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Effect of glycation of albumin on its binding to renal brush-border membrane vesicles: influence of aging in rats

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Abstract

Aging is associated with the loss of preferential urinary excretion of Amadori-product glycated albumin. We have measured the binding of ^{125}I-labeled glycated albumin to the renal brush-border membrane vesicles from young and old rats to determine whether a specific receptor-mediated endocytosis system may be involved. ^{125}I-Glycated albumin was specifically bound by renal brush-border membrane vesicles in a time- and temperature-dependent manner; the binding was concentration-dependent, saturable and reversible. Scatchard plots gave an apparent dissociation constant K_m of 488 ± 17 nM, and a number of binding sites N of 33.5 ± 3.4 pmol/mg protein/min in membrane vesicles from young (3 months old) rats; the binding of native [^{125}I]albumin, gave a K_m of 1194 ± 200 nM ($P < 2\%$) and N of 82.4 ± 16.3 pmol/mg protein/min ($P < 3\%$). Vesicles from 10-month-old rats had a similar K_m (619.6 ± 135.3 nM) and N (21.91 ± 2.98 pmol/mg protein/min), while those from older (30 months old) rats had significantly increased K_m (1344 ± 237 nM, $P < 3\%$) and N (81.3 ± 10.9 pmol/mg protein/ min, $P < 1\%$) for ^{125}I-glycated albumin binding. ^{125}I-Glycated HSA was not displaced by unlabeled native HSA in less than 100-fold excess and native [^{125}I]HSA was only displaced by a 10-fold excess of unlabeled glycated HSA. The binding of native [^{125}I]HSA was partly inhibited (85%) by unlabeled glycated HSA. Thus, there appear to be two different binding sites, one for glycated and the other for native albumin, lying close together; and the glycation site on albumin is the discriminatory recognition factor.

Keywords: Albumin; Glycation; Binding; Renal brush-border membrane vesicle; Aging

1. Introduction

Glycation of proteins, especially the main circulating protein albumin, normally occurs very slowly under physiological conditions [1,2]. But the impaired glucose tolerance that is associated with aging [3,4] leads to more rapid glycation and a significant increase in the fraction of albumin that is glycated [5]. There is good evidence that glycation alters the physicochemical properties of albumin [6,7], affecting both its functional properties and its renal handling [8]. Glycation of albumin increases its permeability across the glomerular basement membrane [9,10] and impairs its tubular reabsorption [11,12]. The increase in negative charge following glycation [8,13] is believed to be one of the main reasons for its altered recognition [14]. Glycated albumin is not reabsorbed, while native albumin is efficiently reabsorbed by the proximal tubule when the glomerular filtration rate is normal. Indeed, recent studies have shown that this glycated albumin is preferentially excreted in the urine by young normal humans [15] and rats [5,8,16].

This discriminatory excretion, or editing, is gradually lost with aging in humans [15] and rats [5], due to dilution of the excreted glycated albumin with native albumin, while a significant proteinuria develops mainly due to albumin leakage [17]. Several reports claim that the glomerular leakage of albumin with aging results from an alteration in glomerular size-perm selectivity because of increased transcapillary pathways and/or a loss of electrical sieving. It could also be due to a change in capillary hemodynamics [18,19]. Whatever the precise mechanism responsible for this increased glomerular permeability, it could not yield the dramatic decline in the editing ratio that occurs in elderly rats [5] unless there was an increase in the tubular reabsorption of glycated albumin by some alternative system, even when the reabsorptive capacity for native albumin is overloaded. We have therefore postulated the existence of a receptor-mediated endocytosis

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system specific for Amadori-product glycated albumin in the convoluted proximal tubule cells. This system would develop with age and be capable of collecting that ligand, much like several other ligands. For example, aldehyde-modified albumin is taken up by a receptor-mediated process [20]. The basic requirement for endocytosis of glycated albumin is thus a specific site.

This study provides evidence for the existence of specific binding sites for Amadori-product glycated albumin on renal brush-border membrane vesicles that are different from a native albumin binding system. The receptor-mediated endocytosis system is described, and the competition between native and glycated albumins were examined using the rapid filtration technique.

