

Original Paper

N-Acetyl Cysteine Attenuated the Deleterious Effects of Advanced Glycation End-Products on the Kidney of Non-Diabetic Rats

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Key Words

Advanced glycation end-products • RAGE • Oxidative stress • Kidney disease • N-acetyl cysteine

Abstract

Aim: To assess the renal effects of chronic exposure to advanced glycation end-products (AGEs) in the absence of diabetes and the potential impact of concomitant treatment with the antioxidant N-acetyl cysteine (NAC). **Methods:** Wistar rats received intraperitoneally 20 mg/kg/day of albumin modified (AlbAGE) or not (AlbC) by advanced glycation for 12 weeks and oral NAC (600mg/L; AlbAGE+NAC and AlbC+NAC, respectively). Biochemical, urinary and renal morphological analyses; carboxymethyl-lysine (CML, an AGE), CD68 (macrophage infiltration), and 4-hydroxynonenal (4-HNE, marker of oxidative stress) immunostaining; intrarenal mRNA expression of genes belonging to pathways related to AGEs (*Ager*, *Ddost*, *Nfkb1*), renin-angiotensin system (*Agt*, *Ren*, *Ace*), fibrosis (*Tgfb1*, *Col4a1*), oxidative stress (*Nox4*, *Txnip*), and apoptosis (*Bax*, *Bcl2*); and reactive oxidative species (ROS) content were performed. **Results:** AlbAGE significantly increased urine protein-to-creatinine ratio; glomerular area; renal CML content and macrophage infiltration; expression of *Ager*, *Nfkb1*, *Agt*, *Ren*, *Tgfb1*, *Col4a1*, *Txnip*, *Bax/Bcl2* ratio; and 4-HNE and ROS contents. Some of these effects were attenuated by NAC concomitant treatment. **Conclusion:** Because AGEs are highly consumed in modern diets and implicated in the progression of different kidney diseases, NAC could be a therapeutic intervention to decrease renal damage, considering that long-term restriction of dietary AGEs is difficult to achieve in practice.

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Introduction

The advanced glycation end-products (AGEs) are compounds derived from the Maillard reaction, in which reducing sugars react nonenzymatically with amino groups of proteins, nucleic acids or lipids [1]. The interest in AGEs in the pathogenesis of diseases in which hyperglycemia is not present lies in the fact that AGEs can be acquired exogenously through diet and tobacco [2, 3]. High fat and high sugar foods, primarily those processed at low humidity and high temperatures, stored for long periods or containing food additives, are important exogenous sources of AGEs [4, 5] and of advanced lipoxidation products (ALEs) [6]. AGEs and ALEs are present in abundance in Western-diet and their intake is increasing worldwide; diet-derived AGEs are currently recognized as contributors to the body's AGEs pool, to systemic inflammation [4] and to chronic kidney diseases [7].

AGEs may be deleterious by at least two major mechanisms: (1) receptor-independent modifications of the extracellular matrix (ECM) architecture as a result of crosslinking modified proteins, and (2) receptor-dependent modulation of cellular functions. Several AGEs-binding receptors have been identified, including RAGE, AGER1, AGER2, AGER3 and macrophage scavenger receptors 1 and 2 [8]. AGEs-RAGE binding induces activation of MAP kinases and NFκB, as well as NADPH oxidases, resulting in the generation of reactive oxygen species (ROS) and redox imbalance [9-11].

Although the first studies addressing AGEs-RAGE signaling had been focused on its participation in the development of diabetic nephropathy, growing evidence suggests that this signaling pathway may also participate in the pathogenesis of non-diabetic kidney diseases such as those associated with hypertension, obesity, sepsis and lupus [12-14]. Beyond that, genetic RAGE deletion or blockade with neutralizing antibodies was able to prevent the progression of a broad range of kidney diseases [15, 16].

N-acetyl cysteine (NAC) is a prodrug for cysteine, which, in turn, is one of the aminoacids of the tripeptide glutathione (GSH), the most abundant intracellular antioxidant [17] whose effects depend on its direct scavenger action as well as on its ability to provide reducing equivalents for the activity of GSH-peroxidase, a powerful defense against peroxides [18]. Protective effects of NAC have been demonstrated in situations of renal function decline such as the remnant kidney model [19] and senescence [20].

Considering that (1) heat-processed foods are the major components of modern diets [5]; (2) the kidneys are the major site of AGEs removal [21], (3) the involvement of AGEs in non-diabetic kidney diseases [15, 16] and (4) the participation of redox imbalance in AGEs deleterious effects [10], the present study was designed to assess the renal effects of chronic exposure to AGEs in the absence of diabetes mellitus and the potential beneficial impact of concomitant treatment with NAC. The following aspects were evaluated: (1) renal function, morphology and content of carboxymethyl-lysine (CML, an AGE) and macrophages; (2) the renal expression of genes belonging to pathways related to AGEs, fibrosis, oxidative stress, and apoptosis and to the renin-angiotensin system (RAS) and; (3) the renal content of 4-hydroxynonenal (4-HNE, a marker of oxidative stress) and of ROS.

