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Advanced glycation end products impair the scavenger function of rat hepatic sinusoidal endothelial cells

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Abstract

Aims/hypothesis. We have previously reported that advanced glycation end products are eliminated from the circulation mainly by scavenger receptor-mediated uptake in hepatic sinusoidal endothelial cells. Our experiments showed that the degradation of AGE-modified protein after endocytosis in hepatic sinusoidal endothelial cells occurs slowly compared with that of other scavenger receptor ligands. The aim of this study was to investigate further the mechanism whereby AGE-modified protein affects the important scavenger function of hepatic sinusoidal endothelial cells.
Methods. Primary cultures of hepatic sinusoidal endothelial cells were pre-incubated with unlabelled ligand, unbound ligand was washed off, and the endocytic capacity was measured by addition of radiolabelled ligand, and immune electron microscopy.
Results. Pre-incubation with unlabelled AGE-modified bovine serum albumin reduced subsequent endocytosis of radiolabelled scavenger receptor ligands AGE-modified bovine serum albumin, formaldehyde-treated serum albumin, oxidized low density lipoprotein and acetylated low density lipoprotein by 50, 56, 32 and 20%, respectively. Non-scavenger receptor-mediated endocytosis was not affected by pre-exposure to AGE-modified protein. Pre-incubation with a number of non-AGE-ligands did not affect subsequent endocytosis via any of the major endocytosis receptors in hepatic sinusoidal endothelial cells. Incubation in fresh medium for 6 h after pre-exposure to AGE-modified protein almost completely restored normal scavenger receptor-mediated endocytic activity. Quantitative immune electron microscopy showed that the amount of a newly described scavenger receptor for AGE-modified protein is reduced after pre-incubation with AGE-modified protein. Subcellular fractionation showed that pre-incubation with AGE-modified protein delays intracellular transport of scavenger receptor ligands.
Conclusion/interpretation. Endocytosis of AGE-modified protein leads to loss of scavenger receptors and delayed intracellular transport in hepatic sinusoidal endothelial cells. [Diabetologia (2002) 45:1379–1388]

Keywords Advanced glycation end products, hepatic sinusoidal endothelial cells, scavenger receptor mediated endocytosis, endocytic capacity, intracellular transport.

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Abbreviations: AcLDL, Acetylated low-density lipoprotein; AGE, advanced glycation end products; β-AGA, β-acetylglucosaminidase; CSPG, chondroitin sulphate proteoglycan; FSA, formaldehyde-treated serum albumin; HA/S-R, hyaluronan-scavenger receptor; HAGG, heat aggregated γ-globulin; HSA, human serum albumin; KC, Kupffer cell; OxLDL, oxidized low-density lipoprotein; PINP and PIIINP, N-terminal propeptides of type I and III procollagen; SEC, hepatic sinusoidal endothelial cell; SR, scavenger receptor; TCA, trichloroacetic acid; TC, tyraminyl cellobiose.

Hepatic sinusoidal endothelial cells (SEC) constitute the most important cellular site for elimination of soluble waste macromolecules from the circulation of terrestrial vertebrates [1, 2]. The waste product endocytosis receptors expressed by mammalian liver SEC are divided into at least five types according to specificity of ligand binding: the hyaluronan receptor [3, 4], the mannose receptor [5, 6], the collagen α-chain receptor [7], the Fc-γ-receptor [8], and the scavenger receptor (SR). The latter receptor eliminates physiological waste molecules such as N-terminal propeptides of types I (PINP) and III (PIIINP) procollagen [9], and nidogen [10] from the circulation. Proposed atherogenic ligands such as oxidized LDL (OxLDL) [11] and AGE [12] are also cleared by the SR of SEC. Although the presence of SR class A has been established in SEC [13, 14], the uptake of SR ligands in SEC of mice that are genetically deficient in SR class A proceeds normally [15, 16], suggesting that SRs different from SR class A are important for the SEC SR function. A polyclonal antibody raised against a hyaluronan binding protein in rat liver, was also shown to inhibit the endocytosis of SR-ligands in SEC in vitro, indicating that the hyaluronan receptor of SEC is functionally related to the SR [17]. Since the characterization and cloning of SR class A types I and II [18, 19], several other SRs have been identified, and a family of SRs, termed SR class A, B, C, D, E and F, has been identified. SRs are involved in cell adhesion, lipoprotein metabolism and host defence [20, 21, 22, 23, 24].

Advanced glycation end products are compounds formed by non-enzymatic irreversible binding of glucose and reactive dicarbonyl species to body proteins. Long-term incubation of proteins with glucose leads to AGE which are characterized by fluorescence, brown color, and intra- and intermolecular cross-linking. Under pathological conditions, such as diabetes, ageing, atherosclerosis and Alzheimer's disease, accumulation of AGE-modified proteins can lead to tissue damage through a variety of mechanisms. AGE receptors are found in a number of different cell types [25, 26], and include the receptor for AGE (RAGE) [27], galectin 3 [28, 29], lactoferrin-like AGE-binding protein [30, 31], SR class A [32, 33], 80K-H and oligosaccharyltranferase-48 [34, 35], CD 36 [36], and SR class B type I [37]. Until recently little was known about clearance of AGE from the body. It has been shown that AGE protein is efficiently cleared from the circulation by hepatic non-parenchymal cells [38]. In line with this finding, it was demonstrated that intravenously injected AGE protein was taken up in hepatic SEC and to a lesser extent Kupffer cells (KC) [12]. Experiments in our laboratory showed that the degradation of AGE-protein after endocytosis in SEC occurs slowly compared with that of other SR-ligands. Our aim was to investigate further the mechanism whereby AGE protein affects the important scavenger function of SEC.