2. Materials and methods

2.1. Isolation of renal brush-border membrane vesicles

Renal brush-border membrane vesicles (RBBMV) were prepared from the cortexes of individual paired kidneys of young (3 months old) and older (10 and 30 months old) rats killed by decapitation. Studies were performed on male Wistar rats (WAG/Rij), as this strain does not suffer from age-associated nephropathy [21]. The kidneys were removed, decapsulated, placed in ice-cold buffer (300 mM D-mannitol, 12 mM Tris-HCl (pH 7.4)) and processed at 4°C. Membranes were fractionated by the $MgCl_2$ precipitation method [22], and the final brush-border membrane preparation thus obtained was resuspended in 300 mM mannitol, 10 mM Hepes-Tris (pH 7.4). Aliquots (200 μl) of brush-border membranes (3 mg/ml) were stored in liquid nitrogen until used.

2.2. Quantitative protein and enzyme assay

Protein was determined by the Lowry method with BSA as standard [23]. The marker enzymes for preparation assessment were amino peptidase (EC 3.4.11.2) and Na^+/K^+ ATPase (EC 3.6.1.3), whose activities were assayed by the methods of Haase et al. [24] and Heller and Hanahan [25]. The brush-border membrane vesicles were routinely enriched 7.2 ± 0.09 ($n = 7$) times in amino peptidase and 0.45 ± 0.04 ($n = 7$) in Na^+/K^+ ATPase over the homogenate in rats of all three age groups.

2.3. Preparation and purification of Amadori-product glycated albumin

Iodinated human serum albumin ([^{125}I]HSA, specific activity 25 μCi/mg) was from Sorin Biomedica (Italy), human and bovine unlabeled serum albumin (fraction V) were from Sigma (USA), and lysozyme (EC 3.2.1.17) was from Miles (France). Unlabeled HSA was dissolved in phosphate-buffered saline (PBS; pH 7.4) containing 0.02% sodium azide, 1 mM PMSF, 5 mM EDTA, 1 mM NEM and incubated without (native control HSA), or with 0.5 M glucose at 37°C in the dark for 1, 2, 3 or 5 days [26]. The samples were then extensively dialyzed against 0.25 M ammonium acetate/0.05 M $MgCl_2$ (pH 8.5) at 4°C to remove free glucose. Amadori-product glycated albumin (AP-HSA) was separated from nonglycated albumin (native HSA) by affinity chromatography on a column of phenyl-boronate (Glyco-Gel B from Pierce) [27] and the percentage of glycated albumin determined according to the manufacturer. The extent of glycation was determined by measuring the amount of formaldehyde released by periodate oxidation of c_1 hydroxyls in the Amadori-product form of the glycated albumin, using the method of Ahmed and Furth [28]; results are expressed as mol HCHO/mol albumin. The percentage of glycated albumin

