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Albumin-bound fatty acids induce mitochondrial oxidant stress and impair antioxidant responses in proximal tubular cells

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Albumin induces oxidative stress and cytokine production in proximal tubular cells (PTECs). Albumin-bound fatty acids (FAs) enhance tubulopathic effects of albumin *in vivo*. We proposed that FA aggravation of albumin-induced oxidative stress in PTECs might be involved. We hypothesized that mitochondria could be a source of such stress. Using a fluorescent probe, we compared reactive oxygen species (ROS) production after exposure of PTECs to bovine serum albumin (BSA) alone or loaded with oleic acid (OA-BSA) (3–30 g/l for 2 h). There was no difference in cellular albumin uptake, but OA-BSA dose-dependently induced more ROS than BSA alone ($P<0.001$). OA-BSA-induced ROS was significantly alleviated by mitochondrial inhibition, but not by inhibitors of nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) oxidase, xanthine oxidase, or nitric oxide synthase. Gene expression analysis showed that neither the NADPH oxidase component p22phox nor xanthine oxidase was induced by BSA or OA-BSA. OA-BSA, in contrast to BSA, failed to induce mitochondrial manganese superoxide dismutase 2 (SOD2) expression. OA-BSA showed a greater capacity than BSA to downregulate heme oxygenase-1 mRNA expression and accentuate inflammatory cytokine mRNA and protein. Supplementation of SOD activity with EUK-8 reduced ROS, and interleukin-6 protein expression was suppressed by both mitochondrial inhibition and SOD augmentation. Thus, in PTECs, FAs accentuate albumin-induced oxidative stress and inflammatory cytokine expression via increased mitochondrial ROS, while frustrating protective antioxidant responses.

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KEYWORDS: albumin; oleic acid; proximal tubular cells; mitochondria; oxidative stress; superoxide dismutase

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Proximal tubular epithelial cells (PTECs) treated with excess albumin produce numerous pro-oxidant and proinflammatory substances.[1–6] There is evidence that such albumin-stimulated PTEC activity contributes to tubulointerstitial inflammation *in vivo*. For example, in human nephrotic kidneys, interleukin 8 (IL-8) mRNA and protein localized mainly to tubular epithelial cells, and the distribution of infiltrating interstitial leukocytes approximated with IL-8-expressing tubules.[7] Similarly, in proteinuric rats, osteopontin was upregulated in proximal tubules with adjacent interstitial inflammatory infiltrates.[8] These findings indicate that PTECs are not merely structural elements but are active players in pathophysiologic processes. Oxidative stress plays a key role in these events. In PTECs exposed to albumin, increased intracellular production of reactive oxygen species (ROS) was required for activation of nuclear factor kappaB and expression of IL-8[7] and macrophage chemoattractant protein 1.[9]

Some effects of albumin on PTECs are not owing to the molecule itself, but rather to fatty acids (FAs) bound to it. Excess PTEC fibronectin production was induced by oleic acid-complexed albumin but not by pure albumin.[10] FA presented to PTECs via albumin is efficiently taken up, leading to such effects as altered cellular growth,[11] disturbed metabolism,[11,12] increased apoptosis,[13] and the release of lipid metabolites with immunologic[14] and biochemical[15] properties that may be pathologically important. The concept of albumin-bound FA toxicity is supported by *in vivo* evidence. In rodents, we[16] and others[17,18] have found that albumin-bound FAs aggravate albumin-induced nephropathic effects, including renal cortical apoptosis,[17] tubulointerstitial inflammation,[16–18] and glomerular injury.[16] Moreover, nephrotic patients with the relatively benign minimal change disease (MCD) have more than threefold lower urinary albumin FA content than individuals with non-MCD varieties of nephrosis that tend to run a more aggressive course.[19] These observations together indicate that albumin-bound FA exerts extra deleterious effects on PTECs,

on top of those of albumin alone. However, information about these processes remains limited, and in particular it is not known whether albumin-bound FA can increase oxidative stress.

We employed an *in vitro* protein overload model of cultured PTECs to explore two questions relating to the idea that albumin-bound FAs exaggerate oxidant effects of albumin on PTECs. First, we postulated that FAs increase the ROS-inducing capacity of albumin. We tested this hypothesis by comparing ROS production after exposure to bovine serum albumin (BSA) either alone or loaded with oleic acid (OA-BSA). We found that cells exposed to OA-BSA produced up to 2.5-fold more ROS than those treated with BSA alone. Second, we explored the source of OA-BSA-induced ROS in these cells. Evidence from another cell type suggests that FAs stimulate ROS production from the mitochondria.[20] In vascular endothelial cells exposed to lipids, there is an important mitochondrial contribution to ROS.[21] Thus, we hypothesized that mitochondria may also be a major source in PTECs upon treatment with albumin-bound FAs. Our findings supported this hypothesis. We went further to assess the responses of cellular antioxidant defence mechanisms to the FA-induced disturbance of redox balance, and to explore whether the resultant excess oxidant stress is of functional consequence in terms of cellular proinflammatory transcriptional and translational activity. The present study reveals a failure in the mitochondrial antioxidant response as a mechanism by which FAs bound to albumin can induce oxidative and inflammatory stresses in PTECs.

