

Original Article

Addition of oleic acid to delipidated bovine serum albumin aggravates renal damage in experimental protein-overload nephrosis

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Abstract

Background. Non-esterified fatty acids (NEFA) carried on albumin may have a causal role in the development of chronic proteinuria-induced nephropathy. To investigate whether NEFA aggravate renal structural damage, we studied the effects of NEFA addition to delipidated bovine serum albumin (BSA) in protein-overload nephropathy.

Methods. Three groups of Wistar rats received daily intraperitoneal injections (3 weeks) of either 1 g NEFA-free BSA (BSA-0), or NEFA-free BSA with three (BSA-3) or six (BSA-6) molecules oleic acid added per BSA molecule. An additional group received saline injections only (SAL). Renal damage was evaluated by immunohistochemistry and RT-PCR.

Results. Interstitial and glomerular α -smooth muscle actin (α -SMA, marker of myofibroblast transformation) expression were higher in BSA-3/6 than in saline-injected controls ($P < 0.001$). Glomerular macrophage influx and desmin (marker of glomerular epithelial cell damage) expression were higher in all BSA-injected rats than SAL ($P < 0.001$). Interstitial macrophage influx was elevated in BSA-0/3 ($P < 0.05$) and BSA-6 ($P < 0.001$) compared to SAL. Addition of six molecules of oleic acid to BSA revealed higher interstitial and glomerular α -SMA expression ($P < 0.001$), increased interstitial macrophage numbers ($P < 0.001$) and enhanced glomerular desmin expression ($P < 0.05$) compared to BSA-0. RT-PCR revealed higher glomerular α -SMA mRNA expression in BSA-3/6 than SAL ($P < 0.001$ and 0.05, respectively), interstitial α -SMA mRNA was elevated in BSA-6 ($P < 0.05$). Interstitial TGF- β 1 mRNA expression was significantly higher in BSA-3 than SAL ($P < 0.05$).

Conclusions. These data show that addition of oleic acid to NEFA-free BSA aggravates renal damage, suggesting a role for NEFA in the pathogenesis of proteinuric nephropathies.

Keywords: albuminuria; non-esterified fatty acids; oleic acid; protein-overload; proteinuric nephropathies

Introduction

Progression to end-stage renal disease is the final common pathway of chronic proteinuric nephropathies. The degree of proteinuria is associated with the rate of progression of renal disease [1,2]. However, the underlying mechanism by which filtered proteins cause renal damage is still unclear. Many authors postulate that albumin, which is the most prominent protein in the glomerular ultrafiltrate, induces renal injury [1,3]. Filtered albumin is reabsorbed by receptor-mediated endocytosis by proximal tubular cells [3], which leads to activation of intracellular signalling pathways. The resulting increased synthesis and secretion of pro-inflammatory mediators attract and activate macrophages, a process ultimately leading to interstitial fibrosis [4].

It is not known whether albumin activates these processes directly or indirectly through molecules carried on albumin. Albumin is the major carrier of non-esterified fatty acids (NEFA) in the circulation and recent studies suggest that these NEFA carried on albumin play an important role in the initiation of renal damage [5,6]. Normally, ~ 0.7 molecule of NEFA is bound to each albumin molecule, but this ratio is markedly raised in the nephrotic syndrome [2]. Consequently, proximal tubular cells are exposed to high concentrations of NEFA carried on albumin in proteinuric nephropathies.

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In vitro experiments have revealed that exposure of proximal tubular epithelial cells to NEFA-loaded albumin leads to enhanced apoptosis when compared to NEFA-free albumin [7]. Furthermore, oleate, the most abundant NEFA in human serum [8,9], induces fibronectin secretion of proximal tubular cells *in vitro*, an effect that was not seen after addition of other NEFA to albumin [8]. *In vivo* studies in experimental protein-overload nephropathy, in which repeated intraperitoneal injections of albumin increase the permeability of the glomerular barrier, with subsequent development of proteinuria and impairment of renal function [10], revealed that albumin loaded with a mixture of NEFA causes more tubulointerstitial injury than NEFA-free albumin [5,6]. However, up to now all these *in vivo* studies compared regular NEFA-carrying albumin with delipidated albumin. In addition to fatty acids, other compounds with the potential to induce renal damage may have been removed from albumin during the delipidation procedure.

To investigate the involvement of fatty acids carried on albumin in the pathogenesis of renal disease, we compared delipidated albumin with fatty acid-loaded delipidated albumin in experimental protein-overload nephropathy. Based on its reported capacity to induce fibronectin production, we chose to selectively load delipidated albumin with one single NEFA, oleic acid.

Subjects and methods

Induction of protein-overload proteinuria

Protein-overload nephropathy was induced in adult male Wistar rats (Harlan, Horst, Netherlands), weighing 221 ± 2 g (mean \pm SEM). Rats were housed in a light- and temperature-controlled environment, and had free access to water and standard rat chow. All rats received intraperitoneal injections five times a week for three consecutive weeks under anaesthesia (isoflurane). Rats were injected with either 3.4 ml of saline (SAL, $n=8$), 1 g of fatty acid-free bovine serum albumin (BSA; Sigma-Aldrich, Zwijndrecht, Netherlands, catalog no. A-3803) without addition of oleic acid (BSA-0, $n=8$) or with addition of three (BSA-3, $n=8$) or six (BSA-6, $n=8$) molecules of oleic acid (Sigma-Aldrich, Zwijndrecht, Netherlands, catalog no. O-1008) per BSA molecule. All procedures were approved by the Committee for Animals Experiments of the University of Groningen and the Principles of Laboratory Animal Care (NIH publication no. 85-23) were followed.