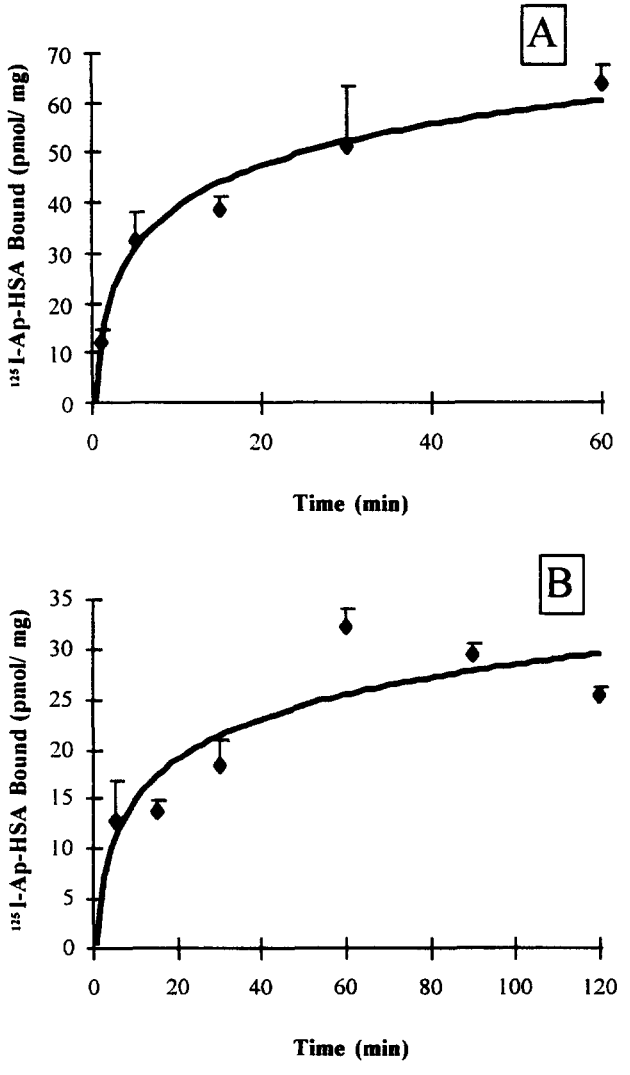

Fig. 1. Time course of [^{125}I]AP-HSA binding to renal brush-border membrane vesicles from young (3-month-old) rats. Membrane vesicles (50 μg/ml) were incubated at 37°C (A) or 4°C (B) with [^{125}I]AP-HSA (14.9 nM) until indicated time. Data show specific binding and represent mean ± S.E. of three assays carried out in triplicate.

depended on the incubation time, and varied from 56.40 ± 4.65 (1 day) to 85.8 ± 10.55 (2 days), 92.30 ± 3.11 (3 days) and 96.15 ± 1.86 (5 days) ($n = 3$). The mean extent of glycation was 2.40 ± 0.57 mol HCHO/mol albumin for the whole period of incubation, while control HSA had 0.79 ± 0.09 mol HCHO/mol albumin. Subsequent experiments used glycated labeled and unlabeled HSA incubated for 5 days. Samples of glycated albumin were prepared by boronate chromatography, dialyzed against kinetic buffer (10 mM Hepes-Tris, 150 mM NaCl (pH 7.4)), concentrated in an Amicon cell, and the protein content measured. Aliquots of [^{125}I]AP-HSA and [^{125}I]HSA (5 μg) were analyzed by SDS-PAGE electrophoresis on 10% acrylamide gel and autoradiographed.