RESULTS

FA binding has no effect on albumin uptake by HK-2 cells

Electro-immunodiffusion assays (Table 1) showed similar albumin uptake by HK-2 cells after BSA or OA-BSA treatment for 2 h, in a dose-dependent manner. Similar uptake was also visualized by immunohistochemistry (not shown). The results are comparable with previous findings in similar cells.[22–24]

Table 1 | Albumin uptake after exposure to albumin with or without bound OA

	Treatment: albumin concentration (g/l)	Cellular albumin content after 2 h (μg BSA/mg total cellular protein)
Control	0	0
BSA	3	12.3 (2.7)*
	15	32.4 (2.3)*,#
	30	45.2 (2.4)*,#
OA-BSA	3	13.6 (1.8)*
	15	24.8 (2.5)*,#
	30	42.0 (6.9)*,#

BSA, bovine serum albumin; OA, oleic acid.
Mean (s.e.m.) of three independent experiments (μg albumin/mg total cellular protein).
*$P<0.001$ vs control,
#$P<0.05$, ##$P<0.01$ vs 3 g/l.
There were no significant treatment or time differences.

OA-BSA induces more ROS than BSA

Incubation of HK-2 cells with OA-BSA for 2 h resulted in markedly and significantly greater ROS production than BSA alone, at every concentration tested. OA-BSA induced ROS dose-dependently (up to fourfold increase from control level), whereas BSA induced moderate (up to 1.5-fold) ROS increase, which attained significance only at the highest concentration (Figure 1a). Cell viability was not impaired by 2-h BSA or OA-BSA exposure (3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) release assays; Figure 1b).

The property of albumin as an excellent carrier for many hydrophobic substances (besides FAs) raises the concern that it could acquire unknown contaminants in the course of commercial preparation and purification processes. To confirm that the effects we observed were not influenced by such contamination, we repeated some experiments using a different formulation of low endotoxin (0–2.0 EU/mg), low FA (0–0.2 mg/g) BSA (SERVA Electrophoresis GmbH, Heidelberg, Germany). As shown in Table 2, the pattern of ROS production induced by the SERVA product was similar to that of the Sigma product described in the above paragraph.

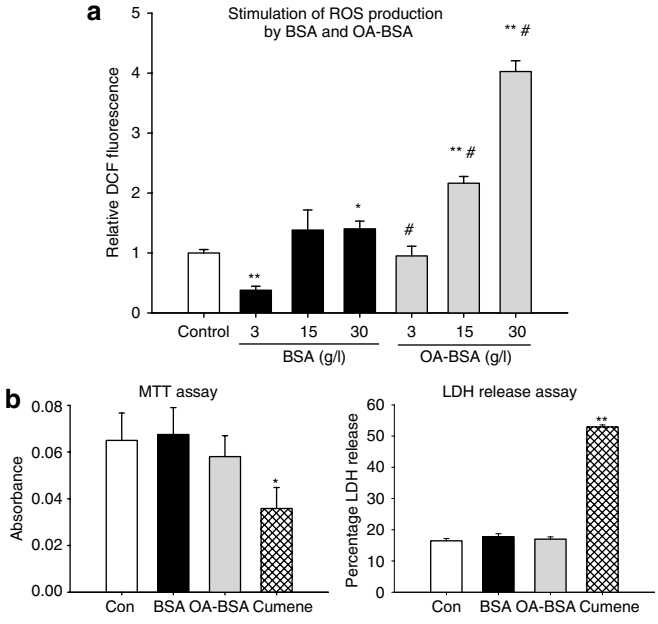

Figure 1 | Effects of BSA and OA-BSA on cellular ROS production and viability. (**a**) ROS production after exposure to albumin (BSA) alone or enriched with oleic acid (OA-BSA) in proximal tubular epithelial cells (PTECs), measured by DCF fluorescence normalized to control values (a.u.). *$P<0.05$, **$P<0.001$ vs control (untreated) cells; #$P<0.001$, OA-BSA vs corresponding BSA concentration. Mean (s.e.m.) of at least three independent experiments is shown. (**b**) MTT and LDH release assays. Cells exposed to cumene hydroperoxide for the same duration were used as positive control. *$P<0.05$, **$P<0.001$ vs all other groups. Mean (s.e.m.) of three independent experiments is shown.

