\(\alpha\)-Lipoic acid reduces expression of vascular cell adhesion molecule-1 and endothelial adhesion of human monocytes after stimulation with advanced glycation end products

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ABSTRACT

Advanced glycation end products (AGEs) have been identified as relevant mediators of late diabetic complications such as atherosclerotic disease. The endothelial migration of monocytes is one of the first steps in atherogenesis and monocyte–endothelial interaction itself is linked to the expression of adhesion molecules like vascular cell adhesion molecule-1 (VCAM-1). Recently, stimulation of VCAM-1 by AGEs has been demonstrated. Since endothelial stimulation by AGEs is followed by generation of oxygen free radicals with subsequent activation of nuclear transcription factor \(\kappa\)B, we investigated the influence of \(\alpha\)-lipoic acid on the expression of VCAM-1 and monocyte adherence to endothelial cells in vitro by means of cell-associated chemiluminescence assays and quantitative reverse transcriptase polymerase chain reaction using a constructed recombinant RNA standard. We found that \(\alpha\)-lipoic acid was able to decrease the number of VCAM-1 transcripts from 41.0 ± 11.2 to 9.5 ± 4.7 RNA copies per cell in AGE-stimulated cell cultures. Furthermore, expression of VCAM-1 was suppressed in a time- and dose-dependent manner by \(\alpha\)-lipoic acid as shown by chemiluminescence endothelial cell assay. Pretreatment of endothelial cells with 0.5 mM or 5 mM \(\alpha\)-lipoic acid reduced AGE-induced endothelial binding of monocytes from 22.5 ± 2.9% to 18.3 ± 1.9% and 13.8 ± 1.8% respectively. Thus, we suggest that extracellularly administered \(\alpha\)-lipoic acid reduces AGE-albumin-induced endothelial expression of VCAM-1 and monocyte binding to endothelium in vitro. These in vitro results may contribute to the understanding of a potential antioxidative treatment of atherosclerosis.

INTRODUCTION

Advanced glycation end products (AGEs) are believed to play an important role in micro- and macrovascular complications of diabetes mellitus [1]. They have been characterized as irreversible products of a slow and complex reaction of the aldehyde or keto group of sugars with the terminal amino group of proteins [2]. Several

Key words: advanced glycation end products, \(\alpha\)-lipoic acid, vascular cell adhesion molecule-1.
Abbreviations: AGE, advanced glycation end product; DMEM, Dulbecco's modified Eagle's medium; HUVEC, human umbilical vein endothelial cell; mAb, monoclonal antibody; NF-\(\kappa\)B, nuclear transcription factor \(\kappa\)B; RLU, relative light units; RT–PCR, reverse transcriptase–polymerase chain reaction; VCAM-1, vascular cell adhesion molecule-1.
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studies have demonstrated that elevated levels of AGEs are found in the serum of patients with diabetes [3], and in patients with renal failure or receiving haemodialysis treatment [4,5]. The cellular effect of AGEs is mediated by specific receptors, and one of these, the receptor for AGE (RAGE), has been identified on endothelial cells, monocytes/macrophages, mesangial cells, neurons and smooth muscle cells [6–11]. Binding of AGEs to this receptor induces the generation of oxygen free radicals [12,13] which activate nuclear transcription factor κB (NF-κB). Activated NF-κB induces regulation of many genes after its translocation to the nucleus. For example, expression of vascular cell adhesion molecule-1 (VCAM-1) is regulated by NF-κB and therefore, intracellular oxidative stress induced by AGEs seems to be very important for the understanding of the pathogenesis of vascular disease in diabetes mellitus [6,13].

There are a lot of important intracellular antioxidant defence mechanisms like the glutathione redox system, the vitamin C/vitamin E cycle and the z-lipoic acid/dihydrolipoic acid redox pair. Impairment of these defence mechanisms in diabetes mellitus has been described by several authors [14,15]. Furthermore, the ability of z-lipoic acid, a cofactor in the z-ketoglutarate dehydrogenase complex, to reduce lipid peroxidation, to quench radicals, to regenerate vitamins C and E (in its reduced form, dihydrolipoic acid), to increase intracellular levels of glutathione and to prevent glycation of serum albumin has been well documented [16–22].

