Oxidant stress in renal inflammation: mechanisms and remedies

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Iwadi ọna l'ati gb'ogun ti apapọju ẹya-atẹgun akanpa to nşe ẹdọ-kidinrin l'eşe
(pẹlu ẹda ni şoki l'edee Yoruba)

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(met een samenvatting in het Nederlands)

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Contents

Abbreviations

ABFA	albumin-bound fatty acid
Ang II	angiotensin II
AT-1	angiotensin II type 1 receptor
AT-2	angiotensin II type 2 receptor
BoC11	C11-Bodipy 581/591
BSA	bovine serum albumin
CCCP	carbonyl cyanide 3-chlorophenylhydrazone
DCF	dichlorofluorescein
FA	fatty acid
GFR	glomerular filtration rate
GSH	glutathione
GSH Px or GPx	glutathione peroxidase
GSSG	oxidised glutathione
H2DCF-DA	2`,7`-dichlorodihydrofluorescein diacetate
H_2O	water
H_2O_2	hydrogen peroxide
HO-1	heme oxygenase-1
IL	interleukin
LDH	lactate dehydrogenase
L-NAME	Nω-nitro-L-arginine methyl ester
MTT	3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide
NA	nicotinic acid (niacin)
NADPH	nicotinamide adenine dinucleotide phosphate
NO	nitric oxide
NOS	nitric oxide synthase
O_2	molecular oxygen
$^{\cdot}O_2^-$	superoxide anion
OA	oleic acid
OA-BSA	oleic acid-loaded bovine serum albumin
$^{\cdot}OH$	hydroxyl ion
ONOO-	peroxynitrite
PTEC	proximal tubular epithelial cell
ROS	reactive oxygen species
ROT	rotenone
SOD1	copper-zinc (cytosolic) superoxide dismutase
SOD2	manganese (mitochondrial) superoxide dismutase
Tempol	4-hydroxy-2,2,6,6-tetramethyl piperidine-1-oxyl
TTFA	2-thenoyltrifuoroacetone
UUO	unilateral ureteral obstruction
XO	xanthine oxidase

Chapter 1

General introduction:
Oxidant stress in renal inflammation

Reactive oxygen species in health and disease

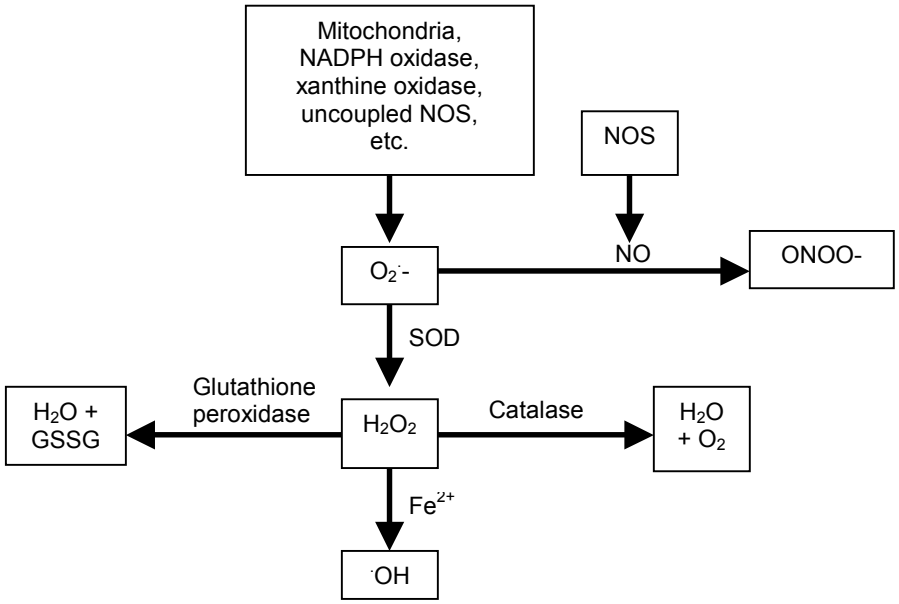
In the course of normal cellular metabolism, the generation of energy through aerobic processes involves the four-electron reduction of molecular oxygen (O_2) to water. Partially reduced oxygen metabolites may result as by-products of these and other metabolic reactions. These partially reduced metabolites include the superoxide anion ($O_2^{\cdot}$-), and hydrogen peroxide (H_2O_2), which are respectively the one- and two-electron metabolites of O_2. In the presence of transition metals, H_2O_2 can be further converted to the hydroxyl ion ($^{\cdot}OH$). These metabolites are extremely reactive, and for this reason they are referred to as reactive oxygen species (ROS). Contrary to initial belief that these molecules were merely toxic by-products that lack physiologic function, current opinion increasingly recognises that ROS have importance in normal cellular signaling and regulation [1-8]. ROS function as oxygen tension sensors via a mechanism involving HIF-1 [5, 6] that helps to regulate the control of ventilation and erythropoietin production. They mediate CGMP-dependent functions like vascular tone regulation [8], and are fundamentally important to normal immune function, as neutrophil phagocytic performance is heavily dependent on their ability to generate ROS "bursts". ROS mediate signal transduction from membrane receptors in various physiological processes [1, 3, 8], and they modulate other redox-sensitive transcription factors such as NF-kB, AP-1 [2, 8, 9], p53, Myb, [2], and STAT [3]. H2O2 is specifically involved in PDGF, insulin and TNF-α signaling via cysteine oxidation [6].

However, in situations where normal regulatory mechanisms fail to prevent a build-up of excess ROS, injurious effects are liable to follow. Under physiologic conditions, a complex network of reactions maintains the homeostatic balance between pro-oxidant and anti-oxidant forces in the cells and tissues (Figure 1). *Pro-oxidant* refers to factors that produce or enhance production of ROS, or that oppose their removal; while *antioxidant* factors are those that either suppress ROS production or support their removal. During normal cellular and organ function, the net physiologic effect of these opposing forces is that ROS production and removal are balanced, preventing a build-up of ROS to toxic levels. *Oxidant stress* is the term used to describe a state of unfavourable redox balance in which pro-oxidant factors gain the upper hand over anti-oxidant factors, leading to a net increase in ROS, with an attendant increase in risk of cellular and tissue damage.

On the *pro-oxidant* side, in addition to the aerobic metabolic processes described above, which takes place within the mitochondria, other major cellular sources of ROS

include the membrane-bound oxidoreductase enzyme NADPH (or NADH) oxidase, as well as other enzymes like hypoxanthine/xanthine oxidoreductase, cyclooxygenase, and lipoxygenase. Another enzyme, nitric oxide synthase (NOS), in the presence of the co-factor tetrahydrobiopterin produces the antioxidant molecule nitric oxide (NO), which normally serves as a physiological counterbalance to ROS. However, under conditions of inadequate substrate availability (tetrahydrobiopterin deficiency), NOS enters an "uncoupled" state, in which it becomes a net ROS producer. In the physiological state ROS production outside the mitochondrial oxidative metabolic system, such as through the activity of NAD(P)H oxidase, is closely regulated and directed at normal cellular functions. When such enzymes are abnormally over-stimulated in the presence of injurious stimuli, or when the cellular mechanisms for disposing of ROS are damaged, ROS becomes excessive and the state of oxidant stress ensues.

Figure 1. General cellular redox scheme



Among the most important *antioxidant* components of the normal physiologic redox network, the enzyme superoxide dismutase (SOD) converts superoxide to hydrogen peroxide, which is in turn disposed of by either or both of two other enzymes, catalase and glutathione peroxidase. Molecules such as glutathione and thioredoxin are major cellular reductants, while α-tocopherol is a lipid-phase antioxidant that is especially active against

hydroxyl radical activity. Nitric oxide (NO) represents another powerful antioxidant resource; in health, a relative balance between ROS and NO is an important element of normal redox homeostasis. However, there is a characteristic of NO that serves to emphasise the very complex, and still incompletely understood interrelationships within the cellular physiologic redox networks. NO, on reaction with superoxide, forms peroxynitrite ($ONOO^-$). Although this may be a way in which NO combats oxidant stress, helping to "mop up" excess superoxide and thus reduce the risk of eventual downstream conversion to the extremely reactive hydroxyl ion, it seems paradoxical that $ONOO^-$ is itself a powerful ROS with unclear physiologic roles and potential deleterious effects [4].

In the presence of excess ROS, virtually all cell components are liable to oxidant damage, but the most common cellular structural targets of oxidative damage are proteins, lipids and DNA. Among the resultant biochemical effects are protein inactivation, lipid peroxidation and DNA mutations [6], respectively manifesting functionally as impairment of enzyme function, disruption of cellular integrity, and an unstable genome [3]. Oxidatively damaged molecules are not merely inert, disused material, but they have pathophysiologic and practical importance of their own. On the one hand, there is evidence that oxidatively modified cellular components, such as oxidised lipoproteins [10, 11], actually possess reactive properties; as such they become participants in a vicious circle of progressive oxidant injury: damaged cellular molecules helping to amplify and propagate oxidation of yet more cell components. On the other hand, they may serve a useful experimental (and increasingly clinical) purpose as possibly quantifiable markers of oxidant-mediated injury, and in this way they have been exploited for the purpose of monitoring oxidant stress and investigating potential therapeutic measures.

Oxidant stress is considered one of the chief mechanisms behind the normal process of ageing, and extensive evidence in the medical literature indicates that a failure of normal redox balance is involved in a wide range of pathophysiologic conditions in virtually every organ system [8]. To cite a few examples, oxidant stress is involved in *in vitro* or *in vivo* models of hepatic damage, excess ROS being both a mediator and a consequence of upregulated pro-inflammatory cytokine expression [12, 13]. ROS appear to mediate apoptotic neuronal injury [14], and antioxidant treatment relieves ischaemic brain injury in experimental stroke [15]. Impaired ROS-dependent p53 stress sensitivity may be a factor in malignant cellular behaviour of leukaemia [16]. Other conditions linked with increased oxidant stress include cardiac ischaemia [17], systemic metal toxicity [18, 19] and metabolic

derangements [20-22]. Similarly, evidence increasingly points to the crucial role of oxidant stress-related mechanisms in many pathophysiologic processes in cardiovascular disease [23], connected with a range of highly ROS-sensitive signaling pathways and gene expression patterns [24-27]. For example, ROS are central to the pathophysiology of atherosclerosis. They mediate angiotensin (Ang) II-induced activation of MAP kinase pathways that lead to vascular smooth muscle cell hypertrophy and proliferation [1]. Oscillatory shear stress, a mechanical factor that predisposes to atherosclerosis, provokes endothelial cell ROS production [28]. ROS in turn mediate oxidation of LDL, local inflammation [29, 30], and vascular smooth muscle dysfunction [31]; in addition ROS contribute to various forms of vascular endothelial cell dysfunction by increasing vascular endothelial permeability and leukocyte adhesion [32]. Redox-sensitive activation of transcription factors within circulating leucocytes themselves, may further predispose to inflammation, adhesion and migration [33].

In other cardiovascular pathophysiologic settings, ROS are mediators of cardiomyocyte mitochondrial impairment and cell death during ischaemia reperfusion injury [17, 34]; and oxidant stress is currently considered to be both a cause and a consequence of arterial hypertension [34]. Although the exact role of ROS in the pathogenesis and maintenance of blood pressure elevation are not altogether clear, it is associated with hypertension of diverse pathogenetic mechanisms [18, 35-43]. As a consequence of hypertension, NADPH oxidase-dependent excess ROS may promote endothelial dysfunction via decreased NO availability, and stimulates signaling pathways that ultimately enhance media hypertrophy [44].

Importance of oxidant stress in renal disease

The role of oxidant stress in renal injury is increasingly recognised [45-47]. Major ROS-producing enzymes including NADPH [48-50] and NADH [48, 51] oxidases, and xanthine oxidase [48], are expressed in the kidney. The antioxidant defensive elements like superoxide dismutase, glutathione peroxidase, and catalase [52] are also present. The expression and activities of these molecules under physiologic conditions, as well as their regulatory and functional responses in both absolute and relative terms when challenged by local or systemic noxious stimuli, are issues of current and major biomedical research interest. As with other organs, within the kidneys important physiologic functions are ascribed to oxygen radicals, such as regulating NO-dependent actions on the renal

microvasculature and helping to maintain normal vasomotor tone [53]. However, under pathologic conditions, when regulatory mechanisms fail to keep ROS levels within physiologic limits, these radicals can mediate myriad renal injurious effects [53, 54]. Evidence of increased oxidant stress and/or oxidant-dependent pathophysiologic mechanisms has been observed in numerous experimental renal injury models. Some examples are briefly described below.

Chronic renal insufficiency models. Oxidant stress involvement in the pathophysiology of chronic renal disease and the uremic syndrome is now widely acknowledged [10, 34, 39]. Specific redox derangements identified in animal models of chronic renal failure include upregulation of NADPH oxidase, downregulation of superoxide dismutase, and decreased NO availability [55], as well as increased susceptibility during catalase deficiency [56]. Irrespective of the underlying renal pathology, during uraemia ROS mediate such effects as increased cytokine expression, increased complement activation, and neutrophil priming, which all work together to promote vascular inflammation, endothelial injury, platelet activation, and vascular smooth muscle proliferation and migration [57, 58].