Materials and methods

Materials. $Na^{125}I$, Sephadex G-25 (PD-10), γ-globulin, Gelatin Sepharose 4B, Healon (hyaluronan), and Percoll were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). Fibronectin was purified from human plasma on a column of Gelatin Sepharose 4B as described by the manufacturer. Bacterial collagenase and Complete Mini (protease inhibitor cocktail) were from Boehringer Mannheim (Mannheim, Germany); BSA, mannan, cycloheximide, digitonin, MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide), and culture medium, RPMI 1640, supplemented with 20 mmol/l sodium bicarbonate, 0.006% (w/v) penicillin and 0.01% (w/v) streptomycin were from Sigma Chemical (St.Louis, Mo., USA); human serum albumin (HSA) was from Octapharma (Ziegelbrücke, Switzerland); collagen was from Collagen Biomaterials (Palo Alto, Calif., USA); culture dishes were from Falcon (Becton Dickinson, Plymouth, UK). Formaldehyde-treated BSA (FSA) was prepared as described [39]. Heat aggregated γ-globulin (HAGG) was prepared by heating γ-globulin in phosphate buffered saline (PBS) at 63°C for 1 h. Chondroitin sulphate proteoglycan (CSPG), prepared from bovine nasal cartilage, was donated by Dr. H. Pertoft, University of Uppsala, Sweden. LDL (d.=1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of fresh plasma from normolipidaemic subjects after overnight fasting, and dialysed against 0.15 mol/l NaCl/1 mmol/l EDTA pH 7.4. EDTA was removed from the LDL solution by dialysis against PBS. LDL was oxidized in 5 μmol/l $CuSO_4$ for 24 h at 37°C. Oxidation was arrested by chilling on ice, and adding 0.5 mol/l EDTA to a final concentration of 1 mmol/l. Finally, OxLDL was concentrated, and dialysed against 0.15 mol/l NaCl/5 mmol/l EDTA at 4°C. Acetylated LDL (AcLDL) was prepared by chemical modification of LDL with acetic anhydride as described [38].
Immune reagents. AGE-BSA was localized on thawed cryosections using mouse anti-AGE monoclonal antibody, clone no. 6D12 (Kumamoto Immunochemical Laboratory, Kumamoto, Japan), whose major immunological epitope is N^{ε}-(carboxymethyl)lysine [40], and rabbit anti-cow albumin polyclonal antibody (Dako A/S, Glostrup, Denmark). Rabbit anti-SEC hyaluronan-scavenger-receptor (HA-S-R) polyclonal antibody was a kind gift from Dr. P. McCourt, University of Tromsø, Norway. Protein A-gold complexes were purchased from Drs. G. Postuma and J.W. Slot, University of Utrecht, The Netherlands.

Animals. Male Sprague Dawley rats from Harlan (Blackthorn, Bicester, UK) were kept under controlled conditions and fed a standard diet (B & K, Nittedal, Norway) and water ad libitum. The experimental protocols were approved by the Norwegian Ethics Committee for Research on Animals.

Preparation of AGE-BSA. AGE-BSA was prepared exactly as described [38]. Reduced AGE-BSA (rAGE-BSA) was prepared by slowly adding 1/5 volume of 500 mmol/l $NaBH_4$ in 0.5 mol/l NaH_2PO_4/Na_2HPO_4 buffer pH 7.4, and incubated for 1 h at room temperature. Buffer was changed to PBS by use of Vivaspin MWCO 10 kD (Vivascience, Lincoln, UK).

Labelling techniques. Proteins dissolved in PBS were labelled with ^{125}I either by a direct reaction using Iodogen (Pierce Chemicals, Rockford, USA) as described [41] or by conjugating the protein with ^{125}I-tyraminyl cellobiose (TC) [5]. Iodogen was used for ^{125}I-labelling of TC. Radiolabelled protein was separated from free ^{125}I by gelfiltration through a PD-10 column and radioactivity measured in a γ-counter (Cobra II,

Packard, Meriden, USA). The resulting specific radioactivity was approximately 25 kBq/μg protein (directly labelled protein) or 17 kBq/μg protein (^{125}I-TC-labelled proteins). OxLDL and AcLDL were labelled with ^{125}I as described [42] to a specific radioactivity of approximately 17 kBq/μg protein.

Isolation and culture of liver cells. Preparation of pure cultures of functionally intact SEC from a rat liver has been described [43].

Endocytosis studies in vitro. SEC were cultured (1×10^6 cells per culture) in 2-cm^2 wells and maintained in serum-free RPMI 1640 medium at 37°C and 5% CO_2. After washing, cultures were supplied with 200 μl fresh medium containing 1% (v/v) HSA, inhibitors and trace amounts (30 ng) of radiolabelled proteins. Incubations carried out for 1 h at 37°C to measure endocytosis were terminated by transferring the media, along with one wash (500 μl) with PBS, to tubes containing 20% (w/v) trichloroacetic acid (TCA) (750 μl). TCA precipitates only intact protein or intermediate degradation products of high molecular weight [9]. The extent of degradation was calculated by measuring acid-soluble ligand and presented as percentage of total radioactivity. Cell-associated ligand was quantified by measuring the amount of label solubilized in 1% (w/v) SDS. Endocytosis were defined as the sum of degraded acid soluble radioactivity, and cell associated radioactivity. Experiments with cycloheximide were done by pre-incubation of SEC cultures in medium only, or medium with 100 μg/ml AGE-BSA for 3 h at 37°C. Cells were washed and incubated with 100 μmol/l cycloheximide in medium or medium only for 3 h at 37°C prior to the endocytosis experiment as described. The antibody inhibition study was carried out by pre-incubation of cultured SEC with medium containing IgG from control and immune serum at indicated concentrations for 30 min at 37°C prior to endocytosis experiments as described.

MTT-assay. The cytotoxicity was analysed by an intracellular reduction of a tetrazolium salt (MTT) to a coloured formazan product which is an indicator of cell viability. Cultured SEC (1×10^5 cells per culture) in 0.3-cm^2 wells, were incubated with 100 μmol/l cycloheximide in medium or medium only for 3 h at 37°C, washed, and incubated for 2 h at 37°C with 100 μl MTT-solution (solution of 5 mg/ml was diluted 1:10 in medium). 50 μl of the MTT-solution was removed, and 100 μl stop solution (isopropanol with 0.04 M HCl) was added. The plate was shaken for 30 min, and color was measured with an ELISA reader (Novapath Mini Reader, Biorad, Oslo, Norway) at 570 nm with the reference filter set to 630 nm. All MTT-assays were done in triplicate.