Albumin solutions

BSA solutions (30%) were prepared using phosphate buffered saline (PBS, pH 7.2). After addition of the oleic acid, the solutions were incubated for 2.5 h at 37°C (with gentle shaking). After filtration (0.22 μ m Millex GP filter unit; Millipore S.A.S. Molsheim, France) solutions were frozen (-20°C) until time of injection. Total fatty acid spectra in the BSA solutions were determined by gas-chromatography

Table 1. Quantification of fatty acid contents of different BSA solutions

Name	Number of C atoms and double bonds	BSA-0		BSA-3		BSA-6	
		mmol/l	%	mmol/l	%	mmol/l	%
Palmitic acid	C16:0	<0.05 ^a	–	0.15	1.2	0.27	1.1
Stearic acid	C18:0	–	–	<0.05	0.2	<0.05	0.1
Vaccenic acid	C18:1 ω 7	–	–	0.33	2.6	0.66	2.6
Oleic acid	C18:1 ω 9	–	–	11.65	94.6	23.63	94.9
Linoleic acid	C18:2 ω 6	–	–	0.10	0.8	0.21	0.8
Eicosenoic acid	C20:1 ω 9	–	–	0.07	0.6	0.12	0.5

^aIn BSA-0 only minor quantities of palmitic acid were detected, while other fatty acids were not detected.

as previously described [11]. Commercially acquired BSA-0 contained no detectable amounts of fatty acids, with the exception of a trace amount of palmitic acid (Table 1). Both BSA-3 and BSA-6 contained approximately the amount of oleic acid that was added (13.5 and 27 mmol/l, respectively) to the fatty acid-free BSA, i.e. 11.7 and 23.6 mmol/l, while other fatty acids were only detected in minor quantities. Endotoxin levels in BSA solutions were measured by chromogenic limulus test and were similar in all the solutions (0.25–2.5 ng/ml).

Clinicopathological parameters

Body weight was measured every week. Urine was collected weekly by placing the rats for 24 h in metabolic cages with access to drinking water only. Blood samples (100–150 μ l) were taken on days 5, 9 and 16 from the tail vein under isoflurane anaesthesia. At the end of the observation period, the rats were anaesthetized with isoflurane, a 2 ml blood sample was taken by canulation of the aorta, and the kidneys were perfused *in situ* with saline and removed. Immediately after perfusion, a coronal tissue slice through the midportion of the kidneys was snap-frozen in isopentane and stored at -80°C for reverse transcriptase polymerase chain reaction (RT-PCR) analysis. Another coronal tissue slice was fixed in 4% paraformaldehyde and processed for paraffin embedding. Paraffin sections were used for immunohistochemistry and were stained with periodic acid-Schiff to evaluate renal damage.

Measurements in blood and urine

Urinary and plasma protein was measured using the TPU method (Dade Behring, Leusden, Netherlands). Plasma and urine total creatinine levels were determined colorimetrically by a commercial kit (Creatinine 555-A; Sigma-Aldrich, Zwijndrecht, Netherlands). Total plasma triglycerides were measured using a colorimetric enzymatic assay (Triglycerides INT 336-10; Sigma-Aldrich). Total plasma cholesterol was measured using a commercial kit (InfinityTM Cholesterol Liquid Reagent 401-25p; Sigma-Aldrich). Total plasma glucose concentrations were measured using the glucose oxidase method (YSI Glucose Analyzer; YSI Inc., Yellow Springs, OH). Total plasma NEFA levels were measured using a colorimetric enzymatic NEFA kit (Wako Chemicals USA, Inc., Richmond, VA, USA).

Immunohistochemistry

Paraffin sections (4 µm) were dewaxed and subjected to heat-induced antigen retrieval by overnight incubation in 0.1 M Tris-HCl buffer at 80°C. Endogenous peroxidase was blocked with 0.075% H₂O₂ in PBS for 30 min. Alpha-smooth muscle cell actin (α -SMA, a marker of myofibroblast transformation) was detected using a mouse monoclonal antibody (clone 1A4; Sigma Chemical Co., St Louis, MO, USA). Monocytes/macrophages were detected using a mouse monoclonal antibody (ED1; Serotec, Kidlington, UK). Desmin, a marker for glomerular visceral epithelial cell damage, was detected using a mouse monoclonal antibody (clone DE-R-11; Novocastra Laboratories Ltd, Newcastle, UK). Antibodies were incubated for 60 min at room temperature. Binding of the antibody was detected using sequential incubations with peroxidase-labelled (PO-) rabbit anti-mouse and PO-labelled goat anti-rabbit antibody (both from DakoCytomation, Glostrup, Denmark) for 30 min. Antibody dilutions were made in PBS supplemented with 1% BSA and 1% normal rat serum. Peroxidase activity was developed by using 3,3'-diaminobenzidine tetrachloride for 10 min. Sections were counterstained with haematoxylin. For all immunohistochemical staining, controls in which the primary antibody was replaced by PBS and appropriate isotype controls were included; these were consistently negative.

