Glycated and carbamylated albumin are more “nephrotoxic” than unmodified albumin in the amphibian kidney

M.-L. Gross, G. Piecha, A. Bierhaus, W. Hanke, T. Henle, P. Schirmacher, and E. Ritz

1Institute of Pathology, University of Heidelberg, Heidelberg; 2Institute of Zoology, Technical University, Karlsruhe; 3Institute of Food Chemistry, Technische Universität, Dresden; and 4Department of Internal Medicine, University of Heidelberg, Heidelberg, Germany

Submitted 17 June 2010; accepted in final form 1 March 2011

Gross M, Piecha G, Bierhaus A, Hanke W, Henle T, Schirmacher P, Ritz E. Glycated and carbamylated albumin are more “nephrotoxic” than unmodified albumin in the amphibian kidney. Am J Physiol Renal Physiol 301: F476–F485, 2011. First published March 2, 2011; doi:10.1152/ajprenal.00342.2010—There is increasing evidence that proteins in tubular fluid are “nephrotoxic.” In vivo it is difficult to study protein loading of tubular epithelial cells in isolation, i.e., without concomitant glomerular damage or changes of renal hemodynamics, etc. Recently, a unique amphibian model has been described which takes advantage of the special anatomy of the amphibian kidney in which a subset of nephrons drains the peritoneal cavity (open nephrons) so that intraperitoneal injection of protein selectively causes protein storage in and peritubular fibrosis around open but not around closed tubules. There is an ongoing debate as to what degree albumin per se is nephrotoxic and whether modification of albumin alters its nephrotoxicity. We tested the hypothesis that carbamylation and glycation render albumin more nephrotoxic compared with native albumin and alternative albumin modifications, e.g., lipid oxidation and lipid depletion. Preparations of native and modified albumin were injected into the axolotl peritoneum. The kidneys were retrieved after 10 days and studied by light microscopy as well as by immunohistochemistry [transforming growth factor (TGF)-β, PDGF, NF-κB, collagen I and IV, RAGE], nonradioactive in situ hybridization, and Western blotting. Two investigators unaware of the animal groups evaluated and scored renal histology. Compared with unmodified albumin, glycated and carbamylated albumin caused more pronounced protein storage. After no more than 10 days, selective peritubular fibrosis was seen around nephrons draining the peritoneal cavity (open nephrons), but not around closed nephrons. Additionally, more intense expression of RAGE, NF-κB, as well as PDGF, TGF-β, EGF, ET-1, and others was noted by histochemistry and confirmed by RT-PCR for fibronectin and TGF-β as well as nonradioactive in situ hybridization for TGF-β and fibronectin. The data indicate that carbamylation and glycation increase the capacity of albumin to cause tubular cell damage and peritubular fibrosis.

proteinuria is associated with nephrotoxicity (25). For a long time, it has been a matter of debate whether albumin per se or alternative serum proteins are mainly responsible for the adverse effect of proteinuria on renal progression. It has been well documented that in diabetes selective albuminuria, i.e., albuminuria in the absence of other proteins in the urine, is associated with the risk of progressive renal disease (17, 22, 34).

Address for reprint requests and other correspondence: M.-L. Gross, Institute of Pathology, INF 220/221, 69120 Heidelberg, Germany (e-mail: Marie-Luise.Gross@med.uni-heidelberg.de).

The tubulointerstitial space plays an important role in the progression of renal disease. Tubuli and interstitium occupy a substantial percentage of kidney volume (19). It has been emphasized that in contrast to tissue cultures where the morphological and functional changes of epithelial cells appear very quickly (4), the development of renal lesions caused by proteinuria overload in experimental animals takes weeks (5). It has been argued that cellular infiltrations, particularly of macrophages and T cells, play an important role in the genesis of interstitial fibrosis. Tubular epithelial cells contribute to this process through the epithelial-to-mesenchymal transition (EMT). During the inflammatory phase, such activated fibroblasts are stimulated to produce collagenous matrix after stimulation by cytokines, such as TGF-β, EGF, or ET-1 (28).

Several investigators proposed the hypothesis that it is protein uptake into tubuloepithelial cells rather than the presence of albumin and other proteins in the tubular urine which induces an inflammatory phenotype and causes renal damage (24, 25, 27, 29, 37). Such studies further documented that plasma proteins other than albumin cause more intensive tubular cell damage, e.g., complement factors (18), iron-containing proteins, specifically ferritin (35), and others.