Table 2 | ROS measurement after treatment of HK-2 cells with a different albumin formulation

Albumin content (g/l)	BSA	OA-BSA
3	1.5 (0.2)	2.4 (0.2)**,##
15	1.9 (0.2)**	3.3 (0.4)**,#
30	2.3 (0.4)*	3.6 (0.5)**,##
0 (control)	1.0 (0.03)	

BSA, bovine serum albumin; HK-2, human kidney-2; OA, oleic acid; ROS, reactive oxygen species.
Mean (s.e.m.) of three independent experiments.
*$P<0.01$, **$P<0.001$ vs control.
#$P<0.05$, ##$P<0.01$, BSA vs OA-BSA.

OA-BSA-induced ROS is alleviated by mitochondrial inhibitors

In further experiments, cells were exposed to OA-BSA (30 g/l) for 2 h. In intervention studies to probe possible sources of ROS (Figure 2), OA-BSA-induced increase was significantly attenuated by the mitochondrial respiratory chain blockers rotenone (ROT) (complex I inhibitor) and carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (respiratory uncoupling agent), but not by 2-thenoyltrifuoroacetone (complex II inhibitor). The specific nicotinamide adenine dinucleotide phosphate hydrogenase (NADPH) oxidase inhibitor apocynin (APO) had no significant effect. *N*ω-nitro-L-arginine methyl ester and allopurinol also had no effect, excluding uncoupled nitric oxide synthase or xanthine oxidase as ROS sources.

p22phox and xanthine oxidase genes are not induced by OA-BSA

To probe whether BSA or OA-BSA induce pro-oxidant transcriptional changes that could influence cellular ROS sources, we performed reverse transcription-polymerase chain reaction (PCR) studies on the NADPH components p22phox (cytosolic component) and rac1 and rac2 (membrane-bound components). We also tested the xanthine oxidase gene. Although rac1 and rac2 were both induced by either treatment, the vital enzyme component p22phox was not. There was no change in xanthine oxidase expression. Illustrative PCR bands and semiquantitative densitometry of gene expression are shown (Figure 3a).

Mitochondrial antioxidant SOD2 gene induction, seen with BSA, fails with OA-BSA

We assessed the transcriptional response of major cellular antioxidant enzymes (Figure 3b). In cells treated with BSA alone, there was notable upregulation of manganese superoxide dismutase (SOD2) despite the comparatively mild ROS increase. In marked contrast to BSA, OA-BSA failed to induce SOD2. Expression of other redox enzyme genes, copper–zinc superoxide dismutase (SOD1) and glutathione peroxidase, was not significantly regulated by either treatment.

ROS increase is ameliorated by EUK-8, a synthetic SOD analog

To explore the functional implication of the antioxidant gene expression pattern noted above, we tested whether

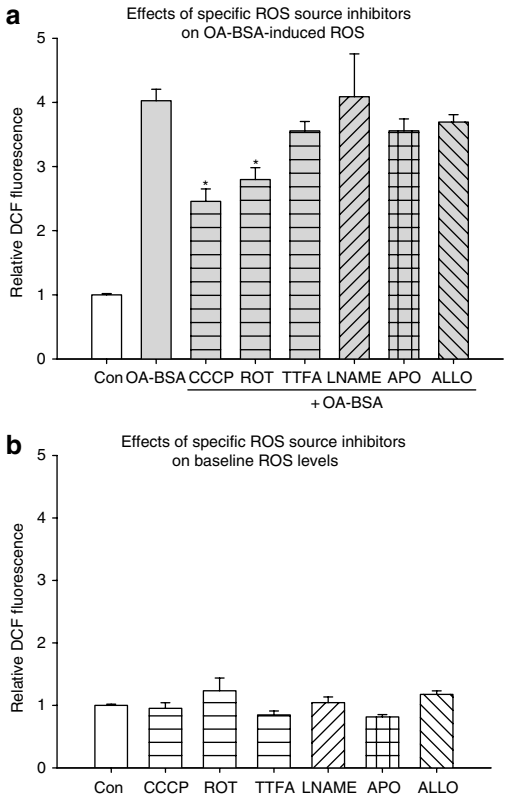

Figure 2 | Effects of inhibitors of ROS sources on stimulated and basal cellular ROS production. (**a**) ROS production by HK-2 cells after OA-BSA treatment in the presence or absence of blockers of potential ROS sources. Mitochondrial inhibitors used were CCCP (uncoupling agent), rotenone (ROT) (complex I), and 2-thenoyltrifuoroacetone (complex II). *N*ω-nitro-L-arginine methyl ester is a nitric oxide synthase inhibitor, APO an NADPH oxidase inhibitor, and allopurinol (ALLO) a xanthine oxidase inhibitor. *$P<0.001$ vs OA-BSA. Mean (s.e.m.) of at least three independent experiments is shown. (**b**) Effects of the above-named intervention agents on baseline (control) cellular ROS levels.

supplementation of SOD might alleviate ROS induced by OA-BSA. We used the saleno-manganese SOD mimetic agent EUK-8, which indeed markedly alleviated ROS (Figure 4).