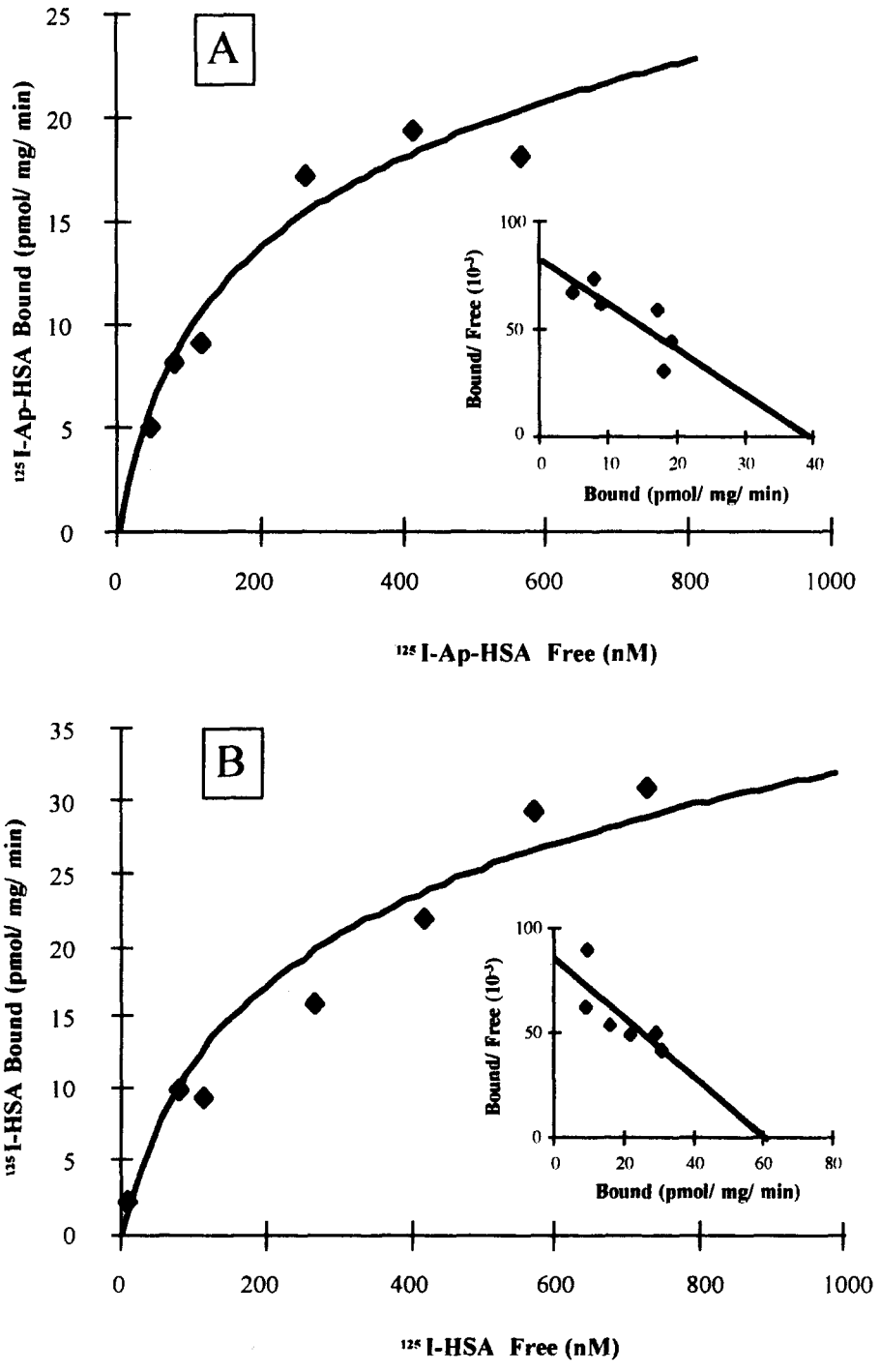

Fig. 2. Specific binding of [^{125}I]AP-HSA (A) and ^{125}I-HSA (B) (pmol/mg protein/min) to renal brush-border membrane vesicles from young (3-month-old) rats. Each point is the mean of five representative assays carried out in triplicate. Inset: Scatchard plot (B/F as a function of B).

2.4. Binding [^{125}I]AP HSA and [^{125}I]HSA to RBBMV

Binding was measured by the rapid filtration technique [29]. All assays were performed in BSA-coated glass tubes (10 g/l) and the filters were presoaked in stop solution (kinetic buffer + 2% BSA) to reduce non-specific retention of labeled ligand on the filter. About 5 μg renal brush-border membrane vesicles from paired batches of kidneys were suspended in 50 μl kinetic buffer (10 mM Hepes-Tris, 150 mM NaCl (pH 7.4)) and preincubated for 10 min at 37°C or 4°C for time course study. Ligand (labeled AP-HSA) plus 13.7 nM cationic lysozyme (total volume 100 μl) was then added to initiate the binding and incubation was continued until the indicated time. The binding kinetic parameters were determined in assays performed at 37°C for 5 min (final ligand labeled AP-HSA and HSA concentration 74–800 nM). The reaction was stopped by adding 2 ml ice-cold stop solution, and the mixture was rapidly