Materials and Methods

Ethics statement

All experimental protocols were conducted in accordance with the guidelines of the Brazilian College for Animal Experimentation (COBEA) and were approved by the Institutional Animal Care and Research Advisory Committee (University of São Paulo Medical School - CAPPesq Protocol #002/14). Experiments were performed with male Wistar rats (mean weight: 180.53 ± 30.73 g). The rats were obtained from colonies from the central animal facility of the University of São Paulo Medical School and housed under standard conditions (constant temperature of 22°C, 12-h dark-light cycle, and relative humidity of 60%) with free access to standard rat chow (Nuvilab CR-1, Nuvital Nutrientes S/A, Curitiba, Brazil) and tap water. Body weight (BW; g) was monitored weekly and percentual body weight gain was calculated using the following equation: (initial BW - final BW)/initial BW.

Advanced glycated albumin preparation

Rat serum albumin modified by advanced glycation (AlbAGE) was prepared as previously described [22]. Briefly, fatty acid and endotoxin free rat serum albumin (Sigma-Aldrich, Taufkirche, Germany) was incubated *in vitro*, for 4 days, under nitrogen atmosphere, at 37 °C, in the dark, under sterile conditions in a water bath shaker, with 10 mM glycoaldehyde (Sigma) dissolved in phosphate buffered saline (PBS) and EDTA (pH 7.4). Control rat serum albumin (AlbC) was incubated with PBS only. After extensive dialysis, samples were sterilized with the use of a Millipore 0.22- μ m filter and frozen at -80 °C until treatments were performed. All samples contained < 50 pg endotoxin/mL as determined by the chromogenic Limulus Amebocyte Lysate (LAL) (Cape Cod, Falmouth, USA).

Experimental protocol

The animals were randomly assigned to one of the following groups and treated for 12 weeks: 1) Control group (AlbC, $n = 9$, unmodified rat serum albumin, 20 mg/kg/day, intraperitoneally [i.p.]), 2) N-acetyl cysteine group (AlbC+NAC, $n = 9$, 600 mg/L in water, *ad libitum*) 3) AGE group (AlbAGE, $n = 11$, AGE-modified rat serum albumin, 20 mg/kg/day, i.p.) and 4) AGE plus NAC group (AlbAGE + NAC, $n = 9$), as previously described [23]. The animals' body weight was measured weekly to adjust the albumin dose accordingly.

After treatment, rats were anesthetized and perfused with PBS (10 mM sodium phosphate buffer containing 0.15 M NaCl, pH 7.4) at room temperature through the abdominal aorta using a peristaltic perfusion pump (Masterflex Easy Load Pump, Cole-Parmer, Illinois, USA). One kidney was removed and quickly snap frozen and stored at -80°C until molecular biology analyses; the other kidney was fixed by perfusion with 4% formaldehyde and processed for histological analyses.

Biochemical and urinary analyses

Two days before euthanasia, the animals were allocated in metabolic cages for 24-h urine collection; they were food deprived for 12-h for blood samples collection. Blood samples were collected from the caudal vein and centrifuged at 1,500 rpm for 10 min. The plasma was used for measurements of glucose, using a glucometer (ACCU-CHEK®; Roche, Basel, Switzerland) and insulin, by enzymatic immuno assay (rat/mouse insulin ELISA kit, Millipore, Bedford, USA). The concentrations of sodium in urine was determined by flame photometry (9180 Electrolyte Analyzer, Roche, Mt. Wellington, Auckland, New Zealand). The urinary and plasmatic concentrations of creatinine and the urine excretion of proteins were analyzed by commercial kits (Labtest, Minas Gerais, Brazil) according to manufacturer's guidelines.

Systolic blood pressure (SBP) measurement

SBP was measured at the end of the treatment, in conscious and resting animals, using a noninvasive tail-cuff plethysmography method (BP-2000 SERIES Blood Pressure Analysis System™; Visitech Systems Inc., Apex, USA), as previously described [24]. Briefly, the tail artery was dilated by placing the animal for 20 min into a thermostatically controlled plastic holder. The pulse was detected by passing the tail through a tail-cuff sensor, which was attached to an amplifier. SBP (mmHg) was calculated as a mean of six consecutive cuff inflation-deflation cycles, performed when a clear initial and constant pulse could be detected.

Morphological and immunohistochemical analyses

For morphological analyses, 4 μ m histological sections were stained with hematoxylin and eosin (HE) and examined under a light microscope (Eclipse 80i, Nikon, Tokyo, Japan). To access the planar glomerular area, 50 glomerular tufts outer edges *per animal* were traced on a video screen and the areas were determined by a computerized morphometry program (NIS-Elements D, Nikon). For immunohistochemical analyses, 4 μ m kidney sections were subjected to staining with anti-desmin antibody as a marker of podocyte injury (Abcam, Cambridge, UK), anti-CML as a marker of AGE (Santa Cruz Biotechnology, Texas, USA), anti-CD68 antibody as a marker of macrophage infiltration (AbD Serotec, Oxford, UK), and anti-4HNE as a marker of oxidative stress (Abcam, Cambridge, UK). The tissue sections were deparaffinized and incubated with 20% goat serum in PBS for 60 min for nonspecific protein binding blockade. Then, sections were incubated with primary antibodies (1:500 for anti-desmin, 1:50 for anti-CML, 1:50 for anti-CD68, and 1:100 for anti-4HNE), overnight at 4°C. In the next day, the reaction products were detected using the avidin-biotin-peroxidase complex (Vector Labs, Burlingame, USA) and the sections were counterstained with methyl

green (Amresco, Ohio, USA), dehydrated and mounted with Permount (Fisher Scientific, Fair Lawn, USA). Desmin expression was qualitatively evaluated in 30 glomerulus in each one of the experimental conditions [25] and CML and 4-HNE contents were qualitatively evaluated in 10 fields in each experimental condition. The mean number of CD68-positive cells (macrophages) infiltrating the renal cortical tubulointerstitium was obtained by evaluating 30 – 40 grid fields (measuring 0.087 mm² each) and by calculating the mean counts *per* kidney [24].