Diabetes. Experimental diabetic nephropathy is a model of renal injury in which oxidant stress involvement is relatively well elucidated. Increased ROS levels are stimulated directly or indirectly by high glucose levels [59-63], an effect that may be aggravated by other factors, such as deficiency of trace elements like selenium [64]. These oxygen radicals mediate pathophysiologic events via activation of signal transduction cascades and transcription factors that favour extracellular matrix remodelling [65], glomerular mesangial expansion, inflammation and tubulointerstitial fibrosis [66]. Although several ROS-producing enzymes, including NADPH oxidase and uncoupled NOS [63], have been suggested to be the sources of increased renal ROS in the diabetic state, Brownlee's hypothesis [20, 67], which is currently gaining much attention, proposes that overproduction of superoxide by the mitochondrial electron transport chain is the central biochemical event linking together all the underlying mechanisms of hyperglycaemia-induced damage in kidneys and other organs. Oxygen radicals are postulated to stimulate a series of events, including polyol pathway activation and intracellular accumulation of advanced glycation end-products. These events in turn trigger a subsequent wave of damage-effector cascades that finally result in pro-inflammatory, vasomotor, angiogenetic and other deleterious consequences.

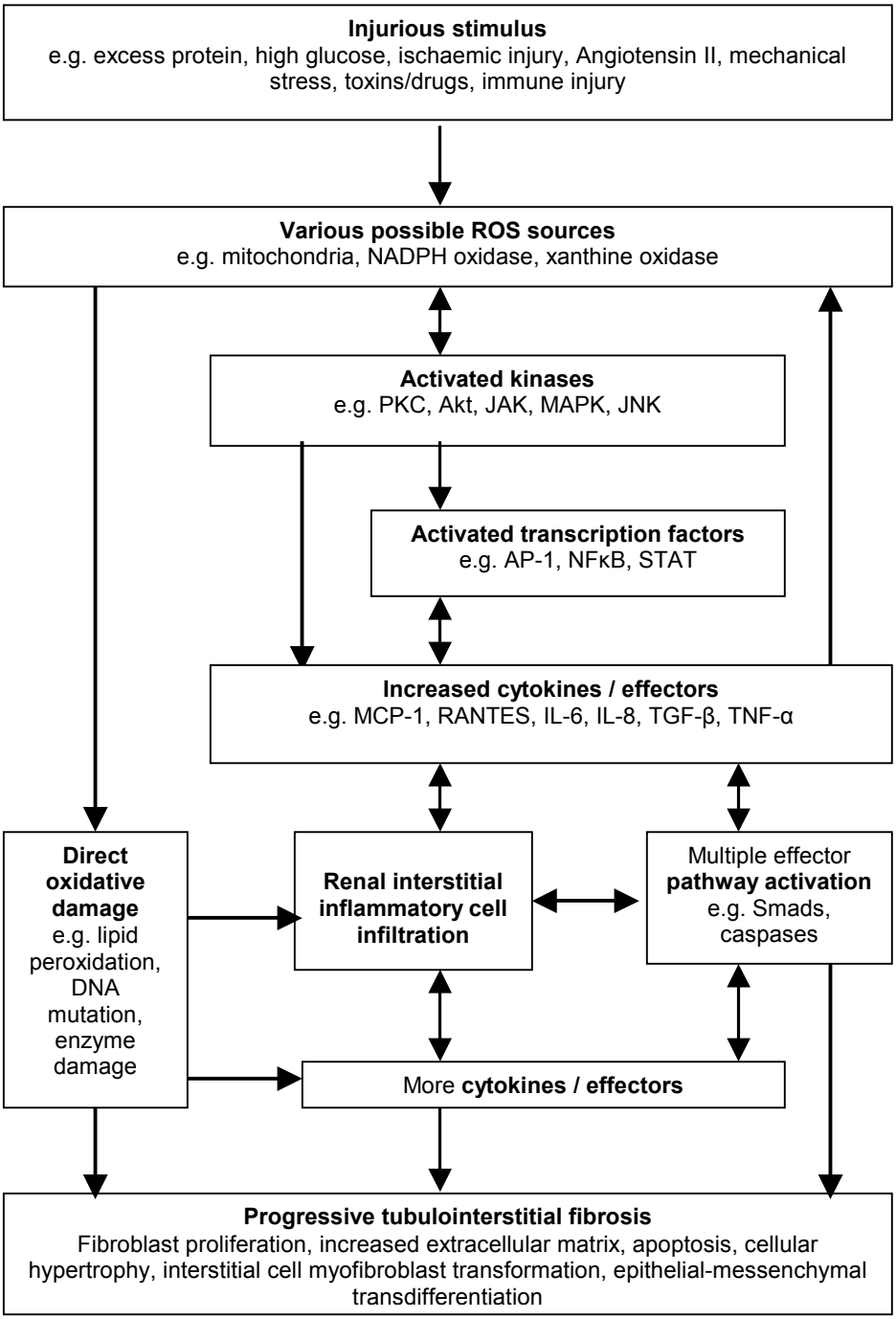
Increased Angiotensin II. There is a close interaction between oxidant stress and nephropathic effects of Ang II [68, 69]. Ang II-mediated, ROS-dependent effects include renal tubular cellular hypertrophy [70, 71] and dose-dependent hypertension, increased renal vascular resistance, and diminished glomerular filtration rate [72]. In conjunction with Ang II [73], oxidant stress plays an important role in the genetically determined hypertension and renal and other tissue injury that are characteristic of the spontaneously hypertensive rat (SHR) [74, 75]; indeed, that perinatal antioxidant supplementation significantly reduces adult blood pressure in SHR [76] suggests that a complex oxidant stress state is at the core of pathological gene-environment interactions that may "programme" increased blood pressure in these animals [77].

Other examples. Apart from the major examples described above, other renal injury models associated with evidence of oxidant stress involvement include anti-Thy 1.1 glomerulonephritis [78], aldosterone-induced injury [79], renal ischaemia/reperfusion injury [80, 81], and various toxic nephropathies [82-84]. Others include renovascular disease [85], myoglobin-induced injury [86], trace element deficiency [64], and iron overload [87]. In further examples, oxidant stress, manifested in the form of diminished tissue NO synthesising capacity, is associated with nephropathic conditions like puromycin nephrosis [88], anti-glomerular basement nephritis [89], cholesterol-induced injury [90], and chronic streptozotocin-induced diabetes [91]. In rats that were chronically fed with antioxidant-deficient diets, nephropathic effects observed included proteinuria, depressed glomerular filtration rate [92], interstitial expansion, and pro-fibrotic gene expression patterns [47]. Moreover, experimentally induced NO deficiency resulted in proteinuria, hypertension and renal injury [93-95]. Finally, increased oxidant stress may mediate some of the natural changes that ultimately predispose to progressive decline of renal performance in the ageing kidney [96]. Oxidant stress involvement in the protein overload model, and in obstructive nephropathy, are described in detail in later chapters of this thesis.

Oxidant stress drives renal inflammation

A principal mechanism through which oxidant stress promotes renal injury is by playing a major role in tubulointerstitial inflammation (Figure 2). Tubulointerstitial inflammatory cell influx is a key feature in the initiation and perpetuation of many forms of renal injury [97, 98], and end-stage renal disease is characterised by a persistent state of chronic renal and systemic inflammation [99]. The initial drivers of renal interstitial inflamma-

Figure 2. Integrated schema illustrating the crucial roles of oxidant stress and interstitial inflammatory cell influx in progressive tubulointerstitial injury



tory infiltration are the increased expression of chemokine receptors in resident kidney cells, and the stimulation of these resident cells to actually produce chemotactic factors; these changes occur as early responses to the injury as part of the initial disease onset process, and macrophages represent a particularly important population of inflammatory cells involved [98]. Proximal tubular cells in particular, under pathophysiologic conditions, can be induced to express powerful pro-inflammatory activity including the production of a series of cytokines [100-102]. There is evidence that these processes are ROS-dependent, particularly in the setting of proteinuric disease [103-105]. Additionally, ROS are important in signal transduction for several transcription factors, including nuclear factor kappa B (NF-κB) [53, 54], which in turn stimulates transcription of chemotactic cytokines like MCP-1 and RANTES.

Following their initial recruitment, infiltrating inflammatory cells take up active roles in establishing and propagating the pathophysiologic process [102]. Through direct and indirect actions, they further aggravate the inflammatory process by provoking more oxidant stress and themselves producing more cytokines, including TGF-β and PDGF [105], leading to a self-sustaining vicious cycle of mutually amplifying inflammation and oxidant stress. These inflammatory cell-mediated events continually activate multiple injury-effector pathways and promote pro-inflammatory gene expression, all acting together to enhance progressive injury [34, 102, 106-110]. For example, ROS-mediated activation of the transcription factor AP-1 may instigate TGF-β signalling, which aggravates inflammation, promotes extracellular matrix (ECM) growth and tubular epithelial messenchymal transition (EMT), and thus contributes to tubulointerstitial fibrosis. TGF-β1 has also been shown to stimulate PAI-1 in mesangial cells, which decreases plasmin and inhibits ECM degradation [66]. Furthermore, via adverse effects on cell cycle regulation, ROS may contribute to tubular cell hypertrophy [10].

Monitoring renal inflammation and oxidant stress

In general, inflammation monitoring is accomplished either through direct *in vivo* assessment of tissue inflammatory cell infiltration, or (both *in vivo* and *in vitro*) by estimation of the *potential for inflammation* e.g. as measured by cellular or tissue expression levels of pro-inflammatory cytokines, including transforming growth factor beta (TGF-β) or interleukin (IL)-6. The expression of anti-inflammatory molecules, such as heme oxygenase-1 (HO-1), or of transcription factors that are known to support pro-inflammatory pathways, such as NF-

κB, also provide valuable indications of inflammatory status. In the present studies, we employed both the direct and indirect approaches. We variously assessed the expression of IL-6, IL-8, or HO-1 as appropriate. In *in vivo* protein overload studies especially, we made use of tubulointerstitial macrophage count as the marker of renal inflammation. Tubulointerstitial macrophage infiltration plays a critical role in the initiation and maintenance of progressive renal injury [98]. Macrophage detection was based on immunohistochemical staining for the specific mouse macrophage antigen F4/80. F4/80 is the preferred antigen for detecting mouse macrophages because of its superior specificity; unlike antibodies against other macrophage markers like Mac-1, Mac-2, Mac-3 and CD68, antibodies against F4/80 clearly distinguished macrophages from $FSP1^+$ fibroblasts [111]. F4/80 immunohistochemistry is a tested method that has been previously employed by other workers [112, 113].

Monitoring of oxidative stress can be pursued using several different strategies, both *in vivo* [23, 114-116] and *in vitro* [117-120] (Figure 3). The most direct strategy is to attempt actual measurements of radical species such as superoxide and hydrogen peroxide [121]. However, direct monitoring of oxidative stress, particularly *in vivo*, has proved an enduring challenge for biomedical researchers. In both cells and intact tissue, and under various experimental conditions, a wide range of techniques and approaches has been employed but there is as yet no single “gold standard” of measurement. Among methods that are used are fluorescence-based assays [122-130] chemiluminescence [119, 124, 125, 131], and electron spin resonance [119, 132, 133]

A less direct oxidant stress monitoring strategy is to assess the pathobiologic impact of oxidant stress by detecting and quantifying tissue or cellular structural markers of oxidant injury, such as oxidised proteins, lipids, or damaged DNA. Markers for protein damage include protein carbonyls and advanced oxidation protein products; for lipids, malondialdehyde, various other aldehydes, oxidised low-density lipoprotein (LDL), and F2 isoprostanes; and for DNA, 8-hydroxy-2’-deoxyguanosine. These markers serve as biologic “footprints” of oxidant injury, and by appropriate selection they could be used as stress measures at all levels of experimentation, ranging from the most basic cellular set-up to assays of blood or urine that may offer insights into whole-body oxidant stress status. We have previously utilised this approach in our laboratory; we developed a ratio-fluorescence assay for detecting oxidised lipids in *in vitro* and *ex vivo* settings, using the redox-responsive fluorescence-shift properties of the lipophylic probe C11-Bodipy 581/591 [93, 134-138].

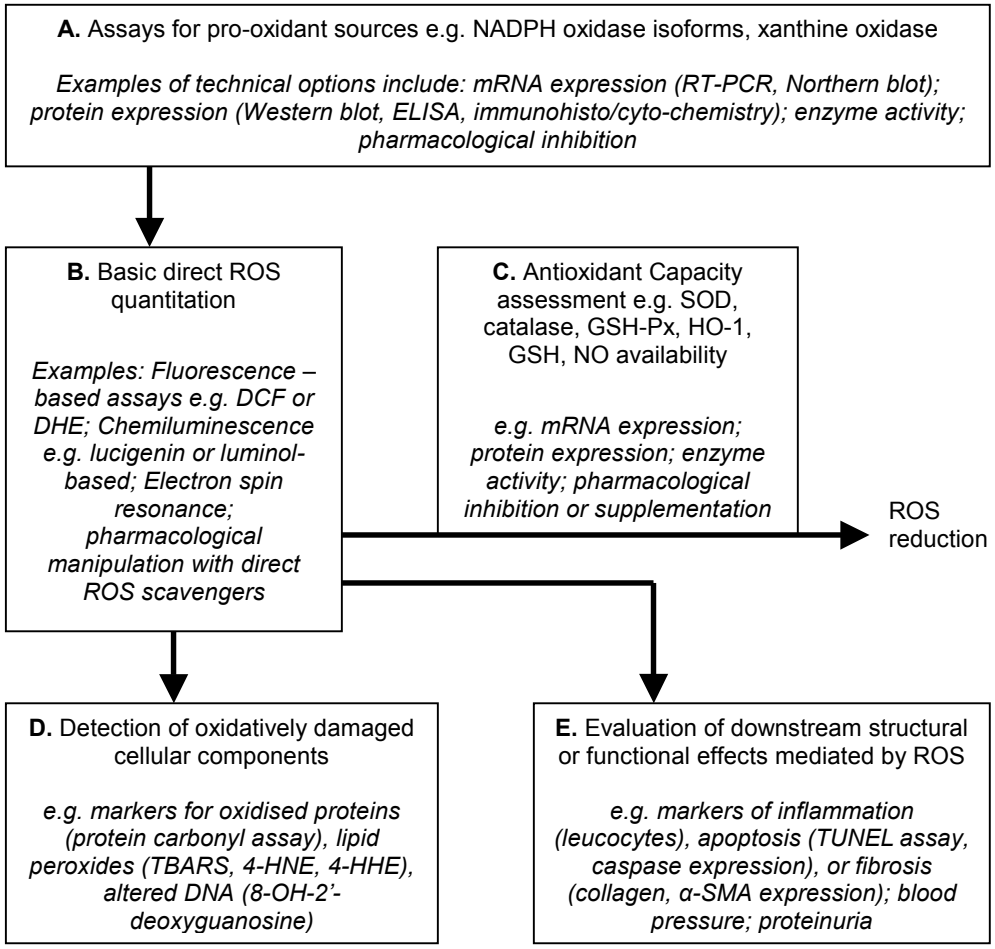
Among other ways of monitoring oxidant stress, molecular techniques could be employed to measure the gene or protein expression of redox-relevant molecules, such as ROS-producing enzymes or ROS-scavenging enzymes. These represent indirect means of assessing cellular, tissue or even whole-organ capacity or potential for generating or combating oxidant stress. Yet another option is to use functional assays; for example, monitoring the activities of enzymes that are either pro-oxidant or anti-oxidant. Lastly, pharmacological manipulations could be adopted at a whole-organ or whole-animal level, using *ex vivo* or *in vivo* methods. In this case, the study design should be based on previously known evidence to assess whether a particular pharmacological intervention would show specific predicted effects. As an example, if previous studies have suggested that a particular pro-oxidant enzyme mediates a certain ROS-dependent effect, such as tissue inflammation, then specifically targeted pharmacological blockade of the enzyme should be expected to suppress that particular effect.