The expression of glomerular α -SMA was measured using computerized morphometry (average of fifty glomeruli per kidney). The image of a given glomerulus present on the screen was traced with a cursor along Bowman's capsule. Interstitial α -SMA was also measured using computerized morphometry. For that purpose, fifty interstitial fields were randomly selected; vessels and glomeruli were excluded from measurements. The renal sections were evaluated moving from cortex to medulla and visa versa at a magnification of 200 \times . The total staining was divided by the total surface area and expressed as a percentage. For glomerular measurement of ED1 the number of positive cells per glomerulus was counted (average of fifty glomeruli). For interstitial measurement of ED1 the number of positive cells per field was counted (average of thirty fields per kidney); vessels and glomeruli were excluded from measurements. For glomerular desmin staining, fifty glomeruli were scored semi-quantitatively, by estimating the percentage of desmin-positive glomerular visceral epithelial cells in the outer cell layer of the glomerular tuft. Desmin staining is presented in arbitrary units (0–4). Value 0 accounts for 0–5% staining, 1 for 5–25% staining, 2 for 25–50% staining, 3 for 50–75% and 4 for 75–100% staining.

RNA extraction, reverse transcription and PCR

Fifty glomeruli and thirty interstitial fields were extracted from 5 µm frozen sections by laser dissection (PALM, Bernried, Germany). Total RNA was isolated using an Absolutely RNA Microprep Kit (Stratagene, CA, USA). cDNA was synthesized from 10 µl RNA by oligo(dT) priming (1 h) using a Sensiscript Reverse Transcriptase kit (Qiagen, Westburg BV, Leusden, Netherlands). PCRs were performed in a 30 µl reaction containing 1 µl cDNA and using Taq DNA polymerase (Amersham Biosciences, Roosendaal, Netherlands). Each RNA sample was verified for the absence of genomic DNA by performing RT-PCRs in which the

addition of the reverse transcriptase enzyme was omitted. PCRs were performed for transforming growth factor- β 1 (TGF- β), α -SMA and GAPDH. The following primer sequences were used: TGF- β F 5'-AGA AGA ACT GCT GTG TAC GG-3', TGF- β R 5'-ACC CAC GTA GTA GAC GAT GG-3', α -SMA F 5'-ACG GCA TCA TCA CCA ACT GG-3', α -SMA R 5'-AGA CGC ATG ATG GCA TGA GG-3', GAPDH F 5'-ACT CAG AAG ACT GTG GAT GG-3' and GAPDH R 5'-GTT GCT GTT GAA GTG ACA GG-3'. PCR cycles consisted of 30 s 94°C, 45 s 55°C and 45 s 72°C. The final extension step consisted of 7 min at 72°C. The number of cycles was selected to allow for amplification within the linear range. The number of cycles was: 37 and 41 for TGF- β in glomeruli and interstitium, respectively, 35 and 33 for α -SMA in glomeruli and interstitium, respectively, and 35 for GAPDH in both glomeruli and interstitium. Amplified products were analysed by electrophoresis on a 1.2% agarose gel. Intensity of the bands was measured using a geldetector (Bio-Rad, Veenendaal, Netherlands) and was expressed as a ratio compared to GAPDH.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analyses were performed with GraphPad Prism (version 3.00 for Windows; GraphPad Software, San Diego, CA, USA). Group comparisons were made using one-way ANOVA (with Dunnett's posttest). All BSA-treated groups were compared to the SAL control group. For analysis of the effect of added oleic acid, the BSA-3 and BSA-6 groups were compared to the group that received NEFA-free BSA (BSA-0). A two-sided *P*-value <0.05 was taken to indicate a statistically significant difference.

Results

Clinicopathological parameters

Clinicopathological parameters are shown in Table 2. At sacrifice one rat (group BSA-3) had renal abscesses, and was excluded from further study. All other rats were healthy and growing well (mean 221 \pm 2 g at start of the experiment, 291 \pm 3 g at sacrifice). Plasma cholesterol and fasting triglyceride levels were lower in BSA-injected rats compared to SAL (*P* < 0.05). Fasting glucose levels and creatinine clearances did not differ between any of the groups. Fasting NEFA levels were significantly higher in BSA-3 and BSA-6 injected rats compared to SAL (*P* < 0.05). The BSA injections produced a marked rise in plasma total protein compared to SAL. Urinary protein excretion was higher in all three experimental groups compared to SAL, although this did not reach statistical significance in BSA-3. Between the BSA-treated groups there were no significant differences in clinicopathological parameters.

Immunohistochemistry

Results of staining for markers of both interstitial and glomerular damage are shown in Figure 1.

