Inhibition of human endothelial cell nitric oxide synthesis by advanced glycation end-products but not glucose: relevance to diabetes

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ABSTRACT

Endothelial dysfunction, with decreased NO (nitric oxide) biosynthesis, may play a pathophysiological role in diabetic vasculopathy. The aim of the present study was to determine the relative contributions of glucose and AGE (advanced glycation end-product) accumulation in suppressing NOS-3 (the endothelial isoform of NO synthase). Cultured HUVECs (human umbilical vein endothelial cells) were incubated with different concentrations of glucose, unmodified albumin or AGE-modified albumin for different times. NOS activity was measured from the conversion of L-[3H]arginine into L-[3H]citrulline, and the expression, serine phosphorylation and O-glycosylation of NOS-3 were determined by Western blotting. High (25 mmol/l) glucose, for up to 12 days of incubation, had no effect on the activity or expression of NOS-3, nor on its degree of serine phosphorylation or O-glycosylation, compared with physiological (5 mmol/l) glucose. By contrast, AGE-modified albumin exerted a concentration- and time-dependent suppression of NOS-3 expression in HUVECs at a range of concentrations (0–200 mg/l) found in diabetic plasma; this was evident after 24 h, whereas inhibition of NOS activity was seen after only 3 h incubation with AGE-modified albumin, consistent with our previous observations of rapid suppression of NOS-3 serine phosphorylation and NOS-3 activity by AGE-modified albumin. In conclusion, AGE-modified albumin suppresses NOS-3 activity through two mechanisms: one rapid, involving suppression of its serine phosphorylation, and another slower, involving a decrease in its expression. We also conclude that, in the context of the chronic hyperglycaemia in diabetes, the effects of AGEs on endothelial NO biosynthesis are considerably more important than those of glucose.

INTRODUCTION

Diabetes mellitus, whatever its cause, is characterized by endothelial cell dysfunction, which manifests itself as an impairment of endothelial NO (nitric oxide) generation in response to NO-dependent agonists [1–4]. The most important complications of diabetes relate to vascular disease, both in the micro- and macro-vasculature, and it is believed that endothelial dysfunction may play an important part in the physiology of diabetes-associated vasculopathy [5]. Although dysfunction of endothelial NO generation has been well documented in vivo in
patients with both Type I and Type II diabetes, the underlying aetiology of this remains unclear.

One of the features of diabetes which can be demonstrated in vivo is the presence of oxidative stress [6–9]; an increase in oxygen-derived free radicals will decrease NO availability, since the superoxide anion can scavenge NO by combining with it to form the potent oxidant peroxynitrite. However, chronic oral supplementation with the antioxidant vitamins C and E only partially improves endothelium-dependent dilatation in Type I diabetes, and has very little if any effect on endothelium-dependent dilatation in Type II diabetes [10]. It has therefore been suggested that suppression of endothelial NOS (NO synthase) activity may be more important as a cause of reduced availability of NO in the context of diabetes. Indeed, in diabetes, the endothelial isoform of NOS, NOS-3, may become uncoupled, such that it generates superoxide in preference to NO [11].

HUVECs (human umbilical vein endothelial cells) obtained from umbilical cords following delivery in pregnancies complicated by gestational diabetes, far from exhibiting decreased NO biosynthesis, appear to synthesise increased amounts of NO [12]. It is likely, therefore, that dysfunction of the endothelial NO system in diabetes is not intrinsic to the endothelial cells, but is caused by the extrinsic environment to which the cells are exposed. Among the metabolic abnormalities that are found in the context of diabetes, high glucose and the accumulation of AGEs (advanced glycation end-products), a group of glycated and extensively cross-linked macromolecules formed as a consequence of chronic hyperglycaemia, have been implicated in the causation of endothelial NO dysfunction. However, it has been reported previously [13] that sustained incubation of cultured endothelial cells with high glucose may actually increase NOS activity, and this is consistent with the increase in NOS activity found in HUVECs isolated from gestational diabetic pregnancies [12].