Downregulation of heme oxygenase-1 is enhanced by OA-BSA

For an insight into whether bound FAs might also influence albumin-induced inflammatory effects, we studied the gene expression of cytokines IL-6 and IL-8. Both treatments similarly increased cytokine expression. However, OA-BSA had a greater tendency to suppress the expression of heme oxygenase-1 (HO-1) (Figure 3c), a molecule with important renal antioxidant and anti-inflammatory effects.[25,26]

OA-BSA aggravates oxidant-sensitive IL-6 protein expression

To assess whether the observed effects in gene expression are reflected at protein level, we measured IL-6 in cells treated with BSA or OA-BSA. OA-BSA induced significantly greater cellular expression of IL-6 protein than BSA (Table 3), despite their similar effect at the level of gene expression. We then

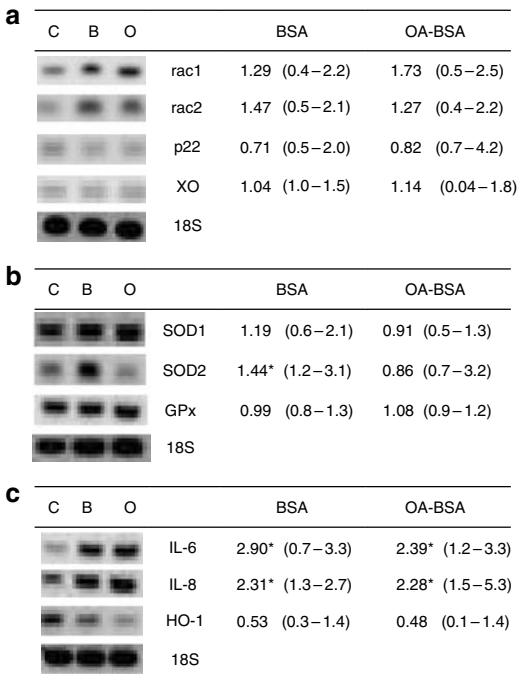

Figure 3 | Illustrative bands from reverse transcription-polymerase chain reaction experiments, normalized to 18S and related to control levels, showing transcriptional changes induced by BSA and OA-BSA. C, control; B, BSA; O, OA-BSA. Densitometric data are shown as median (range) fold difference vs control, 4–5 independent experiments, *$P<0.05$, BSA vs OA-BSA (Friedman). (**a**) Expression pattern of pro-oxidant genes: NADPH oxidase components (rac1, rac2, p22phox) and xanthine oxidase. (**b**) Expression of antioxidant genes: SOD1, SOD2, glutathione peroxidase. (**c**) Expression of genes of downstream mediators: inflammatory cytokines IL-6 and IL-8, and the antioxidant molecule HO-1.

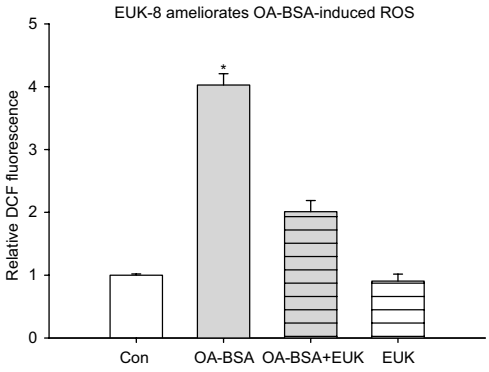

Figure 4 | ROS production by HK-2 cells after OA-BSA treatment in the presence or absence of EUK-8, a manganese SOD mimetic. *$P<0.001$ vs all other groups.

tested whether antioxidant intervention would ameliorate the effect of OA-BSA. The saleno-manganese complex and SOD mimetic agent EUK-8, and the mitochondrial blockers rotenone and CCCP, all reduced IL-6 protein expression to baseline (control) levels. The intervention agents acting alone did not significantly alter baseline levels (not shown).

Table 3 | IL-6 protein expression in HK-2 cells after treatment with BSA or OA-BSA

	IL-6 (ng/mg total cellular protein)
Control	0.96 (0.1)
BSA	1.65 (0.2)*
OA-BSA	2.38 (0.4)**
OA-BSA+EUK-8	1.16 (0.2)
OA-BSA+rotenone	0.90 (0.4)
OA-BSA+CCCP	1.10 (0.4)

CCCP, carbonyl cyanide 3-chlorophenylhydrazone; BSA, bovine serum albumin; IL, interleukin; OA, oleic acid.
Mean (s.e.m.) of up to eight independent experiments.
*$P<0.05$ vs control.
**$P=0.005$ vs control.