Binding and digitonin experiment. Cultured SEC (3×10^6 cells/culture) in 10-cm^2 wells were pre-incubated for 3 h at 37°C with medium only, or medium containing 100 μg/ml AGE-BSA, hyaluronan or FSA. Cells were washed with cold PBS and chilled before they were allowed to bind radiolabelled ligand for 1 h at 4°C (3 kBq added). Cells were also treated with 0.055% (w/v) digitonin for 10 min at 4°C before incubation with radioactive ligand. Unbound ligand was removed, and cell-associated ligand was quantified by measuring the amount of label solubilized in 1% (w/v) SDS.

Subcellular fractionation. Cultured SEC (5×10^6 cells/culture) in 28-cm^2 dishes were maintained in medium at 37°C. Cultures were supplied with 1.5 ml medium only, or medium with 100 μg/ml AGE-BSA, and pre-incubated for 3 h at 37°C. After washing, the cells were pulsed with trace amounts (17 kBq) of radiolabelled proteins for 1 h at 37°C. The cells were washed, fresh medium added, and incubation continued for another 3 h at 37°C. SEC were scraped off in 0.25 mol/l sucrose with protease inhibitors, sonicated for 5 s, and a postnuclear fraction was prepared by centrifugation at 1300×*g* for 10 min at 4°C. A 4 ml sample was layered on top of a 30 ml linear sucrose gradient (1.10–1.32 g/ml). After centrifugation at 76,000×*g* in a Beckman SW28 rotor for 5 h at 4°C, the gradient was divided into 18×2 ml fractions by upward displacement using Maxidens (Lipotec, Liverpool, UK). The densities of the fractions were calculated from the refractive indices [44]. β-acetylglucosaminidase (β-AGA), a lysosomal marker enzyme, was assayed fluorometrically [45].

Preparation of specimen for electron microscopy. Cultured SEC (3×10^6 cells/culture) in 10-cm^2 dishes, were pre-incubated for 3 h at 37°C with medium only or medium with 100 μg/ml AGE-BSA. The cultures were fixed by adding 8% paraformaldehyde in 200 mmol/l HEPES pH 7.4 and stored at 4°C. Fixed cells were gently scraped off the culture dishes and pelleted in 10% gelatin in a Microfuge E (Beckman, UK). After infusion overnight with 2.3 mol/l sucrose the pellet was mounted on a specimen holder and frozen in lN_2.

Immune electron microscopy. Sections were obtained from frozen pellets of SEC using an Ultracut S Ultramicrotome with an FCS cryo chamber (Leica, Vienna, Austria), and sectioned with a diamond knife (Drukker International, Cuijk, The Netherlands) at –90°C. The sections were retrieved in 2.3 mol/l sucrose and methylcellulose (50/50) [46], and transferred to carbon-coated grids. Immune cytochemical labelling was done as described [47]. Antibodies were detected by protein A-gold complexes. The monoclonal anti-AGE antibody 6D12 was used without a bridge between the primary antibody and protein A. A fixative block (1% glutaraldehyde in H_2O) between the first and second marker pair was used for double labelling. The sections were dried and examined in a JEM 1010 transmission electron microscope operating at 80 kV.

Quantitative immune cytochemistry. After pre-incubation of SEC with or without 100 μg/ml AGE-BSA for 3 h at 37°C, the cells were washed, and supplied with medium only. After 0 and 6 h at 37°C, the cells were prepared for electron microscopy. The sections, immune labelled for SEC HA-S-R, were examined in a random fashion, and for each time point (0 and 6 h) four cell profiles were photographed at a magnification of ×25,000. Gold particles were counted. The samples were immune labelled and photographed by different persons. The origin of the sample was unknown to the photographer.

Statistical analysis. The experimental data were analysed by Microsoft Excel software, and ANOVA was used for statistical analysis. Significant difference was established at a p value of less than 0.05.

Results

Effect of pre-incubation with AGE-BSA on SEC scavenger function. Endocytosis of ^{125}I-AGE-BSA was monitored in SEC pre-incubated with AGE-BSA, hyaluronan, mannan, HAGG or collagen for 1–12 h at 37°C (Fig. 1). While pre-incubation with AGE-BSA reduced endocytosis of ^{125}I-AGE-BSA by 28 to 53%, pre-exposure to non-SR-ligands did not affect subsequent SR-mediated endocytosis of ^{125}I-AGE-BSA

Table 1. Effect of preincubation of SEC with AGE and other ligands on subsequent endocytosis via different endocytosis receptors

Radiolabelled ligand	AGE-BSA	rAGE-BSA	FSA	Preincubated ligand			
				Hyaluronan	Mannan	HAGG	Collagen
^{125}I-AGE-BSA	49.9±11.6[b]	69.8±1.2[b]	97.7±0	99.0±1.8	99.8±0.3	100±0	100±0
^{125}I-FSA	41.5±23.1[a]	59.8±5.8[b]	88.1±3.9				
^{125}I-OxLDL	67.9±5.0[b]		111.4±3.2	97.3±2.5			
^{125}I-AcLDL	81.9±5.9[a]	82.8±2.0[b]	89.0±8.7	94.3±15.3			
^{125}I-CSPG	57.5±3.9[b]			98.5±3.3			
^{125}I-Mannan	94.5±1.3[a]				86.2±2.3[a]		
^{125}I-HAGG	92.9±4.4					91.1±15.9	
^{125}I-Collagen	99.2±1.1						94.4±7.9