Recently, we introduced the axolotl kidney as an amphibian model which allows assessment of the associated nephrotoxicity of proteinuria in vivo independently of the potential confounding effect in mammalian kidneys, e.g., glomerular damage, altered hemodynamics, etc. (10). The amphibian kidney has a subset of nephrons which drain the peritoneal cavity so that selective protein loading of proximal tubular epithelial cells is possible in the minority of “open” nephrons which drain the peritoneal cavity, but not in “closed” nephrons which derive tubular fluid only via the glomerular filtrate. Moreover, in protein-overload studies in rodents, the albumin-bound fatty acids may never appear in the glomerular filtrate. Moreover, in protein-overload studies in rodents, the albumin-bound fatty acids may never appear in the glomerular filtrate, because in the circulation fatty acids are loosely bound to albumin (30).

Using this model, the current study was designed to test the hypothesis that glycation or alternative modifications (e.g., carbamylation; delipidation; fatty acids containing) increase the associated nephrotoxicity of albumin. The readout was the intensity of selective peritubular fibrosis around open tubuli draining the peritoneal cavity.

METHODS

Amphibian Kidney as a Model

The kidney of the axolotl (Ambystoma mexicanum) represents an amphibian opisthophros in which ciliated peritomal funnels, so-called nephrostomae, drain the peritoneal cavity. In urodelic amphibians such as the axolotl, such nephrostomae connect to the proximal tubule in close proximity to the glomerulus. In former pilot studies,
we marked the nephron lumen of open nephrons by an intraperitoneal injection of ink and thus provided proof that only open nephrons were loaded by proteins (10). Principles of laboratory animal care were followed in the present study.

**Experimental protocol.** Eighteen-month-old neotenic axolotls of both sexes, weighing between 80 and 120 g, were obtained from the axolotl colony at the University of Indiana. In pilot studies, we did not find sex-specific differences in protein loading of open nephrons. To obtain appropriate numbers of these rare animals, we studied both males and females. The animals were maintained in tanks of aerated tap water at a constant temperature of 18°C with a 12:12-h light (0600–1800)-dark cycle (1800–0600). One week before the study, the animals were randomly allotted to 6 groups of 10 animals each. After randomization, the sex proportion in the six experimental groups varied from one to two females to eight to nine males. Vehicle and saline as a control; group 2 received albumin (25 mg/day); group 4 received carbamylated albumin (25 mg/day); and group 6 received lipid-depleted albumin (25 mg/day).

For 10 days the animals received daily intraperitoneal injections of 0.2 ml vehicle or modified albumin dissolved in isotonic saline. The solutions were endotoxin free as verified by Limulus assay. We reasoned that during the 10-day duration of the study antibody production by these poikilothermic animals was extremely unlikely. In the present study, we used the above dose of unmodified albumin, because pilot experiments had shown no tubular uptake of the lower doses.

Under general anesthesia (3-aminobenzoic acid ethyl ester, A-5040, Sigma, Deisenhofen, Germany; 10 g/l water in the tank), blood was obtained and retrograde perfusion fixation was started via the main heart ventricle, using 3% glutaraldehyde as a fixative for light microscopy. The kidneys were excised and embedded in paraffin or Epon-Araldite.

For immunohistochemistry, animals were perfused with ice-cold isotonic saline. The kidneys were then excised. One part was snap-frozen, and the other part was fixed with 4% formaline. Light microscopy and electron microscopy. Two-micrometer paraaffin sections were stained with a connective tissue stain (Ladewig stain) and examined using light microscopy at a magnification of ×100. The tubulointerstitial changes were quantified by two “blinded” examiners who were unaware in the assignment to the treatment groups. A scoring system was used to compare the slides under study with a set of photos of standardized lesions, i.e., tubular dilatation, protein droplet content of tubular epithelial cells, and peritubular interstitial fibrosis around protein-storing tubules. Non-protein-storing tubules were not included in the scoring process, because in a preceding paper it had been shown by serial sections that protein storage by tubular epithelial cells occurred only in open nephrons (10) (see also Fig. 1).

The scoring system was as follows: 0, no change; 1, minimal change; 2, moderate change; 3, marked change; and 4, very pronounced change.

The tubular dilatation score of 0 corresponded to an average diameter of 50 μm, 1 to 100 μm, 2 to 150 μm, 3 to 200 μm, and 4 to 250 μm or more.

In two randomly selected animals per group, ultrathin kidney sections were cut, stained with lead citrate and uranyl acetate, and assessed using a Zeiss EM 10 at various magnifications.

**Preparation of Modified Albumin Solutions**

**Glycation of albumin.** Advanced glycation end products (AGE)-albumin was prepared by incubating 1 mg/ml BSA (fraction V; A-8806 cell culture tested, low endotoxin, fatty acid free, Sigma-Aldrich) with 200 mM glucose at 37°C for 6 wk in 100 mM phosphate, pH 7.4, 0.5 mM sodium azide under sterile conditions. At the end of incubation, AGE-albumin preparations were dialyzed against 100 mM phosphate for 24 h and 0.9% NaCl for 12 h. Assays against 100 mM phosphate for 24 h and 0.9% NaCl for 12 h.
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for endotoxin showed AGE-albumin preparations to contain undetectable levels of LPS (<10 pmol at a protein concentration of 5 mg/ml).

