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Lysosomal dysfunction induced by changes in albumin's tertiary structure: Potential key factor in protein toxicity during diabetic nephropathy

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ABSTRACT

Aims: Increased amounts of protein, in particular albumin within renal tubular cells (TBCs), induce the expression of inflammatory and fibrogenic mediators, which are adverse prognostic factors in tubulointerstitial fibrosis and diabetic nephropathy (DN). We sought to assess the participation of the thiol-linked tertiary structure of albumin in the mechanism of protein toxicity in a model of TBCs.

Materials and methods: Cultured human renal proximal tubular cells, HK-2, were exposed to isolated albumin from patients with and without DN (Stages 0, 1 and 4). The magnitude of change of the albumin tertiary structure, cell viability (LDH leakage), apoptosis (Annexin V), transdifferentiation and reticulum endoplasmic stress (Western blot and flow cytometry) and lysosomal enzyme activity were assessed.

Key findings: We found that albumin from Stage 4 patients presented > 50% higher thiol-dependent changes of tertiary structure compared to Stages 0 and 1. Cells incubated with Stage 4 albumin displayed 5 times less viability, accompanied by an increased number of apoptotic cells; evidence of profibrogenic markers E-cadherin and vimentin and higher expression of epithelial-to-mesenchymal transition markers α-SMA and E-cadherin and of endoplasmic reticulum stress protein GRP78 were likewise observed. Moreover, we found that cathepsin B activity in isolated lysosomes showed a significant inhibitory effect on albumin from patients in advanced stages of DN and on albumin that was intentionally modified.

Significance: Overall, this study showed that thiol-dependent changes in albumin's tertiary structure interfere with the lysosomal proteolysis of renal TBCs, inducing molecular changes associated with interstitial fibrosis and DN progression.

1. Introduction

Normally, albumin is captured and extensively degraded by renal proximal tubule cells. However, albumin may have a pathogenic role in the progression of renal damage. Increased amounts of protein, in particular albumin, are an independent mediator of the progression and development of chronic renal insufficiency, rather than only a marker of glomerular dysfunction [1–3]. We investigated the thiol-dependent changes of albumin's tertiary structure in the mechanism of protein toxicity in the development of renal tubulointerstitial fibrosis (TIF) and diabetic nephropathy (DN).

DN is characterized by increased albuminuria, which in turn plays a subsequent role in TIF and is considered a factor closely correlated with end-stage renal disease (ESRD) [4–6]. Several pathways have been investigated to understand the nature of protein and albumin's morbid participation in TIF, including activation of the complement cascade [7–10], tubular chemokine and chemoattractant expression [11], NF-κB activation [12], PKC-dependent generation of fibrogenic molecules activated by interleukins [13], apoptosis activation and endoplasmic reticulum stress [14–16]. Additionally, transdifferentiation of epithelial tubular cells to myofibroblasts (EMT) induced by albumin overload contributes to the development of TIF by the interstitial deposit of collagen and fibronectin [17–19]. However, a direct link between protein overload and activation of these metabolic pathways is still missing [20], and the specific mechanisms by which increased protein concentrations in tubular cells result in toxicity remains elusive.

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Recent studies highlight the strong correlation that exists between albumin structural changes and decreased renal filtration rates presented by patients at different stages of renal disease [21,22]. These results established that thiol-linked structural changes of albumin and the redox state of the molecule correlate with the progression of DN. Serum albumin from diabetic patients in advanced stages of DN presented changes suggestive of secondary structure and overt thiol-dependent changes on its tertiary structure that can be detected and quantified [22,23]. These structural changes are explained by considering the remarkable stress that affects the dynamic of thiol groups during diabetes. In addition to redox imbalance, some functional consequences on albumin's structure include instability, reduced resistance to catabolism and altered binding properties. However, the specific mechanism of albumin participation in the pathogenesis of DN must still be elucidated.

We hypothesize that structural changes could play a role in albumin toxicity on renal proximal tubular cells (PTCs). In this study, we found that thiol-dependent changes in the tertiary structure of albumin affect the proteolytic activity of PTCs, producing cellular and molecular changes indicative of protein overload and tubular interstitial fibrosis, including reduced viability, apoptosis, transdifferentiation and endoplasmic reticulum stress. We used a model of human proximal tubular HK-2 cells and isolated albumin from patients with advanced stages of DN, as well as albumin structurally modified.

2. Materials and methods

2.1. Cell culture

Human proximal tubular cells (HK-2) were purchased from American Type Culture Collection (Manassas, VA 20108 USA) and maintained in DMEM/F12 (GIBCO, Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS (Equitech-Bio, Inc. Kerrville, Texas, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin under standard conditions of temperature humidity and CO_2. Cells were trypsinized using 0.25% trypsin-EDTA and incubated in 6-well plates until they reached an average of 80–100% confluence. Leupeptin was used as a protease inhibitor (Sigma, Chemicals, St. Louis, MO, USA), and thapsigargin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) as an endoplasmic reticulum stress positive control.

2.2. Cell viability and apoptosis

Cellular changes of viability were quantitatively assessed by measuring LDH leakage from damaged cells with the use of a colorimetric assay kit (Biovision, Milpitas, CA, USA) according to the manufacturer's instructions, with some modifications. Briefly, after incubation periods and prior to assays, a culture plate was incubated with 1% (V/V) Triton X-100 in FBS-free medium at 37 °C to obtain a positive control with 100% cytotoxicity, adjusting to the absorbance at 495 nm; the results were then reported in absorbance units. A functional positive control was achieved using several sucrose concentrations and determined at 50 mM by 4 h as a control with 50% cytotoxicity. Cell apoptosis was assayed by the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, CA, USA). After incubation periods, trypsinized and washed cells were resuspended in binding buffer and stained with Annexin V-FITC conjugated in dark conditions and then analyzed by flow cytometry.

2.3. Human samples

The protocol for the research project # R-2013-1602-18 was approved by the Ethics and Investigation Committee # 1602 of the General Hospital Regional # 1, Instituto Mexicano del Seguro Social (IMSS), Morelia, Michoacán, México. The investigation was performed in accordance with the principles of the 1995 Declaration of Helsinki and the 2000 Edinburgh revision. The patients signed informed consent forms, and the anonymity of each individual has been preserved. Samples were obtained from the Nephrology Unit at Regional General Hospital 1, Morelia, Michoacán, México. Serum samples obtained by vein puncture were divided into groups according to their estimated glomerular filtration rate (GFR) [24] as follows: the control group (Stage 0) consisted of samples from 17 early-diagnosed diabetic patients with no evidence of structural or functional renal decline; the Stage 1 group included samples isolated from 20 patients with renal damage, defined by abnormalities in the urine and blood tests with a GFR ≥90 mL/min/1.73 m^2; and the Stage 4 group consisted of samples isolated from 20 patients with overt renal damage and a GFR between 15 and 29 mL/min/1.73 m^2. The clinical information of patients grouped by GFR is presented in Table 1.

Table 1
Patients and samples grouped by GFR[a].

Characteristics	Control Stage 0	Initial DN Stage 1	Advanced DN Stage 4
n (male:female)	17 (7:10)	20 (5:15)	20 (9:11)
Age (years)	49 ± 9	47 ± 8	60 ± 9***
BMI[b] (kg m^{-2})	28.5 ± 5.7	32.8 ± 4.6*†	26.6 ± 6.4
Hb_{A1c}[c] (%)	6.1 ± 0.62	7.4 ± 1.1*	7.2 ± 0.32*
Blood creatinine (mg dL^{-1})	0.79 ± 0.19	0.84 ± 0.10**	3.48 ± 1.1***
GFR (mL/min/1.73 m^2)	124.8 ± 45	100.6 ± 9.9**	17.96 ± 3.9***

[a] Glomerular filtration rate (mL/min/1.73 m^2).
[b] Body mass index.
[c] Glycated hemoglobin.
* $p < 0.05$ vs. control.
*† $p < 0.05$ vs. control and advanced DN.
** $p < 0.01$ vs. control.
*** $p < 0.001$ vs. control and initial DN.