DISCUSSION

The present study provides observations suggesting that albumin-bound FAs augment the oxidative capacity of albumin on PTECs via excess mitochondrial ROS, in the face of an impaired mitochondrial ROS-scavenging response. A complex of albumin with OA (OA-BSA) significantly and dose-dependently increased intracellular ROS much more than albumin alone (BSA). OA-BSA stimulation experiments in the presence of various inhibitors implicated mitochondria and not NADPH oxidase, xanthine oxidase, or nitric oxide synthase as the main source of ROS. A manganese SOD mimetic agent, EUK-8, alleviated the OA-BSA-induced ROS increase. Not only did OA-BSA markedly increase mitochondrial ROS, but also adversely affected gene expression of the key mitochondrial antioxidant system, the manganese-containing SOD2. Whereas BSA induced protective SOD2 gene expression, this response was completely absent with OA-BSA. Parallel to increased oxidative stress, OA-BSA aggravated the protein expression of the inflammatory cytokine IL-6 beyond the level of BSA alone. This effect was redressed by both SOD supplementation and mitochondrial inhibition, suggesting that the dual-mechanism oxidant effect of albumin-bound FAs promotes proinflammatory PTEC activity.

There is as yet no broad consensus regarding the specific sites of ROS production on the mitochondrial electron transport chain. Complex I is thought to be important, but complexes II and III are also known to be capable of ROS production, and there are variations between tissues.[27,28] We probed possible ROS contribution from complexes I and II, and from the respiratory coupling step. Only the complex II inhibitor 2-thenoyltrifuoroacetone failed to substantially reduce OA-BSA-induced ROS, suggesting complex I as the key location of ROS production within PTEC mitochondria. In experiments probing other possible ROS sources apart from mitochondria, APO, a specific inhibitor of NADPH oxidase complex assembly,[29] failed to attenuate ROS. Moreover, gene expression studies indicated that the crucial NADPH oxidase component p22phox was not induced by OA-BSA. p22phox plays an essential role in NADPH oxidase function by facilitating transfer of the cytosolic elements to the membrane, enabling catalytic activation.[30] Interestingly,

we found that PTECs robustly express both of the low molecular weight G-protein NADPH oxidase complex components rac1 and rac2. Both molecules tended to be upregulated after OA-BSA treatment, but apparently without functional significance in the absence of p22phox induction. In further inhibition studies of cellular ROS-producing enzymes, neither nitric oxide synthase inhibition with *N*ω-nitro-L-arginine methyl ester nor blockade of xanthine oxidase with allopurinol yielded significant reductions in ROS.

Marked reduction of OA-BSA-induced ROS was observed with EUK-8, a manganese–salen compound that possesses efficient catalytic SOD antioxidant activity.[31,32] Crucially, EUK-8 also has potent catalase activity, precluding the risk of SOD-driven H_2O_2 accumulation. Thus, an SOD/catalase enzymatic axis (driving the reaction sequence $O_2^{\bullet -} \rightarrow H_2O_2 \rightarrow H_2O + O_2$) appears to be the pivotal redox pathway in this OA-BSA model of PTEC protein overload (Figure 5). Gene expression of other cellular antioxidant enzymes, SOD1 and glutathione peroxidase, was not regulated by either BSA or OA-BSA.

Mitochondria produce ROS during the course of normal cellular metabolism, and the local scavenging action of mitochondrial SOD (SOD2) is a major protector of the organelle, and indeed the whole cell, from auto-oxidation (via ROS diffusion into the cytoplasm).[33] Excess mitochondrial ROS can have devastating effects, as illustrated by severe lifespan reductions in SOD2 null mice.[27,33] This contrasts with remarkably milder phenotypes in mice lacking other SOD isoforms. In the present study, OA-BSA increased mitochondrial ROS and suppressed the protective gene induction of SOD2 seen with BSA alone.

To further assess the cellular defensive response to increased oxidative stress, we examined the gene expression of HO-1, a widely distributed enzyme that plays an important role in maintaining cellular redox balance. HO-1 induction is seen as a defensive response to injurious stimuli[25] and is specifically protective of PTECs against various types of oxidant agents.[26] Contrary to our expectation of increased expression as an attempt to counter oxidant stress, we surprisingly observed that HO-1 was down-regulated in PTECs exposed to BSA, an effect that was enhanced by OA-BSA. Thus it appears that, apart from aggravating albumin-induced oxidative stress, albumin-borne FAs also further depress the HO-1 antioxidant protection. However, in contrast to our finding of suppressed HO-1 expression in protein-overloaded human PTECs, it was recently reported that no changes occurred in HO-1 expression in kidneys of rats that received a brief course of albumin injections (7 days), or in cultured rat PTECs that were exposed to albumin.[34] This contrast may be a further example of differences in renal transcriptional HO-1 reaction between humans and other species. Marked differences are known between human and murine renal HO-1 gene responses to various stimuli, including heat shock, hypoxia, and hyperosmolarity.[35]