[a] $p<0.05$ vs control
[b] $p<0.001$ vs control
Cultures of SEC were preincubated with 100 μg/ml unlabelled ligands for 3 h at 37°C, washed, and radiolabelled ligands as indicated in the table were added for 1 h at 37°C (n=3). Results, given as per cent of control ± SEM, are means of three different experiments done in triplicate. 100% corresponds to 40–50% of added ^{125}I-AGE-BSA, ~30% of added ^{125}I-FSA, ~20% of added ^{125}I-OxLDL, ~35% of added ^{125}I-AcLDL, ~20% of added ^{125}I-CSPG, ~10% of added ^{125}I-mannan, ~6% of added ^{125}I-HAGG, and ~20% of added ^{125}I-collagen

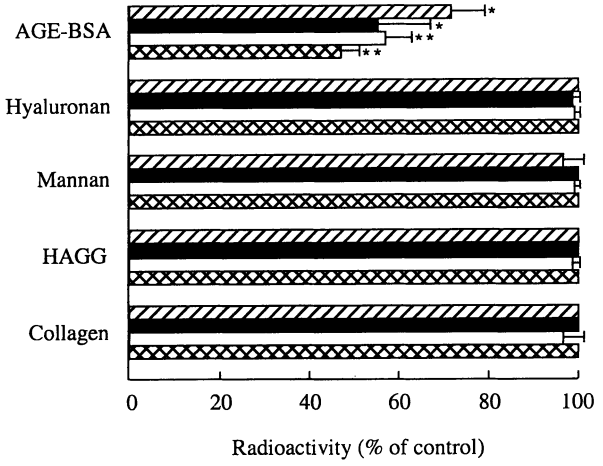

Fig. 1. Effect of pre-incubation of SEC with ligands for different receptors on subsequent endocytosis of ^{125}I-AGE-BSA. Cultures of SEC were pre-incubated with 100 μg/ml AGE-BSA, hyaluronan, mannan, HAGG or collagen in medium for 1 (*shaded bars*), 3 (*black bars*), 6 (*open bars*), or 12 h (*crossed bars*) at 37°C. The cells were washed, and incubation continued with trace amounts of ^{125}I-AGE-BSA (500 Bq) for 1 h at 37°C. Results, given as per cent of control ± SEM, are means of three different experiments done in triplicate. 100% corresponds to 40–50% of added ^{125}I-AGE-BSA. *$p<0.01$ vs control, **$p<0.001$ vs control

Table 2. Dose-response effect of AGE-BSA preincubation on subsequent endocytosis of ^{125}I-AGE-BSA in SEC

AGE-BSA (μg/ml)	Endocytosis of ^{125}I-AGE-BSA
0	100±0
1	96.0±5.7
10	88.8±12.9
50	68.5±8.4[a]
100	49.6±11.6[b]

[a] $p<0.01$ vs control
[b] $p<0.001$ vs control
Cultures of SEC were preincubated with 0–100 μg/ml AGE-BSA for 3 h at 37°C, washed, and incubation continued for another 1 h at 37°C in the presence of trace amounts of ^{125}I-AGE-BSA. Results, given as per cent of control ± SEM, are means of three different experiments done in triplicate. 100% corresponds to 40–50% of added ^{125}I-AGE-BSA

(Fig. 1). To study whether pre-loading of SEC with a specific ligand caused a general change in the scavenger capacity, we measured endocytosis of trace amounts of labelled ligands for different receptors in SEC after pre-incubation with excess amounts of unlabelled ligands for 3 h at 37°C (Table 1). While pre-incubation with AGE-BSA reduced endocytosis of ^{125}I-AGE-BSA and ^{125}I-FSA by 50 and 58%, endocytosis of ^{125}I-OxLDL, ^{125}I-AcLDL and ^{125}I-CSPG was reduced by 32, 18 and 42%. At variance, pre-incubation with FSA reduced uptake of ^{125}I-FSA and ^{125}I-AcLDL by approximately 10%, whereas uptake of ^{125}I-OxLDL was not inhibited. Reduced endocytosis caused by pre-incubation with rAGE-BSA was indistinguishable from that of AGE-BSA. Pre-exposure of SEC to unlabelled AGE-BSA, mannan, HAGG or collagen affected subsequent endocytosis of ^{125}I-mannan, ^{125}I-HAGG and ^{125}I-collagen only slightly. Studies on specificity of binding of ^{125}I-OxLDL to SEC showed that simultaneous incubation with unlabelled AGE-BSA or FSA inhibited endocytosis by 63 and 47%, respectively, while hyaluronan had no inhibitory effect. Pre-incubation of SEC with different concentrations of AGE-BSA showed a concentration dependent inhibition, with 50 and 100 μg/ml AGE-BSA reducing endocytosis of ^{125}I-AGE-BSA by 31 and 50%, respectively (Table 2).

Effect of pre-incubation with AGE-BSA on the SEC SR activity. Next we studied the effect of endocytosis of

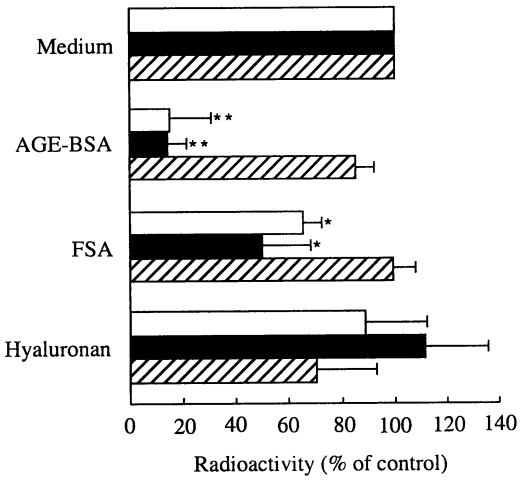

Fig. 2. Effect of pre-incubation with ligands for different receptors on subsequent binding of ^{125}I-AGE-BSA at 4°C. Cultures of SEC were pre-incubated with medium only or medium with 100 μg/ml AGE-BSA, FSA or hyaluronan for 3 h at 37°C. The cells were washed and allowed to bind ^{125}I-AGE-BSA (*open bars*), ^{125}I-FSA (*black bars*), or ^{125}I-CSPG (*shaded bars*) for 1 h at 4°C. Results (cell associated radioactivity), given as per cent of control ± SEM, are means of three different experiments done in duplicate. *p<0.01 vs control, **p<0.0001 vs control

