evaluated. For this purpose two experimental models were used: human endothelial cells transfected to express high glutathione peroxidase levels cultured in hyperglycaemic media, and human endothelial cells cultured in hyperglycaemic media with the antioxidant GSH. Cells grown in 20 mM glucose produced higher values of PAI-1 with respect to controls. The production of PAI-1 was not influenced by hyperglycaemia in transfected cells. GSH in the medium reduced hyperglycaemia-induced PAI-1 over-production, but also reduces the basal production of PAI-1 in the cells grown in normal glucose concentration. These data show that antioxidant defences may reduce hyperglycaemia-induced PAI-1 over-production in human endothelial cells in culture. The hypothesis that oxidative stress may play an important role in the pathogenesis of diabetic complications is then supported by this study.

Key words: Hyperglycaemia, endothelial cells, PAI-1, free radicals, diabetic complications.

Introduction

The nature of the vascular complications of diabetes has fostered long-standing interest in whether and how the disease alters the haemostatic and thrombolytic systems. Notwithstanding contradictory findings, substantial evidence indicates that diabetes is associated with a hypercoagulable state.[1,2] Plasma concentrations of plasminogen-activator inhibitor-1 (PAI-1), a serine protease inhibitor that attenuates fibrinolytic activity and potentiates thrombosis[3] are increased in diabetes mellitus.[4–6] It has been recently reported that endothelial cells cultured in high glucose concentration increase PAI-1 production.[7] The pathogenesis of this phenomenon remains still unclear.[7] It has been also reported that glucose can enolize, producing reduced molecular oxygen, even under physiological conditions.[8] At the same time, it has been shown that hyperglycaemia may alter some endothelial cell functions by generating free radicals.[9,10] This study evaluates the possibility that hyperglycaemia stimulates PAI-1 over-production in human endothelial cells in culture through free radical generation. For this purpose two experimental models were used. The first consists of growing human endothelial cells in hyperglycaemic media transfected with a vector expressing high levels of the antioxidant enzyme glutathione peroxidase (GPx), which protects cells against free radical

Drs Ceriello, Curcio, Pegoraro and Stel are with the Dept. of Clinical and Experimental Pathology and Medicine, Chairs of Internal Medicine, Pathology, and Clinical Pathology, University of Udine, Udine. Dr dello Russo is with the Istituto Immunotrasfusionale, Ospedale Civile di Udine. Drs Amstad and Cerutti are with the Dept. of Carcinogenesis, Swiss Institute for Experimental Cancer Research, Epalinges, Lausanne, Switzerland. Address correspondence to: Dr Antonio Ceriello, Dept. of Clinical and Experimental Pathology and Medicine, Chair of Internal Medicine, University of Udine, P. le S. Maria della Misericordia, 33100 Udine, Italy. Tel: (+39) 432–552842; Fax: (+39) 432–42097.

damage. The second approach was to incubate human endothelial cells in hyperglycaemic media with the antioxidant GSH.

Material and methods

Cell culture

Three different cell cultures were used in this study.

(a) Human endothelial cells from umbilical vein (HUVEC) cultures were established as previously described.[11] Cells were grown in 199 medium (Sigma, St Louis, MO, USA) with 8% foetal calf serum (Gibco Laboratories, Grand Island, NY, USA), endothelial cell growth supplement (50 μg/ml) (Sigma) and heparin (5 μg/ml; a gift from Alfa-Wassermann, Bologna, Italy), in an atmosphere of 5% CO_2 at 36.5°C. Cultures were fed twice a week with fresh medium. At confluence they were passaged using a solution of 20 U/ml collagenase (Worthington, Freehold, NJ, USA), 0.75 mg/ml trypsin (1:300, ICN Biomedicals, High Wycombe, Bucks HP12 3XJ, UK), 2% heat-inactivated dialysed chicken serum (Gibco Laboratories), in Ca^{+}- and Mg^{2+}-free Hanks' balanced salt solution.[12] At every passage cells were checked for their ability to produce factor VIII-related antigen by indirect immunofluorescence microscopy. The mouse monoclonal anti-human factor VIII-related antigen was from Dako Corporation (Carpintera, CA, USA) and the goat anti-mouse IgG, FITC conjugated, from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA).