2.4. Isolation and processing of human albumin (HA)

Albumin was separated and purified by affinity chromatography using Blue Sepharose (GE-HealthCare Bio-Science AB, Uppsala, SW) as described [21,22]. Samples were packed in 10 ml columns washed and equilibrated with 5 bed volumes of starting buffer (50 mM citric acid, 100 mM Na_2HPO_4, pH 3.0). Each diluted sample was filtered with 0.45-μm Millipore membranes, passed through the column and washed with 10 additional bed volumes of starting buffer. The albumin bound to the gel was eluted with the corresponding buffer (50 mM KH_2PO_4 and 1.5 M KCl, pH 7.0), and fractions were dialyzed extensively using 10 kDa cut-off membranes. Each eluted fraction was examined with 4–20% SDS-PAGE gels (TruPage, Sigma-Aldrich, St. Louis, MO, USA) to verify protein integrity. For urine samples, proteins were concentrated using 30 kDa Ultra-15 centrifugal filters (Millipore, Bedford, MA, USA). An approximate volume of 1000 ml was processed to obtain the required albumin amount from controls and Stage 1 patients. The approximate volume to obtain albumin samples from Stage 4 DN patients was 30–200 ml on average. The urine samples were used only to show evidence of structural changes in urine albumin from advances stages of DN.

2.5. Induction of stress and measurement of albumin's tertiary structural changes

Albumin's structural change was measured with the use of a standardized procedure previously described [21,23]. Briefly, the stress produced over albumin's molecule modifies the SS-SH relationship, an essential component of tertiary structure. The molecular response to an insult yields an increased proportion of the reduced state, which can be measured. The postulated mechanism by which the reduced state increases progressively after a stress insult has been established [21], and the response to stress, the response surplus (RS), is an indirect measurement of albumin's structural change produced on its tertiary

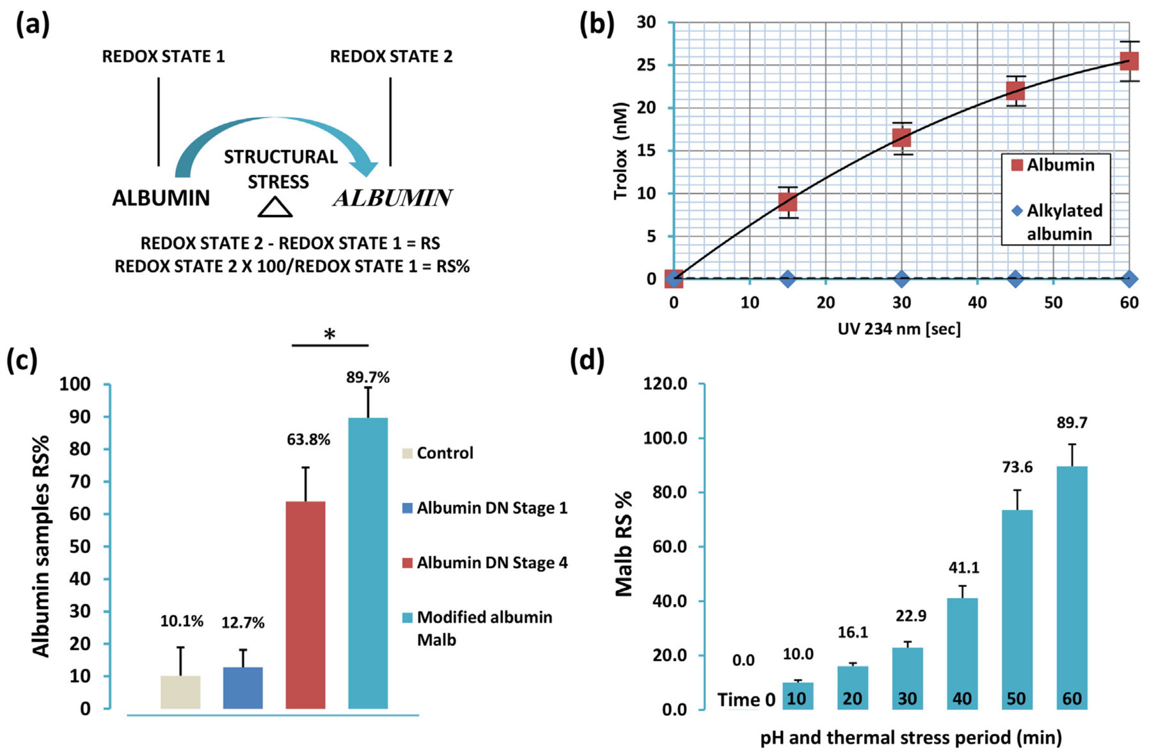


Fig. 1. Magnitude of change in the thiol-dependent tertiary structure of albumin (a): Simplified scheme of the response to stress measurement or RS. (b): Potential relationship established between the reduced state of structurally modified albumin (TEU) and stress duration (red squares). In a second line, the effect produced by alkylation of cysteine residues of albumin on the correspondent reduced capacity (blue diamonds) (c): RS% calculated; values above 50% were obtained in Stage 4 and Malb in relation with albumins from Stage 0 (control) and Stage 1. (d): Time course of RS/stress duration in the process to obtain Malb, as described in the Material and methods section. (*$p < 0.001$ vs. control and Stage 1.) TEU: Trolox equivalent units (nM); Malb: modified albumin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

structure. Albumin's RS was calculated by measuring the reduced state of proteins before and after an induced stress, and the final values (Δ) were reported as percentages. For the RS measurement, subsequent stress was produced with short-wave UV light as follows: albumin (30 µg/µL) in phosphate buffer pH 7.4 was irradiated for 30 s by a 6 W 0.16 A lamp, 3 UVTM-36 (UVP, Upland, CA, USA) with a wavelength adjusted to 254 nm. The energy of the light used in the experiments was 10 mW/cm^2, monitored with a radiometer UVX-25 (UVP, Upland, CA, USA) [21]. The temperature used for the experiments was maintained at 25 °C with a Peltier system. The reduced state of samples was calculated by the redox potential of copper (Cu) and the standard CUPRAC antioxidant capacity assay [25] before and after induced stress and calculated as Trolox equivalent units (nM). Final values of RS were reported as percentages.

2.6. *Alkylation of thiol residues of albumin*

Just after induced stress, alkylation of albumin residues of cysteine with iodoacetamide (Sigma, Chemicals, St. Louis, MO, USA) completely prevents albumin's reduction potential, corroborating the participation of cysteine residues in the RS profile. Albumin samples (100 µg) previously subjected to stress were dissolved in 100 µL ammonium bicarbonate solution (100 mM, pH 8.0). Prepared at the moment and in the former solution, aliquots of 5 µL of iodoacetamide (375 mM) were added to the samples and incubated for 30 min protected from light. After being dialyzed extensively with PBS pH 7.4, samples were processed to complete RS determination as previously described. The effect of alkylation of cysteine residues is described in the next section.

2.7. *Structurally modified albumin (Malb)*

Human albumin solutions (30 mg/mL in citrate buffer) were alkalinized and heated (pH 11.5 and 50 °C) for 1 h with slow agitation, and aliquots obtained by intervals of 10 min. After incubation periods, the pH values of the solutions were adjusted to 5.5 as follows: albumin treated samples and controls maintained at pH 5.5 were dialyzed by 10 kDa cut-off membranes (Millipore, Bedford, MA, USA) and with a citrate/phosphate buffer pH 5.5 as elution buffer. The protein concentration was calculated using the molar extinction coefficient of 36,040 M^{-1} cm^{-1} at 278 nm [26]. The methodological bases to develop focalized changes on the tertiary structure of albumin have been thoroughly described; the denaturation of albumin as performed here takes place in a sequential manner, and large changes in its tertiary structure are produced without morphological changes, loss of α-helix content or aggregation [27–30]. The identity and integrity of albumin were assessed on each sample with the use of 4–20% SDS-PAGE gels (TruPage, Sigma-Aldrich, St. Louis, MO, USA) and densitometric analysis. The final structural stress produced was estimated measuring the RS% of the samples (Fig. 1d), following previously reported procedures.

2.8. *Digestibility of albumin with papain*

To assess the participation of the mechanism of action common to cysteine proteases in relation to inhibition of cathepsin B activity, we performed assays with immobilized papain (PAPAIN-I) prepared with Sepharose activated with cyanogen bromide, according to the procedure described elsewhere [31]. Papain is a prototype cysteine protease and was used here as affordable model to explore the molecular mechanism of enzyme inhibition. Albumin was incubated with papain and filtered (30KU; Millipore, Bedford, MA, USA) to verify the rate of digestibility of modified and nonmodified albumin, as described in detail in the corresponding figures. The albumin concentration was measured with the Lowry's method [32].