Table 2. Clinicopathological parameters at week 3

	SAL	BSA-0	BSA-3	BSA-6
Body weight (g)	289 ± 3.9	295 ± 3.7	298 ± 7.3	288 ± 8.9
Total cholesterol (mmol/l)	1.8 ± 0.13	1.3 ± 0.08 ^a	1.2 ± 0.17 ^a	1.1 ± 0.06 ^a
Fasting triglycerides (mmol/l)	0.5 ± 0.04	0.3 ± 0.03 ^a	0.3 ± 0.03 ^a	0.3 ± 0.01 ^a
Fasting glucose (mmol/l)	4.2 ± 0.2	4.6 ± 0.3	4.3 ± 0.3	5.1 ± 0.5
Fasting NEFA (μmol/l)	484 ± 39	674 ± 37	717 ± 83 ^a	746 ± 58 ^a
Total protein (g/l)	35.6 ± 3	53.8 ± 3.3 ^a	47.4 ± 4.4	58.8 ± 5.4 ^a
Urinary protein excretion (mg/d)	9 ± 0.6	30 ± 6.4 ^a	24 ± 2.5	37 ± 7.4 ^a
Creatinine clearance (ml/min)	1.3 ± 0.04	1.4 ± 0.10	1.4 ± 0.08	1.4 ± 0.07

^a $P < 0.05$ compared to SAL.

Representative photomicrographs of these stainings are shown in Figures 2 and 3. α -SMA was present in the glomerular mesangial regions (Figure 3A and B) and in peritubular interstitial cells (Figure 2A and B). These cells had the morphological characteristics of fibroblasts. α -SMA expression in either the glomeruli or the interstitium did not differ between the SAL and BSA-0 group (Figure 1A and B). α -SMA expression was, however, significantly elevated in interstitium and glomeruli from BSA-3 and BSA-6 compared to SAL ($P < 0.001$).

Monocytes and macrophages (ED1 staining) were present in the interstitium and glomeruli (Figures 2C and D, and 3C and D). Interstitial macrophage influx (Figure 1A) was elevated in the BSA-0, BSA-3 ($P < 0.05$) and BSA-6 groups ($P < 0.001$) compared to SAL. In glomeruli, the macrophage influx (Figure 1B) was significantly higher in all BSA treated rats than SAL ($P < 0.001$). Desmin staining (Figure 3E and F) was massively increased in glomerular visceral epithelial cells in all BSA treated rats compared to SAL ($P < 0.001$, Figure 1B).

To evaluate the additional effect of oleic acid compared to NEFA-free BSA, an analysis with BSA-0 as control for BSA-3 and BSA-6 was carried out (Figure 1A and B). Interstitial α -SMA expression in BSA-3 and BSA-6 was higher than in BSA-0 ($P < 0.001$), as was glomerular α -SMA expression in BSA-6 ($P < 0.001$). Interstitial macrophage influx was higher in BSA-6 compared with BSA-0 ($P < 0.001$), while glomerular macrophage influx did not differ between BSA-0 and BSA-3/BSA-6. Finally, glomerular desmin expression was higher in the BSA-6 group than in BSA-0 ($P < 0.05$).

RT-PCR

Results of RT-PCR for TGF- β and α -SMA are shown in Figure 4. The amount of interstitial α -SMA mRNA in BSA-3 was not statistically different from both SAL and BSA-0 (Figure 4A). Interstitial α -SMA mRNA in BSA-6 was significantly increased compared to SAL ($P < 0.05$). In glomeruli, the differences were more pronounced (Figure 4B). Glomerular α -SMA mRNA was significantly increased in both the BSA-3 and BSA-6 groups compared to SAL ($P < 0.001$ and $P < 0.05$, respectively).

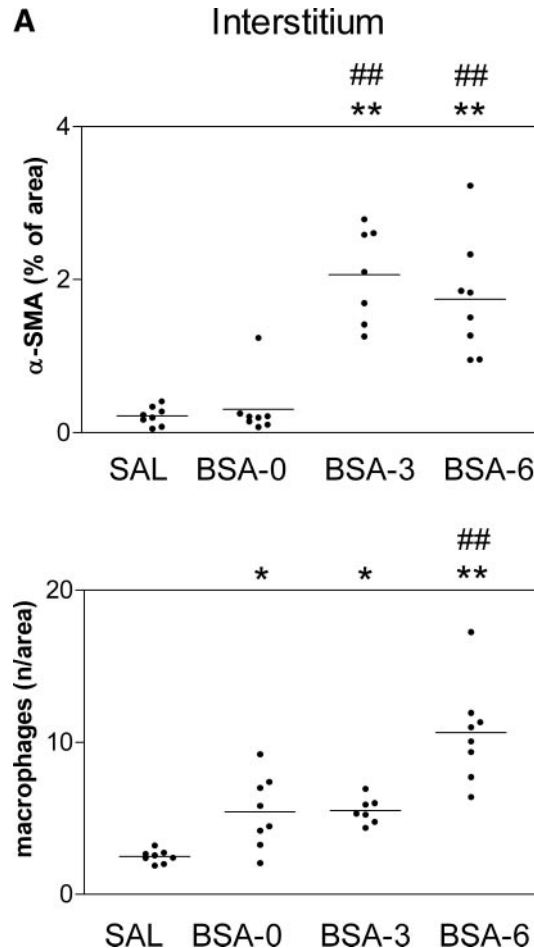


Fig. 1. (A) Interstitial results of immunohistochemistry after 3 weeks of BSA-induced protein-overload. Expression of α -SMA and influx of macrophages. * $P < 0.05$ and ** $P < 0.001$ SAL vs BSA-0/3/6; # $P < 0.05$ and ### $P < 0.001$ BSA-0 vs BSA-3/6. (B) Glomerular results of immunohistochemistry after 3 weeks of BSA-induced protein-overload. Expression of α -SMA, influx of macrophages and expression of desmin. * $P < 0.05$ and ** $P < 0.001$ SAL vs BSA-0/3/6; # $P < 0.05$ and ### $P < 0.001$ BSA-0 vs BSA-3/6.