Intrarenal mRNA expression

The isolation of RNA was performed using the TRIzol LS Reagent (Life Technologies, Carlsbad, USA) and a RNA extraction kit (Qiagen Sciences, Germantown, USA) in a fragment of frozen kidney. The total RNA was quantified by the measurement of the optical density at 260 nm (NanoDrop, Thermo Scientific, Carlsbad, USA) and 2 µg were reverse-transcribed using random hexamers (High Capacity cDNA Reverse Transcription Kit; Life Technologies, Carlsbad, USA) following the manufacturer's instructions. Real-time PCR was performed using the StepOnePlus System (Life Technologies, Carlsbad, USA) and the TaqMan assay system (Life Technologies, Carlsbad, USA). The following inventoried assays were used: *Ace* (Rn00561094_m1), *Ager* (encodes RAGE; Rn01525753_g1), *Agt* (Rn00593114_m1), *Bax* (Rn02532082_g1), *Bcl2* (Rn99999125_m1), *Col4a1* (Rn001482913_m1), *Ddost* (encodes AGER1; Rn01518759_m1), *Nfkb1* (Rn01399583_m1), *Nox4* (Rn00585380_m1), *Ren* (Rn00561847_m1), *Tgfb1* (Rn00572010_m1), *Tnf* (Rn01525859_m1) and *Txnip* (Rn01533891_g1). All quantitative PCR studies were performed using 20 ng of cDNA and all samples were assayed in duplicate. The data were normalized by the *Actb* (Rn00667869_m1) expression (reference gene) and relative levels of mRNA expression were calculated using the comparative cycle threshold (Ct) (2^{-ΔΔCt}) method [26].

Dihydroethidium (DHE) staining

To investigate oxidative stress, frozen, optimal cutting temperature-embedded kidney tissue was cryosectioned into 12-µm-thick sections and placed on a glass slide. The sections were stained with 10 µmol/L DHE solution (Invitrogen, Carlsbad, USA) and slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Images were obtained under microscope system (Eclipse 80i, Nikon, Tokyo, Japan) and fluorescence was detected with a 590 nm long-pass filter. The average DHE fluorescence intensity was calculated from 5 interstitial fields from each animal.

Statistical analyses

Statistical analyses were performed using the JMP software version 8.0 (SAS Institute, Cary, USA). All data are expressed as mean ± standard mean error (SEM). The non-parametric Wilcoxon signed-rank test followed by Tukey's post-test was employed and a *p*-value < 0.05 was considered statistically significant.

Results

Effects of chronic administration of AGEs on metabolic, biochemical and urinary variables

Table 1 displays body and kidney weights, biochemical and urinary variables and SBP in the different studied groups. Chronic exposure to AGEs did not influence body weight gain or SBP. Kidney weight-to-body weight ratio was also similar in all groups, indicating the treatment did not induce kidney hypertrophy. Regarding renal function, there were no differences in the urinary flow rate and in sodium excretion. There were also no changes in the creatinine clearance (Fig. 1A). However, AlbAGE-treated rats presented a significant increase in the urine protein-to-creatinine ratio; the reduction observed by treatment with NAC in the group receiving AlbAGE did not reach statistical significance (AlbC: 1.647 ± 0.104; AlbC+NAC: 2.215 ± 0.171; AlbAGE: 2.555 ± 0.189; AlbAGE+NAC: 1.993 ± 0.234; Fig. 1B).

Effects on kidney morphology and CML content

Figure 2 shows that glomeruli of AlbAGE-treated rats are significantly larger compared to those of AlbC rats. The reduction observed by treatment with NAC in the group receiving AlbAGE did not reach statistical significance (AlbC: 4,589 ± 196.3; AlbC+NAC: 4,999 ± 327.6;

Table 1. Evaluated variables of rats treated for 12 weeks with rat serum albumin (AlbC) or with rat serum albumin modified by advanced glycation (AlbAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlbC+NAC and AlbAGE+NAC, respectively). U_{Na^+} , V, excreted load of sodium; SBP, systolic blood pressure. Data are expressed as mean \pm SEM

Variables	AlbC (n=9)	AlbC+NAC (n=9)	AlbAGE (n=11)	AlbAGE+NAC (n=9)
Initial body weight (g)	187.58 \pm 31.48	178.37 \pm 40.49	174.64 \pm 25.53	184.28 \pm 32.88
Final body weight (g)	486.60 \pm 65.33	501.77 \pm 56.40	486.76 \pm 55.78	509.34 \pm 37.47
Body weight gain (%)	164.80 \pm 14.66	195.80 \pm 26.54	178.90 \pm 13.90	183.40 \pm 15.15
Kidney weight/body weight (mg/g)	3.40 \pm 0.06	3.46 \pm 0.15	3.33 \pm 0.08	3.47 \pm 0.09
Blood glucose (mg/dL)	111.4 \pm 3.71	115.1 \pm 2.39	109.10 \pm 2.94	110.80 \pm 4.25
Plasma insulin (ng/ml)	1.41 \pm 0.28	--	1.40 \pm 0.18	--
Urinary flow (ml/min)	0.008 \pm 0.001	0.010 \pm 0.001	0.009 \pm 0.001	0.009 \pm 0.001
U_{Na^+} , V (μ Eq/ml/min)	1.08 \pm 0.16	1.48 \pm 0.14	1.17 \pm 0.13	0.86 \pm 0.045
SBP (mmHg)	117.20 \pm 3.35	116.50 \pm 5.20	119.50 \pm 6.46	119.80 \pm 6.34