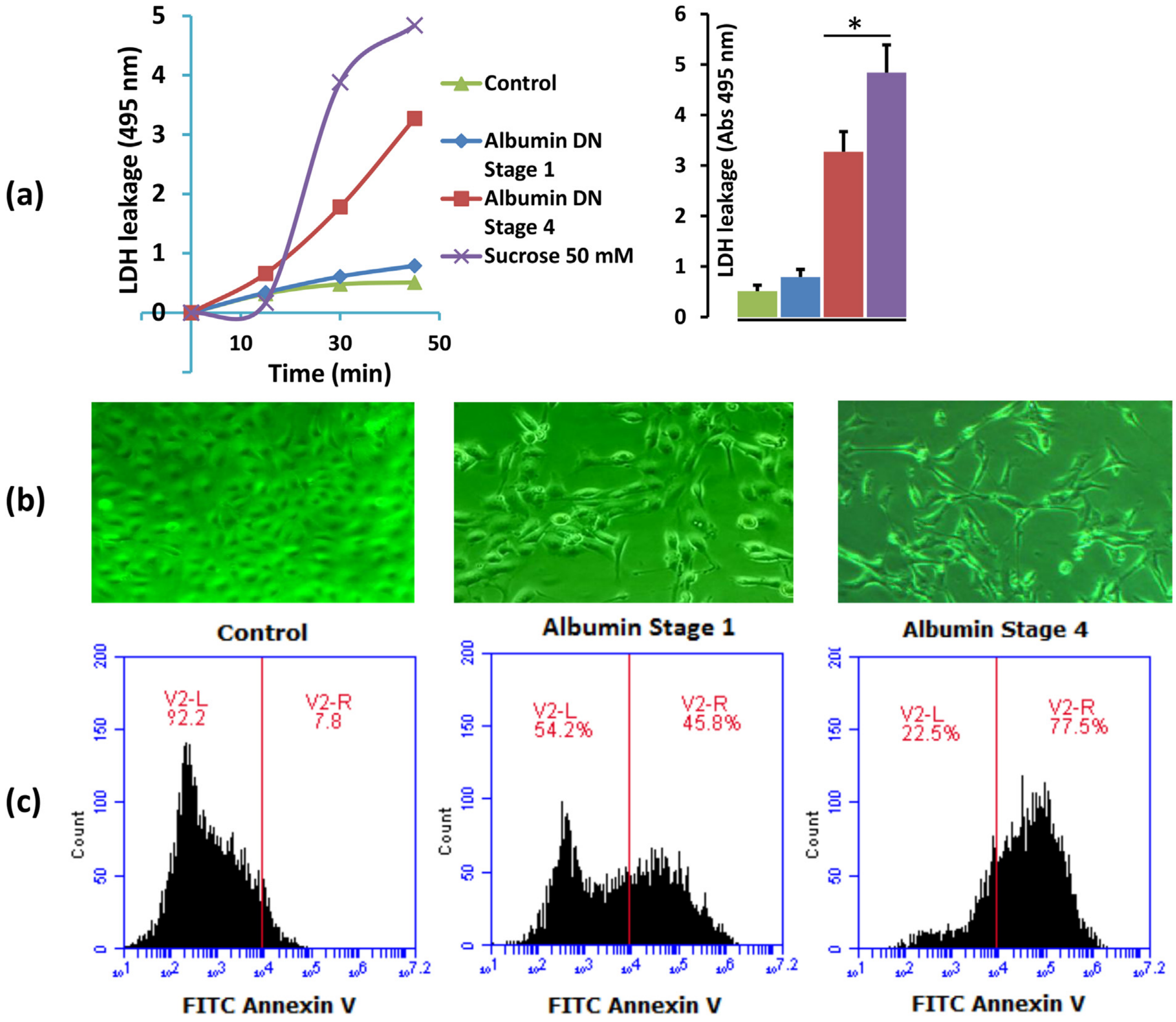


Fig. 2. Effect of albumin on the viability and apoptosis of HK-2 cells. (a) Left: time course of lactate dehydrogenase (LDH) leakage as an index of viability of tubular cells exposed to control (Stage 0), Stage 1 albumin, and albumin from patients with DN Stage 4 after 72 h of incubation with albumin (5 mg/mL) at standard conditions. Center: average ± SD of three experiments after an accumulated effect of 50 min of LDH leakage. Sucrose (50 mM/4 h) was used as a positive control as described in the Materials and methods section. (b): Morphological changes as observed after 72 h of incubation using phase contrast microscopy (500×). Left: normal mosaic morphology of cells grown with control albumin. Center: enlargement of cells and some blebbing in cultures grown with albumin from Stage 1 patients. Right: cells grown with albumin from patients in advanced stages of DN Stage 4; scarce blebbing and more enlargement and spindle shape of cells are observed (c): FITC Annexin V flow cytometric analysis of control tubular cells (left) and cells exposed to Stage 1 albumin (center), and albumin from patients with diabetic nephropathy (DN) Stage 4 (right). Even with an important number of cells FITC Annexin V positives with Stage 1 albumin (38%), a higher proportion of cells (69.7%) results FITC Annexin V positive after incubation with albumin from DN Stage 4 patients. $^{*}p < 0.001$ compared with the control.

2.9. Flow cytometry analysis

Human renal proximal HK-2 cells were washed 3 times with serum-free medium and 3 times with sterile, room temperature PBS, trypsinized, quenched with media containing serum, centrifuged and resuspended with cold HEPES 10 mM pH 7.0, centrifuged and resuspended in paraformaldehyde 4%, and then centrifuged and washed with HEPES. Before the staining procedure, the cells were incubated in 1 x PBS/BSA 0.5%, 0.15 M glycine to block nonspecific protein-protein interactions. Cells were counted and diluted to a minimal concentration of 1×10^{7} cells/mL and processed through flow cytometry analysis. The stained cells were analyzed using a BD Accuri flow cytometer (Becton Dickinson, Ann Arbor, MI, USA). Antibodies used to carry out cytometric analysis were E-cadherin-PerCP mouse against human and vimentin-Alexa 488 mouse against human (BD Biosciences, CA, USA).

2.10. Western blot analysis

Incubated cells were trypsinized, washed in cold PBS and suspended in HEPES buffer solution (pH 7.5, 2 mM EDTA, 1 mM DTT, 0.25 M sucrose, and proteinase inhibitors). Cell suspensions were sonicated, and the supernatant containing the cytosol fraction was obtained by centrifugation at 10,000 ×*g* (15 min, 5 °C). Samples containing 20 μg of cellular proteins were loaded and separated using precast 4–20% SDS-PAGE gels (TruPage, Sigma-Aldrich, St. Louis, MO, USA) and transferred onto PVDF membranes (Thermo Scientific, Rockford, IL, USA). After being blocked for 1 h with 5% milk in TBST, the samples were washed and incubated with primary antibodies in 5% BSA, 1× TBS, and 0.1% Tween 20 at 4 °C overnight. Specific proteins were detected by secondary antibodies conjugated with horseradish peroxidase and detected with enhanced chemiluminescence substrate SuperSignal West Dura Extended Duration Substrate (Thermo Scientific, Rockford, IL, USA). The primary antibodies used here were as follows: BIP HSPA5 (GRP78) rabbit against human (Cell Signaling Technology, Danvers,