Glomerular TGF- β mRNA (Figure 4B) was not altered in all BSA-treated groups compared to SAL. In the interstitium (Figure 4A) TGF- β mRNA in the BSA-3 group was significantly higher than in SAL ($P < 0.05$). Interstitial TGF- β mRNA was slightly decreased in BSA-6 compared to BSA-3.

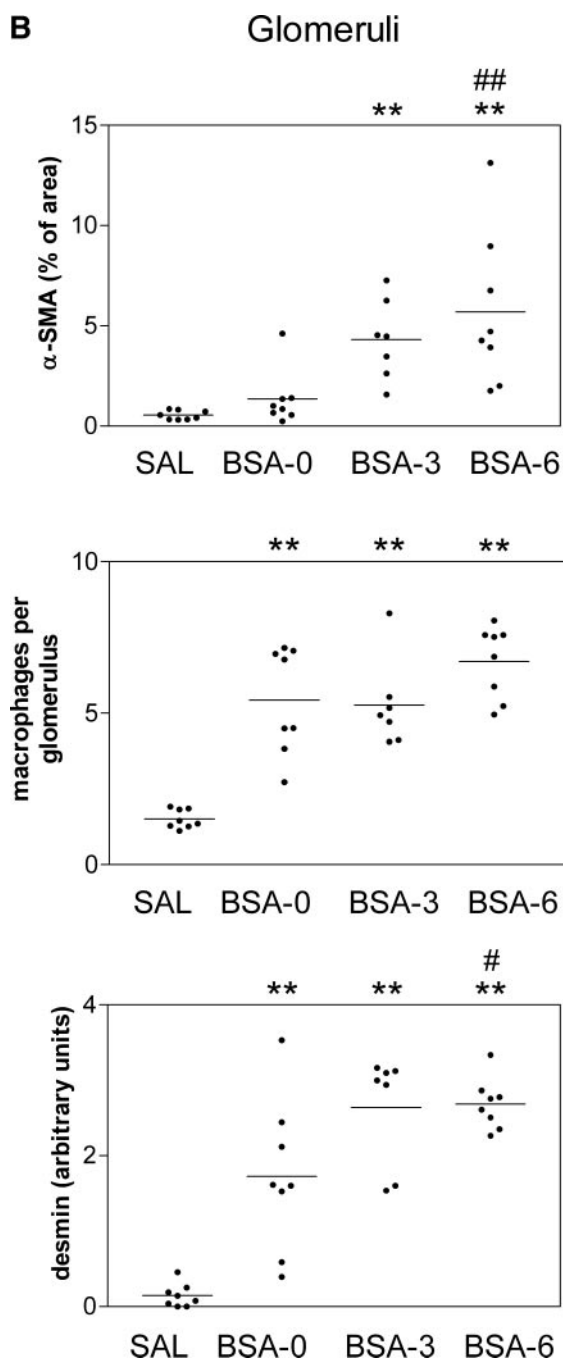


Fig. 1. Continued.

Discussion

The present study was designed to investigate the effect of NEFA carried on delipidated albumin, and in particular one single NEFA, oleic acid, on the development of renal damage in experimental protein-overload nephropathy. In this model, daily intraperitoneal injections of BSA cause proteinuria within 24 h of the first injection. Proteinuria peaks at day 3 and is still elevated at day 14 compared to controls [12]. Urinary protein excretion is accompanied by interstitial influx of macrophages and T-cells, which trigger

interstitial fibroblast proliferation and accumulation of extracellular matrix proteins [10]. This process of renal fibrosis ultimately leads to rapid loss of renal function [4].

Previous studies of Thomas *et al.* [6] and Kamijo *et al.* [5] have provided evidence for the detrimental effects of NEFA bound to albumin on renal structure. In those experiments, a comparison was made between regular lipidated BSA and delipidated BSA. Since the delipidation procedure may also remove other potentially damaging compounds carried by albumin, such as bilirubin, haematin, steroids and hydrophobic organic anions [9], we compared delipidated albumin with NEFA-loaded delipidated albumin. For that purpose, oleic acid was selectively added to the delipidated, NEFA-free, bovine serum albumin at a molar ratio of 1:3 and 1:6 (albumin:oleic acid, respectively). Albumin contains approximately five to seven very high affinity NEFA binding sites. These are formed by hydrophobic clefts into which the hydrocarbon tail of fatty acids can be inserted [9]. The ratios used in this study exceed the normal NEFA load of albumin (0.7 NEFA per albumin molecule), but reflect the ratios that are reported in adult nephrotic syndrome, namely 2.5–6 NEFA per albumin molecule [2].

Consistent with other studies [6,10], all BSA-treated rats revealed a rise in plasma total protein and urinary protein excretion at week 3. No differences in clinico-pathological parameters between the BSA-injected groups were found. Morphologically, focal segmental glomerulosclerosis and interstitial fibrosis were absent after 3 weeks. This was expected based on previous studies in this model [10,12,13], in which only mild interstitial lesions and tubular damage were found after 2–4 weeks, while using a double daily dosage of BSA.