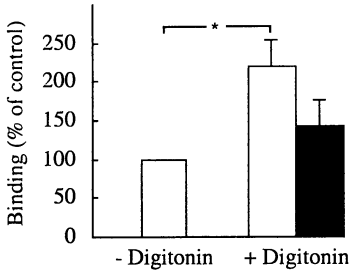

Fig. 3. Effect of digitonin. Cultures of SEC were pre-incubated in medium with (*black bars*) or without (*open bars*) 100 μg/ml AGE-BSA for 3 h at 37°C. The cells were washed, treated with 0.055% digitonin for 10 min at 4°C, and allowed to bind ^{125}I-AGE-BSA for 1 h at 4°C. Results (cell associated radioactivity), given as per cent of control ± SEM, are means of three different experiments done in duplicate. *p<0.01

AGE-BSA on the surface expression of SEC SR. Treatment of SEC with AGE-BSA reduced binding capacity of ^{125}I-AGE-BSA and ^{125}I-FSA by 85%. Pre-incubation with FSA at 37°C inhibited the subsequent binding of ^{125}I-AGE-BSA and ^{125}I-FSA at 4°C by 34 and 50%, respectively, whereas pre-incubation with hyaluronan reduced binding of ^{125}I-CSPG by 30% (Fig. 2). To assess the total cellular pool of receptors after pre-incubation with AGE-BSA, the cells were treated with digitonin, allowing the ligand to establish contact with intracellularly located receptors (Fig. 3). When SEC were pre-incubated with medium only, digitonin treatment resulted in a 2.2-fold increased binding of ^{125}I-AGE-BSA compared with non-treated cells. In contrast, pre-incubation of the cells with AGE-BSA before digitonin treatment did not increase binding of ^{125}I-AGE-BSA compared with the non-treated cells. When the same type of experiment was done with ^{125}I-collagen, digitonin treatment was observed to double the binding, which was unaffected by pre-incubation with AGE-BSA.

Effect of pre-incubation with AGE-BSA on the intracellular transport of endocytosed ligands in SEC. To study the intracellular distribution of SR-ligands in SEC we used subcellular fractionation of SEC following a 1 h endocytosis pulse of ^{125}I-TC-labelled ligands. This adduct is trapped intracellularly in the organelle where degradation of the carrier protein takes place. Centrifugation of SEC homogenates in sucrose gradients showed that ^{125}I-TC-AGE-BSA and ^{125}I-TC-PIIINP were associated with organelles with increasing buoyant densities (Fig. 4). In cells pre-incubated with medium only, ^{125}I-TC-AGE-BSA and ^{125}I-TC-PIIINP were distributed throughout the gradient in organelles banding at approximately 1.10, 1.15 and 1.19 g/ml (Fig. 4A, C). After a 3-h chase the ligands were concentrated in organelles banding at approximately 1.19 g/ml (Fig. 4B, D). When SEC were pre-incubated with AGE-BSA for 3 h before the 1 h pulse of radiolabelled ligands, ^{125}I-TC-PIIINP was concentrated in an organelle banding at approximately 1.15 g/ml, while ^{125}I-TC-AGE-BSA was located in an organelle banding at approximately 1.11 g/ml (Fig. 4A, C). After a 3-h chase the distribution of radiolabelled ligands was similar to the pattern in SEC pre-incubated with medium only (Fig. 4B, D). The intracellular distribution of ^{125}I-mannan (a ligand for the mannose-receptor) was the same in SEC that had been pre-incubated with or without AGE-BSA. β-acetylglucosaminidase, a lysosomal marker enzyme, was identified at 1.2 g/ml.

Endocytosis of AGE-BSA. Studies on endocytosis of SR-ligands showed that the degradation of ^{125}I-AGE-BSA occurs slower than that of ^{125}I-FSA in SEC (Fig. 5). The cell-associated radioactivity after endocytosis of ^{125}I-AGE-BSA and ^{125}I-FSA was approximately 30 and 13% of added radioactivity after 4 h of incubation at 37°C. The amount of degradation product increased with increasing incubation time, with 50 and 72% of added ^{125}I-AGE-BSA and ^{125}I-FSA being degraded after 4 h at 37°C. To examine whether the cells could restore their endocytic ability after degradation and "wash-out" of pre-loaded AGE-BSA, we supplied cells that had been pre-incubated with AGE-BSA with fresh medium and allowed the cells to process AGE-BSA for different time periods before adding ^{125}I-AGE-BSA or ^{125}I-CSPG. With increasing incubation time in medium only, the endocytic capacity increased towards that of control cells (Fig. 6). To study whether the restored endocytic capacity was due to de novo synthesis of receptor protein, we