Various experimental techniques are deployable to fit any chosen oxidant stress monitoring strategy. For example, fluorescence-based techniques are as applicable to direct ROS quantification, as they are to indirect measurements of oxidant stress such as assays of altered lipid, proteins or DNA. Taking this particular example further, considerable flexibility at the laboratory bench is afforded by the availability of different methods for fluorescence quantification, such as microplate fluorimetry, flow cytometry, or microscopy with quantitative image analysis. Whatever oxidant stress monitoring strategy the investigator elects to adopt, and whichever laboratory technique is employed to carry out such a strategy, all approaches have their advantages and disadvantages. The challenge for researchers is to select the strategy and technique(s) that are most appropriate for their particular experimental questions and conditions. The ideal situation might be to use a combination of methods to enhance the robustness of information derivable from experiments.

Towards combating renal oxidant stress

Despite the abundance of reports of increased oxidant stress in kidney diseases, in many instances the mechanisms of oxidant stress are still not yet well understood. The most important questions that remain to be answered regarding the role of oxidant stress in renal pathophysiology relate to the underlying mechanisms of generation of ROS, and the understanding of intrinsic intrarenal responses to such stress. Because of the highly complex nature of biological redox networks, it is crucial that efforts to ameliorate oxidant

Figure 3. Five strategic approaches for oxidant stress monitoring. Various tactical and technical options are possible with any chosen strategy



A - Assessment of ROS sources, e.g. upregulated xanthine oxidase expression may indicate increased oxidant stress due to that enzyme. **B** – Direct ROS measurement. **C** – Assay of antioxidant factors: may indicate the intrinsic tissue antioxidant capacity to respond to increased oxidant stress, e.g. could reveal if a particular pathophysiologic setting is characterised by depressed antioxidant defence or show tissue attempt to counter oxidant stress. Results should be interpreted in the context of the overall data available. **D** – quantifying the "footprints" of oxidant damage. **E** – this facilitates a pharmacological approach to test the functional significance of increased oxidant stress; and to assess potential treatments. If a specific pathophysiologic effect is at least partly ROS-mediated, then an appropriate antioxidant drug should relieve that effect. For example, in an NADPH oxidase-dependent inflammatory or hypertensive condition, pharmacological inhibition of the enzyme should achieve a measurable effect, such as reduction in leukocyte infiltration or blood pressure.

stress should be guided by an understanding of the specific redox disturbances within each particular pathophysiologic setting. In many kidney injury models, such understanding is currently quite limited. For example, in each situation it is useful to determine whether the net increase in oxidant stress is a consequence of enhanced pro-oxidant activity and/or reduced antioxidant capacity.

A range of antioxidant remedies has been tried in various attempts to ameliorate renal injury. Dietary antioxidant supplements are a group that has been tested repeatedly, with variable results. Vitamins C and E both significantly ameliorated diabetes-induced increases in glomerular volume and inflammatory cytokine (TGF-β) expression [139]. On the other hand, neither vitamin E nor probucol, when individually administered, demonstrated any benefit in rats with puromycin aminonucleoside nephrosis [140]; although when given as a combined therapy, the two agents together prevented renal lipid peroxidation and renal fibrosis in uninephrectomised hypercholesterolaemic rats [141]. Among other notable findings, Hahn et al. in a study of the 5/6 subtotal nephrectomy model of glomerulosclerosis, showed that α-tocopherol not only prevented renal injury but indeed reversed established disease in remnant kidneys [142]. The SOD mimetic agents tempol [143] and EUK-134 [144] both reduced renal dysfunction and injury in rats with ischaemia/reperfusion injury, and the hydroxyl radical scavenger MCI-186 (edarabone) demonstrated protective effects in both *in vitro* and *in vivo* models of cisplatin-induced renal injury [145].

However, some of the enthusiasm generated by such promising experimental animal studies has not been sustained by rather mixed clinical outcomes in human patients. For example, in a clinical randomized controlled (but not blinded) trial in haemodialysis patients, the thiol-containing free radical scavenger acetylcysteine showed benefit in reducing cardiovascular events, but did not improve overall mortality [146]. To further complicate the picture, it appears that a therapeutic approach that is beneficial in one situation may indeed be harmful in another, as demonstrated by supplementation of the nitric oxide precursor L-arginine that improved outcomes in ischaemic acute renal failure [147] but aggravated renal injury in an immune glomerulonephritis model [148]. These variable and sometimes conflicting outcomes of antioxidant therapies in renal disease may reflect the complexities of redox physiology. The nature of redox imbalance varies between models, and each situation needs to be understood and specifically appropriate strategies composed. Evidently, much more work is required to resolve these issues. The subject is further discussed later in the present thesis.

Overall thesis approach

The overall hypothesis of this thesis is that *oxidant stress is a crucial element in renal tubulointerstitial inflammation, and relief of oxidant stress may effectively reduce inflammation.* In the studies reported in this thesis, we utilised a variety of indices of oxidant stress. In our *in vitro* work, we adopted a fluorescence-based assay in conjunction with molecular indices (redox-related gene expression analysis), and we placed emphasis on functional confirmation of benefits suggested by observations with the fluorescence-based assays. In our mouse studies we also adopted multiple approaches, including an *ex vivo* fluorescence measurement, a molecular approach using RT-PCR, and most importantly, pharmacological strategies that were based either on our own preliminary data, or on previously documented reports in the literature. For example, based on our finding that protein overload induces mitochondrial oxidant stress and inhibits mitochondrial SOD protection in cultured tubular cells (chapter 2), in mouse experiments we tested whether SOD supplementation or mitochondrial inhibition would ameliorate renal inflammation after protein overload (chapter 4).

Experimental questions addressed in this thesis

Oxidant stress mechanisms in cultured tubular cells (chapter 2)

- Does excess protein exposure provoke oxidant stress in proximal tubular cells? What is the underlying mechanism of such stress: increased pro-oxidant activity, depressed antioxidant capacity, or both? Is such oxidant stress provoked mainly by albumin *per se*, or by albumin-bound fatty acids (ABFA)?
- Are protein-induced tubular cell inflammatory responses dependent on increased oxidant stress? Would antioxidant intervention alleviate such inflammation?
- Protein overload has been previously associated with inflammatory responses in renal tubular cells. Are such responses due mainly to albumin effects or to the effects of ABFA?

Developing a suitable *in vivo* model of renal inflammation due to protein overload (Chapter 3)

- Proteinuria during protein overload in C57BL mice is unimpressive, severely limiting the value of the model in studying the effects of excess tubular protein exposure. Could a different mouse strain be employed as a suitable model of protein overload-induced

proteinuria and renal inflammation, to facilitate *in vivo* study of the effects of excess tubular protein exposure?

Exogenous antioxidants offer protection *in vivo* (chapter 4)

- Does increased oxidant stress contribute to renal inflammation during protein overload in mice? Based on our *in vitro* findings (Chapter 2), could antioxidant therapy (via SOD augmentation or mitochondrial inhibition) reduce such inflammation?
- In renal inflammation induced by protein overload, how important is the contribution of albumin-bound fatty acids (ABFA)? Would systemic reduction of non-esterified fatty acids have a beneficial effect?

Intrinsic renal antioxidant resistance to Angiotensin II-induced injury (chapter 5)

- C57BL/6 mice are known to be resistant to several models of renal injury. In the case of Angiotensin II infusion, is this resistance a function of reduced susceptibility to oxidant stress, and by what mechanism?

UUO model: Apocynin pilot study (chapter 6)

- NADPH oxidase has been previously proposed as a source of oxidant stress in renal injury. In renal inflammatory states, can specific pharmacological inhibition of NADPH oxidase provide functional benefit by alleviating renal inflammatory cell influx?

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Chapter 2

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

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Abstract

Albumin induces oxidative stress and cytokine production in proximal tubular cells (PTEC). Albumin-bound fatty acids (FA) enhance tubulopathic effects of albumin *in vivo*. We proposed that FA aggravation of albumin-induced oxidative stress in PTEC might be involved. We hypothesised that mitochondria could be a source of such stress. Using a fluorescent probe, we compared reactive oxygen species (ROS) production after exposure of PTEC 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, were 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 PTEC, FA accentuate albumin-induced oxidative stress and inflammatory cytokine expression via increased mitochondrial ROS, while frustrating protective antioxidant responses.

Introduction

Proximal tubular epithelial cells (PTEC) treated with excess albumin produce numerous pro-oxidant and pro-inflammatory 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 localised mainly to tubular epithelial cells, and the distribution of infiltrating interstitial leucocytes 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 PTEC are not merely structural elements but are active players in pathophysiologic processes. Oxidative stress plays a key role in these events. In PTEC 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 (MCP-1) [9].

Some effects of albumin on PTEC are not owing to the molecule itself, but rather to fatty acids (FA) bound to it. Excess PTEC fibronectin production was induced by oleic acid-complexed albumin but not by pure albumin [10]. FA presented to PTEC 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 FA 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 three-fold 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 PTEC, 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 PTEC to explore two questions relating to the idea that albumin-bound FA exaggerate oxidant effects of albumin on PTEC. First, we postulated that FA 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 fatty acids stimulate ROS production from mitochondria [20]. In vascular endothelial cells exposed to lipids, there is important mitochondrial contribution to ROS [21]. Thus, we hypothesised that mitochondria may also be a major source in PTEC upon treatment with albumin-bound FA. 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 pro-inflammatory transcriptional and translational activity. The present study reveals a failure in the mitochondrial anti-oxidant response as a mechanism by which FA bound to albumin can induce oxidative and inflammatory stresses in proximal tubular cells.

Methods

Cell culture and model characterisation. We utilised HK-2 cells, immortalised epithelial cells derived from human proximal tubules (obtained from the Department of Pathology, UMC Utrecht). Cells were cultured at 37^{o}C and 5% CO_2, using Gibco RPMI 1640 medium (with 25mM HEPES 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 characterised for proximal tubular epithelial phenotype [22, 23].

Albumin uptake. To establish that HK-2 cells are suitable for albumin overload studies with or without added FA, we quantified BSA uptake. Cells were grown to confluence in 6-well plates, serum-starved for 24 h, exposed to 3-30g/l BSA or OA-BSA for 2 h, and washed. Cells were homogenised, 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 visualise intracellular BSA, cells were grown to confluence on 8-well Lab-Tek chamber slides, (Nalge Nunc, Naperville, IL) 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 oleic acid (OA) as our model FA, being the most abundant FA on urinary albumin [11]; moreover, albumin-bound OA increased ROS in vascular endothelial cells [24] and induced a more pronounced fibrogenic response in PTEC than other FA [10]. To prepare albumin solutions, BSA with very low endotoxin content (<1 ng/mg**)** (Sigma A-9430) was dissolved in phosphate-buffered saline (PBS). For OA-BSA solutions, OA (Sigma O-1008) was added to fresh BSA solutions, mixtures incubated for 2.5 h at 37ºC with gentle shaking, filtered (0.22 μm), aliquoted and stored at -20 ºC. Fatty acid spectra in the solutions were determined by gas chromatography as described by Muskiet *et al*. [25]. Briefly, 100μl of BSA or OA-BSA solution mixed with 1mg butylated hydroxytoluene (BHT, 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 FA like palmitic, linoleic, oleic, stearic and vaccenic acids, while 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 [26].

In the first series of experiments, cells were treated with BSA (3-30g/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 optimal concentration of OA-BSA (at 30g/l) for 2 h with or without co-treatment with various agents chosen to answer specific study questions. Agents employed were apocynin (APO) (1mM), allopurinol (ALLO, 100μM), carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (10μM), 2-thenoyltrifuoroacetone (10μM), rotenone (ROT) (10μM), N-ω-nitro-L-arginine methyl ester (L-NAME) (2mM) (ICN Biomedicals, Aurora, OH), 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 PBS supplemented with 0.9 mM $CaCl_2$, 0.45 mM $MgCl_2$, and 5 mM glucose (PBS+++). 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 pre-incubation 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 dichlorofluorescein (DCF). We detected and quantified DCF fluorescence by microplate fluorospectrometry [27] using a FLUOstar Optima reader (BMG Labtechnologies, Durham, NC, USA) at excitation and emission wavelengths 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 37ºC with 20μM H_2DCFDA in the dark for 30 min, and washed to remove excess probe. Experimental treatments were then commenced (37ºC) 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 (10-500μM) for 30 minutes 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 normalised to corresponding vehicle control (±SEM).