(b) Spontaneously transformed and immortalized human endothelial cells (HEC) were a generous gift from Dr Takahashi. This cell line shows spontaneous phenotypic alterations, without evidence of virus implication, leading an indefinite life span.[13] Although one of the endothelial cell markers, factor VIII-related antigen is negative in this cell line, immunocytochemical staining for the lectin Ulex europaens I and anti-human endothelium as well as glomerular epithelium monoclonal antibody was positive.[13] Angiotensin-converting enzyme activity and PAI-1 production was also demonstrated.[13] This cell line has been used for the study of some physiologically active factors.[13]

(c) HEC transfected to express high levels of the antioxidant enzyme GPx (GPx-HEC): a pD5 vector containing the gpt-resistance cassette was constructed. For this purpose a 2.2 kb *Bam*HI fragment containing the SV40-*Escherichia coli* gpt resistance cassette was removed from vector pMSG described by Lee *et al.*,[14] blunt-ended *Xba*I site of pD5. The pD5 vector, a gift from K. Berkner (Zymogenetics Inc.), contains the adeno 5 major late promoter and the SV40 promoter and enhancer sequences.

A 1 kb cDNA fragment was removed by *Eco*RI digestion from pSPT19-GPx which contains a bovine GPx cDNA insert. The pSPT19-GPx construct was a gift from G. Mullenbach (Chiron, Corp.). The ends were filled in with Klenow enzyme and *Bam*HI linkers were added to the blunt-ended GPx cDNA fragment before ligation into unique *Bam*HI site of the pD5-gpt vector described above.

The pD5-gpt-GPx vector was transfected into HECs according to Chen and Okayma.[15] Resistant clones were selected for 9 days in medium containing 8% foetal calf serum, 250 μg/ml xanthine, 25 μg/ml mycophenolic acid, 2 μg/ml aminopterin, 10 μg/ml thymidine and 15 μg/ml hypoxanthine. The GPx activity in the parent HECs and in the transfectant (GPx-HECs) was determined as described previously.[16] GPx expression was approximately three-fold higher in the GPx-HECs transfectant relative to parent HECs (120 *vs* 37 IU/mg protein).

Study design

(a) To study the effects of GSH on glucose-dependent increase of PAI-1 secretion, HUVEC were plated in 5 and 20 mM glucose, in the absence or in the presence of 3 mM glutathione. This GSH concentration was chosen since it shows the better protection against hyperglycaemia-induced delay in replication time in HUVEC in culture (Curcio *et al.*, unpublished data). HUVEC were plated in 60-mm tissue culture dishes at a cell density of 1.3×10^5 for controls and 1.7×10^5 for cells destined for high glucose treatment.[7] The differential plating density was implemented to study experimental and control cells in the same assay at the same final density. This was otherwise unachievable owing to the replicative delay induced by high glucose,[17] but desirable because PAI-1 production is highly dependent on the growth state of endothelial cells.[18] Cells were kept for 1 week in the experimental conditions. Medium was exchanged every other day and the 24 h conditioned medium was harvested for testing PAI-1 production at day 7 after plating. At the same time, cells were trypsinized and counted.

(b) HEC and GPx-HEC were seeded in 60-mm tissue culture dishes, at a density of about 6×10^4 and cultured as above described for HUVEC. The difference in doubling replication time between HUVEC and HEC accounts for the different plating.[13] Medium for GPx-HEC had the addition of 50 ng/ml Na_2SeO_3, since GPx expression is Se-dependent. As described for HUVEC, cells were grown in two different glucose concentrations, 5 or 20 mM, respectively. Cells were kept for 1 week in the experimental conditions. Medium was exchanged every other day and the 24 h conditioned medium was harvested for testing PAI-1

production at day 7 after plating. At the same time, cells were trypsinized and counted.

For both studies (a) and (b) three different experiments were performed. In each experiment, triplicate dishes were used at each test point. Each dish was assayed for PAI-1 in triplicate. The results of all the wells, for each experimental point, were averaged for statistical analysis.

PAI-1 antigen concentration was evaluated by ELISA (Biopool, Umea, Sweden). This ELISA kit detects active and latent (inactive) forms of the protein.[19] PAI-1 antigen concentration was measured in ng/ml and expressed in ng/10^6 cells/24 h. Statistical analysis was performed by paired and unpaired Student's *t*-test.

Results

At the time of testing, HUVEC counts were not different in the various conditions (Figure 1). In 20 mM glucose, higher values of PAI-1 were found with respect to control ($P < 0.001$; Figure 1). GSH in the medium reversed this phenomenon, ($P < 0.001$ *vs* high glucose alone), but also reduces the HUVEC basal production of PAI-1 in normal glucose concentration ($P < 0.001$ *vs* normal glucose without GSH). Cell counts of HEC or GPx-HEC were not different in normal or high glucose concentrations (Figure 2). After 7 days of culture, in normoglycaemia, GPx-HEC produced less PAI-1 than HEC (Figure 2). HEC in hyperglycaemic conditions produced more PAI-1 than HEC cultured in normal glucose concentrations ($P < 0.001$), while GPx-HEC PAI-1 production was not influenced by high glucose in the medium (Figure 2).