The aim of this study was to characterize the influence of antioxidative treatment with z-lipoic acid on the AGE-mediated expression of VCAM-1 in cultured endothelial cells and, as the functional task of the study, on the endothelial adhesion of human monocytes in vitro after stimulation with AGEs.

**Preparation of AGE-BSA**

The method of preparation of AGE-BSA has been published many times (see [6,13]). Briefly, BSA (Sigma Aldrich) was incubated in PBS with 0.5 M glucose at 37 °C for 6 weeks with 2 μg/ml PMSF, 2.5 mM EDTA and antibiotics as described above. The sample was dialysed against PBS and AGE-BSA was identified by fluorescence spectrometry. The identity of AGE-BSA has been checked by spectrofluorometry. The presence of endotoxin was excluded by a *Limulus polyphemus* assay (Sigma Aldrich). In addition, experiments were performed with commercial glycated albumin (Sigma Aldrich) in equal concentrations. There were no significant differences between commercial and newly synthesized glycated albumin concerning the expression of endothelial adhesion molecules. The experiments reported below were performed with the synthesized glycated albumin.

**Chemiluminescence endothelial cell assay**

The second passages of HUVECs were cultured in microtitre plates coated with gelatin. After the different treatment procedures, cells were fixed with methanol/ethanol (2:1, v/v) for 20 min. Incubation with VCAM-1 mAbs (mouse anti-human, Immunotech, Hamburg, Germany) for 2 h at a concentration of 400 ng/ml and washing steps with PBS were performed. After a second incubation with a chemiluminescent-labelled secondary antibody (flashlight-GxMlgG; Biotrend, Köln, Germany) at a concentration of 25 ng/ml, the photonic emission (relative light units) was measured in a luminometer (EG & Bertold, Bad Wildbad, Germany) and analysed after subtraction of the photonic emission of non-specific binding.

**Adhesion assays**

Blood was drawn from 10 healthy donors and anticoagulated with 5000 i.u. of heparin. First, each blood sample was diluted with an equal volume of PBS. Mononuclear cells were obtained by density gradient centrifugation using Ficoll (Pharmacia). The interface was collected and washed twice with Dulbecco’s modified Eagle’s medium (DMEM). These cells were then incubated in DMEM containing 20% fetal calf serum) with magnetizable polystyrene beads (20×10⁴ beads/ml for 30 min; Dynal, Hamburg, Germany) coated with a primary mAb specific for the CD14 membrane antigen. The cell suspension was placed in a magnetic separator rack (Dynal) and washed twice in DMEM/20% fetal calf serum. Cell number was calibrated using a PC-based cell counter system (Casy 1, Schärfe Systems, Freiburg, Germany). The purity of monocytes achieved by this method was > 98% as documented by microscopical counting after Giemsa staining. The second passages of HUVECs were grown to confluence in microtitre plates coated with gelatin.

### MATERIAL AND METHODS

#### Endothelial cell culture

Human umbilical vein endothelial cells (HUVECs) were isolated according to the method of Jaffé and co-workers [23] by perfusion of the human umbilical veins with 0.1% collagenase for 20 min. The harvested cells were washed with medium 199 and plated into 25-ml flasks coated with gelatin. Cells were cultured in medium 199 containing 20% (v/v) fetal calf serum (Greiner, Frickenhausen, Germany), 5 mg/ml endothelial growth supplement (Sigma Aldrich, Deisenhofen, Germany), 100 units/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Eggenstein, Germany). Purity of endothelial cells was checked by indirect immunofluorescence microscopy using a monoclonal antibody (mAb) against von Willebrand factor. Only early passages were used for subsequent studies.
After co-incubation with the monocytes (100,000 per cm², 45 min), cells were fixed with methanol/ethanol (2:1) for 20 min and blocked with 1% blocking reagent (Boehringer Mannheim, Germany) for 30 min. Incubation with mAbs (mouse anti-human) against pan-leucocyte membrane antigen CD45 (Immunotech) for 2 h at a concentration of 400 ng/ml and washing steps with PBS were performed. After a second incubation with a chemiluminescent-labelled secondary antibody (flashlight-GxMlg; Biotrend) at a concentration of 25 ng/ml, the photonic emission (relative light units) was measured in a luminometer (EG & Bertold, Bad Wildbad, Germany). Percentage of bound monocytes was analysed according to the photonic emission of a standard curve of chemiluminescent-labelled monocytes. Scanning electron microscopy was performed according to standard procedures.