Cell viability. We used lactate dehydrogenase (LDH) release and 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) tests, by spectrophotometry with Ultramark microplate system (Bio-Rad Laboratories, Hercules, CA). (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 PBS. Then for both measurements, 50 μl of a pre-heated assay mix (37°C) containing 9mM pyruvate (Merck, Darmstadt, Germany) and 1.41 mM NADH (ICN, Amsterdam, 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 (0.04N HCl) isopropanol for 15 min with gentle shaking. Absorbance was read at 570nm.

Gene expression studies. We assessed gene expression by semi-quantitative 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 solubilised 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^{o}C. *Reverse transcription* was carried out in batches of 5 μg total RNA per sample, with 1 μg of random hexanucleotide added to total volume of 11 μl. The solution was heated to 70^{o}C for 10 min, and RT performed in 30 μl of reaction volume containing 500 μM dNTP (Ambion Europe, Huntingdon, UK), 20 U RNAseOUT recombinant ribonuclease inhibitor, 1 x first-strand buffer, 10mM DTT, and 200U Superscript II reverse transcriptase (Invitrogen) at 42^{o}C for 2h. The volume was heated to 95^{o}C for 2 min and then immediately cooled on ice. The cDNA samples were stored at –20^{o}C. *Polymerase chain reaction* conditions were optimised for each gene. Typically, PCR was performed in 50μl volumes containing cDNA, 10mM Tris.HCl (pH 8.3), 50 mM KCl, 200 μM dNTP, 1.5 mM $MgCl_2$, 1.25 U *Taq* DNA Ploymerase (Invitrogen), and 125 ng sense and antisense primers (Sigma-Genosys, Haverhill, UK) (Table 1). Negative controls contained a PCR mix without cDNA. PCR was run at 95^{o}C (30s), 55^{o}C (30s), and 72^{o}C (30s) 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). Gels were photographed with Chemidoc XRS system (Bio-Rad) and images analysed 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.

Interleukin-6 ELISA. A human IL-6 sandwich ELISA 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 30g/l BSA or OA-BSA for 2 h in the presence or absence of interventions as specified, and washed. Cells were then homogenised and the supernatants stored at -20°C. For the ELISA procedure, 100 μL capture antibody in coating buffer was added to each well of a 96-well plate, and incubated overnight at 4^{o}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 diluted Biotinylated Detection Antibody in Assay Diluent (incubated at room temperature for 1 h, then washed), 100 μL diluted Av-HRP in Assay Diluent (at room temperature for 30 min and washed), 100 μL TMB substrate (in the dark for 15 min), and finally 100 μL stop solution, followed by absorbance reading at 450 nm.

Table 1. 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

Statistics. Data are shown as mean (s.e.m.) unless otherwise indicated. Differences among groups were analysed 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 $p<0.05$.

Results

FA binding has no effect on albumin uptake by HK-2 cells. Electro-immunodiffusion assays (Table 2) 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 visualised by immuno-histochemistry (not shown). The results are comparable with previous findings in similar cells [28-30].

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), while BSA induced moderate (up to 1.5-fold) ROS increase that attained significance only at the highest concentration (Fig 1a). Cell viability was not impaired by 2-hour BSA or OA-BSA exposure (MTT and LDH release assays, Fig 1b).

Table 2. Albumin uptake after exposure to albumin with or without bound oleic acid (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.

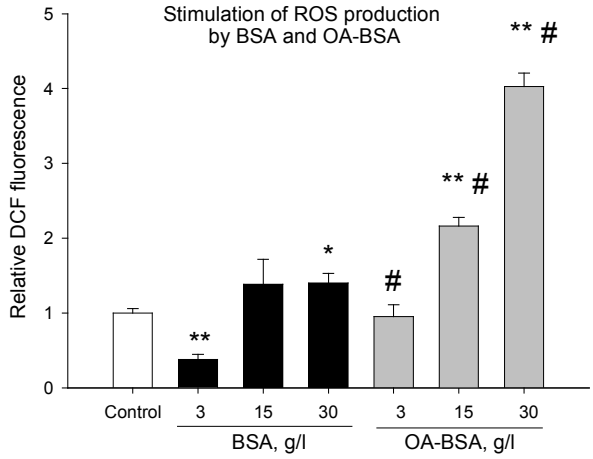
The property of albumin as an excellent carrier for many hydrophobic substances (besides fatty acids) raises the concern that it could acquire unknown contaminants in the course of commercial preparation and purification processes. To confirm that the effects we observed were not influenced by such contamination, we repeated some experiments using

Table 3. ROS measurement after treatment of human kidney-2 (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.

(A)



(B)

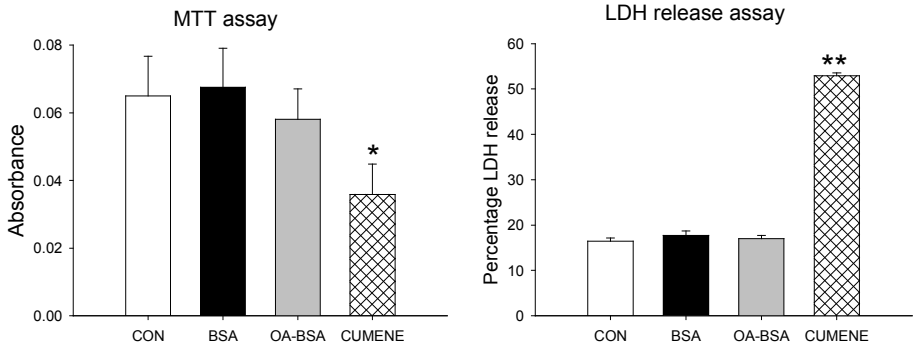


Figure 1. Effects of BSA and OA-BSA on cellular ROS production and viability. (A) ROS production after exposure to albumin (BSA) alone or enriched with oleic acid (OA-BSA) in proximal tubular epithelial cells (PTEC), measured by DCF fluorescence normalised to control values (a.u.)**.** *p<0.05, **p<0.001 vs. control (untreated) cells; #p<0.001, OA-BSA vs. corresponding BSA concentration. Mean (s.e.m.) of at least three independent experiments. **(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.

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a different formulation of low-endotoxin (0-2.0 EU/mg), low-fatty acid (0-0.2 mg/g) BSA (SERVA Electrophoresis GmbH, Heidelberg, Germany). As shown in Table 3, the pattern of ROS production induced by the SERVA product was similar to that of the Sigma product described in the above paragraph.

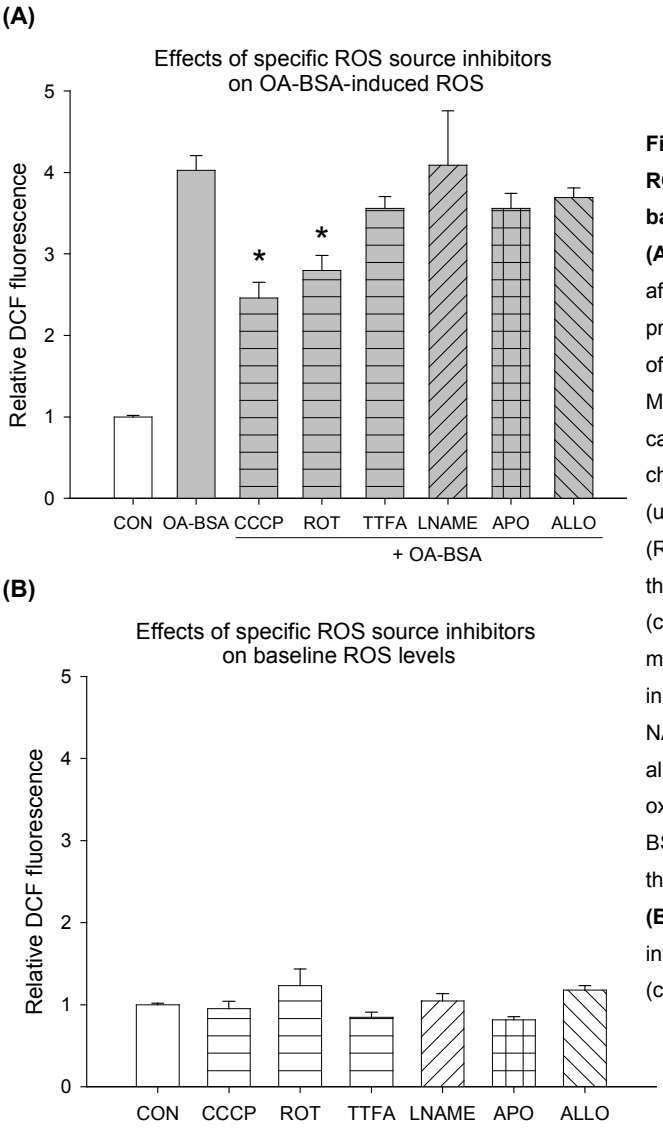

Figure 2. Effects of inhibitors of ROS sources on stimulated and basal cellular ROS production. (A) ROS production by HK-2 cells after OA-BSA treatment in the presence or absence of blockers of potential ROS sources. Mitochondrial inhibitors used were carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (uncoupling agent), rotenone (ROT) (complex I), and 2-thenoyltrifuoroacetone (TTFA) (complex II). Nω-nitro-L-arginine methyl ester (L-NAME) is a NOS inhibitor, apocynin (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. **(B)** Effects of the above-named intervention agents on baseline (control) cellular ROS levels.

OA-BSA-induced ROS is alleviated by mitochondrial inhibitors. In further experiments cells were exposed to OA-BSA (30g/l) for 2 hours. In intervention studies to probe possible sources of ROS (Fig. 2), OA-BSA-induced increase was significantly attenuated by the mitochondrial respiratory chain blockers rotenone (ROT), (complex I inhibitor) and CCCP (respiratory uncoupling agent), but not by TTFA (complex II inhibitor). The specific NADPH oxidase inhibitor apocynin (APO) had no significant effect. L-NAME

and allopurinol also had no effect, excluding uncoupled nitric oxide synthase (NOS) or xanthine oxidase as ROS sources.

	Gene	*BSA*	*OA-BSA*
(A)	**rac1**	1.29 (0.4-2.2)	1.73 (0.5-2.5)
	rac2	1.47 (0.5-2.1)	1.27 (0.4-2.2)
	p22	0.71 (0.5-2.0)	0.82 (0.7-4.2)
	XO	1.04 (1.0-1.5)	1.14 (0.04-1.8)
	18S		
(B)	**SOD1**	1.19 (0.6-2.1)	0.91 (0.5-1.3)
	SOD2	1.44* (1.2-3.1)	0.86 (0.7-3.2)
	GPx	0.99 (0.8-1.3)	1.08 (0.9-1.2)
	18S		
(C)	**IL-6**	2.90* (0.7-3.3)	2.39* (1.2-3.3)
	IL-8	2.31* (1.3-2.7)	2.28* (1.5-5.3)
	HO-1	0.53 (0.3-1.4)	0.48 (0.1-1.4)
	18S		

Figure 3. Illustrative bands from RT-PCR experiments, normalised 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 is 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.

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 RT-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 semi-quantitative densitometry of gene expression are shown (Fig. 3a).

Mitochondrial antioxidant SOD2 gene induction, seen with BSA, fails with OA-BSA. We assessed the transcriptional response of major cellular antioxidant enzymes (Fig. 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, were not significantly regulated by either treatment.

ROS increase is ameliorated by EUK-8, a synthetic SOD analogue. To explore the functional implication of the antioxidant gene expression pattern noted above, we tested whether 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 (Fig 4).

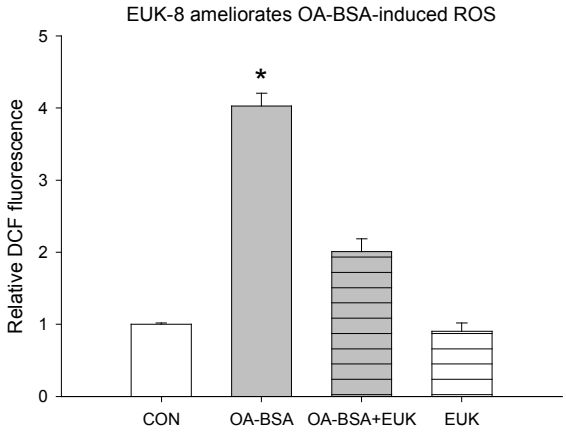

Figure 4. ROS production by HK-2 cells after OA-BSA treatment in the presence or absence of EUK-8, a manganese SOD mimetic. *p<0.001 vs. all other groups.

Downregulation of heme oxygenase-1 is enhanced by OA-BSA. For an insight into whether bound FA 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) (Fig 3C), a molecule with important renal anti-oxidant and anti-inflammatory effects [31, 32].

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 4), despite their similar effect at the level of gene expression. We then 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 4. 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)

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 FA augment the oxidative capacity of albumin on PTEC 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 NOS 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, it also adversely affected gene expression of the key mitochondrial antioxidant system, the manganese-containing SOD2. While 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 FA promotes pro-inflammatory 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 [33, 34]. We probed possible ROS contribution from complexes I and II, and from the respiratory coupling step. Only the complex II inhibitor TTFA 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, apocynin, a specific inhibitor of NADPH oxidase complex assembly [35], 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 [36]. Interestingly, we found that PTEC robustly express both of the low molecular weight G protein NADPH oxidase complex components rac1 and rac2. Both molecules tended to upregulation 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 NOS inhibition with L-NAME 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 [37, 38]. 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 $O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow H_2O+O_2$) appears to be the pivotal redox pathway in this OA-BSA model of PTEC protein overload (Fig. 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) [39]. Excess mitochondrial ROS can have devastating effects, as illustrated by severe lifespan reductions in SOD2 null mice [33, 39]. 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.