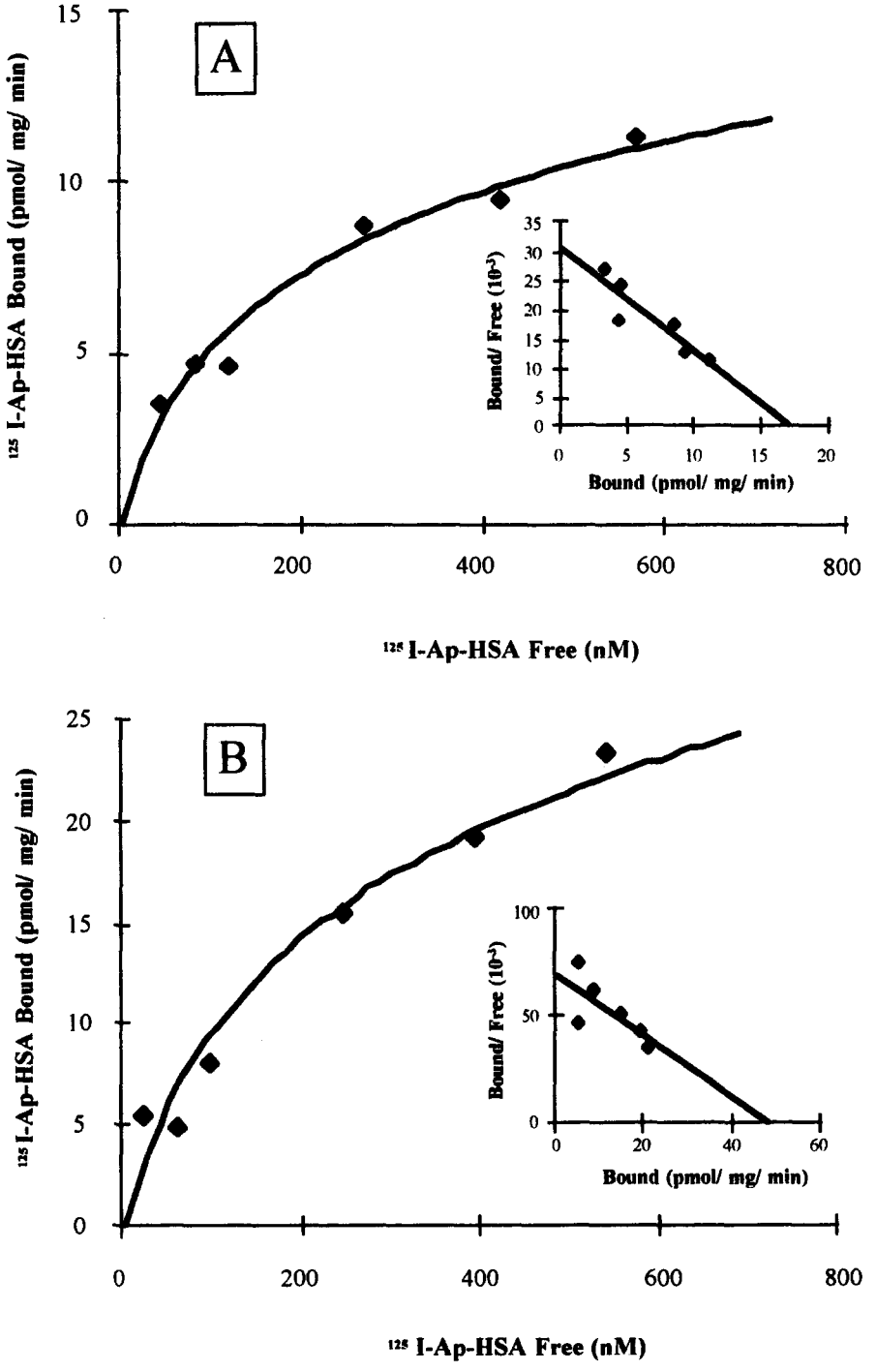

Fig. 3. Specific binding of [^{125}I]AP-HSA to renal brush-border membrane vesicles from 10-month- (A) or 30-month- (B) old rats. Each point is the mean of five representative measurements carried out in triplicate. Inset: Scatchard plot (B/F as a function of B).

filtered (0.45-μm pore size, cellulose filter type HAWPO 25; Millipore) under vacuum. The tubes were rinsed with 4 ml ice-cold stop solution and the rinse passed through the filter. The filters were processed for liquid scintillation counting. Specific binding was obtained by subtracting the blank value from an assay in which the renal brush-border membrane vesicles were preincubated with excess unlabeled ligand (500-times the ^{125}-I-labeled ligand concentration) before adding ^{125}I-ligand (non-specific binding) from the total binding data. At least two experiments on the binding of [^{125}I]AP-HSA and [^{125}I]HSA by brush-border membrane vesicles from young rats (3-month) were performed in parallel.

The competitive binding of [^{125}I]AP-HSA with HSA, [^{125}I]HSA with AP-HSA and AP-HSA with [^{125}I]HSA was also investigated. ^{125}I-Ligand (298 nM) was incubated for 5 min at 37°C with 5 μg RBBMV. Unlabeled AP-HSA or HSA (at 1-, 10-, 100-, and 1000-fold concentrations) was then added and incubation continued for a further 5 min. The reaction was stopped and the radioactivity on filters was counted as above. Each assay was run in triplicate.

2.5. Calculations

Means, standard errors and regression analyses were calculated by the method of least squares and used for statistical analysis. Differences among means were tested for statistical significance ($P < 0.05$) using Student's paired t-test.

3. Results

3.1. Preparation and purification of AP-glycated HSA

Phenyl-boronate agarose was used to separate glycated from nonglycated human albumin (HSA). 87% of the ^{125}I-labeled HSA incubated with 0.5 M glucose for 5 days was glycated and bound by the phenyl-boronate column. Less than 1.9% of the control ^{125}I-labeled HSA was bound. The bound material was used as labeled AP-glycated HSA; it contained over 2.40 mol glucose/mol albumin, while the unbound fraction (less than 0.80 mol glucose/mol albumin) was used as native labeled HSA. Autoradiogaphy of labeled samples (5 μg) separated by SDS-PAGE electrophoresis revealed a single 66 kDa band HSA monomer; larger and smaller bands were not detected with this amount (data not shown).