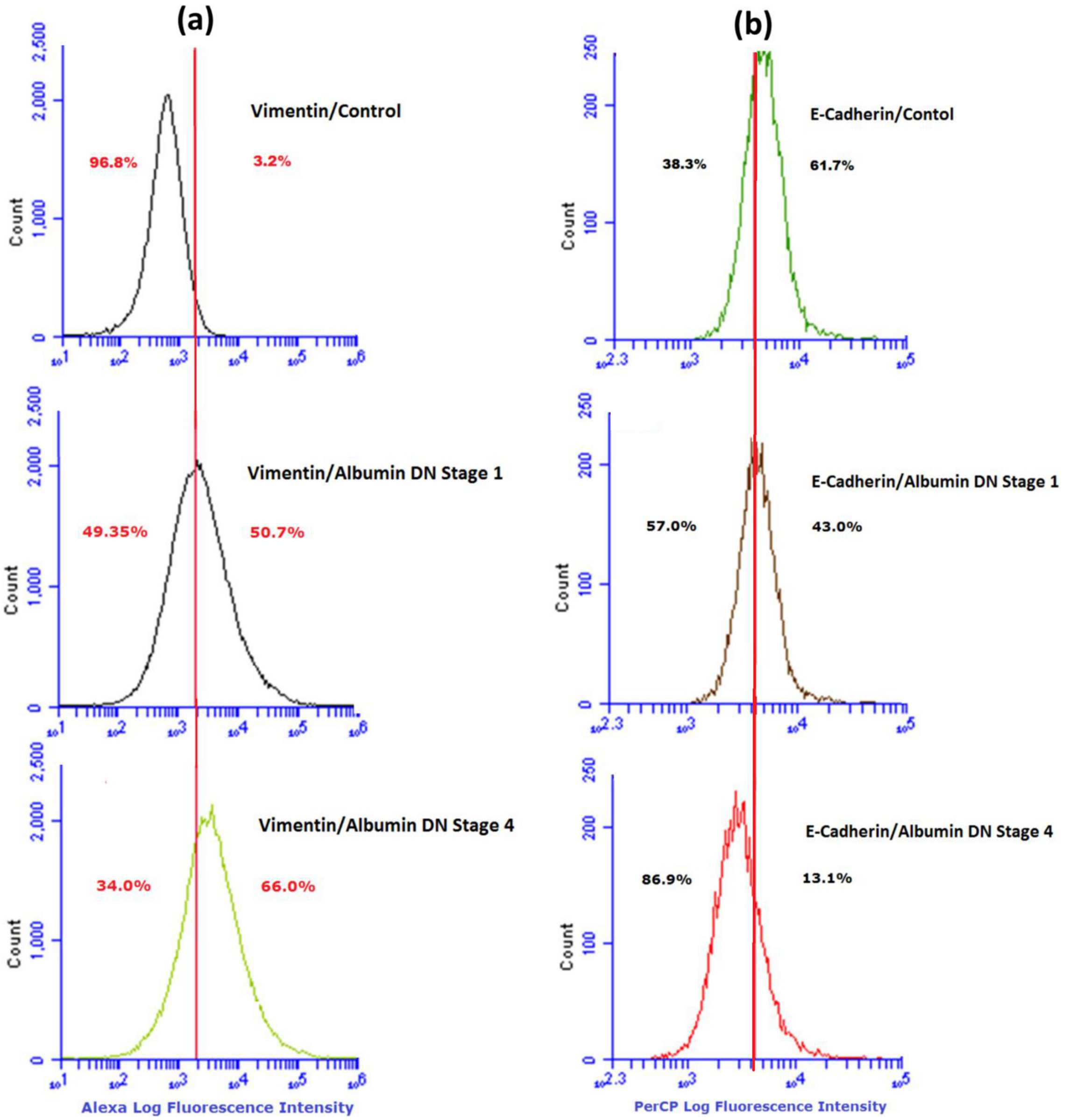


Fig. 3. Effect of albumin on the expression of profibrogenic markers in HK-2 cells. (a): Flow-cytometric histograms of HK-2 cells that were incubated with control albumin (Stage 0), Stage 1 albumin and albumin from Stage 4 patients for 72 h, labeled with Alexa-conjugated vimentin. (b): Representative flow-cytometric histograms of HK-2 cells that were incubated with control albumin (Stage 0), albumin from Stage 1 and albumin from Stage 4 patients for 72 h, labeled with PerCP-conjugated E-cadherin. At the end of incubation period, cells were washed with serum-free medium and trypsinized, centrifuged and resuspended in 10 mM HEPES pH 7.0 for analysis. A decrease of approximately 18% and nearly 50% of cells positive for E-cadherin was observed in Stage 1 albumin and albumin from DN Stage 4 patients respectively vs. control. For vimentin, an increase in positive cells of 50% and 60% on average for Stage 1 albumin and albumin from DN patients Stage 4, respectively, was observed. Cells were treated with albumin (5 mg/mL) as described in the Materials and methods section.

MA, USA), ACTA2 (α-SMA) mouse against human (Thermo Scientific, Rockford, IL, USA), and E-cadherin mouse against human (Sony Biotechnology, San Jose, CA, USA). The internal standard was β-actin (Cell Signaling Technology, Danvers, MA, USA).

2.11. Lysosomal fractions

Trypsinized and washed HK-2 cells were disrupted by sonication (25 kHz, 100 W X4 pulse of 5 s each in ice-cold sucrose 25 M). The crude lysosomal fractions were prepared by differential centrifugation, including low and medium speed at 1000 × *g*, 5000 × *g* and 20,000 × *g*. From the supernatant, final purified fractions were obtained by the separation of rough ER/mitochondria with the use of calcium chloride (8 mM final concentration) and low-speed centrifugation at 5000 × *g* for 15 min. The supernatants were concentrated with the use of centrifugal filters (30 kDa) and resuspended in an ice-cold buffer. The purified fractions were assayed for protein concentration and enzymatic activities of acid phosphatase, β-*N*-acetylglucosaminidase and cathepsin B. The integrity of lysosomes was assessed using Neutral Red dye as described [33]. Briefly, the dye appears concentrated in the lysosomes, and its uptake can be followed through the absorbance shift from 460 to 510 nm under lysosomal acidic conditions.

2.12. Cathepsin B activity measurement

Enzyme activity was quantified using a fluorometric assay and carbobenzoxy-Arg-Arg-7-amino-4-methylcoumarin as standard, following the principles of Barret and Kirschke [34]. To a 2.5 mL citrate

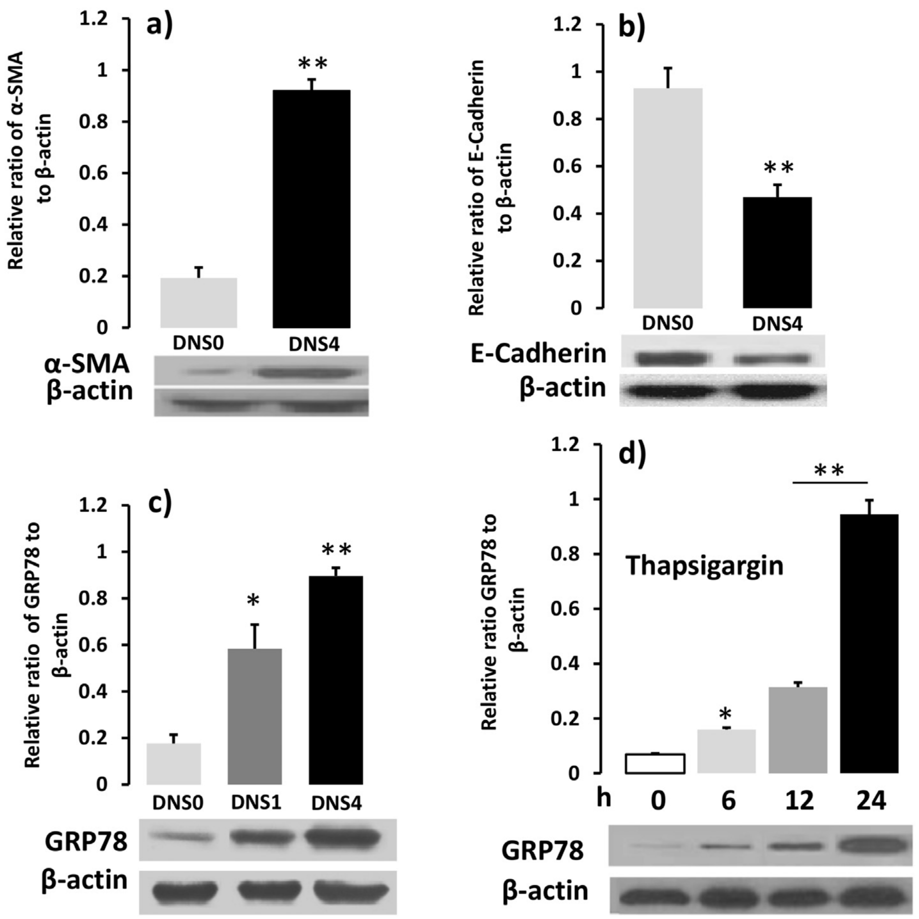


Fig. 4. Effect of albumin on the expression of epithelial-to-mesenchymal transition marker proteins α-SMA/E-cadherin and ER stress marker protein GRP78 in HK-2. a) Western blot for protein expression and densitometric analysis relative to β-actin of α-SMA of cells exposed 48 h to albumin from DN patients Stage 0 (DNS0) and patients with diabetic nephropathy Stage 4 (DNS4); b) Western blot and densitometric analysis of E-cadherin of cells exposed 48 h to albumin from DN patients Stage 0 (DNS0) and patients in Stage 4 of DN Stage 4 (DNS4); c) Western blot for protein expression and densitometric analysis of GRP78 of cell exposed 48 h to albumin from DN patients Stage 0 (DNS0), patients in Stage 1 (DNS1) and patients in Stage 4 of DN (DNS4); d) ER stress positive control with the use of thapsigargin (1 μM) on the expression of protein GRP78. Values are means ± SEM of three determinations of each group. β-Actin served as the control. $^*p < 0.01$ and $^{**}p < 0.001$ compared with S0 and time 0 h for GRP78 expression. α-SMA: alpha smooth muscle actin; GRP78: 78 kDa glucose-regulated protein. Cells were treated with albumin (5 mg/mL) as described in the Materials and methods section.