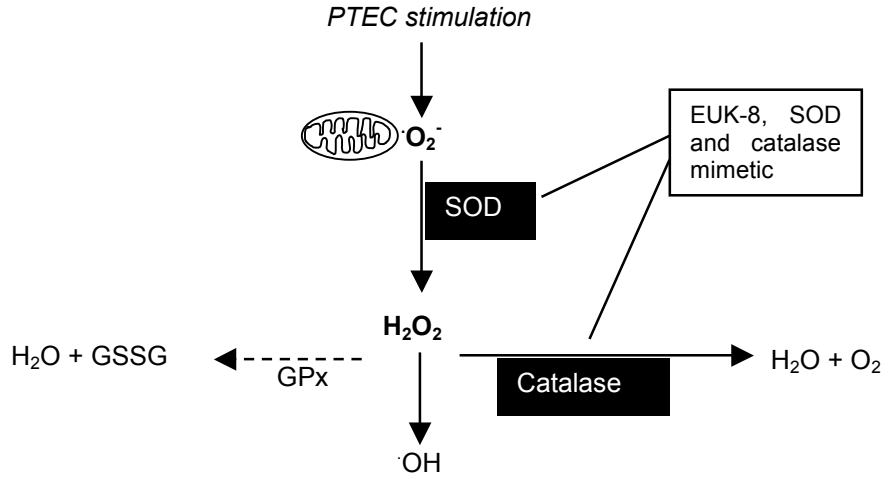

Figure 5. Schema of ROS pathways of interest. Abbreviations: O_2^-, superoxide anion; H_2O_2, hydrogen peroxide; GPx, glutathione peroxidase; GSSG, oxidised glutathione; H_2O, water; O_2, molecular oxygen; $\cdot OH$, hydroxyl ion.

To further assess the cellular defensive response to increased oxidative stress, we examined the gene expression of heme oxygenase-1 (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 [31] and is specifically protective of PTEC against various types of oxidant agents [32]. Contrary to our expectation of increased expression as an attempt to counter oxidant stress, we surprisingly observed that HO-1 was downregulated in PTEC exposed to BSA, an effect that was enhanced by OA-BSA. Thus it appears that, apart from aggravating albumin-induced oxidative stress, albumin-borne FA also further depress the HO-1 antioxidant protection. However, in contrast to our finding of

suppressed HO-1 expression in protein-overloaded human PTEC, 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 PTEC that were exposed to albumin [40]. 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 [41]. 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, this data suggests that the critical effect of albumin-bound FA in the promotion of PTEC pro-inflammatory 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, emphasising the importance of SOD. FA may play a crucial role in the translational control of IL-6 expression, and albumin-bound lipids could escalate pro-inflammatory tendencies within PTEC 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 FA can induce oxidative stress and oxidant-dependent changes in proximal tubular cells. 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 pro-inflammatory 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 FA that depress the cellular defence against ROS.

Acknowledgements

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Chapter 3

In Mice, Proteinuria and Renal Inflammatory Responses to Albumin Overload are Strain-Dependent

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Abstract

Background. The availability of genetically-modified mice has increased the need for relevant mouse models of renal disease, but widely-used C57BL/6 mice often show resistance to proteinuria. 129/Sv mice are considered more sensitive to certain renal models. Albumin-overload, an important model of proteinuric disease, induces marked proteinuria in rats but barely in C57BL/6 mice. We hypothesized that albumin-overload would induce more proteinuria in 129S2/Sv than C57BL/6J mice.

Methods. Male and female C57BL/6J and 129S2/Sv mice received bovine albumin (BSA) for 11 days. Control groups received saline injections. Injected BSA was immunohistochemically localized to study intrarenal handling of overloaded protein. Renal macrophage infiltration (F4/80 immuno-staining) and glomerular ultrastructure (electron microscopy) were assessed.

Results. The BSA-treated groups were similarly hyperproteinemic at D11. Proteinuria differed widely. In C57BL/6J mice, it remained unchanged in females but significantly though mildly increased in males (from 3±1 to 8±2 mg/d, $P<0.05$). In 129S2/Sv, proteinuria was marked in both males and females (4±1 to 59±14, and 0.6±0.2 to 29±9 mg/d respectively, both $p<0.01$). Proteinuria was accompanied by tubulo-interstitial macrophage infiltration in 129S2/Sv mice. Injected BSA was visualized within glomeruli in both strains and in the urinary space and tubules of 129S2/Sv but not C57BL/6J mice, indicating much greater glomerular leakage in the former. No glomerular macrophages or ultra-structural differences were detected.

Conclusion. There are major strain differences in the proteinuria and renal inflammatory response of mice to albumin overload, which are not due to structural variation in the filtration barrier but possibly to functional differences in glomerular protein permeability.

Introduction

Albumin overload is a well-known model of renal tubulointerstitial disease in rats[1]. Strain and gender related differences in magnitude of the proteinuric response to albumin overload have been reported, but females and males of all tested rat strains (Wistar, Sprague-Dawley, DA, and PVG) became significantly proteinuric [2]. Given the increasing recognition of an independent causal role of proteinuria in tubulointerstitial inflammation and ultimately in renal fibrosis [3], the albumin overload model represents an outstanding platform for in vivo investigation of the impact of excess protein load on tubular cells in a primarily non-hemodynamic and non-immunologic setting. The replication of this model in mice, with the attendant possibility to employ genetically modified strains, would be very useful in characterizing the specific impacts of defined genetic traits on the pathophysiologic processes involved. However, in recent reports of protein overload in C57BL/6 mice [4, 5], although effects such as gene expression alterations were observed, the proteinuric response was not impressive, which may significantly limit the potential for exploitation of aspects of the model. This is especially disappointing because of the popularity of the C57BL/6 strain as a background for transgenic and knockout mouse models.

In contrast to the C57BL/6, the 129S2/Sv mouse strain appears to be remarkably responsive to inducement of chronic renal disease. Proteinuria and marked glomerulosclerosis are seen in male 129S2/Sv mice 9 weeks after 5/6 nephrectomy, while male C57BL/6J mice show no proteinuria and only mild sclerosis 24 weeks after the same degree of renal ablation [6]. Similarly, male 129/Sv mice are more susceptible to development of hypertension and renal failure after deoxycorticosterone acetate (DOCA) plus salt than C57BL/6 mice [7]. In rats differences in proteinuria are noted between strains after protein overload [2], and we hypothesised that this is true also of mice. Such differences would have potentially important implications in the development of genetically modified strains, as identical genetic modifications can result in mice with massive phenotypic differences depending on their original genetic background [8]. Thus we conducted experiments to determine whether 129S2/Sv mice would show more proteinuria in response to albumin overload than C57BL/6J mice. Because male gender is generally associated with more proteinuria than female gender, and chronic renal disease progresses more rapidly in males [9], we expected a more pronounced strain difference in males. We found as expected, that 129S2/Sv had much more proteinuria than C57BL/6J despite similar elevations in plasma albumin levels. We conducted electron microscopic examination of

kidneys from both strains, to seek possible glomerular structural differences that could help explain the disparity in proteinuria, but we found none. Finally, we analysed renal interstitial macrophage infiltration to determine whether the proteinuria difference is of functional pathologic significance in terms of susceptibility to renal inflammation after protein overload.

Methods

Animal treatments. We employed male and female C57BL/6J and 129S2/Sv mice, age 14 -18 weeks (Harlan Nederland, Horst, The Netherlands). The mice were maintained on a standard diet (RMH-TM; Hope Farms, Woerden, The Netherlands) and tap water *ad libitum*. For experiments, animals were housed in pairs in cages in a room maintained at 22^{O}C, 60% humidity with a 12/12-hour light/dark cycle. The Utrecht University board for studies on experimental animals approved the protocol. Male and female mice of both strains received intraperitoneal (IP) injections of low-endotoxin bovine serum albumin (BSA, A-9430) (Sigma Chemical Co., St. Louis, MO) (dissolved in saline) for 9 days over an 11-day period. BSA was administered five days per week on a stepwise incremental dose regimen [4], rising from 2 mg/g body weight on the first day (D1) to the maximum dose of 10 mg/g on D5, which was thereafter maintained. Mice in strain, gender and age matched control groups received IP saline injections of corresponding volumes. Proteinuria was measured at baseline (D0), and subsequently at D4 and D11 of treatment, in 16-hour urine samples collected during overnight placement in metabolic cages. At termination (D11), mice were anaesthetized and blood samples collected by aortic cannulation. Kidneys were harvested and samples either fixed in 4% formaldehyde, or snap-frozen in liquid nitrogen and stored at –80^{o}C until analysed. In urine and plasma, total protein was measured by the Bradford method (Bio-Rad Laboratories, Munich, Germany), and BSA and mouse serum albumin (MSA) by immunoelectrophoresis using rabbit anti-bovine or anti-mouse antibodies (Nordic Immunological Laboratories, Tilburg, Netherlands). Plasma urea was measured enzymatically (Elitech, Sees, France), and plasma oncotic pressure by membrane osmometry (Amicon 10PK, Millipore, Billerica, MA USA). Systolic blood pressure was measured in conscious mice by the tail-cuff method (IITC, San Diego, CA, USA).

Renal histology and immunohistochemistry. Formaldehyde-fixed, paraffin-embedded kidney tissue was stained using standard procedures with haematoxylin and eosin and periodic acid-schiff for light microscopic examination by a renal pathologist in

blinded fashion. For intrarenal visualization of injected BSA, kidney sections were deparaffinized, blocked with endogenous buffer/hydrogen peroxide, boiled in 10 mM citrate buffer (pH 6.0), and incubated with rabbit anti-BSA antibody (Nordic Immunological Laboratories, Tilburg, Netherlands, 1:3000 in 5% NGS/PBS), followed by horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (Powervision) (Klinipath, Leiden, Netherlands). They were developed with Nova Red (Vector, Burlingame CA, USA) and counterstained with haematoxylin. Semi-quantitative assessment of the degree of renal cortical BSA staining was carried out in blinded fashion, using a cumulative scoring system with points awarded for the presence of BSA within Bowman's space or in the parietal epithelium ("evidence of BSA leakage" - 2 points); presence of BSA in normal tubules ("weak tubular staining" - 4 points); and BSA in dilated / damaged tubules ("strong tubular staining" - 8 points). To assess renal macrophage infiltration, frozen kidney sections were dried, fixed with acetone, blocked, and incubated with a rat antibody against the mouse macrophage antigen F4/80 [10] (Serotec Benelux, Oxford, UK). Sections were further incubated with horseradish peroxidase-conjugated rabbit anti-rat and swine anti-rabbit antibodies (DakoCytomation BV, Herverlee, Belgium). They were developed with Nova Red and counterstained with haematoxylin. F4/80-positive cells per high power field were counted in blinded fashion.

Electron microscopy. Male and female C57BL/6J and 129S2/Sv mice were treated with saline or BSA, anaesthetized as described, and perfused for 3 min at 200 mmHg with a solution of 3% glutaraldehyde and 0.1% picric acid in 0.1M cacodylate buffer. Kidney slices were immersed overnight in the same solution, and then transferred into 0.1M cacodylate buffer until further processing. Kidney tissue blocks were dehydrated and embedded by standard procedures. Semithin sections (1 μm) were cut on an ultramicrotome, then stained and examined by light microscopy. From areas of interest selected in the semithin sections, ultrathin sections were cut using a diamond knife and studied with a Philips 301 electron microscope.

Statistics. Results are expressed as mean ± SEM. For multiple group comparisons, data was analyzed by ANOVA, Kruskal-Wallis ANOVA on ranks, or repeated measures ANOVA as appropriate, followed by the Student-Newman-Keuls or Dunn's post-hoc test as applicable. For comparisons of two groups, Student's t-test was used (rank sum test or t-test with Welch correction were employed where the data was not normally distributed). Statistical significance was accepted at p values < 0.05.