Addition of oleic acid to NEFA-free BSA had no effect on urinary protein excretion but markedly enhanced the expression of both interstitial and glomerular markers of renal damage, such as α -SMA and desmin. This was accompanied by increased accumulation of renal macrophages, which is considered the first step in the development of renal fibrosis [6]. Consistent with elevated protein expression of α -SMA and desmin, and higher macrophage influx in BSA treated rats, RT-PCR revealed higher α -SMA and TGF- β mRNA expression in BSA-injected rats. Addition of oleic acid to BSA further elevates α -SMA mRNA expression compared to NEFA-free BSA, although statistical significance was not reached.

We present clear differences in markers of interstitial and glomerular damage between NEFA-free BSA and BSA loaded with oleic acid. Although the fatty acid content of the BSA solutions was determined prior to i.p. injection, and fasting NEFA plasma levels were elevated in both BSA-3 and BSA-6 compared to SAL, we could not prove that the fatty acids bound to albumin actually reached the kidney. However, a previous study of Ghiggeri *et al.* [14] suggests that the lower NEFA content of urinary albumin may explain the milder disease progression in patients with minimal change nephropathy compared to patients with other

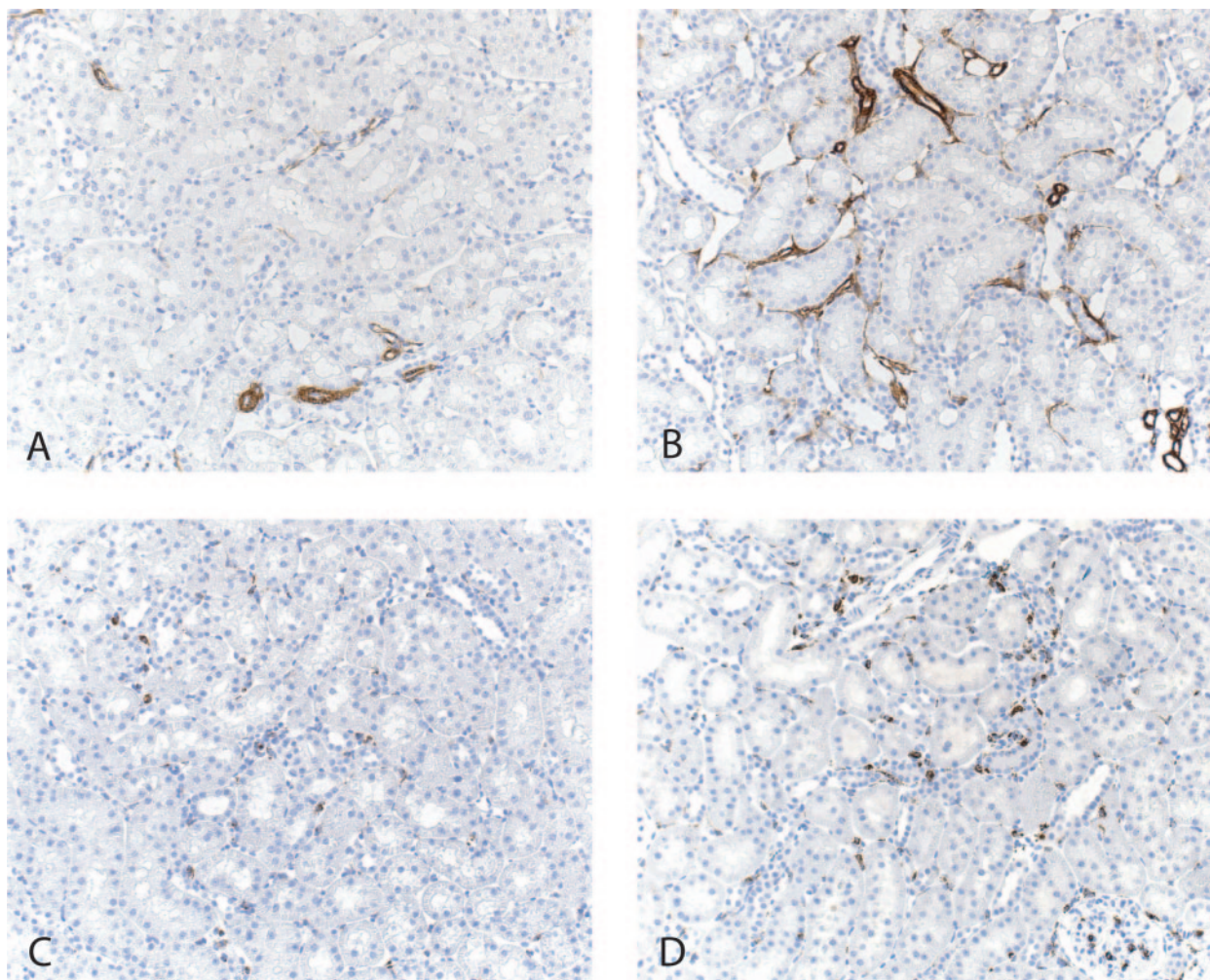


Fig. 2. Results of immunohistochemical staining after 3 weeks of BSA-induced protein-overload. Representative photomicrographs of interstitial sections are shown. α -SMA expression in the delipidated, NEFA-free, BSA (BSA-0) injected group (A) and the BSA with addition of six molecules of oleic acid per molecule BSA (BSA-6) group (B). Influx of macrophages in the BSA-0 group (C) and the BSA-6 group (D). Original magnifications are 200 \times .

nephrotic conditions. In addition, we presume that the differences are blurred due to the fact that the injected NEFA-free BSA picks up free NEFA from the circulation. NEFA are bound to albumin with a $t_{1/2}$ measured in milliseconds [9].