Fatty acids containing and depletion of albumin. Fatty acids containing (BSA fraction V, A-4503) and lipid-poor albumin (BSA fraction V, A-6003) were purchased from Sigma.

Carbamylation of albumin. Albumin (5 mg/ml of protein in 0.15 M NaCl, 0.01% EDTA) was diluted with 0.3 M sodium borate, pH 8.0, to 1.5 times the original volume. Potassium cyanate (20 mg/ml of albumin) was added, and the mixture was incubated at 37°C for 2 h following the procedure described by Weisgraber et al. (33). Excess reagents were removed by dialysis at 4°C against 0.15 M NaCl, 0.01% EDTA, pH 7.0, for 36 h before the albumin preparations were used in the binding ligand degradation assays.

Measurement of carboxymethyllysine. Carboxymethyllysine (CML) was measured as the corresponding trifluoroacetylated methyl esters via gas chromatography/mass spectrometry (GC/MS) after acid hydrolysis. GC/MS was performed on a HP 6890 Plus GC system with an HP 5973 mass-selective detector, using an HP-5 phenylmethylsiloxane capillary column (Agilent Technologies, Waldbronn, Germany). Protein samples were hydrolyzed for 23 h at 110°C in the presence of 6 N hydrochloric acid (under a nitrogen atmosphere). After addition of the internal standard (N-α-carboxymethylornithine), the hydrolysate was dried in vacuo, dissolved in 2 ml of 2 N methanolic HCl, and kept at 65°C for 30 min. After this, the solution was dried in vacuo, dissolved in 500 μl of dichloromethane and 1 ml trifluoroacetic acid anhydride, and kept at room temperature for 1 h. After drying under nitrogen, the residue was dissolved in 300 μl of dichloromethane and subjected to GC/MS analysis. Conditions of GC and MS have been described elsewhere (1)

Measurement of pentosidine, lysine, and arginine. Protein samples were hydrolyzed for 23 h at 110°C in the presence 6 N hydrochloric acid under a nitrogen atmosphere. The hydrolysate was dried in vacuo and dissolved in 0.2 N sodium citrate buffer, pH 2.2. Amino acid analysis was performed on an Alpha Plus amino acid analyzer (LKB-Biochrom, Cambridge, UK), using a stainless steel column (150 × 4 mm) filled with ion-exchange resin DC4A-spec (sodium form, Benson, Reno, NV). The composition of elution buffers, ninhydrin reagent, as well as running conditions were described elsewhere (11). Lysine and arginine were quantified via amino acid analysis and ninhydrin detection by using external standards. Pentosidine was measured via direct fluorescence detection before ninhydrin derivatization as described elsewhere (12).

Immunohistochemistry

For immunohistochemistry, the antibodies listed in Table 1 were used. These antibodies are widely used for tissue of Xenopus laevis, which is a close relative of the axolotl (13, 20). Cryostat sections of Xenopus laevis were used. These antibodies are widely used for tissue of Xenopus laevis, which is a close relative of the axolotl (13, 20). Cryostat sections of Xenopus laevis were used.

Table 1. Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Species</th>
<th>Dilution</th>
</tr>
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<tbody>
<tr>
<td>NF-κB</td>
<td>Santa Cruz Biotecnology, Santa Cruz, CA</td>
<td>Sc-1410</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Santa Cruz Biotecnology, Santa Cruz, CA</td>
<td>Sc-146</td>
</tr>
<tr>
<td>IL-8</td>
<td>Abca, Hiddenhausen, Germany</td>
<td>Ab-7747</td>
</tr>
<tr>
<td>PDGF-AB</td>
<td>Upstate, Hamburg, Germany</td>
<td>No 06-127</td>
</tr>
<tr>
<td>EGF</td>
<td>Santa Cruz Biotecnology, Santa Cruz, CA</td>
<td>Sc-1343</td>
</tr>
<tr>
<td>ET-1</td>
<td>Bio Trend, Cologne, Germany</td>
<td>No 4113-0915</td>
</tr>
<tr>
<td>RAGE</td>
<td>R&amp;D Systems, Wiesbaden, Germany</td>
<td>AF-1145</td>
</tr>
<tr>
<td>B-catenin</td>
<td>Santa Cruz Biotecnology, Santa Cruz, CA</td>
<td>Sc-7199</td>
</tr>
<tr>
<td>Clusterin</td>
<td>Santa Cruz Biotecnology, Santa Cruz, CA</td>
<td>Sc-7870</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>Sigma, St. Louis, MO</td>
<td>A 5691</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Sigma, St. Louis, MO</td>
<td>T 9026</td>
</tr>
<tr>
<td>AGE (advanced glycation end products)</td>
<td>BioLogo, Kronshugen, Germany</td>
<td>AGE102-0.2</td>
</tr>
</tbody>
</table>

Two investigators blinded with respect to the diagnosis used a semiquantitative scoring system for the analysis (light microscopy, magnification ×200). Only those tubules were considered for scoring which showed definite protein storage. The mean calculated concordance for the scores between the two investigators was from k = 0.71 to 0.86. As described previously (2, 10, 30), the intensity of staining was ranked on an arbitrary grading scale: 0, no staining; 1, faintly positive staining; 2, positive staining involving up to 50% of the field of view; 3, positive staining involving >50%; and 4, positive staining of all structures within the field of view.