Quantitative reverse transcriptase–polymerase chain reaction (RT–PCR)

Endothelial cells were harvested by digestion with 0.05% trypsin–EDTA and the cell number was determined in a cell counter (Casy 1, Schärfe Systems). RNA was isolated using silica-gel-based membranes (Qiagen, Santa Clarita, CA, U.S.A.) and its concentration was measured photometrically (Pharmacia Biotech, Freiburg, Germany).

The internal standard was constructed by synthesizing (Pharmacia, Freiburg, Germany) two oligonucleotides (‘triple primers’) of approximately 60 bases containing sequences for the T7 promoter, the target gene (VCAM-1), the spacer gene (glutathione transferase-GSTM4 gene) and a poly(dT) tail. The recombinant RNA forward primer contained the T7 promoter sequence, the VCAM-1 forward (5'-TAATACGACTCACTATAGGCGGGGAGCTACAGCCTCTTT-3') and reverse primer (5’-CTGTGTCTCTCGGTCTCCGGCT-3’), 1.25 mmol of MgCl₂ and 2.5 units of Taq DNA polymerase.

After agarose gel (1.5% plus ethidium bromide) electrophoresis, quantification by PC-based densitometry was performed. The calculated amount of specific mRNA was related to the cell number in order to determine the number of RNA copies per cell.

Scanning electron microscopy

Fixation, dehydration, critical-point drying, and sputtering (polaron equipment) of the electron microscopy samples were performed according to standard methods with subsequent analysis by a stereoscan MK 250 (Cambridge) electron microscope.

Statistical analysis

Values are expressed as means±S.D. The statistical significance was assessed by non-parametric analysis (Mann–Whitney U-test).

RESULTS

Since AGEs have been reported to induce oxygen free radicals after binding to their receptor [12,13], we investigated whether α-lipoic acid was able to influence the NF-κB-related expression of VCAM-1 on human endothelial cells after stimulation with AGE-BSA. HUVECs were pretreated with α-lipoic acid in various concentrations (0.05–10 mM) and with different pre-incubation periods (0–24 h). Endothelial expression of VCAM-1 antigen was assessed by a chemiluminescence assay [photonic emission of bound mAbs was measured over a period of 5 s and defined as relative light units (RLU)]. Untreated HUVECs revealed 66.7±14.5 RLU (Figure 1) whereas the photonic emission of AGE-BSA-stimulated cells (1 μM; 5 h) was raised to 1014.0±179.0 RLU (P < 0.0001). Pretreatment of HUVECs with 10 mM α-lipoic acid suppressed VCAM-1 expression to baseline levels independent of the duration of antioxidative treatment. A preincubation period of 24 h was required to suppress VCAM-1 in endothelial cells if α-lipoic acid was used at a concen-
HUVECs were stimulated with 1 μM AGE-BSA for 5 h (A). Antioxidative treatment with 10 mM, 5 mM, 0.5 mM or 0.05 mM α-lipoic acid started 0–24 h before AGE stimulation. Untreated HUVECs (C) served as a control. The time- and dose-dependent influence of α-lipoic acid is apparent. Statistical analysis was assessed by non-parametric analysis (Mann–Whitney U-test).

Figure 2  Quantitative RT–PCR for VCAM-1 by competition of the target sequence (T) with a recombinant RNA standard (S)
Each lane represents the competition of an aliquot of the RNA sample (lower row: 205 bp) with a dilution of the recombinant RNA standard (upper row: 165 bp). The dilution factor of the standard (895 x 10⁸ copies) is shown above each lane. An increasing number of VCAM-1 transcripts shifts the intensity of target sequence (T) products (lower row) to the right because of better quantitative competition with the following higher concentration of standard (S).