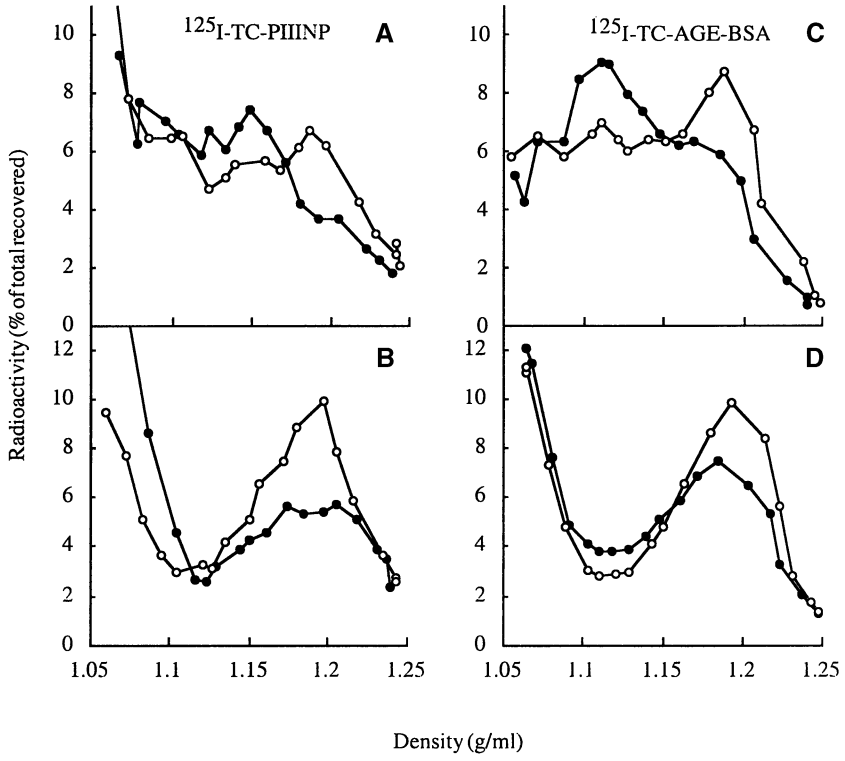

Fig. 4A–D. Intracellular transport of SR-ligands in SEC. Cultures of SEC were pre-incubated with medium only (*open symbols*), or medium with 100 µg/ml AGE-BSA (*filled symbols*) for 3 h at 37°C. The cells were washed, and incubation continued for another 1 h at 37°C in the presence of ^{125}I-TC-PIIINP or ^{125}I-TC-AGE-BSA. After 0- (**A**, **C**) and 3-h (**B**, **D**) chase at 37°C, the postnuclear fraction was fractionated in a sucrose gradient. Results are presented as % of total recovered radioactivity in the gradient as a function of the density of the fraction. The data are representative of four independent experiments

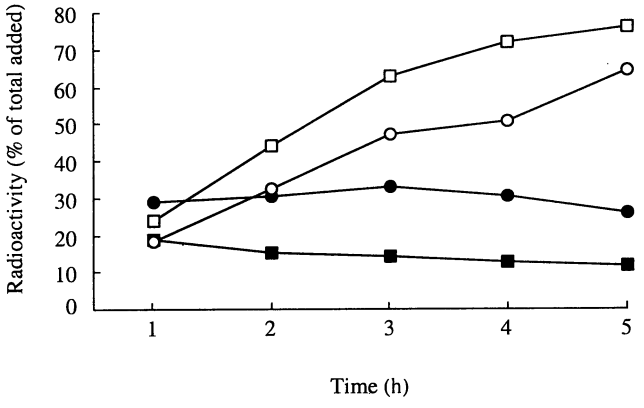

Fig. 5. Kinetics of degradation of endocytosed ^{125}I-AGE-BSA and ^{125}I-FSA. Cultures of SEC were incubated with ^{125}I-AGE-BSA (*circles*) or ^{125}I-FSA (*squares*) for 1–5 h at 37°C. The results are presented as cell-associated radioactivity (*filled symbols*) and acid-soluble radioactivity (*open symbols*). Results, given as per cent of total added radioactivity, are the mean of two different experiments performed in triplicate. Variation was <10%

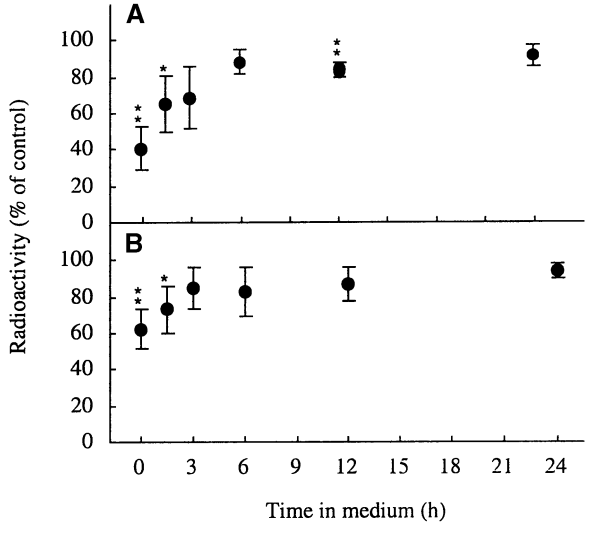

Fig. 6A, B. Rate of restoration of SR-mediated endocytosis in SEC after pre-incubation with AGE-BSA. Cultures of SEC were pre-incubated with medium only, or medium with 100 µg/ml AGE-BSA for 3 h at 37°C. The cells were washed and incubated with medium only for 0, 1.5, 3, 6, 12 and 24 h, before ^{125}I-AGE-BSA (**A**) or ^{125}I-CSPG (**B**) was added for 1 h 37°C. Results, given as per cent of control ± SEM, are means of three different experiments done in triplicate. 100% corresponds to 40–50% of added ^{125}I-AGE-BSA and ~20% of added ^{125}I-CSPG. *p<0.05 vs control, **p<0.01 vs control

treated AGE-BSA-pre-loaded SEC with cycloheximide (Fig. 7). Although cycloheximide treatment itself inhibited endocytosis by 15%, pre-incubation with AGE-BSA followed by cycloheximide treatment inhibited restoration of endocytosis by 27%. Cell via-

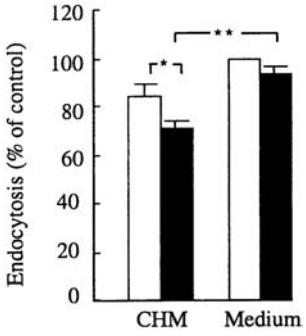

Fig. 7. Effect of cycloheximide on restoration of AGE-mediated reduced SR-endocytosis. Cultures of SEC were pre-incubated with medium only (*open bars*), or medium with 100 µg/ml AGE-BSA (*black bars*) for 3 h at 37°C. The cells were washed, incubated with medium only or medium with 100 µmol/l cycloheximid (CHM) for 3 h at 37°C, before ^{125}I-AGE-BSA was added and incubation continued for another 1 h at 37°C. Results, given as per cent of control ± SEM, are means of three different experiments done in triplicate. 100% corresponds to 40–50% of added ^{125}I-AGE-BSA. *p<0.02, **p<0.005