We have demonstrated previously [14] that AGE-modified albumin rapidly decreases NOS activity in HUVECs (within 30 min of incubation), and that this effect is mediated through CML [ε-(carboxymethyl)-lysine, the major AGE adduct found in vivo] residues acting via the AGE-R1 receptor on the endothelial cells, giving rise to a decrease in serum phosphorylation of NOS-3. However, it is not clear whether AGE-modified albumin may, in the longer term, have effects on endothelial cell NOS-3 expression and above the effects on its phosphorylation.

The aim of the present study was to determine both the short- and longer-term effects of glucose and AGE-modified albumin, at a range of concentrations found in the circulation of patients with diabetes, on NOS activity and NOS-3 expression in HUVECs. In particular, we wished to determine the relative effects of glucose and AGE-modified albumin on this, since both will be elevated in diabetic patients, particularly where glycaemic control is poor.

**MATERIALS AND METHODS**

**Synthesis of AGE-modified albumin**

AGE-modified albumin was prepared by incubating BSA (20 g/l, fraction V, low endotoxin) with 50 mmol/l glucose in PBS containing 1 mmol/l EDTA for 12 weeks at 37 °C under sterile conditions. The mixture was then extensively dialysed against multiple changes of PBS to remove unbound glucose. We confirmed the absence of endotoxin (measured as < 0.015 endotoxin units/ml) using the Limulus Ameobocyte Lysate assay (Charles River Laboratories). CML content of AGE-modified albumin, determined by ELISA as described previously [14], was found to be 150 CML units/mg of protein, whereas unmodified albumin contained 1.95 CML units/mg of protein. We therefore examined the effects of 50, 100, and 200 mg/l AGE-modified albumin (corresponding to 7500, 15 000, and 30 000 CML units/l) on HUVECs, since these CML concentrations are representative of those found in the plasma of diabetic patients [14].

**HUVEC isolation and culture**

Fresh umbilical cords (n = 10) were obtained after delivery of healthy babies to healthy normotensive mothers either by vaginal delivery or by elective Caesarean section. In particular, cords from mothers with gestational diabetes were excluded from the present study. Informed consent was obtained in all cases. The study conformed to the standards set by the Declaration of Helsinki (2000) of the World Medical Association, and was approved by the Institutional Review Board for Human Studies of Nanjing University Medical School and by the St Thomas’ Hospital (London) Research Ethics Committee. The umbilical vein was cannulated and flushed with 30 ml of warm Dulbecco’s PBS, after which the cord was clamped at the distal end and the vessel was filled with medium 199 with added collagenase (Type II, EC 3.4.24.3; 1 g/l) until mildly distended. After incubation at 37 °C (15 min), the cord was unclamped and the digest drained. The vessel was gently massaged and flushed through with a further 30 ml of Dulbecco’s PBS, and the digests were pooled. The resultant endothelial cell suspension was centrifuged (400 g for 5 min), and the cell pellet was resuspended in medium 199 with Earle’s salts supplemented with 500 international units/ml penicillin, 500 mg/l streptomycin, 1.25 mg/l fungizone, 2 mmol/l glutamine, 20 % fetal bovine serum, 0.01 % heparin (Grade 1A) and 120 mg/l endothelial cell growth supplement (from bovine neural tissue). This suspension was seeded into a 25 ml gelatin-coated culture flask.

Monolayer cultures of HUVECs were grown at 37 °C in an atmosphere of 95 % air and 5 % CO2. At
confluence, cells were detached from the substratum by brief exposure to trypsin/EDTA (2 min at 37°C), pelleted (400 g for 5 min) and were passaged at a split ratio of 1:3. The presence of a pure population of HUVECs was confirmed both by their characteristic cobblestone morphology under phase-contrast microscopy and by positive direct immunofluorescence staining for von Willebrand factor and negative immunofluorescence staining for smooth muscle α-actin.