3.2. Binding of [^{125}I]AP-HSA by renal brush-border membrane vesicles: effect of age

In these experiments binding refers to the specific binding. [^{125}I]AP-HSA bound to renal brush-border membrane vesicles; binding occurred rapidly at 37°C and increased with time up to 60 min (Fig. 1A). At 4°C, the binding of [^{125}I]AP-HSA by RBBVM was appreciably slower and did not reach a plateau after 120 min of incubation, corresponding to 50% of the maximum binding at that time (Fig. 1B). Nevertheless, the initial binding rate was quite rapid even at 4°C, as can be seen by the earliest time point. Subsequent experiments focused on how the parameters of

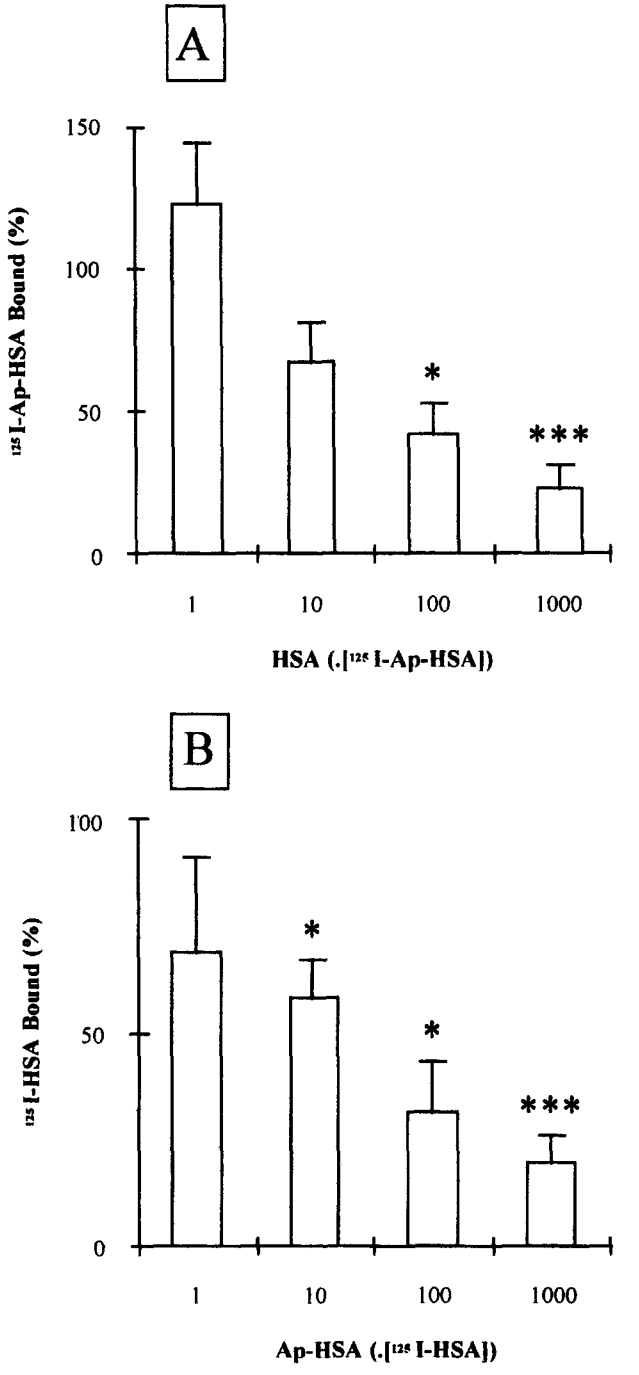

Fig. 4. Displacement of [^{125}I]AP-HSA from binding sites on renal brush-border membrane vesicles by increasing concentrations of native HSA (A) and displacement of ^{125}I-HSA by AP-HSA (B). Data showing residual binding ligand expressed as a percentage of specific binding without competitor (100%) were determined from triplicate assays of three experiments and expressed as mean ± S.E.

[^{125}I]AP-HSA binding by RBBMV at 37°C for 5 min, varied with age.