Semiquantitative RT-PCR for Renal Fibronectin and TGF-β Expression

Total RNA was isolated from kidneys using peq Gold RNA Pure (peq Lab, Erlangen, Germany) according to the manufacturer’s instructions. RNA was checked for degradation of total RNA on a 1% agarose gel. One microgram of RNA from each animal was reverse transcribed with the help of a First Strand cDNA Synthesis Kit for RT-PCR (Roche Diagnostics, Mannheim, Germany) with random primer (final concentration 3.2 μg). One microgram of RNA and random primer was incubated at 65°C for 10 min, followed by cooling at 4°C. Then, the reaction mix (buffer, RNase inhibitor, RT, dNTP) was added. The complete mixture was incubated as proposed by the manufacturer. To ensure the fidelity of mRNA extraction and RT, all samples were subjected to PCR amplification with oligonucleotide primers specific for the constitutively expressed gene GAPDH to normalize the data. All PCR reactions were performed using a LightCycler-Fast Start DNA Master+ SYBR Green I Kit (Roche Diagnostics) and quantified.

The primer sequences for the fibronectin gene were as follows: sense 5-ATT GCG TAC TCC CAA CTT CG-3 and antisense 5-ACA TGC TTC CAC GAG TC-3.

The primer sequences for the TGF-β gene were as follows: sense 5-CAG GAG CCT GCC CAT ATT TA-3 and antisense 5-GCT GTT CGA TTT TGG GTG TT-3.

The cycling conditions for fibronectin were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles for 95°C for 10 s, 64°C for 10 s, and 72°C for 14 s using LightCycler.

The cycling conditions for TGF-β were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles for 95°C for 10 s, 56°C for 10 s, and 72°C for 12 s using LightCycler.

The fluorescent product was collected at 72°C at the last step of each cycle.

Western Blots (Fibronectin, TGF-β, Collagen I)

Tissue was homogenized in solubilizing buffer (pH 7.5, 0.1% Triton X-100, 10 mM EDTA, 50 mM HEPES, 4 mM EGTA, 10 mM NaPO4, 100 mM glycerol phosphate, 25 mM sodium fluoride, 1 mM sodium orthovanadate) and Protease Inhibitor Cocktail (Roche). The protein extracts were separated on a 6 and a 15% SDS-polyacrylamide
gel and transferred to polyvinylidene difluoride membranes (Immunoblot-PVDF, Bio-Rad).

Immunodetection was performed with rabbit polyclonal anti-fibronectin (1:100, 4°C overnight, F-3648, Sigma-Aldrich), rabbit polyclonal anti-TGF-β1 (1:100, 4°C overnight, sc-146, Santa Cruz Biotechnology), and rabbit polyclonal anti-collagen (1:200, 4°C overnight, 2150–0020, Biotrend).

A lysate of HepG2 cells served as a positive control (used for fibronectin) and mouse anti-β-actin antibody (1:15,000, MP Biomedicals) was used as a loading control. Incubation with primary antibody was followed by incubation with a 1:2,000 dilution of goat anti-rabbit or goat anti-mouse horseradish peroxidase-labeled antibody (Amer- sham, Bioscience). Visualization was performed with ECL+ (Amer- sham, Bioscience).

Nonradioactive In Situ Hybridization of Axolotl Fibronectin and TGF-β

A 215-bp cDNA fragment of fibronectin cDNA was obtained from a PCR product using axolotl-specific primers (see above) after reverse transcription of axolotl kidney total RNA.

For in situ hybridization of TGF-β1, a 168-bp fragment of the cDNA was obtained from a PCR product using primers from Gallus gallus, because axolotl-specific primers were not available.

The segment encoding positions 156–370 of the axolotl fibronectin gene was inserted into the pGEM-T easy vector system (Promega Biotech, Madison, WI). For preparation of the antisense probe, the plasmid was linearized with NcoI and transcribed using Sp6 bacterio- phage RNA polymerase. Linearization of the plasmid with Bsal and transcription with T7 RNA polymerase generated the sense RNA probe. Digoxigenin-labeled RNA probes were synthesized using a DIG RNA labeling mix (Roche).

The preparation for the probes of TGF-β1 was done in a parallel fashion.