OA-BSA significantly escalated cellular IL-6 protein mass beyond the level induced by BSA alone. As IL-6 gene expression was increased to a similar degree by the two treatments, these data suggest that the critical effect of albumin-bound FAs in the promotion of PTEC proinflammatory capacity may be at the level of gene translation rather than transcription. OA-BSA-induced IL-6 was suppressed by CCCP and ROT, confirming the key role of mitochondria-derived oxidant stress, as well as by EUK-8, emphasizing the importance of SOD. FAs may play a crucial role in the translational control of IL-6 expression, and albumin-bound lipids could escalate proinflammatory tendencies within PTECs by driving the gene translation of IL-6 and possibly other cytokines, via ROS-dependent mechanisms. Such effects may at least partly explain our[16] and others[17,18] observations that FA-loaded albumin causes more renal damage *in vivo* than albumin alone.

In summary, our findings offer new insights into mechanisms by which albumin-bound FAs can induce oxidative stress and oxidant-dependent changes in PTECs. Bound oleic acid aggravates albumin-induced oxidative stress via increased mitochondrial ROS production and frustration of protective SOD2 transcriptional response, indicating a major pro-oxidant shift in mitochondrial redox balance that may promote proinflammatory cytokine mechanisms. The HO-1 response, another important protective mechanism, is also impaired. These observations support the notion of albumin as a 'Trojan horse', transporting into tubular cells harmful FAs that depress the cellular defence against ROS.

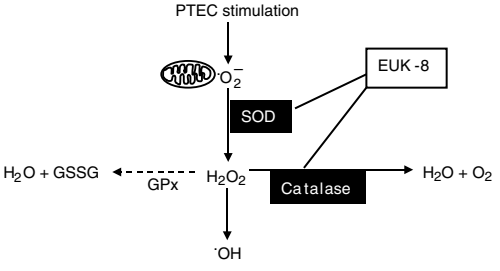

Figure 5 | Schema of ROS pathways of interest. Abbreviations: $^{\bullet}O_2^-$, superoxide anion, H_2O_2, hydrogen peroxide; GPx, glutathione peroxidase; GSSG, oxidized glutathione; H_2O, water; O_2, molecular oxygen; $^{\bullet}OH$, hydroxyl ion.

MATERIALS AND METHODS

Cell culture and model characterization

We utilized HK-2 cells, immortalized epithelial cells derived from human proximal tubules (obtained from the Department of Pathology, UMC Utrecht, The Netherlands). Cells were cultured at 37°C and 5% CO_2, using Gibco Rosewell Park Memorial Institute 1640 medium (with 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid buffer and L-glutamine) (Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum, sodium bicarbonate, 100 U/ml penicillin, and 100 μg/ml streptomycin. HK-2 cells are well characterized for proximal tubular epithelial phenotype.[36,37]

Albumin uptake

To establish that HK-2 cells are suitable for albumin overload studies with or without added FAs, we quantified BSA uptake. Cells were grown to confluence in six-well plates, serum-starved for 24 h, exposed to 3–30 g/l BSA or OA-BSA for 2 h, and washed. Cells were homogenized, and the supernatant stored at −20°C. Total protein was measured by the Bradford method. BSA was measured by immunoelectrophoresis using a rabbit anti-BSA antibody (Nordic Laboratories, Tilburg, The Netherlands). Results are expressed as BSA relative to total protein. To visualize intracellular BSA, cells were grown to confluence on eight-well Lab-Tek chamber slides (Nalge Nunc, Naperville, IL, USA), treated as above, and stained with a rabbit anti-BSA antibody (Nordic) and a goat anti-rabbit secondary antibody (Powervision) (Klinipath, Leiden, The Netherlands).