Results

Strain and gender differences in proteinuria. At D0, in both strains proteinuria was higher in males than in females; C57BL/6J 2.7±0.4 vs. 0.6±0.1 mg/d (P<0.05) and 129S2/Sv 3.4±0.5 vs. 0.6±0.1 mg/d (P<0.05). Within each gender, there were no differences between strains. At the end-point, all BSA-treated groups were similarly hyperproteinemic (Fig 1a and b, and Table 1), but proteinuria differed widely between the two strains. In response to BSA, proteinuria massively increased in male 129S2/Sv but not significantly in C57BL/6J mice (Fig 1c); and while female 129S2/Sv mice developed highly significant proteinuria, in their C57BL/6J counterparts protein excretion completely failed to increase (Fig 1d). Proteinuria in both male and female 129S2/Sv was already significant by D4, and

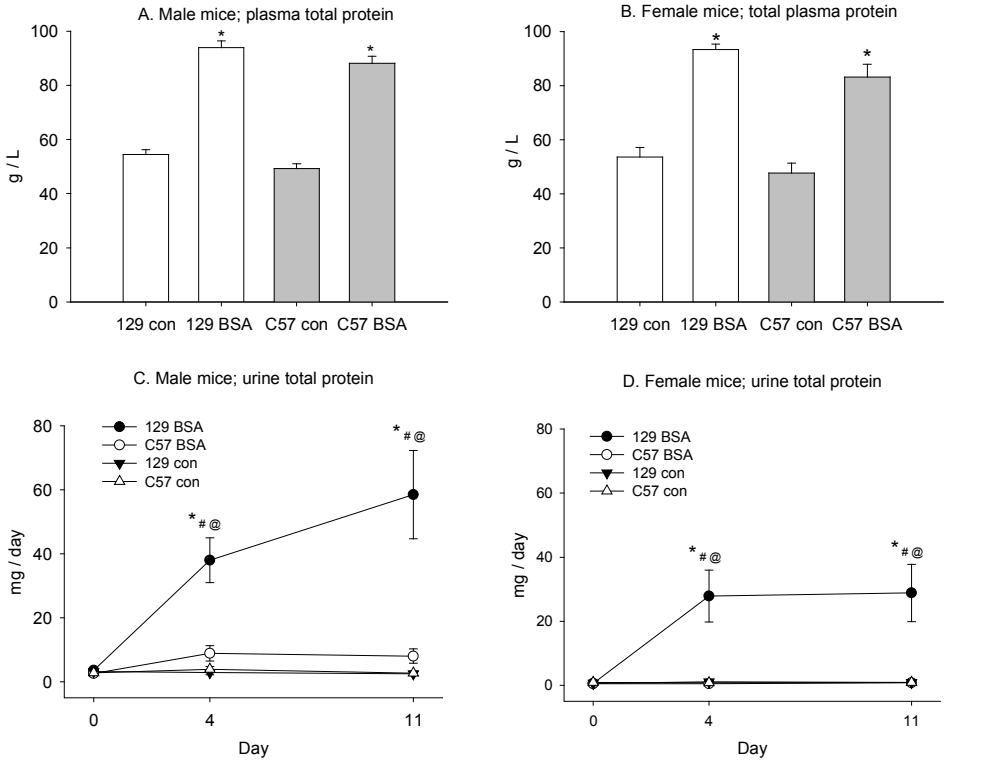

Figure 1. The effect of BSA treatment on plasma and urine protein levels. Plasma total protein levels in male **(A)** and female **(B)** mice after 11-days of BSA; and proteinuria in the same groups **(C, D)** during the course of treatment. * p< 0.001, BSA vs. Control (con); # p< 0.001, 129S2/Sv vs. C57BL/6J mice; @ p< 0.001 vs. D0. Abbreviations: 129 (129S2/Sv mice), C57 (C57BL/6J mice), con (control saline-injected mice).

Table 1: Plasma measurements following albumin treatment over 11 days. (Abbreviations: TP – total protein; BSA - bovine serum albumin; MSA - mouse albumin; COP - colloid oncotic pressure; ND - not determined)

	129S2/Sv				**C57BL/6**			
	Male		**Female**		**Male**		**Female**	
	BSA	Saline	BSA	Saline	BSA	Saline	BSA	Saline
n	*16*	*13*	*7*	*7*	*11*	*9*	*9*	*5*
TP (g/L)	94 (2)*	54 (2)	93 (2)*	54 (3)	88 (3)*	49 (2)	83 (5)*	48 (4)
MSA (g/L)	29 (1) * $ #	33 (1) $ #	37 (2) * #	45 (2)	45 (4)	42 (3)	43 (1)	50 (2)
BSA (g/L)	34 (1)	-	39 (2)	-	29 (2)	-	35 (4)	-
Urea (mmol/l)	10.9 (0.7)	10.1 (1.4)	8.1 (1.2)	9.2 (0.9)	10.5 (0.6) *	8 (1)	ND	ND
COP (mmHg)	25.4 (0.7)* $ #	14 (0.7)	29.4 (2.6)*	14 (1)	28.6 (1.7) *	15 (1)	25 (2) *	18 (1)

Mean (SEM); *p<0.05 BSA vs. saline; # p<0.05 C57BL/6J vs. 129S2/Sv; $ p<0.05 male vs. female

was sustained or slightly increased thereafter. Urine BSA and MSA were measured in BSA-treated males. In 129S2/Sv males mouse albumin accounted for 23% of total urine protein (BSA 32±7, MSA 14±3, total protein 59±13 mg/d; n=10). In C57BL/6J males mouse albumin accounted for only 3% of total urine protein (BSA 5.6±0.3, MSA 0.4±0.1, total protein 10.4±1.2 mg/d; n=4).

No strain effect on renal function and blood pressure. Albumin treatment for 11 days had no major effect on renal function (plasma urea, Table 1). Systolic blood pressures were measured by the tail cuff method in male BSA-treated mice and did not differ between strains (mmHg, 97.5±3.8 in 129S2/Sv vs. 103.8±4.4 in C57BL/6J, p=0.322).

Intrarenal visualisation of injected BSA. Immunohistochemical staining permitted us to visualize the injected BSA and determine its location within the nephron at the experimental end-point, giving an insight into renal handling of the exogenous protein (Fig. 2). In 129S2/Sv mice, BSA was prominent within the Bowman's space and the renal tubules (Panel A). However, in C57BL/6J mice (Panel B), while the intravascular presence of BSA within the glomerular tufts was obvious, very little of it was seen elsewhere including

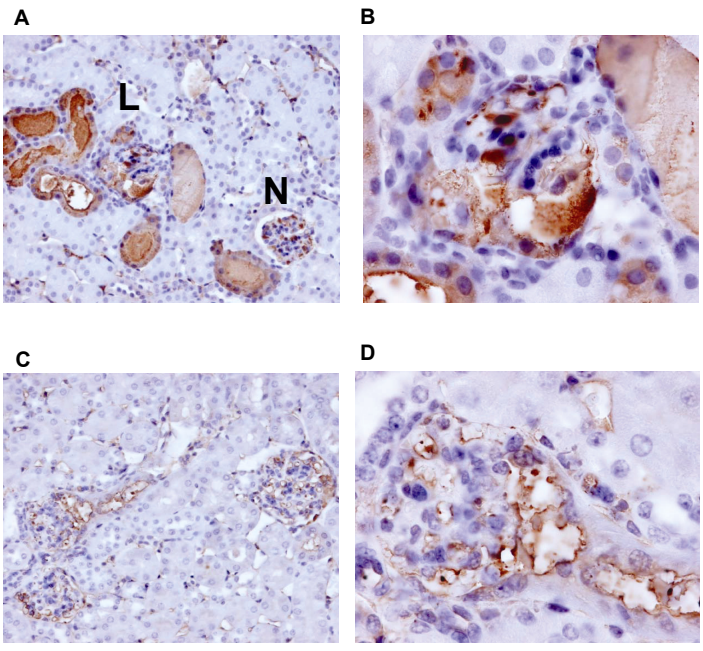

Figure 2. Visualization of renal handling of injected BSA. A: 129S2/Sv mice. Apparently normal (N) and nearby leaking (L) glomeruli with contiguous BSA-filled tubules (200 x). **B:** 129S2/Sv mice. Detail of a leaky glomerulus with a "pool" of BSA in the urinary space (600 x). **C:** C57BL/6J mice. Tubules are largely BSA-free (200 x). **D:** C57BL/6J mice. Detail of positively stained glomerular tuft with little or no BSA visualized in the urinary space (600 x).

the urinary space and tubular lumina. Assessment of intrarenal BSA load by semi-quantitative estimation of cortical BSA staining revealed a significant strain difference, with staining much higher among 129S2/Sv mice (arbitrary units, 40.9 ± 11.5 vs. 4.6 ± 1.6 in C57BL/6J mice, $p < 0.01$).

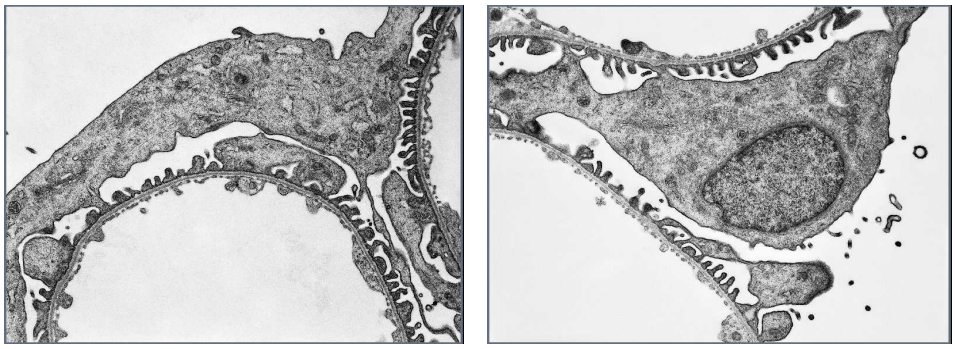

Figure 3. Electron microscopy. Micrographs showing detailed glomerular ultrastructure including podocyte cell body, podocyte foot processes, glomerular basement membrane and endothelium, from male mice injected with BSA for 11 days. **Left panel:** 129S2/Sv, **right panel:** C57BL/6J. There are no remarkable structural variations that could account for the marked difference in proteinuria response. The final magnification for both micrographs is 15000x.

Table 2: Tubulointerstitial macrophage count (F4/80 positive cells).

		129S2/Sv	C57BL/6J
Male	BSA	63 (5) #	53 (3)
	Saline	21 (3)	44 (4) *
Female	BSA	62 (5) #	57 (4) #
	Saline	21 (5)	36 (4)

Mean (SEM); * $p<0.05$ 129S2/Sv vs. C57BL/6J, # $p<0.05$ BSA vs. saline. There were no significant gender differences.

Renal morphology. Routine histology revealed no remarkable glomerular or tubular injury (not shown). On electron microscopy, glomerular ultrastructure was similar between strains after BSA treatment (Fig 3). In addition, tubular and glomerular

appearances after BSA injections were not different from saline-treated animals, and there were no gender differences (not shown). Immunohistochemistry for the mouse macrophage antigen F4/80 revealed that BSA treatment induced significant tubulo-interstitial macrophage infiltration in 129S2/Sv but not C57BL/6J mice, even though baseline F4/80 counts were slightly higher in C57BL/6J mice (Table 2 and Fig 4). Glomerular macrophages were not observed in any mice.

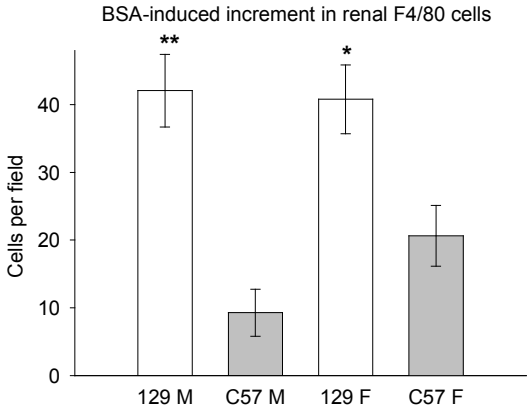

Figure 4. Net influx of tubulointerstitial macrophages after BSA treatment. Net influx of tubulointerstitial macrophages after BSA treatment (corrected for mean values found after saline treatment in each group). F4/80 positive cells were counted per 0.24mm^2 field. * $p< 0.05$, ** $p < 0.01$, 129S2/Sv vs. C57BL/6J. Abbreviations: 129 (129S2/Sv mice), C57 (C57BL/6J mice), M (male), F (female).

Discussion

Protein-overload proteinuria eventually leads to tubulointerstitial injury in rats [1]. We investigated this model in mice to probe the feasibility of protein-overload studies in genetically modified strains. Daily intraperitoneal administration of endotoxin-free BSA in two mouse strains, C57BL/6J and 129S2/Sv, increased plasma protein concentration similarly from about 55 g/L to more than 90 g/L. Nevertheless, only in 129S2/Sv mice did this lead to severe proteinuria, accompanied by significant tubulointerstitial macrophage influx. This strain difference was observed in males and females, with little gender effect. Electron microscopic appearances in mice treated or not treated with BSA did not differ between strains. Immuno-histochemistry revealed that in 129S2/Sv mice much more BSA crossed the glomerular basement membrane (GBM), resulting in prominent staining in Bowman's space and protein-filled tubule sections. Besides having practical implications, these findings suggest major differences in the sieving coefficient of the glomerular basement membrane for albumin within a single species.

Many experimental models of progressive renal injury are associated with proteinuria. However, in C57BL/6J mice it is notoriously difficult to induce proteinuria in such models. For example, in a remnant kidney model practically no increase in proteinuria as well as blood pressure was observed in C57BL/6J mice [6]. In contrast, in the same model in rats, marked proteinuria accompanied uremia and hypertension [11]. Similarly, C57BL/6J mice on DOCA-salt only had mild hypertension and failed to develop proteinuria [7], while NOS inhibition with LNNA also failed to induce proteinuria or much renal injury in C57BL/6J wild type [12] or transgenic mice on C57BL/6J background [13]. Given the increasingly apparent independent role of proteinuria in renal inflammation and fibrosis [3], it is plausible that the marked resistance to proteinuria in the C57BL/6J mouse is a key factor in its comparative insensitivity to well-defined models of progressive renal injury. This notion is supported by our finding that, unlike in 129S2/Sv, BSA treatment in C57BL/6J mice not only failed to provoke proteinuria, but also did not induce substantial interstitial macrophage infiltration.