Paraffin sections (5 μm on adhesive slides = Superfrost plus), were deparaffinized with Xylol and hydrated. After deproteinization with proteinase K (10 μg/ml) and refixation in 4% paraformaldehyde/PBS (pH 7.0, 20 min), the sections were acetylated [10 min, 0.5% acetic anhydride (vol/vol) in 0.1 mol/l triethanolamine, pH 8.0] to reduce nonspecific background staining. Sections were dehydrated in graded alcohols, defatted in chloroform (5 min), and air-dried. The probes were prehybridized in buffer containing 40% formamide for 30 min at 60°C in a moist chamber and then hybridized with 250 pg/μl of labeled probe in buffer as before for 17 h at 60°C.

Samples for TGF-β1 were not prehybridized; hybridization took place with 250 pg/μl of labeled probe in buffer containing 40% formamide for 17 h at 56°C.

Posthybridization steps included removal of unbound probe and two subsequent washes in 2× SSC/50% formamide (30 min each, 60°C), RNase A treatment (20 μg/ml, 0.5 mol/l NaCl, 10 mmol/l Tris-HCl, pH 8.0, 1 mmol/l EDTA, 30 min 37°C), and washes in 1× SSC (37°C, 10 min and 2× 5 min, RT). After posthybridization washes, the sections were equilibrated in buffer 1. Nonspecific background staining was blocked by incubation for 1 h with 0.5% blocking reagent/buffer 1 (Roche).

The hybridization products were visualized by enzyme-linked immunoassay using alkaline phosphatase-conjugated sheep anti-digoxi- genin-Fab fragments (1:300, 750 U/ml, Roche). Following incubation at 4°C overnight, unbound conjugate was removed by two washes in buffer 1, then equilibrated in buffer 3. Nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate served as a chromogen. A negative control was performed using a sense RNA probe.

The last steps were identical to those for the TGF-β1 analysis.

Statistics

Immunohistochemical scores are given as the median and 25 and 75 percentiles, respectively. Data for Western blotting, RT-PCR, and in situ hybridization are given as means ± SD. After testing for normal distribution, the Kruskal-Wallis test or one-way ANOVA was chosen, followed by Duncan’s multiple-range test to test for differences between groups. The results were considered significant when P was <0.05.

RESULTS

Animal Data

The animals in the different groups showed no differences in body weight (average 62.1 ± 6.2 g) and kidney weight (average 0.29 ± 0.05 g).

Chemical Analysis of the Albumin Preparations

As far it was detectable, carbamylated albumin showed the highest percentage of derivatized lysine and arginine (Table 2). CML showed the highest CML pentosidine content.

Albumin Loading and Renal Histology

There were no changes in glomerular histology in any of the groups.

Compared with saline controls, clusters of adjacent dilated tubules with protein sludge within the lumen and numerous protein droplets within tubular epithelial cells were seen compared with saline controls. Such clusters were interspersed between perfectly normal tubuli. Selectively around protein storing tubuli, broad bands of collagen-positive fibrotic tissue (as identified by Ladewig staining) were seen, but no infiltrat- ing cells were detected. The histological scores are summa- rized in Table 3.

When the different groups of treated animal were compared, the number of protein droplets in tubular epithelial cells, tubular dilation, and early tubular fibrosis around protein-storing tubuli were significantly more pronounced in animals injected with glycated albumin compared with either saline controls or animals treated with high-dose unmodified albu- min. The findings were less pronounced in animals exposed to carbamylated, fatty acid-containing and lipid-depleted albu- min.

A unique feature of the amphibian kidney is apparently the storage of protein by collecting duct epithelial cells.

Table 2. Chemical analysis of albumin preparations

<table>
<thead>
<tr>
<th>Albumin Preparation</th>
<th>Derivatized Lysine, %</th>
<th>Derivatized Arginine, %</th>
<th>CML, mg/100 g</th>
<th>Pentosidine, mg/100 g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>17.4</td>
<td>0.04</td>
</tr>
<tr>
<td>Glycated albumin</td>
<td>13.2</td>
<td>9.1</td>
<td>27.3</td>
<td>0.196</td>
</tr>
<tr>
<td>Carbamylated albumin</td>
<td>64.3</td>
<td>5.7</td>
<td>0.28</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Fatty acid-containing albumin</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>0.04</td>
</tr>
<tr>
<td>Lipid depleted albumin</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>Not detectable</td>
<td>0.05</td>
</tr>
</tbody>
</table>

CML, carboxymethyllysine.

AJP-Renal Physiol • VOL 301 • SEPTEMBER 2011 • www.ajprenal.org
changes in collecting duct epithelial cells, i.e., protein droplets, dilatation, and peritubular fibrosis, went in parallel with the finding in proximal tubules. Compared with animals exposed to unmodified albumin, animals with glycated albumin had significantly higher scores in protein storage and peritubular fibrosis.