Fig. 1. Effect of 12-weeks treatment with control albumin (AlbC) or albumin modified by glycation (AlbAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlbC+NAC and AlbAGE+NAC, respectively) on creatinine clearance (Panel A, in mL/min/kg) and urine protein-to-creatinin ratio (Panel B, arbitrary units). Values are expressed as mean \pm SEM of 9-11 animals *per* group.

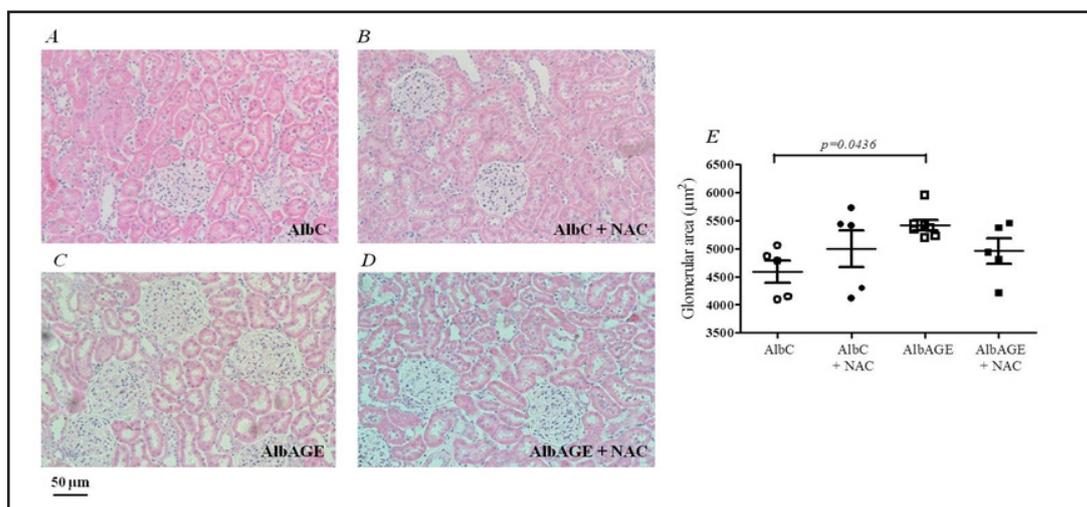
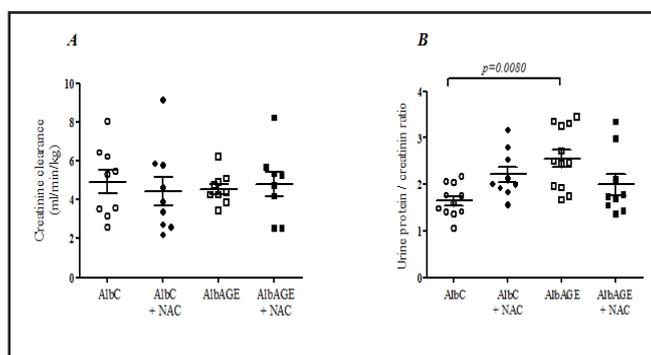


Fig. 2. Effect of 12-weeks treatment with control albumin (AlbC) or albumin modified by glycation (AlbAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlbC+NAC and AlbAGE+NAC, respectively) on renal morphology. Representative photomicrograph of kidney sections stained with hematoxylin and eosin from rats receiving AlbC (Panel A), AlbC+NAC (Panel B), AlbAGE (Panel C) and AlbAGE+NAC (Panel D). Statistical analysis of the glomerular area (Panel E). Values are expressed as mean \pm SEM of the mean area of 50 glomerular tufts outer edges of 5 animals *per* group. Bar = 50 μ m, magnification \times 20.

AlbAGE: 5,416 \pm 95.0; AlbAGE+NAC: 4,956 \pm 222.2 μ m²). Desmin staining, an indicator of podocyte injury, did not change in a qualitative analysis (data not shown). A qualitative

Fig. 3. Effect of 12-weeks treatment with control albumin (AlbC) or albumin modified by glycation (AlbAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlbC+NAC and AlbAGE+NAC, respectively) on renal carboxymethyl-lysine content. Representative photomicrograph of kidney sections from rats receiving AlbC (Panel A), AlbC+NAC (Panel B), AlbAGE (Panel C) and AlbAGE+NAC (Panel D). Magnification $\times 20$.