Specific binding, defined as the difference between binding without (total binding) and with (nonspecific binding) excess unlabeled glycated albumin (500-times), increased in a dose-dependent and saturable manner (Fig. 2A). Scatchard analysis gave a linear plot, indicating one affinity class of receptor (Fig. 2A, inset). The apparent dissociation constant (K_m) and the number of binding sites (expressed as binding capacity, N) were 488 ± 17 nM and 33.5 ± 3.41 pM/min/mg RBBMV from 3-month-old rats ($n = 9$), respectively. The binding of [^{125}I]HSA (Fig. 2B, inset) gave a K_m of 1194 ± 200 nM ($P < 0.01$) and N of 82.4 ± 16.27 pM/mg/min ($P < 0.02$) ($n = 7$). Whereas the binding of [^{125}I]AP-HSA by membrane vesicles from 10-month-old rats did not significantly change, with K_m of 619.6 ± 135.3 nM and N of 21.91 ± 2.98 pM/mg/min ($n = 6$) (Fig. 3A, inset), those from older (30-month) rats had increased K_m (1344 ± 237 nM, $P < 0.02$) and N (81.3 ± 10.9 pM/mg/min, $P < 0.01$) ($n = 5$) (Fig. 3B, inset).

For competitive experiments, RBBMV from young rats were preincubated with [^{125}I]AP-HSA for 5 min, native HSA (1-, 10-, 100- or 1000-fold) was then added and incubation continued for 5 min. [^{125}I]AP-HSA was not displaced from its binding sites by less than a 100-fold greater concentration of cold HSA (Fig. 4A). Similarly, unlabeled AP-HSA did not displace [^{125}I]HSA at less than 10-times concentration (Fig. 4B), indicating that there are different binding sites for AP-glycated albumin and native albumin. Nevertheless, preincubating RBBMV with increasing concentrations of unlabeled AP-HSA inhibited the binding of native [^{125}I]HSA by 85% ($P < 0.007$) as soon as the concentrations of the two ligands were equal (Fig. 5). These data suggest that the two binding sites are close together, with the binding of AP-HSA partly overlapping the neighboring binding site of native HSA.

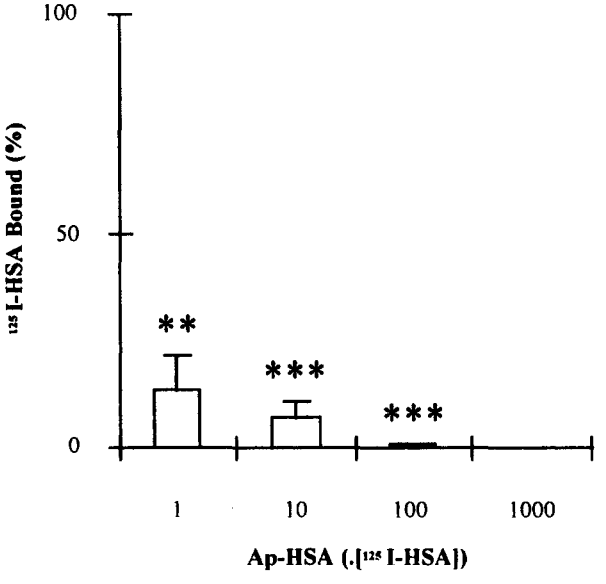

Fig. 5. Inhibition of [^{125}I]HSA binding to renal brush-border membrane vesicles by AP-HSA: RBBMV were preincubated with increasing amount of unlabeled AP-HSA (1-, 10-, 100- or 1000-fold excess) for 5 min before adding a constant amount (298 nM) of [^{125}I]HSA and incubation for 5 additional minutes. Results are expressed as percentage of specific binding of control. Data are means ± S.E. of three determinations from triplicate assays. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$: significantly different from control.

4. Discussion

We have reported that Amadori-product glycated albumin is preferentially excreted by young rats. This phenomenon of editing, is gradually lost with aging [5]. We have now attempted to elucidate this phenomenon by postulating the existence of a receptor-mediated endocytosis system specific for glycated albumin. We suggest that the system is developed by proximal tubular cells and is capable of collecting glycated albumin downstream of the glomerulus in old rats.