Cell treatments

Chemicals and reagents were obtained from Sigma Aldrich (Zwijndrecht, The Netherlands) unless otherwise indicated. We selected OA as our model FA, being the most abundant FA on urinary albumin;[11] moreover, albumin-bound OA increased ROS in vascular endothelial cells[38] and induced a more pronounced fibrogenic response in PTECs than other FAs.[10] To prepare albumin solutions, BSA with very low endotoxin content (< 1 ng/mg) (Sigma A-9430) was dissolved in phosphate-buffered saline. For OA-BSA solutions, OA (Sigma O-1008) was added to fresh BSA solutions, mixtures incubated for 2.5 h at 37°C with gentle shaking, filtered (0.22 μm), aliquoted, and stored at −20°C. FA spectra in the solutions were determined by gas chromatography as described by Muskiet *et al.*[39] Briefly, 100 μl of BSA or OA-BSA solution mixed with 1 mg butylated hydroxytoluene (an antioxidant) and 50 mg margaric acid (17:0, internal quantification standard) was injected into a gas chromatograph. In samples of solutions that we used for experiments, BSA contained very low levels (< 0.025 mmol/l) of long-chain FAs like palmitic, linoleic, oleic, stearic, and vaccenic acids, whereas OA content was very high in OA-BSA, ∼1.7 mmol/l. Thus, although during the preparation protocol we added OA to BSA solutions at a molar ratio of 6/1 OA/BSA, the actual uptake of OA by BSA corresponded to ∼3.6/1 OA/BSA, consistent with findings that albumin has only three high-affinity, primary long-chain FA binding sites, and other weaker, secondary sites with lower affinity.[40]

In the first series of experiments, cells were treated with BSA (3–30 g/l) alone or complexed with oleic acid (OA-BSA), and ROS measured for 2 h with the dichlorofluorescein (DCF) assay (described below). In subsequent experiments, cells were exposed to an optimal concentration of OA-BSA (at 30 g/l) for 2 h with or without co-treatment with various agents chosen to answer specific study questions. Agents employed were APO (1 mM), allopurinol (ALLO, 100 μM), CCCP (10 μM), 2-thenoyltrifuoroacetone (10 μM), rotenone (ROT) (10 μM), *N*-ω-nitro-L-arginine methyl ester (2 mM) (ICN Biomedicals, Aurora, OH, USA), and EUK-8 (10 μM) (Calbiochem, Merck, Nottingham, UK). Concentrations employed were determined during pilot studies. Stock solutions were diluted to final working concentrations with phosphate-buffered saline supplemented with 0.9 mM $CaCl_2$, 0.45 mM $MgCl_2$, and 5 mM glucose (phosphate-buffered saline + + +). Control (untreated) cells for each intervention were incubated with corresponding vehicle solutions. With agents that generated excessive background signal during pilot experiments (APO, EUK-8), the protocol was modified by overnight preincubation and thorough washing before measurements.

ROS measurement

Intracellular ROS were measured with the membrane-permeable fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Invitrogen/Molecular Probes, Merelbeke, Belgium). Upon deacetylation following cell entry, the molecule is retained intracellularly. On reaction with ROS, it yields fluorescent DCF. We detected and quantified DCF fluorescence by microplate fluorospectrometry[41] using a FLUOstar Optima reader (BMG Labtechnologies, Durham, NC, USA) at excitation and emission wavelengths of 485 and 520 nm. HK-2 cells were grown to confluence in 96-well flat-bottom microplates (Corning, NY, USA), serum-starved for 24 h, incubated at 37°C with 20 μM H_2DCFDA in the dark for 30 min, and washed to remove excess probe. Experimental treatments were then commenced (37°C) and DCF fluorescence was monitored at 1–2 min intervals during treatment. Fluorescence slopes were computed per well. Background correction was by assay of nonspecific fluorescence from identically treated but unlabelled cells. In validation experiments, HK-2 cells treated with cumene hydroperoxide (10–500 μM) for 30 min showed dose-dependent increases in DCF signal (data not shown). In general, experiments were carried out at least in triplicate, and repeated at least twice. Results are expressed as mean fluorescence slope ratios normalized to corresponding vehicle control (±s.e.m.).

Cell viability

We used LDH release and MTT tests, by spectrophotometry with Ultramark microplate system (Bio-Rad Laboratories, Hercules, CA, USA). (a) *LDH assay:* To measure released LDH, incubation buffer was aspirated from each well and transferred to a fresh microplate. To measure residual cellular LDH, cells were lysed with 0.1% Triton X-100 in phosphate-buffered saline. Then for both measurements, 50 μl of a pre-heated assay mix (37°C) containing 9 mM pyruvate (Merck, Darmstadt, Germany) and 1.41 mM NADH (ICN, Amsterdam, The Netherlands) was added per well and absorbance quickly read at 340 nm. We computed total LDH, and calculated the released LDH as a percentage of total. (b) *MTT assay:* Incubation buffer was replaced with 0.5 mg/ml MTT (Sigma) and cells further incubated for 90 min at 37°C. MTT was aspirated and the formazan product released by incubation with acidified (0.04 N HCl) isopropanol for 15 min with gentle shaking. Absorbance was read at 570 nm.