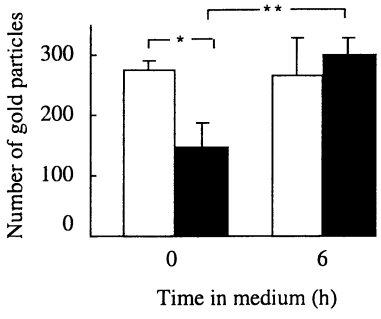

Fig. 8. Quantitative immune electron microscopy. Cultures of SEC were pre-incubated in medium only (*open bars*) or medium with 100 µg/ml AGE-BSA (*black bars*) for 3 h at 37°C. The cells were washed, and after 0- and 6-h incubation in medium only the cells were fixed. For detection of the receptor, ultrathin cryosections were labelled with anti-SEC HA-S-R antibody and protein A-gold. For each time point (0 and 6 h) four cell profiles were photographed in a random matter, and gold particles representing the receptor was counted. The samples were blinded upon labelling and counting. *p<0.025, **p<0.011

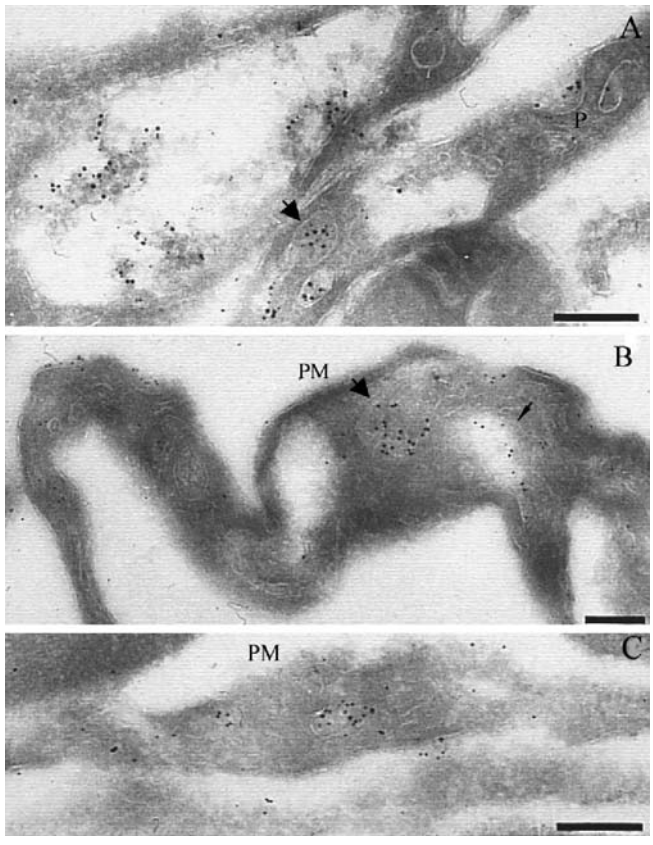

Fig. 9A–C. Immune electron microscopic localization of AGE-BSA and HA-S-R in SEC. Cultures of SEC were pre-incubated with 100 µg/ml AGE-BSA for 3 h at 37°C and fixed immediately. For detection, thawed ultrathin cryosections were labelled with anti-AGE, anti-BSA or anti-SEC HA-S-R and protein A-gold. **A** Cells were double-labelled with anti-AGE (*small gold*) and anti-BSA (*large gold*); ligand is colocalized in pits (*p*), and in intracellular electrondense (*arrow*) and electronlucent vesicle. **B** Double-labelling with anti-AGE (*large gold*) and anti-SEC HA-S-R (*small gold*) showed that AGE was located in electrondense multivesicular vesicle (*large arrow*), and colocalized with the receptor in an electronlucent vesicle (*small arrow*). AGE and HA/S-R also colocalized in pits at the plasma membrane (*pm*). **C** Double-labelling with anti-BSA (*small gold*) and anti-SEC HA-S-R (*large gold*) showed that the receptor and ligand were colocalized. Scale mark: 200 nm

bility of cycloheximide-treated SEC measured by MTT-assay showed that the treatment was not toxic to the cells. The pool of SRs in SEC after uptake of AGE-BSA was also examined by use of quantitative immune cytochemistry. Anti-HA-S-R-antibodies inhibited uptake of ^{125}I-AGE-BSA by 44% (Table 3). As only approximately 10% of the IgG molecules reacts specifically, the concentration of anti-SEC HA-S-R antibodies used in this experiment was 1–100 µg protein/ml. Quantitative immune cytochemistry, using anti-SEC HA-S-R, showed that the amount of labelling in SEC after continuous uptake of AGE-BSA for 3 h at 37°C was about 50% of that observed in SEC pre-incubated in medium only (Fig. 8). After a 6-h incubation in medium only, the amount of labelling of anti-SEC HA-S-R was approximately equal in cells pre-incubated with AGE-BSA or medium only.

Electron microscopic examination of SEC pre-incubated with AGE-BSA. To visualize the intracellular transport of endocytosed AGE-BSA, SEC pre-incubated with AGE-BSA were examined by detection on thawed ultrathin cryosections by the use of anti-AGE, anti-BSA and anti-SEC HA-S-R (Fig. 9). This study showed that AGE and BSA, after 3 h continuous uptake of AGE-BSA, colocalized in pits at the cell surface and in intracellular electronlucent vesicles (Fig. 9A). Double-labelling for AGE and SEC HA-S-R,

Table 3. Effect of anti-SEC HA/S-R antibodies on endocytosis of ^{125}I-AGE-BSA

Anti SEC HA/S-Rc (mg/ml)	Endocytosis of ^{125}I-AGE-BSA
0	100.0
0.01	89.8
0.1	75.3[a]
1	56.0[b]

[a] $p<0.05$ vs control
[b] $p<0.001$ vs control
Cultures of SEC were preincubated with 0–1 mg/ml anti-SEC HA-S-R for 30 min before incubation with trace amounts of ^{125}I-AGE-BSA (500 Bq) for 1 h at 37°C. Results, given as per cent of control, are the mean of two different experiments done in triplicate. Variation was less than 10%. 100% corresponds to 40–50 % of added ^{125}I-AGE-BSA. Incubation with nonimmune IgG did not affect endocytosis of ^{125}I-AGE-BSA

or BSA and SEC HA-S-R showed colocalization in pits at the cell surface and in intracellular electronlucent vesicles. AGE was also localized in a multivesicular compartment (Figs. 9B, C).