Filtered albumin is reabsorbed by proximal tubular cells via receptor-mediated endocytosis by two receptors, cubulin and megalin [3]. After internalization in lysosomes, albumin is degraded to amino acids, which are returned to the circulation [2,3]. In protein-overload, there is increased protein delivery to the proximal tubule, which is associated with over-absorption of proteins. In proximal tubular cells of nephrotic patients, this is morphologically seen as vacuolization [2], which disturbs the cellular architecture and thereby likely its function. The currently held hypothesis proposes that protein-overload of proximal tubular cells activates tubular-dependent pathways of interstitial inflammation and fibrosis [4]. Evidence is available, both *in vitro* and *in vivo*, that vasoactive and

inflammatory genes such as endothelin-1, monocyte chemoattractant protein-1 and chemotactic cytokine RANTES (Regulated upon Activation, Normal T Cell Expressed and Secreted) are upregulated in proteinuric conditions [15].

During albumin degradation in proximal tubular cells, NEFA are released intracellularly, where they can play a role as energy source, membrane components and precursor of lipid mediators. In addition, they can regulate the activity of many cellular processes, including membrane receptor activation, cell differentiation and gene expression [16]. When an uncontrolled influx of NEFA into proximal tubular cells occurs, these cells may keep their intracellular level of NEFA low by incorporating them into a variety of complex lipids such as triglycerides, diglycerides (diacylglycerol, DAG) and phospholipids [17]. Indeed, it has been shown *in vitro* in opossum kidney cells that most NEFA, for example oleate, were metabolized to triglycerides [2]. Elevated levels of DAG activate