**Effect of glucose and AGE-modified albumin on NOS activity in HUVECs**

HUVECs at approx. 50% confluence at passage 2 were incubated with AGE-modified or unmodified albumin (0–200 mg/l), or with two different concentrations of glucose (5 or 25 mmol/l), in the culture medium for different time periods (3–48 h for AGE-modified albumin and unmodified albumin, and 3 h to 12 days for glucose), following which they were washed three times with BSS [balanced salt solution; 125 mmol/l NaCl, 5.4 mmol/l KCl, 16.2 mmol/l NaHCO₃, 15 mmol/l Hepes, 1.0 mmol/l NaH₂PO₄, 0.8 mmol/l MgSO₄, 1.8 mmol/l CaCl₂ and 5.5 mmol/l glucose (pH 7.6)]. After addition of 5 ml of fresh BSS, cells were scraped and transferred into Eppendorf tubes (1 ml of cell suspension per tube), and NOS activity was determined in the absence or presence of 10⁻⁴ mol/l histamine (which activates NOS maximally in HUVECs) from the rate of conversion of L-[³H]arginine into L-[³H]citrulline as described previously [14].

**Effect of glucose and AGE-modified albumin on NOS-3 protein expression in HUVECs**

HUVECs at approx. 50% confluence at passage 2 were incubated with unmodified or AGE-modified albumin (0–200 mg/l) or vehicle for 3–48 h. Other HUVECs were incubated in culture medium containing 5 mmol/l glucose, or in culture medium supplemented with glucose to a final concentration of 25 mmol/l, for 3 or 12 days. At the end of all incubations, cells were lysed by sonication in 0.5 ml of lysis buffer [25 mmol/l Tris/HCl (pH 7.6), 150 mmol/l NaCl, 1 mmol/l PMSE, 1 mg/l aprotinin, 10 mg/l leupeptin, 1 mmol/l EDTA, 50 mmol/l NaF, 1 mmol/l sodium orthovanadate and 1% Triton-X]. This was left on ice for 30 min and subsequently diluted with an equal volume of TBS [Tris-buffered saline; 25 mmol/l Tris/HCl (pH 7.6), 150 mmol/l NaCl] containing 5 g/l BSA, 2 mmol/l CaCl₂ and 0.02% sodium azide. Debris was pelleted at 15000 g for 15 min, and the resulting supernatant (HUVEC lysate) was stored at −80°C. In a portion of the HUVEC lysate, NOS-3 was immunoprecipitated, as described previously [14], and the resulting immunoprecipitates were also stored at −80°C.

Lysates or immunoprecipitates were boiled in an equal volume of SDS/PAGE sample buffer [0.1 mol/l Tris/HCl (pH 6.8), 16% glycerol, 3.2% SDS and 64 mmol/l dithiothreitol] for 5 min and Western blotted for NOS-3 as described previously [14]. Bands thus revealed were analysed by scanning densitometry (Pharmacia ImageMaster, version 2.0 software).

Where indicated, membranes were then submerged in stripping buffer [62.5 mM Tris/HCl (pH 6.7), 2% SDS and 100 mmol/l 2-mercaptoethanol] at 50°C for 30 min with gentle agitation. After being washed twice (for 10 min each) in TBS-Tween (TBS containing 0.1 % Tween 20), membranes were blocked overnight in blocking buffer (5 % non-fat dry milk in TBS) at 4°C, followed by incubation for 2 h with one of the following mouse-derived antibodies: anti-(phospho-NOS-3) (Calbiochem-Novabiochem), anti-O-linked N-acetyl-glucosamine (Calbiochem-Novabiochem), anti-CML (a gift from Daniel Ruggiero, Diabetic Microangiopathy Research Unit, Villeurbanne, France) or anti-α-tubulin (Calbiochem-Novabiochem), each diluted 1:10000 in blocking buffer at room temperature. Membranes were then washed extensively in TBS-Tween, followed by incubation for 30 min at room temperature with goat anti-mouse HRP (horseradish peroxidase)-conjugated IgG (Dako) diluted 1:10000 in TBS-Tween containing 5 % non-fat dry milk. The membranes were then washed, developed and scanned as before.

**Statistical analysis**

All results are presented as means ± S.E.M. of six experiments. Data were analysed by repeated-measures ANOVA, using StatView version 5.0.1, with post-hoc testing using Tukey’s test. Statistical significance was taken as P < 0.05 (two-tailed).