Discussion

Decreased fibrinolysis has been reported in diabetes mellitus.[1,2] The alteration in fibrinolysis seems to be essentially linked to a consistent increase in PAI-1.[4–6] A role of hyperinsulinaemia in causing an increase in PAI-1 in diabetic patients has been proposed.[5] Because insulin has no effect on PAI-1 production by vascular endothelium but stimulates PAI-1 production by hepatocytes,[22] it has been proposed that impaired fibrinolysis in diabetes reflects increased liver synthesis of PAI-1 induced by hyperinsulinism.[5,20] This is a worthy pathogenetic construct because it provides a possible mechanism for the greater elevation in PAI-1 observed in NIDD compared with IDDM patients.[4,6] It does not, however, readily explain the increased PAI-1 activity documented also in IDDM patients, who do not exhibit portal hyperinsulinaemia.[4,6]

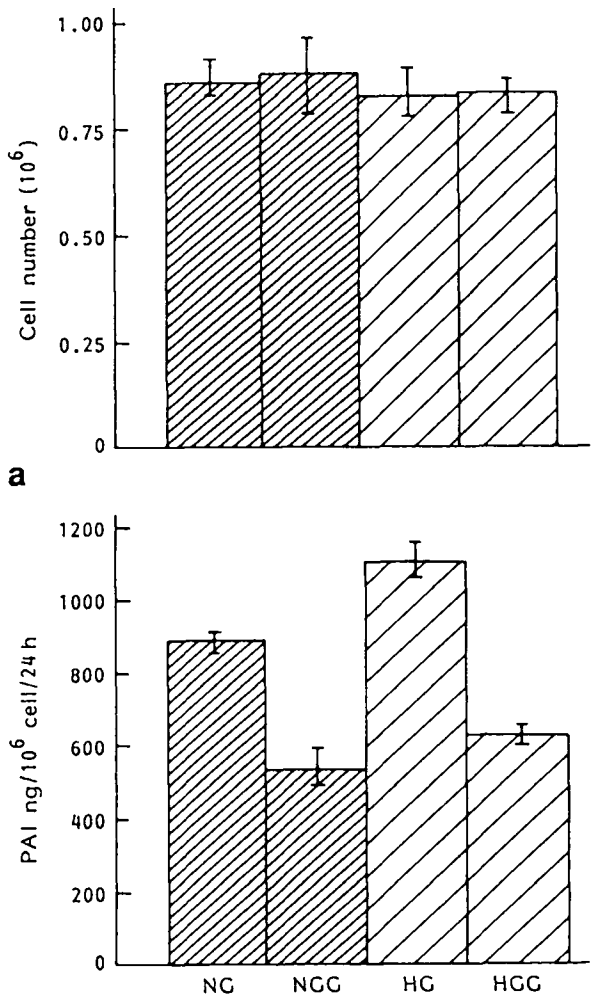

Figure 1. HUVEC cell counts and PAI production in 5 or 20 mM glucose and with or without added GSH. Panel (a) shows HUVEC cell counts expressed as mean ± SE, 7 days after plating (see details in Methods). Values were not statistically different in the various conditions. Panel (b) shows PAI measured in the medium expressed as ng/10^6 cells/24 h ± SE, 7 days after plating (see details in Methods). In 20 mM glucose, higher values of PAI were found compared with controls ($P < 0.0001$). GSH reversed the phenomenon ($P < 0.0001$ *vs* 20 mM glucose alone). PAI basal production in 5 mM glucose was also reduced ($P < 0.0001$ *vs* 5 mM glucose without GSH). NG, 5 mM glucose; NGG, 5 mM glucose + 3 mM GSH; HG, 20 mM glucose; HGG, 20 mM glucose + 3 mM GSH.