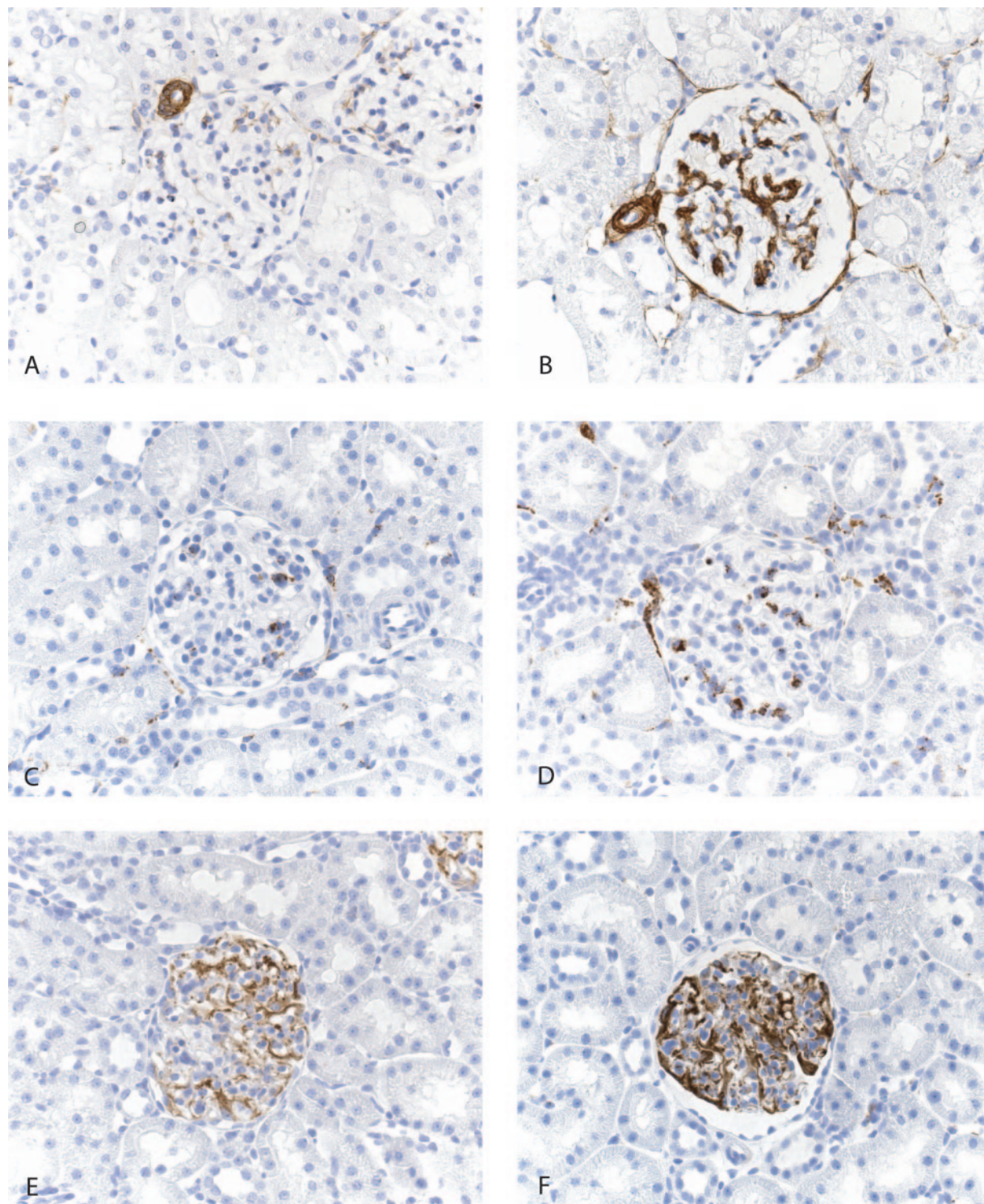


Fig. 3. Results of immunohistochemical staining after 3 weeks of BSA-induced protein-overload. Representative photomicrographs of glomerular sections are shown. α -SMA expression in the delipidated, NEFA-free, BSA (BSA-0) injected group (A) and the BSA with addition of six molecules of oleic acid per molecule BSA (BSA-6) group (B). Influx of macrophages in the BSA-0 group (C) and the BSA-6 group (D). Desmin expression in the BSA-0 group (E) and the BSA-6 group (F). Original magnifications are 400 \times .

protein kinase C (PKC), which has been implicated as a key signalling event in cell growth and proliferation through activation of two transcription factor families, nuclear factor kappa-B (NF- κ B) and activating

protein 1 (AP-1). So, elevated levels of NEFA can alter cell growth and proliferation via PKC [18].

Except for effects on cells in general, NEFA have a number of effects on mitochondria in particular.

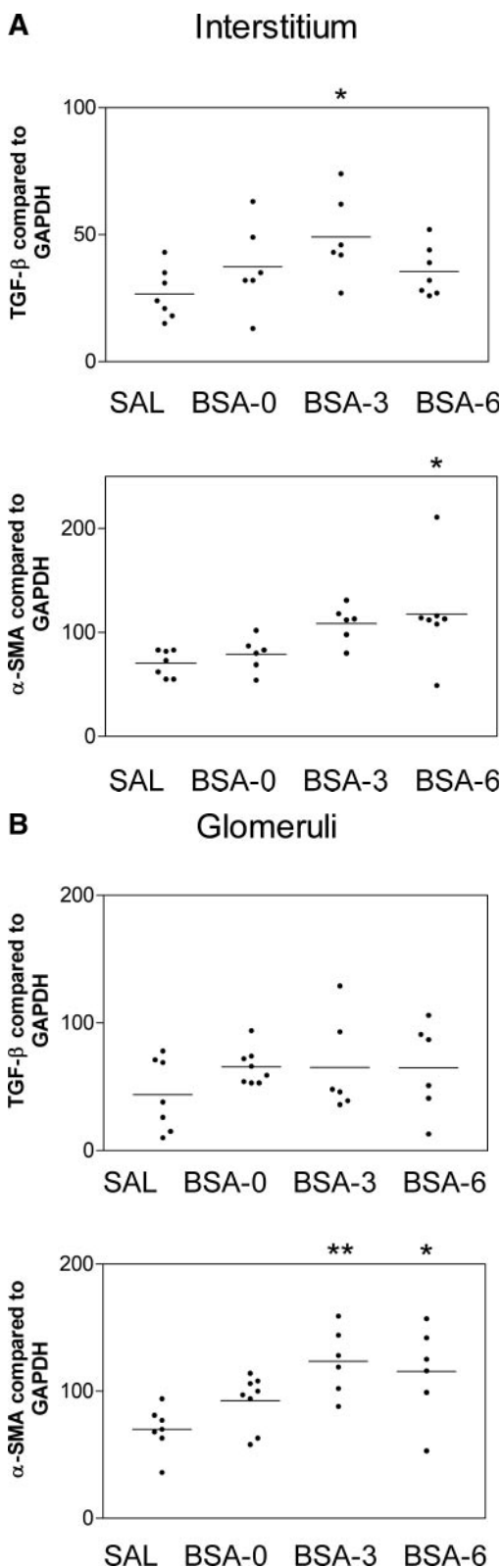


Fig. 4. (A) Interstitial results of RT-PCR after 3 weeks of BSA-induced protein-overload. Interstitial TGF- β and α -SMA are expressed as ratio compared to GAPDH. * $P < 0.05$ SAL vs BSA-0/3/6. (B) Glomerular results of RT-PCR after 3 weeks of BSA-induced protein-overload. Glomerular TGF- β and α -SMA are expressed as ratio compared to GAPDH. * $P < 0.05$ and ** $P < 0.001$ SAL vs BSA-0/3/6.

NEFA are respiratory substrates, which feed electrons into the mitochondrial energy-conserving respiratory chain [19]. Upon an increased availability of NEFA, the respiratory chain can generate more oxygen-free radicals, which can have adverse effects on proximal tubular cells [20].

Evidence for the important role of NEFA is also available in minimal change disease, in which the urinary albumin has a markedly lower NEFA content, compared to urinary albumin from patients with other nephrotic conditions [14]. This low NEFA content may explain the generally non-progressive nature of this condition.

In conclusion, we provide evidence that addition of oleic acid to delipidated, NEFA-free, BSA markedly enhanced the expression of inflammatory and fibrotic markers of interstitial and glomerular damage in our experimental model. Further studies are needed to investigate the precise effects of NEFA on proximal tubular cells and the pathways that are activated.

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Conflict of interest statement. None declared.

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