**Immunohistology**

After intraperitoneal injection of carbamylated or glycated albumin, staining for PDGF-AB in the epithelial cells of albumin-loaded tubuli was significantly increased compared with saline control (Table 4).

**Table 4. Immunohistochemical staining of open tubuli and interstitium, respectively (score 0–4)**

<table>
<thead>
<tr>
<th>Protein storage</th>
<th>Saline Control</th>
<th>Unmodified Albumin</th>
<th>AGE-Albumin</th>
<th>Delipidated Albumin</th>
<th>Fatty Acid-Containing Albumin</th>
<th>Carbamylated Albumin</th>
<th>Kruskal-Wallis Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubuli</td>
<td>0.28 (0.14–0.31)</td>
<td>0.42 (0.36–0.58)</td>
<td>0.79 (0.77–0.95)</td>
<td>0.57 (0.33–0.60)</td>
<td>0.48 (0.35–0.71)</td>
<td>0.75 (0.71–0.83)</td>
<td>0.001</td>
</tr>
<tr>
<td>Interstitium</td>
<td>0.04 (0.00–0.08)</td>
<td>0.15 (0.03–0.21)</td>
<td>0.41 (0.30–0.44)</td>
<td>0.22 (0.15–0.49)</td>
<td>0.13 (0.02–0.34)</td>
<td>0.22 (0.19–0.27)</td>
<td>0.023</td>
</tr>
<tr>
<td>RANTES</td>
<td>0.48 (0.36–0.55)</td>
<td>0.24 (0.21–0.33)</td>
<td>0.10 (0.10–0.17)</td>
<td>0.18 (0.15–0.20)</td>
<td>0.48 (0.26–0.54)</td>
<td>1.32 (1.05–1.36)</td>
<td>0.001</td>
</tr>
<tr>
<td>Tubuli</td>
<td>0.10 (0.00–0.06)</td>
<td>0.05 (0.00–0.14)</td>
<td>1.00 (0.00–1.00)</td>
<td>0.10 (0.00–0.24)</td>
<td></td>
<td>0.00</td>
<td>0.004</td>
</tr>
<tr>
<td>Interstitium</td>
<td>0.28 (0.06–0.49)</td>
<td>0.04 (0.00–0.12)</td>
<td>0.48 (0.39–0.50)</td>
<td>0.69 (0.39–0.87)</td>
<td>0.96 (0.90–1.06)</td>
<td>1.20 (1.07–1.42)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

After injection of carbamylated albumin, staining for the AGE receptor (RAGE) was significantly more pronounced in the albumin-storing tubules, but not in collecting ducts or the surrounding interstitium compared with the groups injected with saline, glycated, lipid-depleted, fatty acid-containing or high-dose albumin. Expression of AGE was significantly increased in the AGE (see Fig. 2) albumin-injected animals compared with all intervention groups.

The protein expression of NF-κB (see Fig. 3) and TGF-β (see Fig. 4) was significantly higher in tubular epithelial cells of animals injected with glycated albumin and carbamylated albumin compared with control animals and animals injected
with unmodified albumin, lipid-depleted albumin, or fatty acid-containing albumin, respectively. There was no difference between the tubular epithelial cells of animals injected with saline, unmodified albumin, fatty acid-containing albumin, or lipid-depleted albumin.

The expression of NF-κB in the surrounding interstitial space was higher in the group injected with carbamylated and glycated albumin compared with the control group and the group injected with unmodified albumin (see Fig. 3).

More intense staining for EGF and endothelin-1 was also seen in the tubular epithelial and interstitial cells of animals injected with carbamylated albumin compared with the other groups.

In animals injected with carbamylated albumin, staining of tubular epithelial cells for IL-8 was significantly more intense compared with the other experimental groups.

In the tubular epithelial cells, staining for clusterin was significantly more pronounced in animals injected with carbamylated albumin and tended to be higher in animals injected with glycated albumin. Tubular staining with tubulin showed significantly increased expression in all modified albumin intervention groups compared with control and unmodified albumin.

Interstitial α-smooth muscle actin staining was significantly elevated in AGE- and carbamylated albumin compared with the other groups (see Fig. 5).

Staining for cathepsin was significantly more pronounced in animals injected with carbamylated albumin compared with all other experimental groups.

**RT-PCR of Fibronectin and TGF-β**

Fibronectin expression was significantly increased ($P < 0.05$) by semiquantitative RT-PCR in the kidneys of animals injected with glycated albumin (score $3.9 \pm 1.67$) compared with animals injected with carbamylated albumin ($1.125 \pm 0.24$), unmodified albumin ($1.3 \pm 0.54$), lipid-depleted albumin ($1.7 \pm 0.56$), enriched albumin ($1.2 \pm 1.08$), or saline ($1.6 \pm 0.76$).