Discussion

AGE-modified proteins are eliminated from the circulation by SR-mediated endocytosis predominantly in SEC [12]. We show that endocytosis of AGE-protein reduces subsequent SR-mediated endocytic capacity in SEC. This effect of AGE-BSA is not a general inhibitory effect on the endocytic pathway, because degradation of ligands that are recognized by other receptors was only marginally affected by prior exposure of SEC to AGE-BSA. Moreover, the phenomenon cannot be explained merely as a ligand–receptor competition effect, since endocytosis experiments with radiolabelled ligands were carried out in fresh medium that contained no unlabelled AGE-BSA. These findings, along with the observation that pre-incubation with ligands specific for receptors other than SR did not affect subsequent endocytosis of radiolabelled ligands for SR or other receptors, suggest that the observed phenomenon is not caused by a cytotoxic effect of AGE-BSA. Instead, we interpret the results to mean that pre-exposure to AGE-BSA imposes a selective reduction of SR-mediated endocytosis in SEC.

Treatment of SEC with digitonin, allowing both the intracellular and cell surface pools of SR to bind ligand, showed that the cells carried an intracellular reservoir of SR amounting to about 55% of the total cellular pool, whereas 45% was located on the cell surface. Binding experiments at 4°C with SEC that had not been treated with digitonin (to avoid binding to intracellular SR) showed that pre-exposure to AGE-BSA reduced binding capacity by 85%. Repeating the same experiment with digitonin-treated cells reduced SR-binding by 37% compared to digitonin-treated cells that had not been pre-incubated with AGE-BSA. Quantitative immune electron microscopy exposing both intracellular and surface associated pools of SR to anti-SEC HA-S-R antibody indicated that pre-incubation with AGE-BSA reduced the content of SR in SEC, confirming the results obtained by binding at 4°C and digitonin treatment. These observations indicate that endocytosis of AGE-BSA in SEC leads to loss of SR activity, which is the direct cause of the decreased SR-mediated endocytosis. We speculate that the cause of this receptor consumption is that AGE-BSA impairs the normal mechanism for dissociation of ligand from receptor in the early endosomal compartment. The undissociated receptor-ligand complex could then travel together down the endocytic pathway, preventing the receptor from recycling to the surface. This will lead to degradation of both ligand and receptor, and thus receptor consumption. The finding that prolonged incubation (6 h) of SEC in AGE-BSA-free medium largely normalized the ability to endocytose AGE-BSA, along with the observation that cycloheximide inhibited this restoration, indicate that de novo protein synthesis was necessary for SR to reappear on the surface of SEC.

Subcellular fractionation of SEC has been previously used to study the intracellular transport of ^{125}I-TC-FSA, a ligand for the SR [48, 49]. Using the same technique we observed that the SR-ligands ^{125}I-TC-AGE-BSA and ^{125}I-TC-PIIINP accumulated in an organelle in the early part of the endocytic pathway of SEC that had been pre-exposed to AGE-BSA. In cells that had not been pre-incubated with AGE-BSA, transport proceeded readily all the way to the lysosomes. This indicates that pre-incubation with AGE-BSA results in delayed intracellular processing of SR-ligands in SEC.

Immune electron microscopy showed that AGE and BSA colocalized in electronlucent intracellular vesicles and in pits at the cell surface, showing that the AGE adduct and the protein moiety of AGE-BSA are transported simultaneously along the endocytic pathway. Using specific markers of endocytic organelles in SEC, a report [50] showed that electronlucent vesicles carrying ligand along the inner aspect of the vesicle membrane represented early sorting endosomes, whereas electrondense vesicles, with ligand distributed throughout the lumen, represented late endosomes-lysosomes. Based on these morphological criteria we observed that after a 3-h continuous uptake of AGE-BSA, the AGE, BSA, and the SEC HA-S-R colocalized in electronlucent vesicles that could be characterized as early endosomes. We also observed AGE and BSA, but not HA-S-R, distributed in the lumen of electrondense vesicles defined as late endosomes-lysosomes [50]. These results show that AGE-BSA is endocytosed via receptors located in pits, with the receptor-ligand complex being transported first to early endosomes. The ligand is then transported fur-

ther to late endosomes-lysosomes. The observation of HA-S-R in cells pre-incubated with AGE-BSA was limited to the cell surface and early endosomes. The observation that the receptor antigen colocalized with AGE or BSA at these sites, suggested that the disappearance of receptor antigen from the later parts of the endocytic pathway was due to receptor degradation rather than ligand masking. The very limited immune gold-staining of AGE or BSA at the cell surface suggested a restricted recycling of ligand. We conclude that the uptake of AGE-BSA in SEC impairs intracellular transport of SR-ligands and reduces the number of receptors for these ligands, supposedly due to their degradation in the endosomal-lysosomal compartment.

Our findings are compatible with the idea that exposure of SEC to high concentrations of AGE proteins in diabetic patients can reduce the clearance of SR ligands from the circulation. This could allow atherogenic substances (OxLDL and AGE protein, that are both SR ligands) to escape hepatic clearance, which could increase the risk of developing cardiovascular complications. The concentration of AGE-BSA used in this study is higher than what would be expected in a physiological situation, therefore our experimental system might not be adequate. However, in patients with even slightly increased concentrations of AGE in the circulation, SEC will be constantly supplied with AGE proteins, allowing high concentrations of intracellularly accumulated AGE. Accordingly, the blood plasma concentration of AGE can be considerable lower than in the presently described in vitro system, and yet lead to a marked exposure of SEC to AGE.

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