**RESULTS**

**Effect of glucose and AGE-modified albumin on HUVEC NOS activity**

NOS activity was determined in HUVECs following incubation with physiological (5 mmol/l) or high (25 mmol/l) glucose concentrations, or with different concentrations of AGE-modified albumin or unmodified albumin (0–200 mg/l), for different times (between 3 h and 12 days for glucose, and between 3 and 48 h for AGE-modified albumin and unmodified albumin). NOS activity was determined from the rate of conversion of L-[³H]arginine into L-[³H]citrulline and was corrected for protein content. NOS activity was not different in cells treated with either high or low glucose, or for different time periods (Figures 1A and 1B). By contrast, AGE-modified albumin inhibited HUVEC NOS activity as early as 3 h in a concentration-dependent fashion, and the inhibitory effect increased as a function of time.
Indeed, AGE-modified albumin inhibited both basal NOS activity and the increase in NOS activity in response to stimulation with $10^{-5}$ mol/l histamine in a concentration- and time-dependent fashion, whereas unmodified albumin did not affect either basal or histamine-stimulated NOS activity (Figures 1C and 1D).

**Effect of glucose and AGE-modified albumin on NOS-3 protein expression in HUVECs**

NOS-3 protein expression was determined in HUVECs by Western blotting following incubation for different time periods with physiological (5 mmol/l) or high (25 mmol/l) glucose concentrations, or with different concentrations of AGE-modified albumin or unmodified albumin (0–200 mg/l). NOS-3 expression was not different in cells exposed to low or high glucose for 3 or 12 days (Figure 2A), or cells incubated with unmodified albumin (200 mg/l) for 48 h (Figure 2B). By contrast, AGE-modified albumin incubation resulted in a decreased expression of NOS-3, which was concentration- and time-dependent, but was only evident after 24 h of incubation (Figures 2C–2F). No change was seen in the expression of the housekeeping protein α-tubulin with these incubations (results not shown).

NOS-3 undergoes post-translational O-glycosylation, which has been reported previously [15] to be increased with a reciprocal decrease in serine phosphorylation at residue 1177 of NOS-3 in bovine aortic endothelial cells treated with high glucose concentrations for 2 days. We therefore wished to determine whether similar changes occur in human endothelial cells in response to high glucose. Therefore NOS-3 was immunoprecipitated from HUVECs following incubation for 3 or 12 days in physiological (5 mmol/l) or high (25 mmol/l) glucose concentrations, and the immunoprecipitates were immunoblotted for O-linked N-acetylglucosamine and for phosphoserine. A band at 135 kDa was identified, corresponding to O-glycosylated NOS-3 (Figure 3A), but the degree of O-glycosylation was not different between the two concentrations of glucose or the two time points studied (Figure 3B). Likewise, we determined the degree of serine phosphorylation of immunoprecipitated NOS-3 following incubation with low or high glucose for 3 and 12 days. Although a band was again detected
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Figure 2 Effects of glucose, unmodified albumin and AGE-modified albumin on NOS-3 protein expression in HUVECs

NOS-3 expression is shown in HUVECs treated with (A) physiological (5 mmol/l) or high (25 mmol/l) concentrations of glucose for 3 or 12 days, (B) unmodified albumin (200 mg/l) or corresponding vehicle for 48 h, (C) AGE-modified albumin (AGE-albumin) at different concentrations (0–200 mg/l) for 48 h, and (D) AGE-modified albumin at a fixed concentration of 200 mg/l for different time periods (0–48 h). Results are shown as means ± S.E.M. of six experiments in each case. **P < 0.001 compared with control (zero concentration or time). ##P < 0.05 and ###P < 0.01 compared with the previous concentration or time point. (E) A representative immunoblot showing the effect of different concentrations of AGE-modified albumin on NOS-3 expression at the 48 h time point. Lane 1, 200 mg/l unmodified albumin; lane 2, vehicle; lanes 3–5, 50, 100 and 200 mg/l AGE-modified albumin respectively. (F) A representative immunoblot showing the effect of 200 mg/l AGE-modified albumin incubated for different times on NOS-3 expression. Lane 1, 200 mg/l unmodified albumin for 48 h; lanes 2–7, AGE-modified albumin incubated for 0, 3, 6, 12, 24 and 48 h respectively.

at 135 kDa (Figure 3C), corresponding to NOS-3, the degree of serine phosphorylation was not different between the two concentrations of glucose or the two time points studied (Figure 3D).