Our BSA staining studies revealed that despite marked and similar increases in plasma protein concentrations, leaked protein was prominently stained in 129S2/Sv mice, but practically absent in C57BL/6J mice. Any substantial rise in plasma albumin level, irrespective of whether it increases or has little effect on the sieving coefficient, should result in at least a proportional increase in tubular albumin reabsorption in order to avoid negative nitrogen balance. In rats tubular albumin reabsorption capacity is apparently operating close to its maximum because administration of BSA immediately results in a selective proteinuria that comprises both exogenous and endogenous albumins [14]. The absence of BSA in practically all nephrons in the C57BL/6J mice suggests that the strain difference we describe is related to differences in glomerular permselectivity and not to differences in tubular reabsorption. What factors could be responsible for such a difference? Electron microscopic analysis did not indicate any structural variations in the filtration barrier that could account for the wide disparity in proteinuria. Systemic blood pressure was similar in BSA-treated male mice, indicating no strain differences in systemic contribution to pro-filtration hydrostatic forces. Nevertheless, we cannot exclude the possibility of intrarenal angiotensin-dependency in explaining the proteinuria difference, because mice are polymorphic for renin genes [15]. While some strains, such as C57BL/6J and BalbC mice, possess only one renin gene, Ren-1, which is expressed in the kidneys, most other strains have an additional renin gene, Ren-2, which is mostly expressed in the submaxillary gland. Such strains include 129S2/Sv, the Swiss mouse, and various wild mice such as *M. musculus, M. hortulanus,*

and *M. domesticus*. In diabetic rats the decreased number of anionic sites on the GBM is corrected by ACE inhibition [16]. However, our pilot experiments with losartan at a high dose (150 mg/L) failed to abrogate the strain difference in glomerular permselectivity, suggesting that yet other factors are involved.

Since there are no obvious ultrastructural differences to explain the wide gap in proteinuria response, it is very likely that electrical or molecular factors within the GBM are at play. There may well be significant inter-strain variations in electrostatic forces within the membrane, because even subtle differences in composition may influence biophysical characteristics like “fixed” charge and pI, causing disparities in albumin sieving coefficients that could underlie the observed difference in proteinuria. There is also the possibility of genetic heterogeneity between mouse strains in the molecular make-up of the slit diaphragm, which is one of the key determinants of glomerular permeability [17]. The slit diaphragm possesses a highly complex organisation comprising structural as well as biochemical signalling properties, with structural components including the membrane proteins nephrin and podocin, the cytoplasmic “adaptor” molecule CD2AP, and the adhesion proteins Neph-1 and FAT-1 [18]. Genetically modified mice lacking each of these proteins develop massive proteinuria, and mutations of genes coding for nephrin, podocin, and CD2AP have been implicated in specific forms of human glomerular disease [18]. In rats, experimental disruption of the nornal interaction between nephrin and Neph-1 led to proteinuria in the absence of foot process abnormalities [19]. Thus, it is attractive to speculate that differential expression of crucial slit diaphragm proteins, or genetically determined variations in important protein-protein interactions, may render certain strains of mice (such as the 129S2/Sv variety) more susceptible to proteinuria than others, even with preserved glomerular ultrastructure.

Judging by urinary composition, filtration of mouse albumin appears to be restricted even more efficiently than bovine albumin in glomeruli of the C57BL/6J strain. It is known that albumin variants having different isoelectric points (pI) differ in GBM permeability [20]. Using Web-based resources, we obtained the amino acid sequences of BSA *(http://ca.expasy.org/cgi-bin/niceprot.pl?P02769)* and MSA *(http://ca.expasy.org/cgi-bin/niceprot.pl?P07724)*, then employed a sequence-based pI calculator *(http://www.embl-heidelberg.de/cgi/pi-wrapper.pl)* (all accessed September 2005) to determine that the approximate pI of MSA is 5.41 while that of BSA is 5.50. This implies that BSA will carry a

weaker negative charge at physiologic pH, favouring its filtration and explaining at least in part, why it is more readily excreted than MSA in both strains.

The protein overload model in 129S2/Sv mice offers the additional attraction of providing possibilities for studying proteinuric disease with or without tubular injury, based on a range of severity levels. In our model of relatively brief BSA administration, we observed pronounced proteinuria with preserved renal function and absence of histological injury.

In summary, we have demonstrated a marked strain difference in the response of mice to albumin overload. In terms of development of proteinuria, and tubulointerstitial macrophage infiltration, 129S2/Sv mice are much more responsive than C57BL/6J mice. This difference is not accompanied by inter-strain variation in glomerular ultrastructure. Glomerular leakage of injected BSA in the 129S2/Sv strain indicates the importance of glomerular protein permeability as a key determinant of susceptibility to proteinuria.

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Conflict of interest statement

No conflicts of interest.

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Chapter 4

In proteinuric mice, fatty acid-induced renal inflammation is alleviated by tempol or mitochondrial inhibition

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Submitted

Abstract

Background. Albumin-bound fatty acids (ABFA) aggravate albumin-induced renal interstitial inflammation, but underlying mechanisms are not clear. We previously found that in cultured proximal tubular cells, oleic acid-rich bovine serum albumin (OA-BSA) stimulated much more mitochondrial reactive oxygen species (ROS) production than albumin alone (BSA). OA-BSA also suppressed protective antioxidant responses. We hypothesised that increased antioxidant capacity might alleviate renal inflammatory effects of ABFA *in vivo*.

Methods. Male 129S2/Sv mice were treated for 18 days with IP injections of BSA (10 mg/g/day) or corresponding doses of OA-BSA (OA:BSA molar ratio ~4:1). Control mice received saline injections. In addition to OA-BSA, some mice received the SOD mimetic agent tempol (10 mmol/L in drinking water) or the mitochondrial Complex 1 inhibitor rotenone (600 ppm in powdered chow). We measured proteinuria, and assessed renal macrophage infiltration by immunohistochemical detection of cells expressing the mouse macrophage antigen F4/80. To assess the influence of lowering non-esterified fatty acids (NEFA), a separate group of mice received a nicotinic acid derivative (Niaspan; 500 mg/L in water) during OA-BSA treatment.

Results. In the first series of experiments, proteinuria induced by BSA and OA-BSA was not significantly different (BSA, 61±12 vs. OA-BSA, 94±15, vs. control mice, 6±1 mg/day, p=NS), but renal macrophage count (F4/80 cells per field) was markedly higher in OA-BSA (103±8) than after BSA (63±5, p=0.003), confirming the added inflammatory impact of ABFA. In further experiments with OA-BSA-treated mice, tempol (TPL) and rotenone (ROT) had no significant effect on urinary protein. However, TPL markedly alleviated OA-BSA-induced renal macrophage influx by 75% ($p<0.001$), showing that inflammation induced by ABFA is largely ROS-dependent and can be suppressed by augmenting SOD activity. Treatment with ROT was also beneficial; macrophage influx was modestly but significantly reduced by 34% ($p<0.05$), suggesting that mitochondrial oxidant stress partly contributes to the inflammation. Nicotinic acid treatment reduced NEFA ($P<0.05$) and significantly alleviated OA-BSA-induced renal inflammation, further confirming the role of ABFA.

Conclusion. These findings imply that in proteinuric states, albumin-bound fatty acids increase renal inflammation as judged by macrophage influx. Dramatic inhibition of macrophage influx by SOD mimetic, tempol, indicates a pivotal role for free radicals in the inflammatory response. Diminished inflammation during rotenone indicates significant mitochondrial contribution to oxidant stress. Lowering NEFA may be beneficial in proteinuric states.

Introduction

Renal interstitial inflammatory cell infiltration is a prominent and consistent characteristic of protein overload nephropathy, induced in rodents by exogenously administered protein in the absence of primary disease [1-5]. This indicates that during proteinuric disease, increased tubular protein traffic per se - irrespective of the mechanism of leakage - is a promoter of renal inflammation. In support of the notion that proteins possess direct renal inflammatory capacity, a plethora of pro-inflammatory effects have been observed in cultured proximal tubular epithelial cells (PTEC) exposed to excess amounts of protein [6-10].

There has been much interest in dissecting the mechanisms by which excess protein leakage provokes renal inflammation and injury. An emerging possibility is that the inflammatory effect of proteinuria may not be due solely to excess albumin, which is the most abundant filtered protein, but rather more to the nephrotoxicity of large amounts of fatty acids (FA) transported into the tubular system by binding to albumin [11, 12]. In recent studies comparing the effects of exposing experimental animals to large doses of either FA-poor or FA-loaded albumin, those treated with FA-loaded albumin exhibited significantly greater degrees of tubulointerstitial inflammation and other features of injury [13-15]. In addition, in vitro data suggest that albumin-bound fatty acids (ABFA) exert pro-fibrotic [16] and apoptotic [17] effects on PTEC. Moreover, nephrotic patients with the relatively benign minimal change disease (MCD) have much lower urinary albumin FA content than individuals with non-MCD varieties of nephrosis that tend to run a more aggressive course [18]. These data support the view that during proteinuria, ABFA induce extra nephropathic effects over and above those of albumin alone.

However, there is limited information about mechanisms by which ABFA might promote renal interstitial inflammation. We previously probed this question in a cell-based model, comparing the effects of treating cultured PTEC with either FA-poor albumin (BSA), or albumin that was loaded with oleic acid (OA-BSA). OA-BSA induced a much higher level of oxidant stress, derived from increased mitochondrial production of reactive oxygen species (ROS) coupled with suppressed cellular antioxidant responses. OA-BSA also induced significantly greater protein expression of the inflammatory cytokine interleukin-6 (IL-6), an effect that was powerfully suppressed by SOD supplementation and virtually abolished by mitochondrial respiratory chain inhibition [19]. These findings led us to

hypothesise that renal inflammation induced by ABFA may be oxidant-dependent, and may be alleviated by pharmacological supplementation of SOD and/or inhibition of mitochondrial ROS.

The present study was conducted to test the above hypothesis. First, we treated separate groups of mice with either BSA or OA-BSA, and confirmed that OA-BSA induced more renal inflammation. In further experiments, OA-BSA treatment was accompanied by pharmacological interventions targeted either at boosting antioxidant capacity or at dampening mitochondrial ROS production. To test whether augmentation of antioxidant function would suppress renal inflammation, we administered the SOD mimetic agent 4-hydroxy-TEMPO (tempol) during OA-BSA treatment. To assess a possible role of mitochondrial ROS, we exposed mice to OA-BSA in the presence of the respiratory Complex I inhibitor rotenone. Finally, we employed nicotinic acid to probe the possibility that sustained systemic lowering of non-esterified fatty acids [20] might ameliorate the renal inflammation induced by OA-BSA.

Methods

Mice. Experiments were conducted with male 129S2/Sv mice, age 14-18 weeks (Harlan Nederland, Horst, The Netherlands). The Utrecht University board for studies on experimental animals approved the protocol. Chemicals and reagents were obtained from Sigma-Aldrich (Zwijndrecht, Netherlands) unless otherwise indicated. Mice were maintained on a standard diet (RMH-TM; Hope Farms, Woerden, The Netherlands) and tap water *ad libitum* and housed in pairs in cages in a room maintained at 22^{O}C, 60% humidity with a 12/12-hour light/dark cycle. 16-hour urine samples for proteinuria measurements were collected overnight in metabolic cages. At termination (D18), mice were injected with an anaesthetic cocktail (46.7mg/ml ketamine, 8mg/ml xylazine, and 0.067mg/ml atropine) at 0.1 ml/20mg IP, and blood samples collected. Kidney samples were either fixed in 4% formaldehyde, or snap-frozen in liquid nitrogen for storage at –80^{o}C until analysed.

Protein overload protocol. Low-endotoxin bovine serum albumin (BSA) (Sigma catalog number A-9430) was dissolved in physiologic saline at a concentration of 330 g/L. The low FA content of this BSA was previously confirmed in our laboratory (Ishola et al, KI 2006, in press). BSA was complexed with oleic acid (OA) (Sigma O-1008) as previously

described, resulting in oleic-acid-loaded albumin (OA-BSA) with OA:BSA molar ratio of ~3.7:1 [19]. To induce protein overload, mice were given intraperitoneal (IP) injections of either BSA or OA-BSA over 18 days, five days per week on a stepwise incremental dose regimen, rising from 2 mg/g body weight on the first day (D1) to the maximum 10 mg/g on D5 which was thereafter maintained. The model was based on a regimen established by Eddy et al. [21]. Previously we found that 129S2/Sv mice subjected to this regimen demonstrated far more proteinuria than similarly treated C57Bl/J mice [1]. Matched control mice received vehicle (physiologic saline) injections.

Pharmacological interventions during OA-BSA treatment. In additional groups of mice, IP injections of OA-BSA were accompanied by systemic administration of either the SOD mimetic agent tempol (Sigma-Aldrich, 17,614-1) dissolved in drinking water (10 mMol/L), or the mitochondrial respiratory chain Complex I inhibitor rotenone (Sigma R-8875), mixed with powdered chow (600 ppm). A separate group was given an extended-release form of nicotinic acid (Niaspan®, Merck BV, Amsterdam, The Netherlands) dissolved in drinking water (500 mg/L). Administration of all intervention medications were commenced 72 hours before starting OA-BSA, and maintained throughout the course of OA-BSA treatment. It was ascertained in pilot series that the medications did not influence water and food intake.

Biochemical measurements. Urine total protein measurement was with the Bradford method (Bio-Rad Laboratories, Munich, Germany). Plasma urea was measured enzymatically (Elitech, Sees, France). Enzymatic kits were also used to measure plasma cholesterol and triglycerides (Elitech) and NEFA (Wako Chemicals, Neuss, Germany).

Renal histology and immunohistochemistry. Formaldehyde-fixed, paraffin-embedded kidney tissue was stained using standard procedures with haematoxylin and eosin and periodic acid-schiff for light microscopic examination by a renal pathologist in blinded fashion. To assess renal macrophage infiltration, frozen kidney sections were dried, fixed with acetone, blocked and incubated with a rat antibody against the mouse macrophage antigen F4/80 [22] (Serotec Benelux, Oxford, UK). Sections were further incubated with horseradish peroxidase-conjugated rabbit anti-rat and swine anti-rabbit antibodies (DakoCytomation BV, Herverlee, Belgium). They were developed with Nova Red and counterstained with haematoxylin. F4/80-positive cells per high power field (area 0.245 mm^2) were counted in blinded fashion.