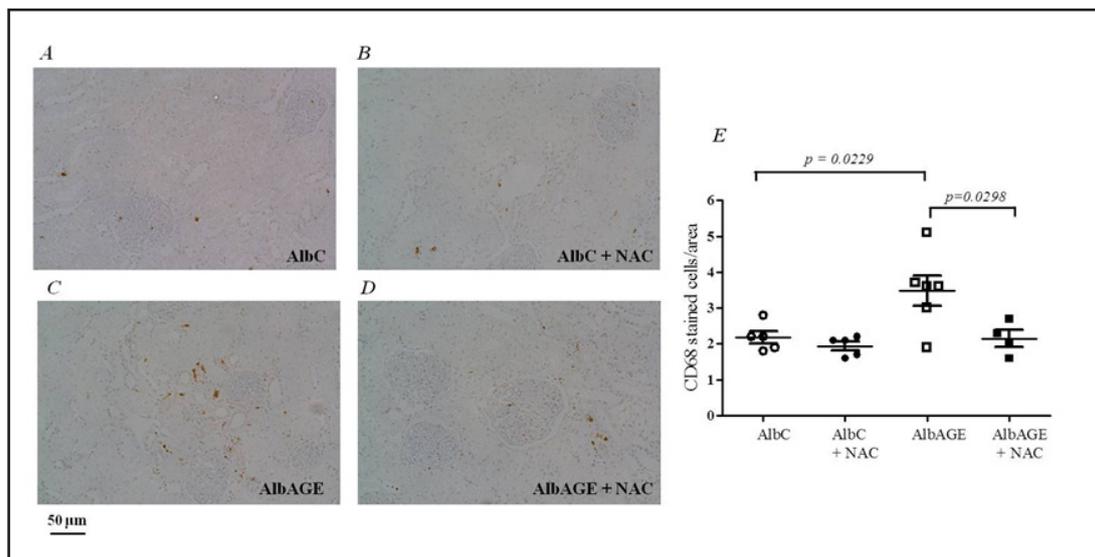
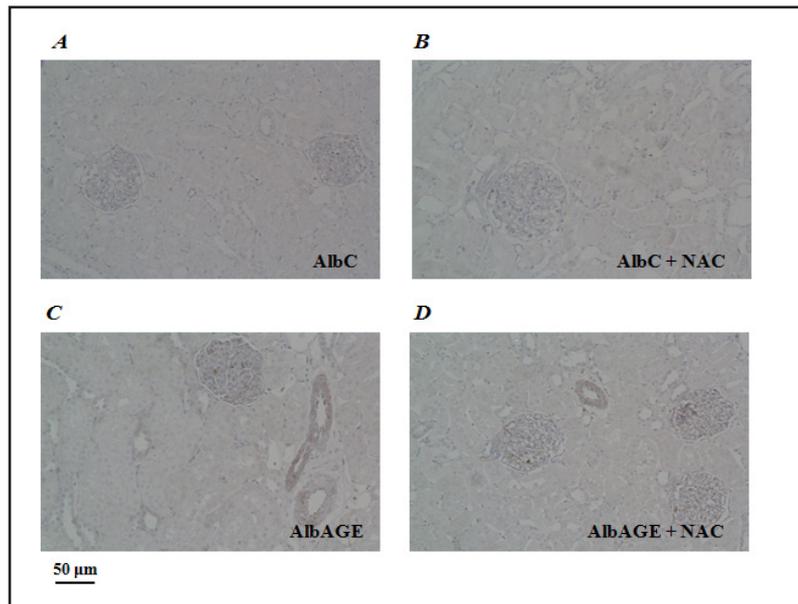


Fig. 4. Effect of 12-weeks treatment with control albumin (AlbC) or albumin modified by glycation (AlbAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlbC+NAC and AlbAGE+NAC, respectively) on macrophage infiltration. Representative photomicrograph of kidney sections stained for CD68 from rats receiving AlbC (Panel A), AlbC+NAC (Panel B), AlbAGE (Panel C) and AlbAGE+NAC (Panel D). Statistical analysis (Panel E, stained cells/area). Values are expressed as mean \pm SEM of 30 grid fields of 5 animals *per* group. Scale bar = 50 μ m, magnification $\times 20$.

analysis of the CML staining revealed a higher content of this AGE in the group receiving AlbAGE in comparison to the group receiving AlbC, which was only slightly reduced by treatment with NAC (Fig. 3).

Effects on macrophage infiltration

Another feature of kidney disease is a high degree of inflammation, which is characterized by macrophage infiltration that contributes to renal injury. Macrophage infiltration was examined in tubulointerstitium using CD68 staining. The number of CD68-positive cells