The results described here demonstrate the existence of this binding system on renal brush-border membrane vesicles (RBBMV), describe its main features and analyze its evolution with aging. Binding of Amadori-product glycated albumin (AP-HSA) showed a time- and temperature-dependent increase; it occurred rapidly at 37°C and noticeably slower at 4°C. These properties are typical of protein binding by renal tubules. Although the RBBMV used in these experiments are vesicular in nature, the observed association represented binding of AP-HSA to the external face of the membrane vesicles rather than the uptake (binding plus internalization). Indeed the properties of the recognition sites for AP-HSA agree well with one of the in vivo functions of the renal brush-border membranes, that of mediating reabsorption of filtered small proteins by the tubules [30]. But this reabsorptive function implies a mechanism for internalizing proteins at the brush-border membrane surface that would be an energy-dependent process. The use of isolated membrane vesicles provides a simple experimental system devoid of the complexities of a whole-cell system and thus excludes the energy metabolism-dependent process permitting only investigation of the binding of proteins, even if intravesicular transport of small molecules such as glucose [31] or amino acids [32] has been reported. Numerous data strongly support this insertion [33], showing in addition that the degradative capacity of the RBBMV for proteins is minimized in these experimental conditions [34]. Accordingly, data reported here refer to membrane binding.

Amadori-product glycated albumin is specifically bound by RBBMV from young rats with a number of binding sites, N of 33.5 ± 3.4 pmol/mg/min and an apparent dissociation constant K_m of 488 ± 17 nM. These values are comparable to those for the renal glomerular mesangial

cell system which binds glycated albumin with two affinity classes of receptor (K_m values 960 nM and 74 nM) [35]. These binding proteins have been found on several cell types, especially phagocytic cells like human monocytes [36] and rat macrophages [37], but also on murine aortic endothelial cells [38,39]. The native albumin binding sites on our system had lower affinity, with a K_m of 1194 ± 200 nM and an N (V_m) of 82.4 ± 16.3 pmol/mg/min, indicating that epithelial tubular cells have binding sites different from the cellular sites of interaction with native albumin in their ability to selectively bind AP-glycated albumin.

The specificity of these glycated albumin binding sites is evidenced by the competition experiments with native albumin and the lack of displacement by native albumin. Glycated albumin bound to RBBMV was partially displaced (15%) by a 100 × concentration of native albumin, which is a very unphysiological ratio. These data strongly suggest that the specificity of AP-glycated albumin binding is due to a domain in glucose-modified albumin, and is supported to the contrary by previous studies showing that albumin binds to cell sites that recognize domains of the albumin molecule [40,41]. This assertion is likewise supported by data on the ligand specificity of the cell receptor for glycated albumin. The glucose-modified domain defined by its immunoreactivity with a monoclonal antibody as the principal site of albumin glycation is responsible for receptor recognition, since blocking the glycated albumin epitope with that monoclonal antibody prevents its binding to cells [35]. That study also showed that the specificity of AP-glycated albumin binding is due to a domain not present in other glucose-modified protein, emphasizing the discriminatory capacity of these binding sites. The second important element is that AP-glycated albumin can block the binding of native albumin to RBBMV (85%). This result is inconsistent with precedent data and indicates that the two binding sites are probably close together. The bound glycated albumin may overlap the binding sites for native albumin. This raises the question of whether this phenomenon is responsible for age-related albuminuria, which occurs well before the albumin endocytosis system becomes saturated [42].

The binding experiment results also reveal an age-related increase in both the maximum binding capacity (N) (+140%) and the apparent dissociation constant (K_m) (+175%) beyond 10 months, which appears to be an age threshold for this strain of rat [5]. These data indicate two changes. One is the biological adaptation of these tubular cells to eliminate the increased amounts of glycated albumin by increasing the number of binding sites. This implies the up-regulation of the gene encoding for this binding protein. The other is the decrease in affinity for the ligand with aging, which might be a direct consequence of changes in the receptor environment, such as glycation of cell membrane proteins.

Although there is good evidence for AP-glycated albumin binding sites on phagocytic cells like the macrophages and monocytes responsible for scavenging the glycated albumin, this has biological importance, more especially as Amadori-products are sources of noxious advanced glycation end products, recent data also indicate that glycated albumin stimulates monocytes to release TNF and IL-1. These cytokines may play a role in the tissue remodeling that occurs in the development of vascular complications with aging [36]. It is also now well established the noxious effect of AP-glycated albumin on glomerular mesangium expansion [35], cause of the capillary occlusion. The location of binding sites for glycated albumin on capillary endothelial cells may be important for the increased permeability or enhanced transcytosis of the glycated protein. This microvascular leakage of glycated albumin can contribute to the pathogenesis of microangiopathy [41]. Thus, the ubiquity of binding sites for glycated albumin seems to have effects that vary with the type of cell. The physiological or pathological relevance of these newly-described binding sites on renal brush-border membrane vesicles remains to be established.

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