Gene expression studies

We assessed gene expression by semiquantitative reverse transcription-polymerase chain reaction. Cells were grown to confluence in six-well plates and rested in serum-free medium for 24 h. After experimental exposures for 2 h, cells were solubilized in TRIzol reagent (Invitrogen). *Total RNA extraction* from TRIzol solution was performed using the procedure recommended by the manufacturer. Extracted RNA was dissolved in distilled water, yields determined with a UV Mini 1240 spectrophotometer (Shimadzu, Duisburg, Germany), and stored at −80°C. *Reverse transcription* was carried out in batches of 5 μg total RNA per sample, with 1 μg of random hexanucleotide added to a total volume of 11 μl. The solution was heated to 70°C for 10 min, and RT performed in 30 μl of reaction volume containing 500 μM deoxynucleoside triphosphate (Ambion Europe, Huntingdon, UK), 20 U RNAseOUT recombinant ribonuclease inhibitor, 1 × first-strand buffer, 10 mM dithiothreitol, and 200 U Superscript II reverse transcriptase (Invitrogen) at 42°C for 2 h. The volume was heated to 95°C for 2 min and then immediately

Table 4 | Primer sequences for RT-PCR

Gene name (abbreviation)	Primer sequence	
	Forward	Reverse
Xanthine oxidase (XO)	CTCGCCATCTTTATTCAAAC	ACTTCATCTCAATGCCAATC
rac1 (rac1)	GAGACGGAGCTGTAGGTAAA	ATCTGTTTGCGGATAGGATA
rac2 (rac2)	CTTCCTCATCTGCTTCTCC	TCTTCTCCTTCAGTTTCTCG
p22phox (p22)	CTTTGGTGCCTACTCCATT	GGCCCGAACATAGTAATTC
Superoxide dismutase 1 (SOD1)	CAATGTGACTGCTGACAAAG	AATTACACCACAAGCCAAAC
Superoxide dismutase 2 (SOD2)	TAGCATTTTCTGGACAAACC	CTTATTGAAACCAAGCCAAC
Glutathione peroxidase (GPx)	ACTACACCCAGATGAACGAG	CGAAGAGCATGAAGTTGG
Interleukin-6 (IL-6)	CCTCTTCAGAACGAATTGAC	CTCAAACTCCAAAAGACCAG
Interleukin-8 (IL-8)	CTGCGCCAACACAGAAATTA	ATTGCATCTGGCAACCCTAC
Heme oxygenase 1 (HO-1)	TTGCTGTAGGGCTTTATGC	CTGCATTTGAGGCTGAGCC
18S (18S)	AGTTGGTGGAGCGATTTGTC	TATTGCTCAATCTCGGGTGG

RT-PCR, reverse transcription-polymerase chain reaction.

cooled on ice. The cDNA samples were stored at −20°C. *Polymerase chain reaction* conditions were optimized for each gene. Typically, PCR was performed in 50 μl volumes containing cDNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 200 μM deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, 1.25 U *Taq* DNA polymerase (Invitrogen), and 125 ng sense and antisense primers (Sigma-Genosys, Haverhill, UK) (Table 4). Negative controls contained a PCR mix without cDNA. PCR was run at 95°C (30 s), 55°C (30 s), and 72°C (30 s) over 30 or 35 cycles, using a PTC-200 DNA Engine (MJ Research, Watertown, MA, USA). PCR products were run on a 2% agarose gel containing ethidium bromide (17 μl/l agarose; MP Biomedicals, Irvine, CA, USA). Gels were photographed with Chemidoc XRS system (Bio-Rad) and images analyzed with Quantity One software (Bio-Rad). Negative controls showed no bands. Results are presented as 18S-corrected intensities relative to control values for each gene.

IL-6 enzyme-linked immunosorbent assay

A human IL-6 sandwich enzyme-linked immunosorbent assay kit (Biolegend Inc., San Diego, CA, USA) was used. Cells were grown to confluence in six-well plates, serum-starved for 24 h, exposed to 30 g/l BSA or OA-BSA for 2 h in the presence or absence of interventions as specified, and washed. Cells were then homogenized and the supernatants stored at −20°C. For the enzyme-linked immunosorbent assay procedure, 100 μl capture antibody in coating buffer was added to each well of a 96-well plate, and incubated overnight at 4°C. After washing and blocking, 100 μl of suitably diluted standards and samples were added to appropriate wells, incubated at room temperature for 2 h, and thoroughly washed. In subsequent steps, to each well were added 100 μl of diluted Biotinylated Detection Antibody in Assay Diluent (incubated at room temperature for 1 h, then washed), 100 μl of diluted Av-horseradish peroxidase in Assay Diluent (at room temperature for 30 min and washed), 100 μl of 3,3′,5,5′-tetramethyl benzidine substrate (in the dark for 15 min), and finally 100 μl of stop solution, followed by absorbance reading at 450 nm.

Statistics

Data are shown as mean (s.e.m.) unless otherwise indicated. Differences among groups were analyzed using one-way or two-way analysis of variance as appropriate; other statistical tests are indicated where used. Statistical significance was accepted at the level of $P<0.05$.

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