We also determined whether incubation of HUVECs with high glucose or with AGE-modified albumin gave rise to AGE modification of NOS-3. For this purpose, following 3 or 12 day incubations of HUVECs with 25 mmol/l glucose or 200 mg/l AGE-modified albumin, NOS-3 was immunoprecipitated and the immunoprecipitates were probed for CML using an antibody which we have characterized previously [14] as being highly specific for CML-modified residues. Under these conditions, no CML band was identified (results not shown).

DISCUSSION

The principal finding from the present study is that AGEs have an important inhibitory effect on endothelial cell NOS activity and expression, which is concentration- and time-dependent, whereas glucose itself has little, if any, effect on endothelial NOS. We have demonstrated previously [14] that the effects of AGE-modified albumin, prepared as described in the present study, on HUVEC NO biosynthesis are mediated specifically by CML moieties in the macromolecule (as demonstrated by blockade of the effects using an anti-CML antibody) and occur through interaction with the endothelial AGE-R1 receptor. In the present experiments, AGE-modified albumin was used at concentrations which are found in the circulation of patients with diabetes, as described previously [14]. Endothelial dysfunction, particularly with respect to NO biosynthesis, is a universal finding in both Type I and Type II diabetes [1–4], and this may be involved in the pathogenesis both of micro- and macro-vascular disease [5], the most important complications of diabetes in humans. Our present study suggests that AGEs, but not glucose, may have an important aetiological role in diabetes-associated endothelial dysfunction. In patients with diabetes, blood glucose levels may vary widely in the short term, depending on a variety of factors, including eating patterns, drug therapy and exercise levels. By contrast, AGEs are relatively long-lived macromolecules, whose levels reflect long-term glycaemic control, and therefore provide an
important mechanism whereby such long-term glycaemic control, as opposed to short-term fluctuations in blood glucose, can have important effects on endothelial NO generation. Although our approach was an in vitro study, the results have important implications for the pathogenesis of diabetic vasculopathy in vivo.

In the present study, the effects of AGE-modified albumin on HUVEC NOS activity and expression have been studied at different times up to 48 h, whereas those of high glucose were studied up to 12 days. This is because, in initial experiments, no effect of high glucose was seen up to 2–3 days; we therefore wished to examine the time course of any effects of high glucose for longer time points. By contrast, the effects of AGE-modified albumin were seen in the much shorter term, and we have shown previously [14] that incubation of HUVECs with AGE-modified albumin for 3 days or longer results in a significant degree of cell toxicity and death. It has previously been reported by Cellek et al. [15] that AGEs can act synergistically with endogenous NO to cause apoptosis in cultured human neuroblastoma (SH-SYSY) cells at time points ranging from 24–72 h; however, in HUVECs, we have found that AGE-modified albumin incubation for 48 h, at the concentrations used in the present experiments, caused no detectable cytotoxicity, as assessed by Trypan Blue exclusion [14]. This difference may be attributable in part to differences in apoptotic response to AGE between neuroblastoma and endothelial cells in culture; additionally, however, the AGE concentrations used by Cellek et al. [15] were considerably greater than those used in our experiments.

We have demonstrated previously [14] that short-term (30 min) incubation of HUVECs with AGE-modified albumin at these concentrations gives rise to a decrease in NOS activity associated with a decrease in NOS-3 serine phosphorylation, but no change in NOS-3 expression. Our present results show that, over the longer term, NOS-3 protein expression is also decreased in HUVECs incubated with AGE-modified albumin, and this gives rise to a progressive further decrease both in basal and stimulated NOS activity in these cells. This decrease in NOS-3 expression and activity in response to AGE-modified albumin cannot be attributed simply to AGE-induced cytotoxicity, since these experiments were performed over a 48 h time period, whereas AGE-modified-albumin-induced HUVEC toxicity (at the same concentrations of AGE-modified albumin) is only manifest after 72 h [14].