Quantitative RT–PCR analysis using an internal recombinant RNA standard was performed in order to quantify the single cell-associated number of VCAM-1.

Nevertheless, even if 5 mM α-lipoic acid was administered simultaneously with AGE-BSA, the VCAM-1 signal decreased to 233.1 ± 27.8 RLU (P < 0.0001). The simultaneous treatment of HUVECs with 0.5 mM α-lipoic acid did not decrease the expression of VCAM-1 antigen significantly (912.5 ± 106.9 RLU), but longer pre-incubation periods were able to definitely attenuate the VCAM-1 response of HUVECs to AGE-BSA. The lowest concentration of α-lipoic acid (0.05 mM) used in this study failed to affect VCAM-1 antigen presentation. It should be emphasized that the influence of α-lipoic acid on VCAM-1 increased constantly in a time-dependent manner over 24 h without revealing a maximum value.

Quantitative RT–PCR analysis using an internal recombinant RNA standard was performed in order to quantify the single cell-associated number of VCAM-1.
Table 1  Densitometric analysis of the recombinant RNA standard and the VCAM-1 target sequence of three different samples: untreated HUVECs (control), HUVECs stimulated with 1 μM AGE-BSA and HUVECs treated with 0.5 mM α-lipoic acid for 6 h before AGE-BSA stimulation

<table>
<thead>
<tr>
<th>Control</th>
<th>AGE-BSA (1 μM)</th>
<th>AGE-BSA (1 μM) + α-lipoic acid (0.5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
<td>Target</td>
<td>Standard</td>
</tr>
<tr>
<td>1167</td>
<td>0</td>
<td>1176</td>
</tr>
<tr>
<td>1190</td>
<td>0</td>
<td>616</td>
</tr>
<tr>
<td>559</td>
<td>174</td>
<td>42</td>
</tr>
<tr>
<td>0</td>
<td>543</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2  Influence of α-lipoic acid on VCAM-1 transcripts after stimulation with AGE-BSA

Quantitative RT–PCR analysis of VCAM-1 transcripts was performed in 28 samples (control: n = 12; AGE-BSA: n = 8; AGE-BSA + α-lipoic acid: n = 8) or 140 competitive PCR products respectively. HUVECs were treated with 1 μM AGE-BSA for 45 min with or without preincubation of 0.5 mM α-lipoic acid for 6 h. Unstimulated HUVECs served as a control.

<table>
<thead>
<tr>
<th></th>
<th>VCAM-1 RNA copies/cell</th>
<th>P value (ct. AGE-BSA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.3 ± 3.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>AGE-BSA (1 μM)</td>
<td>41.0 ± 11.2</td>
<td>—</td>
</tr>
<tr>
<td>AGE-BSA (1 μM) + α-lipoic acid (0.5 mM)</td>
<td>9.5 ± 4.7</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

Figure 3  Scanning electron microscopy of a HUVEC monolayer after co-incubation with separated monocytes under control conditions

transcripts. Representative samples are shown after agarose gel electrophoresis (Figure 2) or densitometric analysis (Table 1). The analysis of 28 samples, i.e. 140 competitive RT–PCR reactions (Table 2), detected 4.3 ± 3.1 copies/cell in untreated HUVECs. The VCAM-1 transcription was increased to 41.0 ± 11.2 copies/cell after stimulation with AGE-BSA for 45 min. According to the results of the chemiluminescence assays of VCAM-1 antigen, the amount of detectable VCAM-1 transcripts was reduced after pretreatment of HUVECs with 0.5 mM α-lipoic acid for 6 h (9.5 ± 4.7; P < 0.0001).