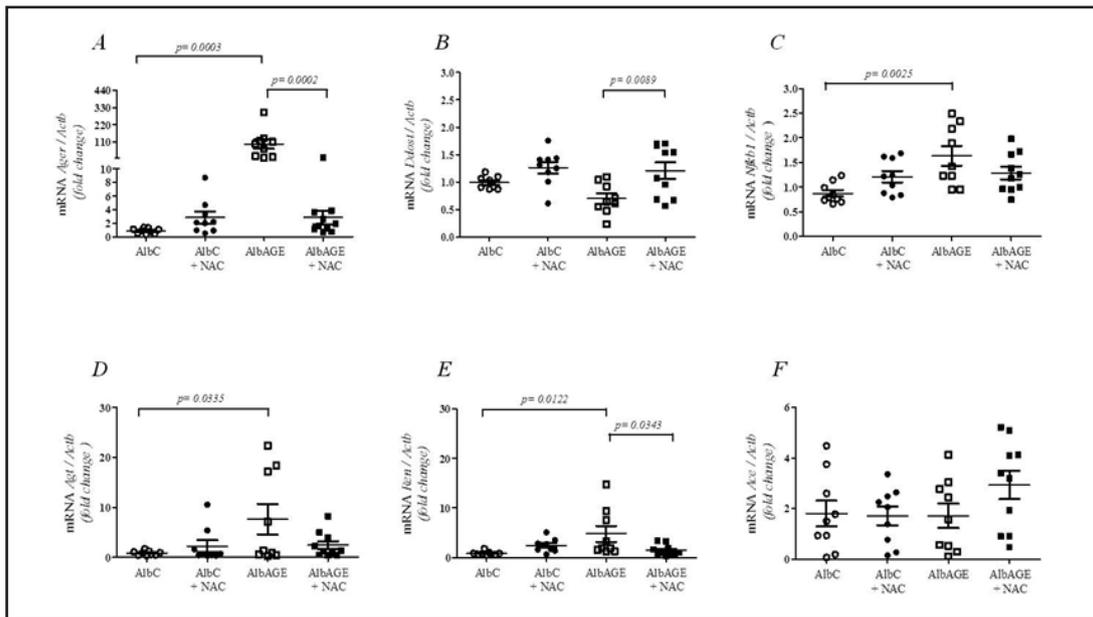


Fig. 5. Effect of 12-weeks treatment with control albumin (AlBc) or albumin modified by glycation (AlBAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlBc+NAC and AlBAGE+NAC, respectively) on renal mRNA expression of: RAGE (*Ager*, Panel A); AGER1 (*Ddost*, Panel B); NFκB (*Nfkb1*, Panel C); angiotensinogen (*Agt*, Panel D); renin (*Ren*, Panel E); and angiotensin-converting enzyme (*Ace*, Panel F). Values are expressed as mean ± SEM of 9-11 animals *per* group.

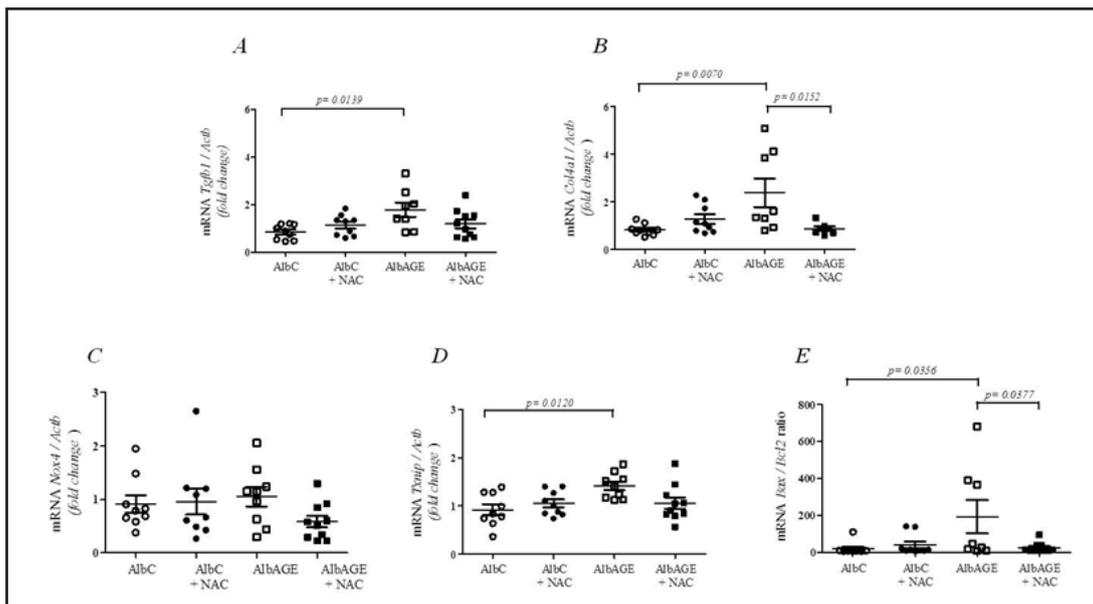


Fig. 6. Effect of 12-weeks treatment with control albumin (AlBc) or albumin modified by glycation (AlBAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlBc+NAC and AlBAGE+NAC, respectively) on renal mRNA expression of: transforming growth factor β (*Tgfb1*, Panel A); collagen type IV, alpha 1 chain (*Col4a1*, Panel B); NADPH oxidase 4 (*Nox4*, Panel C); thioredoxin interacting protein (*Txnip*, Panel D); and *Bax*/*Bcl2* ratio (Panel E). Values are expressed as mean ± SEM of 9-11 animals *per* group.

was significantly higher in tubulointerstitial area from AlBAGE-treated rats in comparison to rats receiving AlBc. Concurrent treatment with NAC significantly decreased AGE-induced macrophage infiltration (AlBc: 2.180 ± 0.174 ; AlBc+NAC: 1.940 ± 0.121 ; AlBAGE: 3.483 ± 0.425 ; AlBAGE+NAC: 2.150 ± 0.233 stained cells/area; Fig. 4).