Other workers have reported previously [16] that elevated glucose concentration gives rise to a suppression of NOS activity, together with an increase in NOS-3 O-glycosylation and a reciprocal decrease in its phosphorylation at Ser1177 in cultured bovine aortic endothelial cells. We therefore wished to ascertain whether this effect also occurs in HUVECs. Our data suggest that, in fact, high glucose has no effect on NOS activity, nor does it affect NOS-3 expression, O-glycosylation or serine phosphorylation, even up to 12 days of incubation. These results need to be replicated in other human endothelial cell lines, but they suggest that effects of glucose on endothelial cell NOS may vary from one species/vascular bed to another. Additionally, in our experiments, the endothelial cells were exposed to high glucose at lower passage number than in reports published previously, at which stage their phenotype would be expected to be closer to that found in vivo.

Srinivasan et al. [17] have reported previously that exposure of cultured aortic endothelial cells, either from mice or humans, to high glucose at similar concentrations to those used in the present study gives rise to a reduction in endothelial cell expression of NOS-3, results which are at variance with the data in the present study in HUVECs. Although this may reflect differences between the effect of glucose in aortic endothelial cells and HUVECs, it is also likely that disparities in the conditions used in the
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experiments are responsible. In the study by Srinivasan et al. [17], aortic endothelial cells were used at passages 3–5, and it is unclear at what stage of confluence the aortic endothelial cells were exposed to high glucose; in the present study, HUVECs were used at earlier passage (passage 2), and all incubations with high glucose (as well as with AGE-modified albumin) were commenced when cells were approx. 50% confluent, since we have observed a significant degree of cell attrition and death if incubations with high glucose are performed in cells approaching full confluency.

Defective generation of NO by the vascular endothelium in diabetes has been attributed to a variety of causes. In the first place, both Type I and Type II diabetes are associated with an increase in oxidative stress in vivo [6–9]; this will result in an increase in NO scavenging by the superoxide anion. Indeed, both glucose and AGEs can induce an increase in generation of reactive oxygen species [18–27]. However, oral antioxidant vitamin therapy reverses endothelial dysfunction to only a small degree in vivo [10], suggesting that the main defect is at the level of NO biosynthesis.

It has been demonstrated previously [28,29] that LDL (low-density lipoprotein) particles are qualitatively different in diabetes, such that LDL from diabetic patients inhibits endothelial function in isolated rabbit aortic rings to a greater degree than the same concentration of LDL from healthy controls. Indeed, we have shown [30] that LDL from diabetic patients exhibits an increase in both glycation and oxidation over that from controls, and that it inhibits NOS activity in cultured HUVECs to a greater degree than LDL from controls. Thus glycoxidized LDL, as is found in the context of diabetes, may contribute to endothelial dysfunction in this disease.

It is likely that endothelial dysfunction in diabetes is multifactorial in origin. Partly it may relate to oxidative stress and the effect of glycated LDL. Indeed, Brownlee [32,33] has proposed that four main molecular mechanisms, namely increased polyol pathway flux, increased hexosamine pathway flux, protein kinase C activation and increased AGE formation, may underpin diabetes-related vascular disease, and all of these mechanisms are triggered by overproduction of reactive oxygen species by the mitochondrial electron transport chain [31–33]. The present results suggest that AGEs contribute significantly to diabetes-associated endothelial dysfunction, thereby supporting their importance as a critical initiating factor in diabetic vasculopathy. Indeed, prevention of AGE formation or action, or therapy to break AGE cross-links, have been shown previously to ameliorate endothelial dysfunction and, in fact, to retard atherosclerosis and to slow the progression of other complications of diabetes in experimental models [34–37]. Our results suggest that, also in humans, prevention or reversal of AGE formation may be of therapeutic benefit in diabetes. Indeed, several agents which interfere with the formation of AGE or AGE precursors bind to tissue AGE receptors, or promote breakdown of AGEs are currently undergoing clinical study in humans with diabetes. Early data suggest that inhibition of AGE formation with pimagedine exerts beneficial effects on the progression of retinopathy and nephropathy in this disease [38]. Whether such therapy leads to an associated improvement in endothelial NO generation remains to be determined.

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REFERENCES