It was a special interest of this study to characterize the functional impact of antioxidative down-regulation of VCAM-1 on monocyte adhesion to endothelium in vitro. Human monocytes were purified using mAbs against the CD14 membrane antigen and co-incubated with confluent endothelial monolayers (Figures 3 and 4). Chemiluminescence analysis revealed that monocyte binding to AGE-BSA-stimulated HUVECs increased to
22.5 ± 2.9% versus 11.2 ± 1.4% in controls (Figure 5). In order to examine the influence of antioxidative treatment on the adhesion of monocytes, HUVECs were incubated with 0.5 mM or 5 mM \( \alpha \)-lipoic acid 6 h before stimulation with AGE-BSA. The percentage of bound monocytes decreased to 18.3 ± 1.9% (\( P = 0.01 \)) and 13.8 ± 1.8% (\( P < 0.0001 \)) respectively.

**DISCUSSION**

Atherosclerotic diseases like coronary heart disease are the major cause of morbidity and mortality in diabetes mellitus [24,25]. Atherosclerosis itself is characterized by focal thickening of the intima of arteries and it is well known that more than 50% of the cells in the lipid core of atherosclerotic plaques are derived from monocytes [26]. Furthermore, the migration of monocytes in the arterial wall is an early step in the pathogenesis of atherosclerosis [27]. This recruitment of monocytes is related to the expression of leucocyte-specific integrins and endothelial adhesion molecules like E-selectin, intercellular adhesion molecule-1 (ICAM-1) or VCAM-1 [28–31]. Although there is widespread agreement that development of atherosclerosis is accelerated in both Type 1 and Type 2 diabetes mellitus, the mechanisms leading to increased monocyte binding to endothelium in diabetes are only partially known. A number of potential mechanisms have been described that may explain the increase in monocyte accumulation in diabetic vessels. For example, increased levels of diacylglycerol due to enhanced glycolysis in hyperglycaemia activate protein kinase C which influences vascular functions and haemodynamic changes in diabetes [32,33]. Furthermore, glycosylation of low-density lipoproteins has been shown to result in increased oxidation, and oxidized low-density lipoprotein stimulates endothelial cells to bind monocytes [34]. In recent years, many groups have focused on the influence of AGEs and oxidative stress on the development of diabetic macrovascular disease [2,35–39]. AGEs result from non-enzymatic glycation of proteins or lipids, initially forming reversible early glycation products (Schiff bases and Amadori products). These early glycation products can undergo further complex molecular rearrangements and become irreversible AGEs [2]. AGEs are found in the plasma and accumulate in the extracellular matrix of the vessel wall in diabetes [2,3]. The early discovery of a macrophage receptor system for the internalization and degradation of AGEs began the speculation about receptor-mediated interactions [40]. Receptors for AGEs (RAGEs) have been characterized on several cell types and it is known that their binding to RAGE induces oxidative stress with subsequent radical-dependent activation of NF-\( \kappa \)-B [13]. Activated NF-\( \kappa \)-B is translocated to the nucleus and initiates changes in vascular homoeostasis and endothelial dysfunction due to its ability to promote synthesis of defence and signalling proteins [41]. Although this study has only been performed with AGE-BSA, convincing data have been published which demonstrate that other free-radical generator systems can also stimulate the expression of VCAM-1 [42,43]. Under physiological conditions, free radicals are rapidly eliminated by antioxidative defence mechanisms, e.g. the glutathione redox system, the vitamin C/vitamin E cycle and the \( \alpha \)-lipoic acid/dihyrolipoic acid redox pair [14,15]. In this context, studies demonstrating the impairment of antioxidative systems [37,38] in diabetes mellitus are of considerable interest.