buffer (pH 5.0) containing substrate with a final concentration of 60 μM, a lysosomal aliquot corresponding to an approximate protein concentration of 100 μg/mL was added to a final volume of 3.0 mL and incubated at 37 °C. Fluorescence of the liberated product 7-amino-methyl-coumarin (AMC) was monitored continuously in a fluorescence spectrometer LS 45 (Perkin-Elmer, Llantrisant, UK) with an excitation wavelength of 370 nm and an emission wavelength of 460 nm. With this base, the effects of modified albumin over the enzymatic activity were evaluated. From assays with different amounts of albumin, the results presented here correspond to the concentration necessary to inhibit the enzymatic activity 20% to normal albumin, as presented in Fig. 7. Apparent *Km* values associated with the cathepsin B activities were determined using various concentrations of the substrate and the corresponding Lineweaver-Burk data transformation. The inhibitory effect of leupeptin on the remaining activity of the lysosomal fraction was calculated using an increasing concentration of the inhibitor. Lysosomes were incubated with the substrate and the inhibitor for 15 min in citrate buffer. The concentration of leupeptin necessary to completely inhibit the enzyme activity was close to 1 μM at established experimental conditions.

2.13. Acid phosphatase activity measurement

Phosphatase activity was measured using a colorimetric assay kit with 4-nitrophenylphosphate as a standard (CS0740, Sigma, Aldrich). Briefly, the incubation mixtures containing aliquots of lysosomal fractions, sodium acetate buffer (pH 4.8) and the substrate were incubated for 60 min at 37 °C, and the effects of albumin (200 μg/mL) over the enzymatic activity were evaluated. After incubation, 4-nitrophenol release was stopped by the addition of NaOH (1 M), and the absorbance was measured at 405 nm. Phosphatase activity was registered in mU/min and reported as mU/mg of protein. One unit of the enzyme produces the hydrolysis of 1 μmol of 4-nitrophenylphosphate per minute to generate 4-nitrophenol at the established experimental conditions.

2.14. N-acetyl-β-d-glucosaminidase activity measurement

The activity was measured using a fluorometric assay and 4-nitrophenyl *N*-acetyl-β-d-glucosaminide (Sigma, Aldrich) as standard. Aliquots of lysosomal fractions (500 μL), diluted in citrate buffer (pH 4.5) and containing 0.26 mM of substrate were incubated for 3 h at 37 °C. With this base, the effects of albumin (200 μg/mL) over the enzymatic activity were evaluated. At the end of the period of incubation, 3.5 mL of glycine buffer (0.5 M, pH 10.4) was added, and fluorescence was measured at 360/455 nm excitation/emission in a fluorescence spectrometer LS 45 (Perkin-Elmer, Llantrisant, UK). Enzyme activity was calculated by the micromoles of 4-methylumbelliferone hydrolyzed per minute at 37 °C and reported as mU/mg of protein.

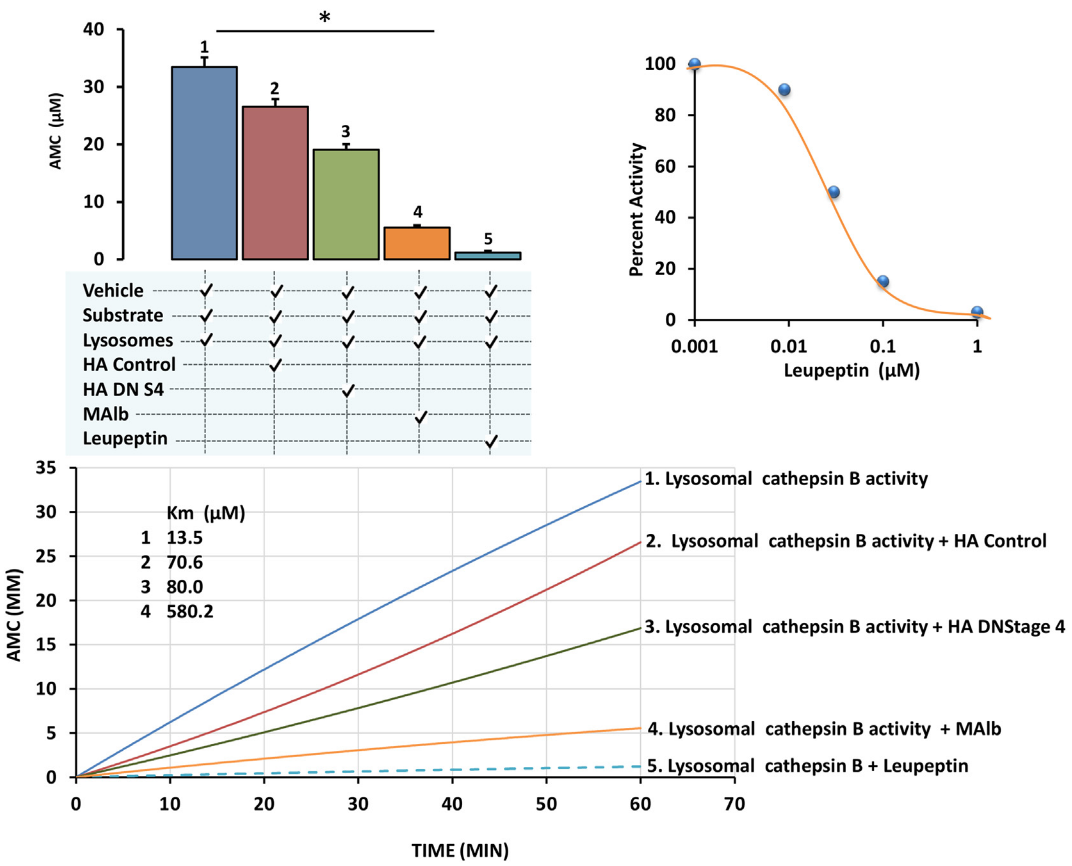
2.15. Statistical analysis

Analysis of the results was performed using the statistical software SPSS 10.0 (IBM, Chicago, IL, USA). Values are expressed as the mean ± SD. Groups were compared using the one-way ANOVA test followed by Dennett's test. Differences were considered statistically significant at $p < 0.01$.

3. Results

3.1. Redox changes determined by RS in albumin samples

Fig. 1a depicts the magnitude of albumin's structural change



	1	2	3	4	5
Vehicle	✓	✓	✓	✓	✓
Substrate	✓	✓	✓	✓	✓
Lysosomes	✓	✓	✓	✓	✓
HA Control		✓			
HA DN S4			✓		
MAlb				✓	
Leupeptin					✓

Fig. 5. Effect of albumin on enzymatic activity of cathepsin B in lysosomal fractions of HK-2 cells. Lysosomal fractions corresponding to a protein concentration of 100 μg/mL were incubated in the presence of albumin (control), albumin from patients with DN Stage 4 and albumin structurally modified (Malb). In the upper graph, the average register ± SD (n = 4) of cathepsin B activity (60 min); lower table corresponds to the sequential scheme of addition. An albumin concentration of 1.5 mg/mL produces an activity reduction of approximately 20% with albumin from patient with Stage 0 (control). Without significant differences with Stage 1 albumin (omitted in the graph). The substrate carbobenzoxy-Arg-Arg-7-amino-4-methylcoumarin was used with a final concentration of 60 μM. The fluorescence of the liberated product 7-amino-4-methylcoumarin (AMC) was registered continuously for 60 min in a fluorescence spectrometer, with an excitation wavelength of 370 nm and an emission wavelength of 460 nm at 37 °C. Apparent Km values associated with cathepsin B activity were determined, including several concentrations of the substrate and the corresponding Lineweaver-Burk data transformation. The upper right-hand graph depicts the leupeptin inhibitory concentration required to suppress the lysosomal protease activity. DN: diabetic nephropathy; HA: human albumin; Malb: modified albumin. *p < 0.001 vs. leupeptin addition.