It has been recently demonstrated that human endothelial cells cultured in high glucose show an imbalance in the antifibrinolytic sense, mainly due to an overproduction of PAI-1.[7] These data are consistent with the finding showing similar results, *in vivo*, when hyperglycaemia is induced in diabetic patients.[21] It has been recently reviewed that hyperglycaemia might activate coagulation generating an 'oxidative stress'.[2]

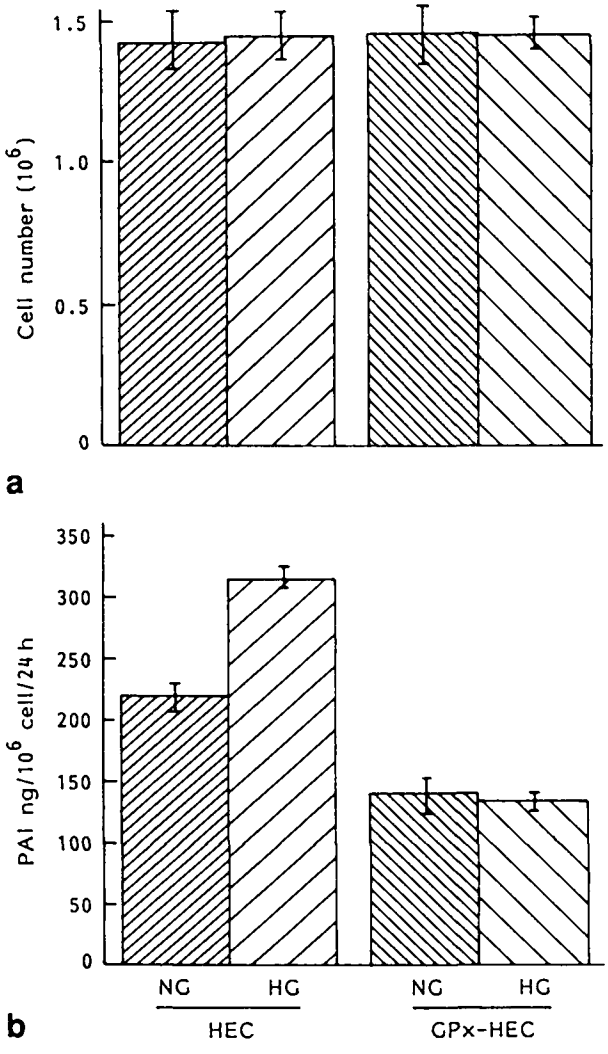

Figure 2. Counts and PAI production in 5 or 20 mM glucose in HEC and GPx-HEC cell populations. Panel (a) shows HEC and GPx-HEC cell counts expressed as mean ± SE, 7 days after plating (see details in Methods). Values were not different in 5 or 20 mM glucose. Panel (b) shows PAI measured in the medium expressed as ng/10^6 cells/24 h + SE, 7 days after plating (see details in Methods). In 5 mM glucose GPx-HECs produced less PAI compared with HECs ($P < 0.001$). In 20 mM glucose, untransfected HECs produced more PAI compared with 5 mM ($P < 0.001$), while PAI production by GPx-HECs was not influenced by 20 mM glucose medium. NG, 5 mM glucose; HG, 20 mM glucose.

At the same time, it has been reported that hyperglycaemia alters some endothelial cell functions through free radical production.[9,10] These data suggest that hyperglycaemia may induce PAI-1 over-production in endothelial cells through a mechanism linked to the generation of free radicals. In this view, it appears plausible that endothelial cells protected against free radicals may not respond to over-producing PAI-1 during the exposure to the hyperglycaemia.

This study shows that, in the presence of high glucose, both human endothelial cell types used in the study upregulate the production of PAI-1, as has been already demonstrated for HUVEC.[7] Hyperglycaemia does not influence PAI-1 production in transfected endothelial cells showing high GPx levels, while the presence of GSH normalizes PAI-1 production induced in HUVEC cultured in hyperglycaemic medium. From these results it is concluded that protection against free radicals protects endothelial cells from hyperglycaemia-induced PAI-1 over-production. This suggests that hyperglycaemia probably acts through the production of free radicals. These data agree with the findings that antioxidants protect endothelial cells against reduced life-span and impaired EDRF production caused by elevated glucose.[9,10] However, our data show that antioxidant defence may decrease PAI-1 production even in control conditions. This result is consistent with the evidence that an imbalance of the antioxidant pathway may alter some cell functions.[22] At the same time, these data may suggest an intriguing explanation of the findings showing that antioxidant supplementation may reduce cardiovascular mortality.[23,24]

This study shows that antioxidant defences may reduce hyperglycaemia-induced PAI-1 over-production in human endothelial cells in culture. The hypothesis that oxidative stress may play a central role in the pathogenesis of diabetic complications is then supported by this study.[25–27]

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