Since AGEs have been shown to induce NF-\( \kappa \)-B activation and expression of VCAM-1 [6], the potential influence of \( \alpha \)-lipoic acid on transcription and expression of VCAM-1 has been investigated in this study using HUVECs. Even if HUVECs never undergo atherosclerotic processes they are a common source of research on atherosclerosis because of their comparable behaviour concerning the expression of adhesion molecules. We were able to demonstrate a time- and dose-dependent effect of \( \alpha \)-lipoic acid on the expression of VCAM-1 antigen in cultured endothelial cells by means of a cell-associated chemiluminescence assay. The highest concentration of \( \alpha \)-lipoic acid (10 mM) suppressed VCAM-1 presentation even when administered simultaneously with AGE-BSA. Furthermore, the results of the experiments performed with 5 mM and 0.5 mM \( \alpha \)-lipoic acid revealed that the increase of the suppression of VCAM-1 response to AGE-BSA continued over time and was not saturable. This does not correlate with the findings of Bierhaus et al. [13] who demonstrated that the suppressing effect of \( \alpha \)-lipoic acid on NF-\( \kappa \)-B activation was reduced after a preincubation period of 8 h. They discussed the loss of \( \alpha \)-lipoic acid inhibitory capacity on the basis of metabolic degradation. However, Bierhaus et al. were working with bovine aortic endothelial cells whereas we performed our studies with human umbilical vein endothelial cells. Thus, the experimental setting is not comparable. It is noteworthy that Bierhaus et al. were able to show that \( \alpha \)-lipoic acid inhibits translocation of NF-\( \kappa \)-B after its inhibitory protein I\( \kappa \)-B has been phosphorylated. They speculate that \( \alpha \)-lipoic acid exerts its inhibitory effect a step behind phosphorylation, e.g. by acting on the phosphorylated NF-\( \kappa \)-B/I\( \kappa \)-B complex or the proposed I\( \kappa \)-B-protease [13].

We constructed a recombinant internal RNA standard according to the method described by vanden Heuvel et al. [44] in order to measure the expression of VCAM-1-mRNA in endothelial cells by means of quantitative RT–PCR which has become a valuable technique for detection and quantification of mRNA levels, especially for products of immunological interest [45]. The number of VCAM-1 transcripts increased from 4.3 ± 3.1 to 41.0 ± 11.2 copies per cell after stimulation with AGE-
vascular disease.

To validate this finding in vivo, we stimulated monocytes in vitro. Increased binding of monocytes in vitro is found in leucocytes isolated from patients with Type 1 and Type 2 diabetes and has been associated with the degree of hyperlipidaemia and hyperglycaemia ([46,47]; T. Kunt, T. Forst, B. Früh, O. Harzer, A. Pfützner, M. Engelbach, H. Löbig and J. Beyer, unpublished work). Furthermore, the effect of AGEs on the endothelial adhesion of monocytes or monocyte-derived cell lines has also been demonstrated [6]. In our study the percentage of bound monocytes increased from 11.2 ± 2.9% in control cultures to 22.5 ± 2.9% in cultures stimulated with AGE-BSA. We were able to show that z-lipoic acid not only decreased the transcription and expression of VCAM-1 but also attenuated the adhesion of monocytes to 18.3 ± 1.9% (0.5 mM z-lipoic acid) and 13.8 ± 1.8% (5 mM z-lipoic acid).

The naturally occurring z-lipoic acid is used in the therapy of diabetic polyneuropathy [19]. Dietary supplied z-lipoic acid is readily absorbed and has been proven to be relatively free of side effects [15]. It has been introduced into polyneuropathy treatment because of its ability to reduce hyperglycaemia-induced neurological failure via improvement of ATP production and energy supply [48]. Nevertheless, neuronal dysfunction in diabetes is also supposed to be mediated by endothelial dysfunction of nutritional capillaries of the nerve fibres [16]. As mentioned already, z-lipoic acid has been demonstrated to be a potent radical scavenger (coenzyme of the pyruvate and z-ketoglutarate dehydrogenase complex) and may therefore contribute to the restoration of oxygen free-radical-related cellular disorders such as endothelial dysfunction due to expression of proteins that are mediated by NF-kB activation, e.g. endothelin-1 or tissue factor [13].

Although these data cannot be extrapolated to in vivo human pathology, our findings have characterized the inhibitory effect of z-lipoic acid transcription on expression of VCAM-1 and on AGE-mediated monocyte adhesion in vitro. Further studies are necessary in order to validate this finding in vivo and the potential role of z-lipoic acid in the treatment of diabetes-associated macrovascular disease.

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