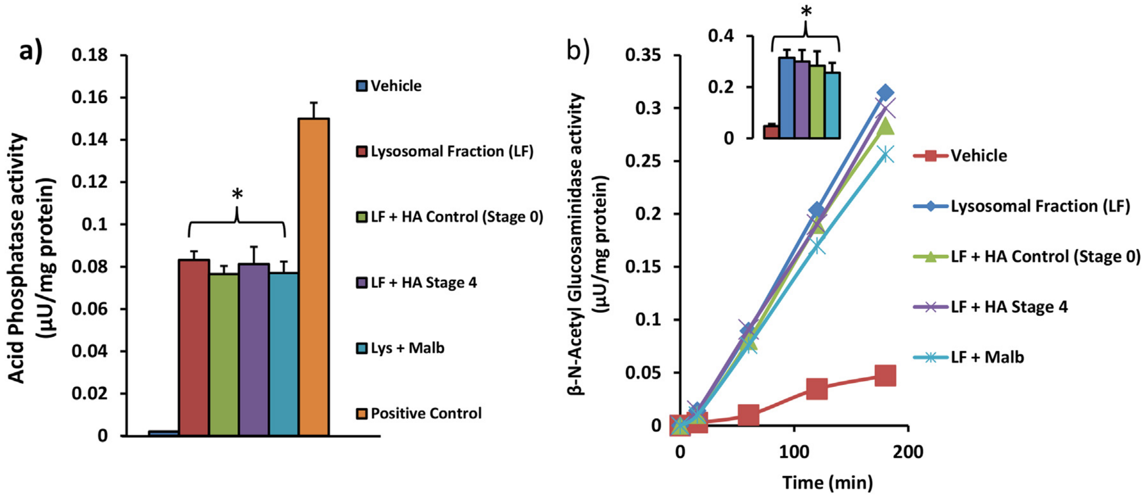


Fig. 6. Effect of albumin on enzymatic activity of acid phosphatase (AF) and β-*N*-acetylglucosaminidase (NAGase). a) Enzymatic activity of AF in lysosomal fractions (LF) of HK-2 cells incubated with control (Stage 0), Stage 4 and Malb by 60 min at 37 °C. Positive control corresponds to lyophilized wheat germ acid phosphatase. *p < 0.001 vs. vehicle and positive control. b) Enzymatic activity of NAGase in lysosomal fractions (LF) of HK-2 cells incubated with albumin control (Stage 0), Stage 4 and Malb by 3 h at 37 °C. control. Details about procedures are in the Materials and methods section of this manuscript. Malb: Modified albumin. *p < 0.001 vs. vehicle; without significant differences between the groups.

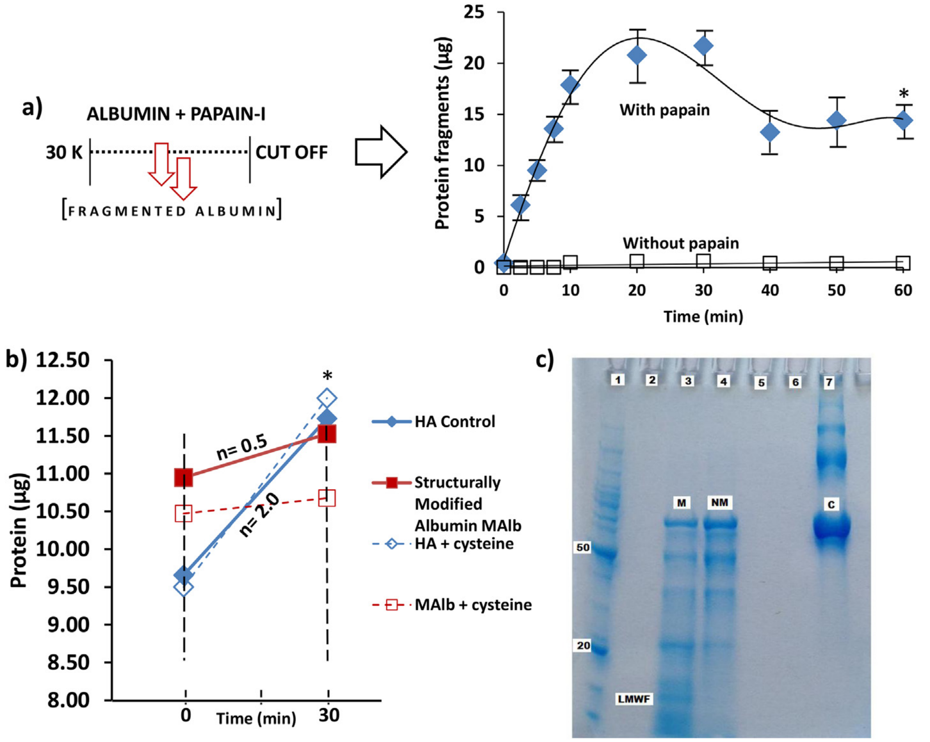


Fig. 7. Evidence that redox changes of albumin affect papain's digestibility. Native and modified albumin were incubated with immobilized papain (PAPAIN-I) and dialyzed with centrifugal filters with 30 kDa molecular cutoff and the filtrate measured as protein fragments as described in the Materials and methods section. a) Prototype assay. Albumin (30 mg/mL) was incubated 30 min with PAPAIN-I (600 μU/mg) and filtered (30KU-15). The albumin fragments were measured by the Lowry's method. Initial value without protein was subtracted of all the fractions. Each point corresponds to the average of three different experiments by period of time. b) Rate of digestibility of commercial albumin (1 mg/mL) by PAPAIN-I (5 U/mL). Slope of lines blue and red represents no modified (NM) and modified albumin (Malb) during 30-min of incubation, corresponding to a rate of 2.0 μg/min and 0.5 μg/min respectively. Dotted colored lines represent the rate of albumin proteolysis by papain in a reducing environment with cysteine 1 mM. c) Polyacrylamide gel electrophoresis of nonmodified (NM) and modified albumin (Malb) incubated with papain. Lines 7 and 1 correspond to control albumin (without papain) (C) and molecular weight marker, respectively. Time 0 corresponds to the time expended until first centrifugation (10 min, 3500 ×*g*, 5 °C). *$p < 0.001$ vs. initial value. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

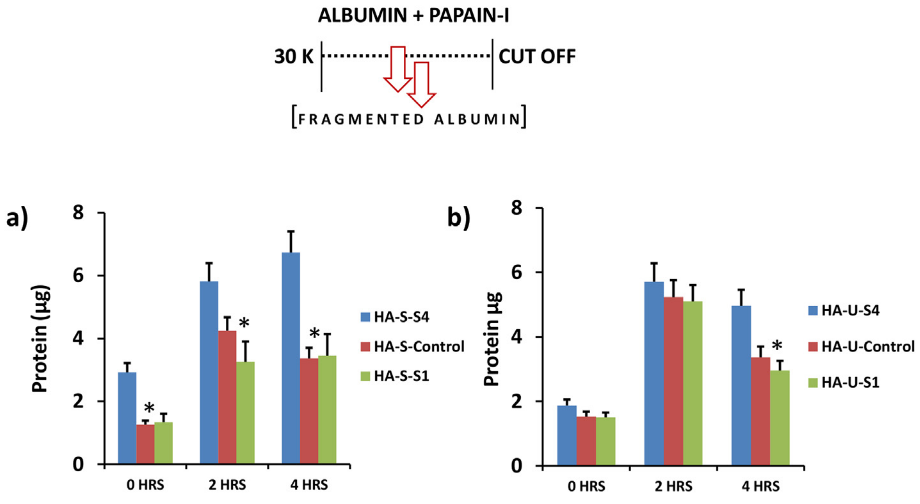


Fig. 8. Effects of serum and urine human albumin (HA) on papain's digestibility. Albumin was incubated with papain (PAPAIN-I) and dialyzed with centrifugal filters with 30 kDa molecular cutoff as described in the Materials and methods section. a) Human albumin (0.1 mg/mL) from serum of patients without DN (HA-S-control), with DN Stage 1 (HA-S-S1) and with DN Stage 4 (HA-S-S4) was incubated with papain (approximately 1 U) and filtered (30KU-0.5); protein fragments were measured in the filtrates; data corresponds to the average of three different experiments by period of time. b) Human albumin from urine (0.1 mg/mL) of diabetic patients without DN (HA-U-control), with DN Stage 1 (HA-U-S1) and with DN Stage 4 (HA-U-S4) were incubated and processed as previously described for serum samples; data correspond to the average of three different experiments by period of time. Albumin from serum and only partially from urine of patients with DN Stage 4 presented reduced digestibility. *$p < 0.001$ when compared with albumin from control and Stage 1. Time 0 corresponds to the time expended until first centrifugation (10 min, 3500 ×*g*, 5 °C).