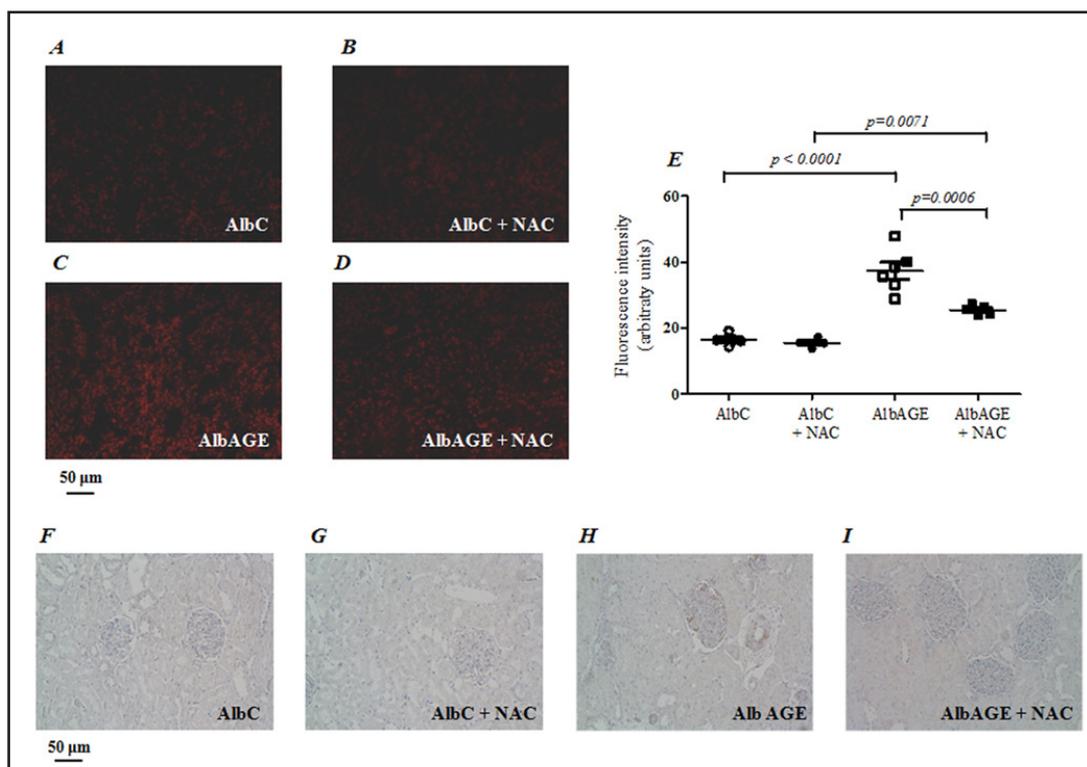


Fig. 7. Effect of 12-weeks treatment with control albumin (AlbC) or albumin modified by glycation (AlbAGE) concomitantly with the antioxidant N-acetyl cysteine (NAC; AlbC+NAC and AlbAGE+NAC, respectively) on the renal content of reactive oxygen species (dihydroethidium [DHE], Panels A-E) and of 4-hydroxynonal (4-HNE, Panels F-I). Representative images of kidney sections from rats receiving AlbC (Panels A and F), AlbC+NAC (Panels B and G), AlbAGE (Panels C and H) and AlbAGE+NAC (Panels D and I). Statistical analysis of the DHE fluorescence intensity (Panel E, in arbitrary units). Values of DHE are expressed as mean \pm SEM of 5 interstitial fields of 5 animals *per* group. Scale bar = 50 μ m, magnification \times 20.

Effects on intrarenal mRNA expression

Several significant differences were observed in the intrarenal mRNA expression in rats chronically exposed to AlbAGE: a 111-fold increase in the expression of *Ager* (encodes for RAGE) (Fig. 5A) and 1.8-fold increase in the expression of *Nfkb1* (Fig. 5C) in comparison to AlbC and a 58% lower expression of *Ddost* (encodes for AGER1) in comparison to AlbAGE+NAC (Fig. 5B). Furthermore, AlbAGE-treated rats exhibited increased expression of *Agt* (8.7-fold) and *Ren* (5.6-fold) (Fig. 5D and 5E, respectively), *Tgfb1* (2.0-fold) and *Col4a1* (3.0-fold) (Fig. 6A and 6B, respectively), *Txnip* (1.5-fold) (Fig. 6D) and *Bax* to *Bcl2* ratio (10-fold) (Fig. 6E) in comparison to AlbC-treated rats. Concurrent treatment with NAC significantly decreased the expression of *Ager* (Fig. 5A), *Ren* (Fig. 5E), *Col4a1* (Fig. 6B) and the *Bax* to *Bcl2* ratio (Fig. 6E) induced by AlbAGE.

Effects on ROS generation in the renal cortex

As shown in Figure 7, *in situ* detection of intracellular ROS with DHE staining demonstrated that chronic AlbAGE exposure increased by 2.2-fold ROS generation in tubular compartments in comparison to AlbC. Concurrent treatment with NAC significantly decreased ROS generation elicited by AlbAGE (AlbC: 16.48 ± 0.77 ; AlbC+NAC: 15.53 ± 0.72 .; AlbAGE: 37.39 ± 2.67 ; AlbAGE+NAC: 25.55 ± 0.69 arbitrary units). Additionally a qualitative analysis of the 4-HNE staining revealed a higher content of this marker of oxidative stress in the group receiving AlbAGE in comparison to the group receiving AlbC, which was notably reduced by treatment with NAC.

Discussion

The main finding of the present study was that concomitant treatment with the antioxidant NAC attenuated some of the deleterious renal effects caused by chronic exposure to AGEs in non-diabetic rats. These results corroborate previous findings that AGEs exert renal detrimental effects *per se*, regardless the presence of hyperglycemia [23, 27, 28], as well as the central role of redox imbalance in this process, as widely known for the mechanism of AGEs-induced cellular injury [11].