TGF-β expression was significantly increased ($P < 0.05$) by semiquantitative RT-PCR in the kidneys of animals treated with glycated ($3.1 \pm 0.8$) and carbamylated albumin ($2.8 \pm 0.9$) compared with animals injected with high-dose albumin ($1.53 \pm 0.7$), lipid-depleted albumin ($1.29 \pm 0.87$), and fatty acid-containing albumin ($1.2 \pm 0.8$) or saline ($1.48 \pm 0.5$).

**Western Blotting (Collagen I, TGF-β, and Fibronectin)**

Western blotting probed with collagen I gave a clear band at the expected molecular sizes ($\sim130$ kDa). Western blotting...
probed with TGF-β gave a clear signal at 12.5 kDa, and fibronectin at the expected sizes (200 kDa) (see Fig. 6).

Nonradioactive In Situ Hybridization of Axolotl Fibronectin and TGF-β1

The expression of fibronectin mRNA was significantly higher \( (P < 0.05) \) in tubular epithelial cells of animals injected with glycated albumin \( (2.07 \pm 0.56) \) and carbamylated albumin \( (1.56 \pm 0.58) \) compared with animals injected with saline \( (0.25 \pm 0.04) \), high-dose albumin \( (0.4 \pm 0.2) \), lipid-depleted albumin \( (1.27 \pm 0.47) \), and fatty acid-containing albumin \( (0.9 \pm 0.47) \).

The expression of TGF-β1 mRNA was significantly higher \( (P < 0.05) \) in tubular epithelial cells of animals injected with glycated albumin \( (0.53 \pm 0.08) \) or carbamylated albumin \( (0.4 \pm 0.26) \) compared with animals injected with saline \( (0 \pm 0) \), high-dose albumin \( (0.06 \pm 0.18) \), fatty acid-containing albumin \( (0.2 \pm 0.03) \), and lipid-depleted albumin \( (0.1 \pm 0.02) \).

DISCUSSION

The above findings were obtained using light microscopy, immunohistology, and RT-PCR. Using peritubular fibrosis as a readout, they confirm the working hypothesis that intraperitoneal injection of endotoxin-free glycated or carbamylated albumin causes more intense tubuloepithelial and interstitial lesions than unmodified albumin and fatty acid-containing or lipid-depleted albumin preparations. Furthermore, the study supports the hypothesis that albumin modified by glycation or carbamylation is strikingly “nephrotoxic.” This conclusion is in excellent agreement with the observation of the limited nephrotoxicity of unmodified albuminuria in minimal change glomerulonephritis (35) and particularly the unique familiar nephropathy Imerlund Gräsbeck syndrome, where selective albuminuria for decades fails to cause deterioration of renal function or advanced renal lesions (35).

It is remarkable that in a cold-blooded animal, albumin injection into the peritoneum causes such fast-appearing impressive pathology of tubular epithelial cells and peritubular fibrosis after no fewer than 10 days. Such lesions were found selectively in the epithelial cells of the open tubuli draining the peritoneal cavity and the surrounding interstitium.

The present study documents that these pathologies are mediated by upregulation of factors involved in the AGE-NF-κB pathway; remarkably, such pathology was seen not only after exposure to glycated but also after exposure to carbamylated albumin. Furthermore, exposure to glycated as well as carbamylated albumin upregulated factors known to be involved, among others, in the genesis of interstitial fibrosis, e.g., PDGF, TGF-β, EGF, and ET-1. Chronic kidney disease with resulting increased fibrosis, regardless of the etiology of the disease, is associated with increased expression of growth

![Fig. 4. Immunohistological staining with transforming growth factor (TGF)-β (magnification ×40). A: control animal with saline ip: no expression of TGF-β. B-D: marked expression of TGF-β (protein level) in animals with ip injection of glycated albumin (B) and carbamylated albumin (C) but not in animals after ip injection of lipid-depleted albumin (D).](http://ajprenal.physiology.org/)

![Fig. 5. Expression of immunohistological staining against α-smooth muscle actin (SMA). Either AGE-albumin- or carbamylated albumin-loaded animals showed an increased expression compared with the other intervention groups. *P < 0.002 vs. saline control, unmodified albumin, lipid-depleted, and fatty acid-containing albumin.](http://ajprenal.physiology.org/)
MCP-1 is known to promote interstitial fibrosis, at least in humans (32). Expression of MCP-1 is found in human tubular epithelial cells in various forms of glomerulopathy (23), e.g., in IgA nephropathy, urinary MCP-1 levels are also correlated with the degree of interstitial fibrosis (8). No significant staining for MCP-1 was found in the axolotl, but the failure to detect it may be due to species difference.

RANTES is another agent which is induced when tubular epithelial cells are exposed to proteins (38). The pathogenetic role is documented by the observation that blocking its effects by Met-RANTES reduces tubular damage in an acute renal rejection model (9). The expression of RANTES was significantly elevated in axolots exposed to carbamylated albumin but not to unmodified or other forms of modified albumin.