measured by the RS parameter before and after an induced stress. The final values obtained were reported as percentages. The linear relationship between reduced state and stress duration is depicted in Fig. 1b (red square). In the same graph, alkylated albumin treated as described previously abrogates the reduced potential of albumin (blue diamonds). Fig. 1c shows the final RS value of albumin separated from control patients, patients with early evidence of DN (Stage 1), patients with advanced DN (Stage 4) and modified albumin (Malb). The separated samples of advanced DN patients presented an RS higher than RS control and Stage 1 patients without evidence of renal damage (53% and 48% respectively) ($p < 0.001$). The time course of intentionally stressed albumin (Malb) is depicted in Fig. 1d. The reduced state of the samples was calculated by the redox potential of Cu after pH-thermal stress and reported as Trolox equivalent units (nM), as shown in the Materials and methods section.

3.2. Albumin from diabetic patients with advanced stages of DN impaired cell viability and induced apoptosis in HK-2 tubular cells

Albumin induces changes in cultured HK-2 cells, which is indicative of cell damage (Fig. 2). HK-2 cells were exposed to isolated albumin from patients with and without DN, and the effects on viability were determined. After 72 h of incubation of serum-free cultures with albumin of Stages 4 patients, viability of cells as measured by LDH leakage was significantly reduced ($p < 0.001$), which is in contrast with Stage 1 and control cultures growing under standard conditions. Similar differences occurred with the cultures grown to confluence and incubated 4 h with 50 mM sucrose (Fig. 2a). In contrast, some changes

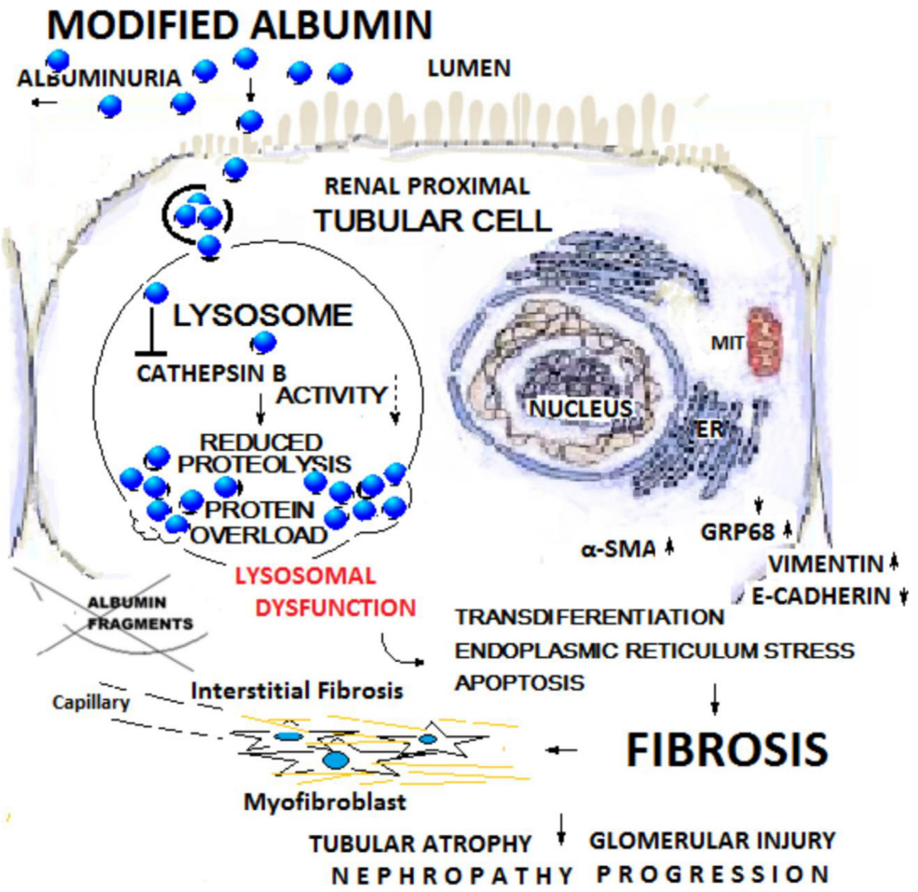


Fig. 9. Possible lysosomal dysfunction produced by albumin over the course of diabetic nephropathy. Proposed mechanism of changes in the tertiary structure of albumin in the accumulation of proteins inside a tubular cell by inhibition of lysosomal proteolytic activity, which triggers events of stress derived in apoptosis, transdifferentiation and ER stress. The stress linked to diabetes, such as oxidative stress associated with oxygen, nitrogen, chloride, Fe and Cu species, hypoxia, uremic toxins, etc., can disturb the stability of the tertiary structure of albumin. A consistent decrease in the rate of catalytic activity of lysosomal cysteine proteases could explain protein overload with the changes described previously. The specific biochemical pathway by which albumin's structural changes modify the action of proteases is not yet established, but the results indicate that a mechanism common to cysteine-proteases is implicated.

compatible with apoptosis were observed, even in cells treated for 72 h with Stage 1 albumin (Fig. 2b). Under the microscope, evident changes in shape with enlargement and some blebbing were observed, in contrast with the control culture, which showed the normal mosaic-shape morphology of HK-2 cells. Less blebbing with more cell enlargement were observed in cells grown with albumin from patients in advanced stages of DN (Stage 4). Under the same context, apoptosis evaluated with flow cytometry reveals that it is possible to find an increase in Annexin V positive cells even in Stage 1 albumin without evidence of changes of renal damage, although the increase was significantly higher in cells grown with albumin from patients with advanced stages of DN (Fig. 2c).

3.3. Albumin from diabetic patients with advanced stages of DN and high RS induces profibrogenic markers, epithelial-to-mesenchymal transition (EMT) and endoplasmic reticulum stress (ERS)

We found evidence that the tertiary structure of albumin induces molecular changes in cultured HK-2 cells, indicative of transdifferentiation and endoplasmic reticulum stress. Evidence of increased expression of vimentin and α-SMA, two profibrogenic markers observed during DN progression was found by flow cytometry (Fig. 3a and b) and Western blotting (Fig. 4a and b), respectively, when TBCs were incubated for 72 h with albumin separated from both patients in Stages 1 and 4. From cytometric analysis, an average reduction of approximately 18% and near 50% for Stage 1 albumin and albumin from DN Stage 4 patients, respectively, was observed in cells positive for E-cadherin (Fig. 3a), and an average increase of 50% and 60% in cells positive for vimentin (Fig. 3b) vs. control was observed for Stage 1 albumin and albumin from DN patients Stage 4, respectively. Incubation of HK-2 cells with albumin from patients with DN increased expression of the ER stress protein GRP78 in relation to Stage 0, with $p < 0.01$ and $p < 0.001$ respectively, as shown in Fig. 4c. A positive control demonstrated a time-dependent overexpression of GRP78 when HK-2 cells were exposed to 1 μM thapsigargin, from 0 to 6 h ($p < 0.01$), 12 and 24 ($p < 0.001$) hours of incubation (Fig. 4d).