There are several studies suggesting that the RAGE signaling pathway is involved in the establishment and progression of different kidney diseases, besides diabetic nephropathy [15, 16]. *In vitro* studies have shown that podocytes are the main RAGE-expressing cells in the glomerulus, where AGEs induce RAS activation [29, 30] and apoptosis [31], among other effects [32]. AGEs also activate RAGE in mesangial cells [33] where they increase expression of transforming growth factor β (TGF β) [33, 34]. Additionally, AGEs have been implicated in diabetic tubulopathy [35].

Although there are many studies investigating pathways activated by AGEs in specific kidney cells, few studies evaluated the renal effects of chronic AGEs administration. McVerry et al. [28] were the first to describe a mouse model of repeatedly infusion of plasma proteins glycated *in vitro*, which determined a pseudodiabetic renal pattern. Vlassara et al. [23] showed that chronic administration of AGE-modified albumin induced glomerular sclerosis and albuminuria in normal rats. The same group also showed that normal mice receiving AGE-modified albumin presented an increase in glomerular ECM and in the expression of growth factors, as well as glomerular hypertrophy [27].

The experimental protocol of the present study is very similar to that discussed above [23], except for the duration of treatment (12 vs. 16 weeks, respectively). We also observed an increase in urinary protein excretion and glomerular hypertrophy. Although we had not detected changes in renal function parameters such as creatinine clearance, urinary flow rate and sodium excretion, or accentuated desmin staining, the results mentioned above are indicative of incipient kidney involvement.

Several of the genes whose expression was evaluated are considered as part of the "AGE-RAGE signaling pathway in diabetic complications", according to KEGG database: *Ager*, *Nfkb*, *Tgfb1*, *Nox*, *Bax* (known to be positively modulated by AGEs) [36-39] and *Bcl2* (known to be negatively modulated by AGEs) [40]. With the exception of *Nox4*, all these genes had their expression modulated by AGEs in the present study.

Despite the differences in the experimental design and in some of the findings, our results are in agreement with those reported by Thomas et al. [41], who showed a close interaction between the AGEs-RAGE pathway and the RAS. After continuous subcutaneous infusion of albumin modified by advanced glycation during 4 weeks, they observed an increased expression of *Agt*, *Ren*, and glomerular hypertrophy (also observed here), besides increased expression of *Ace* and *At1*, lower creatinine clearance, and urinary sodium excretion. In the same study, continuous angiotensin II infusion increased renal accumulation of AGEs [41], demonstrating that the RAS may be activated by AGEs, but also positively feeds back and propagates AGEs formation [10, 42].

Txnip, the gene encoding a protein implicated in redox regulation in several pathological conditions, including hyperglycemia [43], was also upregulated in the kidney of animals exposed to AGEs. Induction of *Txnip* expression after RAGE activation (by another RAGE-ligand, S100B) had been previously demonstrated *in vitro* in retinal endothelial cells [44] and in Schwann cells [45]. Our results suggest that the RAGE-TXNIP axis also participates in the kidney injury induced by AGEs, further contributing to the redox imbalance.

We were not able to find previous studies showing decreased *Ddost* expression in the kidney of animals chronically exposed to AGEs. This gene encodes for AGER1, a receptor responsible for AGEs endocytosis and degradation that restricts pro-oxidative pathways. Cai et al. have demonstrated a higher expression of *Ddost* in non-diabetic mice with a life-long exposure to a low AGEs diet in comparison to those receiving a regular diet. At 24 months,

the former group also exhibited less glomerular sclerosis, fewer renal inflammatory cells, lower expression of *Tgfb1* and of *Col4a1* and lower albuminuria [46], findings similar to the ones observed in our group of animals receiving AGEs and NAC in comparison to those receiving only AGEs. Those authors proposed dietary AGEs restriction as an intervention to decrease tissue damage, including in the kidney, during the aging process [46].

In the present experimental model, NAC decreased ROS generation and macrophage infiltration, expression of the genes encoding RAGE, renin and collagen and the pro-apoptotic expression profile induced by chronic exposure to AGEs. These results widen the spectrum of kidney conditions liable to be positively affected by NAC, as demonstrated for the remnant kidney model [19], acute kidney injury secondary to sepsis [47], nephropathic cystinosis [48], contrast-induced kidney injury [49], bilateral ureteral obstruction [50] and senescence [20].

The mechanism by which NAC attenuated the deleterious renal effects of AGEs is probably related to its ability to increase GSH, the principal intracellular defense against redox imbalance [17]. An *in vitro* study has previously demonstrated in neuronal cells that glycated albumin decreases intracellular GSH, which was prevented by NAC [51]. Thus, part of the ROS generated by RAGE activation must have been scavenged by intracellular GSH in the animals receiving AlbAGE+NAC, as shown by our finding in this group of animals, who presented a lower renal content of ROS. The concomitant decrease in the content of 4-HNE, a product of lipid peroxidation [52], demonstrates that ROS reduction by NAC diminishes oxidation reactions, even though the content of CML has been only slightly reduced. Because we did not measure other AGE compounds besides CML, we cannot definitely establish whether NAC treatment is able to decrease renal AGEs content in our experimental conditions. In uremic animals, NAC was able to prevent the enhancement in plasmatic pentosidine and total AGE induced by chronic kidney disease [53].

In conclusion, NAC treatment attenuates the deleterious renal effects elicited by chronic exposure to AGEs, through the decrease in renal tissue oxidative stress. Because long-term restriction of dietary AGEs is difficult to achieve in practice, NAC could be tested in situations in which AGEs actively participate in the progression of kidney disease.

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Disclosure Statement

The authors have nothing to disclose.

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