In tubular cells, AGE products activate NF-κB via the RAGE in tubular cells (15, 21, 26). In the amphibian kidney, NF-κB expression was upregulated when the tubules were exposed to either carbamylated or glycated albumin. To document that albumin exposed to high glucose concentration had indeed been covalently modified, we measured carboxy-methyllysine, an Amadori product, the concentration of which was significantly increased in the preparation used for intraperitoneal injection.

In the past, increased expression of NF-κB by proximal tubular cells has been shown in kidneys of diabetic animals (16), but it had not been clarified whether this was the result of exposure to high glucose concentration or tubular uptake of modified proteins modified by exposure to high glucose. The present study suggests that the latter is the case.

TGF-β is currently viewed as the key cytokine promoting fibrosis, mediating chemotaxis and transformation of fibroblasts to myofibroblasts, and synthesis of extracellular matrix proteins, e.g., fibronectin and collagen type 1 (28). In the present model, TGF-β is presumably one of the main players in the genesis of peritubular fibrosis, since TGF-β expression was markedly elevated in tubular epithelial cells of axolots after intraperitoneal injection of glycated and carbamylated albumin compared with saline controls. We also found elevated expression of fibronectin mRNA, and this finding was confirmed using nonradioactive in situ hybridization.

One remarkable finding of this study was the observation that peritubular fibrosis around protein-storing tubuli was seen as soon as 10 days after intraperitoneal injection of modified albumin. It is possible that epithelial cell EMT, which has been studied in great detail in mammalian species (36), occurs at a faster rate or more easily in the phylogenetically more primitive amphibian tissue. For instance, amphibian species, including the axolotl, are able to rapidly reconstitute lost extremities (6).

Compared with previous studies on tubular protein storage after glomerular injury or injection of excessive amounts of protein, the present model is unique in that it allows for the selective study of tubular protein uptake without potential artifacts from changes in global renal hemodynamics of peritubular microcirculation or of concomitant glomerular abnormalities by confronting only a minority of tubuli with a protein load. We believe that modified albumin is a trigger for tubular cell injury especially in diabetes, where albuminuria is associated with renal function decline. The results of the axolotl model used

![Western blotting](http://ajprenal.physiology.org/)

**Fig. 6.** Western blotting. Probing with antibodies to TGF-β1, fibronectin, and collagen 1 showed clear bands at the expected molecular size. Lysate from the Hep G2 cell line served as a positive control for fibronectin. Anti-β-actin was used as a loading control.

Factors, leading to the EMT of injured tubular epithelial cells and to progression of the disease (31).

Interestingly, clusterin, a molecule involved in tubular protein uptake, was also upregulated, possibly pointing to positive feedback, i.e., possibly protein exposure increasing protein uptake mechanisms.

These findings are in line with findings in mammals as reported in the literature. Exposure of tubular cells to protein has been shown to trigger expression of proinflammatory factors including ET-1, monocyte chemotactic protein-1 (MCP-1), RANTES, and various adhesion molecules (28). Cytokines are secreted mainly by inflammatory infiltrating and resident tubular epithelial cells, and they play a key role in fibroblast activation and proliferation. One main factor is PDGF: in mammals, PDGF causes influx of macrophages and formation of myofibroblasts (14). This was not observed, however, in the present short-term study on amphibians. In our study, we confirm that PDGF-AB is significantly elevated in tubular epithelial cells exposed to glycated and carbamylated albumin.

Cultured proximal tubular epithelial cells synthesize and secrete ET-1 after stimulation with delipidated albumin, IgG, or transferrin (39). ET-1 triggers interstitial fibrosis, as documented by interstitial fibrosis of ET-1 transgenic mice (3). In the present study, staining for ET-1 was significantly increased in tubular cells in axolotl tubular cells exposed to carbamylated albumin but not in the other intervention groups.
in the present study are limited, in view of the major species difference. It was not possible to gain enough blood in these amphibians to measure blood levels of glycated and carbamylated albumin, and so far there is no method available for measuring blood pressure in these amphibians.

Nevertheless, the observation clearly documents that tubular uptake of proteins triggers tubular epithelial cell activation as well as peritubular fibrosis and that covalent modification of albumin increases its potential to alter the tubular epithelial cell phenotype.

ACKNOWLEDGMENTS

The skilled technical assistance of H. Ziebart, M. Weckbach, Z. Antoni, and D. Lutz is gratefully acknowledged. Special thanks are given to H. Derks, U. Horr, and J. Moyers for graphic design and layout.

GRANTS

The authors are grateful for the generous support of the Else-Kröner-Stiftung (P06/04/A02/04/F0; Eksvrt 162). G. Piecha is a recipient of a fellowship of the International Society of Nephrology.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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