3.4. Albumin from DN patients has inhibitory effects on the proteolytic activity of lysosomes

Fig. 5 shows the observed effect of albumin on proteolytic cathepsin B activity of lysosomal fractions isolated from HK-2 cells. The assays performed involved the successive addition of components of the reaction, leading to the following observations: 1. Addition of a fixed amount of substrate, carbobenzoxy-Arg-Arg-7-amino-4-methylcoumarin, to the reaction mix containing vehicle and lysosomal fractions of HK-2 cells, liberates a constant, time-dependent amount of AMC (33.4 μM), considering 100% activity, in a 60 minute assay. 2. Addition of albumin isolated from Stage 0 patients (HA control) to the reaction mix containing vehicle, substrate and lysosomal fractions of HK-2 cells liberates a constant, time-dependent amount of AMC (26.5 μM), equivalent to 79.4% activity, considering the average 60 minute assay. Assays with samples from Stage 1 patients did not present significant differences with HA control, and this result was omitted for purposes of clarity. 3. Addition of albumin from patients with DN Stage 4 to the reaction mix containing vehicle, substrate and lysosomal fraction of HK-2 cells liberates a constant, time-dependent amount of AMC (19.0 μM), equivalent to 48.1% activity, considering the average 60 minute assay. 4. Addition of modified albumin (Malb/RS = 89.7%) to the reaction mix containing vehicle, substrate and lysosomal fraction of HK-2 cells, liberates a constant, time-dependent amount of AMC (16.8 μM), equivalent to 16.4% activity considering the average 60 minute assay. 5. Addition of 1 μM leupeptin to the reaction mix containing vehicle, substrate and lysosomal fraction of HK-2 cells liberates a constant, time-dependent amount of AMC (1.2 μM) equivalent to 3.3% activity, considering the average 60 minute assay. Lysosomal activities of two additional enzymes, *N*-acetyl-β-d-glucosaminidase and acid phosphatase, did not show significant differences when incubated with normal albumin and albumin from DN Stage 4 patients or Malb (Fig. 6).

3.5. The causative effect of inhibition of albumin on cathepsin B is related to the mechanism of cysteine proteases

Papain was used as a cysteine protease prototype to explore the mechanism of action implicated in the results observed here in relation to inhibition of cathepsin B activity. Fig. 7a shows the results of an assay that uses centrifugal filters with 30 kDa cut-off to verify successively the rate of digestibility of modified (Malb) and nonmodified albumin (NM) by immobilized papain (PAPAIN-I). Fig. 7b shows the differential rate of digestibility of albumin when it is structurally modified and incubated with papain. Moreover, although the reduced state alone is not responsible for producing the inhibitory effect observed, the inclusion of cysteine to the assays modifies to some degree the rate of digestibility (dotted red line). Fig. 7c shows the digestibility presented by papain in normal albumin (NM) in contrast with structurally modified albumin (Malb). Modified albumin inhibits the proteolytic activity of papain and reduces the production of low molecular weight fragments (LMWF), principally those with a MW lower than 20 kDa.

3.6. Papain incubated with albumin from both serum and urine of patients with DN shows evidence of reduced digestibility

To find evidence to support the possible link between human albumin from diabetic patients in advanced stages of DN and inhibition of cysteine proteases, albumin isolated from serum and urine of patients with and without DN was subjected to papain digestion as explained in the Materials and methods and in the previous section. Evidence obtained (Fig. 8) support the notion that changes in the albumin molecule present in serum pass through to urine. After incubation of albumin with immobilized enzyme (PAPAIN-I) and the determination of digestibility and protein measurement, we found that albumin from serum and partially from urine of patients with DN Stage 4 (HA-S-S4 and HA-U-S4, respectively) (Fig. 8a and b) presented reduced digestibility compared to the pairing albumin from patients without evidence of DN (control) (HA-S-control and HA-U-control) and albumin from patients with DN Stage 1 (HA-S-S1 and HA-U-S1) (*$p < 0.001$ for serum and 4 h with urinary albumin when compared with albumin from control and Stage 1).

4. Discussion

Many studies have confirmed that if TBCs are exposed to excessive amounts of protein, a number of inflammatory and fibrogenic mediators indicative of TIF and glomerular damage are produced [6–13]. Although the albumin that appears in urine is the result of the balance between glomerular filtration and tubular reabsorption, there is evidence to support a primary role of renal proximal tubules in the progression of nephron injury. In addition to age-dependent kidney decline, factors associated with hyperglycemia and glucose metabolism, such as glycation and oxidative stress, are implicated in the origin and progression of DN. Glycation and oxidative stress combined produce albumin modifications, including loss of both secondary and tertiary structure [35], impairing its redox properties and thiol group content [36]. Also we previously reported the structural stress and redox changes on albumin produced by partial glycation of the molecule [23]. However, the specific biochemical mechanism by which albumin produces tubular injury is still ignored.

There is evidence that shows that the degradation of reabsorbed proteins is inhibited in the proximal tubule via decreased cathepsin B activity in lysosomes [37,38]. The overload of proteins produced by decreased lysosomal protease activity could explain the excretion of less degraded albumin and the reported reduction of lower molecular weight fragments excreted in urine [39]. We found evidence that structurally modified albumin reduces the cathepsin B-dependent enzymatic activity of isolated lysosomal fractions of HK-2 cells and even the proteolytic capability of papain to produce low molecular weight fragments. The results presented here propose a biochemical mechanism that explains, at least partially, how protein accumulates inside TBCs and how this could promote fibrosis and DN progression.

The consequence of a large number of disulfide bonds in albumin, not only in human albumin but also in serum albumin of various species, facilitates the formation of pairings by disulfide interchange that generate isomeric forms at cysteine pairs that in part account for albumin's notable heterogeneity, as was extensively studied and documented several years ago [40–44]. As a consequence of albumin's heterogeneity and the exacerbated stress imposed by diabetes in the case of DN, isomeric forms of albumin with high levels of changes on the tertiary structure, decreased resistance to catabolism and faster catabolic rates are progressively formed. From here and taking into account previous studies [21,24], we postulate that these isomeric forms prevent albumin proteolysis by direct inhibition of cathepsin B enzymatic activity. In this condition, if the rate of lysosomal proteolysis is compromised, an imminent overload of proteins with consequent renal tubular injury may be produced.

The mechanism that explains how an overload of protein is produced and is detrimental to renal function remains partially ignored or controversial. In a study with animal models of focal segmental glomerulosclerosis, it has been postulated that an acute albumin overload in PTCs can be counteracted with increased lysosomal proteolysis [45]. According to this study, cells respond to increased levels of protein by elevating endogenous enzyme synthesis, which results in the removal of accumulated proteins. However, a similar study sustains that increased proteolysis is not sufficient to maintain normal clearance of proteins and suggests that proteinuria develops as a result of the limited capacity of lysosomes in TBCs to process an increased load of endocytosed proteins [46]. An additional postulate should consider the extended time that it takes diabetes to generate gradual loss of renal function over years, which would overwhelm a compensatory mechanism. Additionally, using a distinct line of research with streptozotocin-induced diabetic rats, there is evidence of decreased activities of lysosomal enzymes including cathepsin B in the kidney and the prevalence of high molecular weight peptides and proteins in the urine, which are indicative of diminished excretion of digested proteins after 30 days of overt diabetes [39].

Despite the expected controversies regarding albumin overload as exposed in the described studies and others, the hypothesis postulated here introduces a differential element: starting from the focal point of a particular change in the tertiary structure of the albumin molecule as a key element in the mechanism that produces protein accumulation inside lysosomes of TBCs by a process that itself explains protein overload (Fig. 9). In addition, in the present study, we employed albumin at lower concentrations in assays with cells, in contrast to the models of protein overload [47–49], supporting the notion that in addition to the protein amount, an additional element plays a leading role in renal albumin processing. Previously, Clavant and Comper noted the importance of albumin's tertiary structure in protein processing in studies with isolated kidneys and suggested the existence of specific recognition events that permit normal albumin to avoid excretion as a mechanism of degradation of altered albumins [50]. However, they recognized the purpose of the cited research was not to determine whether the altered albumin might be responsible for renal disease or albuminuria. In contrast, using TBCs, we did not identify evidence of such a mentioned recognition mechanism, but we did find a morbid consequence of the tertiary structural change of albumin: inhibited catalytic activity of the main kidney lysosomal cysteine protease, protein overload, and the consequent activation of multiple signaling pathways, compatible with TIF. The biochemical mechanism by which the described structural change of albumin modifies the action of cathepsin B has not been established, but the results indicate that the catalytic mechanism of cysteine proteases may be implicated.

5. Conclusion

Our data indicate that thiol-dependent changes in the tertiary structure of albumin interfere with lysosomal proteolytic activity of HK-2 tubular cells, inducing cellular and molecular changes compatible with protein accumulation, interstitial fibrosis and renal damage.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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