Statistics. Data is shown as mean (SEM) unless otherwise indicated. Differences among groups were analysed using appropriate forms of analysis of variance. Statistical significance was accepted at the level of $p<0.05$.

Results

Albumin-bound fatty acid (ABFA) aggravates renal inflammation. In the initial series of experiments, we compared the effects of treating mice with albumin alone (BSA) or loaded with oleic acid (OA-BSA). We selected oleic acid as it is the most abundant urinary ABFA [12]. BSA and OA-BSA similarly increased plasma protein levels (Table 1). OA-BSA induced more proteinuria than BSA, although the difference did not reach significance due to considerable variation within groups (Table 1). The two treatments had no effect on plasma urea levels or renal histological appearance by light microscopy (not shown). However, staining for the specific mouse macrophage antigen F4/80 revealed that mice treated with OA-BSA had significantly higher renal macrophage infiltration than those given BSA alone (Fig. 1).

Table 1. Comparison of effects of BSA and OA-BSA treatment on plasma protein and urinary protein levels

		Proteinuria (mg / day)		Proteinaemia (g/l)
	N	***Baseline***	***Terminal***	
Control	10	3.2 (0.9)	6.2 (1.4)	56 (2)
BSA	7	3.6 (0.5)	61 (12) *#	97 (4) *
OA-BSA	8	2.9 (0.4)	109 (19) *#	89 (2) *

Mean (SEM). *Proteinuria:* * $p<0.001$ vs. control, # $p<0.001$ vs. baseline (2-way repeated measures ANOVA). *Proteinaemia:* * $p<0.001$ vs. control (ANOVA)

Antioxidant treatment with an SOD mimetic agent reduces ABFA-induced renal inflammation. In subsequent experiments we sought to determine whether the observed renal macrophage influx induced by ABFA was related to increased oxidant stress. Some mice were treated with the SOD mimetic agent 4-hydroxy-TEMPO (tempol) alongside OA-BSA. Tempol did not significantly reduce OA-BSA-induced proteinuria (Fig. 2) but markedly reduced renal interstitial inflammatory cell influx by about 75% (Fig. 3). In control mice, tempol tended to reduce urinary protein, but it had no effect on renal macrophage number (Fig. 3).

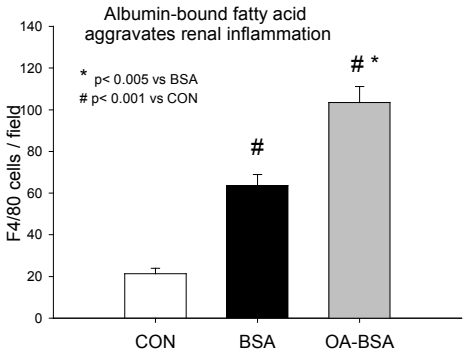

Figure 1. Albumin-bound fatty acid aggravates renal interstitial macrophage influx. CON, control; BSA, albumin; OA-BSA, oleic acid-loaded albumin. There were at least 7 mice in each group.

Mitochondrial inhibition reduces ABFA-induced renal inflammation. We previously found that the inflammatory response induced by ABFA in cultured proximal tubular cells is dependent on increased mitochondrial ROS [19]. To test whether mitochondria also contribute to renal inflammation in the in vivo situation, we co-treated a separate group of mice with OA-BSA and the mitochondrial respiratory chain inhibitor rotenone. Rotenone moderately and significantly reduced OA-BSA-induced renal inflammation by about 35% (Fig. 3), without significantly affecting urinary protein excretion (Fig. 2). Rotenone also had no effect on the renal macrophage population in untreated mice (Fig. 3).

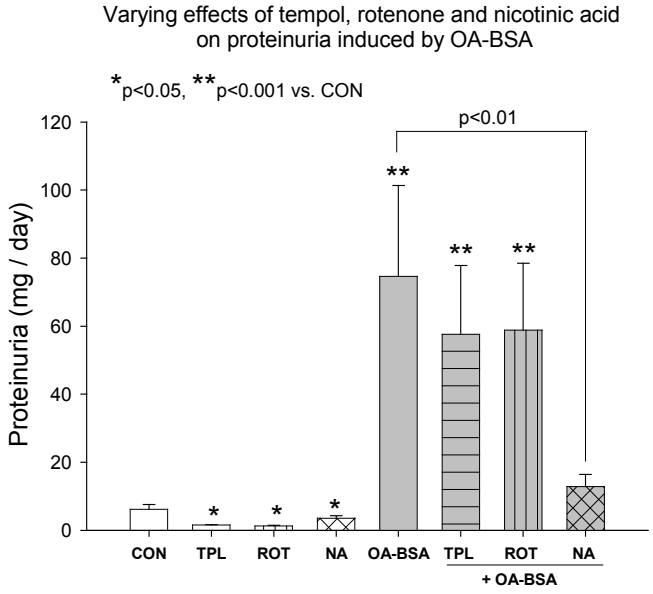

Figure 2. Varying effects of intervention agents on proteinuria induced by OA-BSA treatment.. Antioxidants lack significant effect, but lipolytic therapy markedly reduces proteinuria. There were at least 6 mice per intervention group. CON, control; BSA, albumin; OA-BSA, oleic acid-loaded albumin, TPL, tempol; ROT, rotenone; NA, nicotinic acid.

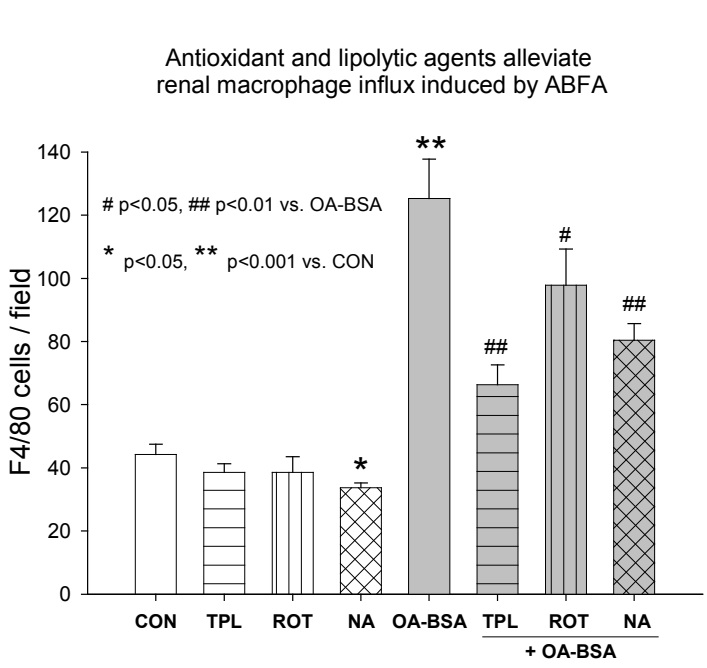

Figure 3. Effects of various interventions on basal and OA-BSA-stimulated renal interstitial macrophage count. Antioxidant therapy with both tempol and rotenone, and plasma NEFA reduction with NA, all alleviate OA-BSA-induced renal interstitial macrophage infiltration. (n = at least 6 per group). CON, control; BSA, albumin; OA-BSA, oleic acid-loaded albumin, TPL, tempol; ROT, rotenone; NA, nicotinic acid.

Nicotinic acid reduces both proteinuria and ABFA-induced renal inflammation. We investigated the impact of ABFA exposure on plasma non-esterified fatty acid (NEFA) levels. As expected, OA-BSA treatment resulted in an increased NEFA level. A sustained-release formulation of nicotinic acid (NA) corrected this increase (Table 2). NA remarkably reduced proteinuria (Fig. 2) and significantly suppressed renal macrophage influx (Fig. 3) induced by OA-BSA treatment. In addition, NA tended to reduce both proteinuria and renal macrophage count in control mice (Fig. 3).

Plasma lipid profile. To gain further insight into systemic lipid metabolism in the OA-BSA model, we carried out additional measurements of plasma lipids (Table 2). Apart from increasing plasma NEFA concentration, OA-BSA significantly reduced plasma cholesterol but had no effect on triglycerides. The reduction in plasma cholesterol was inversely related plasma protein levels ($P<0.01$). Tempol and nicotinic acid had no independent impact on all the measured plasma lipids. In contrast, in mice that received rotenone alone, or that were co-treated with rotenone and OA-BSA, there was a considerable increase in plasma triglycerides.

Table 2. Plasma parameters in mice receiving OA-BSA and/or intervention treatments

	NEFA (mmol/L)	Triglycerides (mmol/L)	Cholesterol (mmol/L)	Total protein (g/l)	Urea (mmol/L)
Control (n = 10)	0.48 (0.06)	0.94 (0.10)	2.85 (0.10)	53(2)	9.2 (0.7)
OA-BSA (n = 10)	2.17 (0.52) **	0.76 (0.11)	1.55 (0.08) **	80 (4) **	8.7 (0.4)
OA-BSA + NA (n = 8)	0.75 (0.07) ##	1.03 (0.16)	1.78 (0.14)	89 (5) **	8.4 (0.4)
OA-BSA + tempol (n = 6)	1.04 (0.07) #	1.25 (0.15)	1.76 (0.06)	74 (3) **	8.8 (0.4)
OA-BSA + rotenone (n = 6)	0.86 (0.17) ##	2.05 (0.30) *	1.98 (0.10)	79 (4) **	10.6 (0.6)
NA (n = 8)	0.57 (0.07)	1.07 (0.17)	3.25 (0.07)	48 (1)	8.4 (0.3)
Tempol (n = 4)	0.46 (0.01)	1.44 (0.33)	3.26 (0.12)	46 (2)	8.7 (0.8)
Rotenone (n = 4)	0.55 (0.04)	2.45 (0.37) *	3.05 (0.17)	45 (3)	11.1 (0.7)

Mean (SEM). * $p < 0.01$, ** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$ vs. OA-BSA. Abbreviations: NA, nicotinic acid; NEFA, non-esterified fatty acids.

Plasma protein and renal function. Plasma protein levels were similarly increased in all BSA and OA-BSA injected groups (Tables 1 and 2), confirming that inter-group differences in proteinuria were independent of proteinaemia. We measured plasma urea levels as an indicator of renal function. Urea was not significantly affected by OA-BSA. Renal function was not altered by antioxidant or lipolytic medication; plasma urea was slightly and not significantly increased in mice that received rotenone (Table 2).

Discussion

Greatly increased renal protein trafficking during proteinuric disease is a key precipitant of interstitial inflammation and injury [23]. The present study was based on the hypothesis that albumin-bound fatty acids (ABFA) contribute to this inflammatory effect via excess ABFA-induced oxidant stress. We employed renal interstitial macrophage infiltration as a marker to compare inflammatory effects of albumin alone (BSA) or loaded with oleic acid (OA-BSA), and used a pharmacological approach to assess the role of oxidant stress. We confirmed that OA-BSA induced significantly more interstitial macrophage infiltration than BSA. The excess macrophage influx was markedly reduced by the SOD mimetic agent tempol, indicating that oxidative stress is a critically important mechanism underlying the renal inflammatory impact of ABFA. Inflammation was also significantly alleviated by the mitochondrial respiratory chain blocker rotenone, indicating involvement of mitochondrial ROS. Finally, lipolytic treatment with nicotinic acid resulted in reduced plasma NEFA and amelioration of both proteinuria and renal inflammation.

Tubulointerstitial inflammatory cell infiltration plays a critical role in progressive renal disease [24]. Our findings are in keeping with previous reports indicating that in protein-overloaded animals, FA-rich albumin stimulates more renal inflammation than albumin alone [13-15] and suggests that this inflammatory effect occurs via an oxidant-dependent mechanism. Renal interstitial inflammation occurs in several proteinuric states, and increased oxidant stress has been observed in such other mechanistically diverse proteinuric models as diabetic nephropathy [25], renal ablation [26], and aldosterone-induced hypertensive renal injury [27], as well as in the human proteinuric condition known as Finnish type congenital nephrotic syndrome [28].

There may thus be an association between proteinuria, interstitial inflammation, and altered redox balance, and the present study addressed the role of ABFA in this relationship. The key questions were whether there is a causal link whereby ABFA are precipitants of inflammation, and whether such inflammation is dependent on oxidant stress. In our previous *in vitro* work, FA-poor albumin provoked a slight increase in ROS production (about 50% above baseline) in renal tubular cells, and cells demonstrated increased mRNA expression of mitochondrial SOD as an apparent antioxidant reaction. But when albumin was loaded with a high FA content, there was a four-fold increase in mitochondrial ROS, accompanied by impaired cellular antioxidant mitochondrial SOD response [19]. These

findings indicated that ABFA induce *in vitro* oxidant stress via a dual mechanism of increased ROS and suppressed antioxidant defence. The results of the present study indicate a similar situation *in vivo*. SOD supplementation was particularly effective in suppressing OA-BSA-induced inflammation. Tempol suppressed interstitial macrophage infiltration in OA-BSA treated mice to a level approaching that of untreated control animals, suggesting that OA-BSA induced functional inadequacy of endogenous antioxidant capacity. Reduced SOD capacity has been previously shown in proteinuric models; kidney expression of both mitochondrial and cytosolic SOD was diminished in chronic renal failure induced by 5/6 nephrectomy [29], and also in a salt-induced proteinuric model [30]. Our previous finding of impaired SOD2 response in proximal tubular cells upon exposure to OA-BSA, together with the present observation that an SOD mimetic alleviates renal macrophage infiltration, suggest that nephropathic effects of ABFA are at least partly a consequence of impaired SOD-mediated anti-oxidant responses.

Importantly with both tempol and rotenone, significant alleviation of renal inflammation was achieved despite the fact that neither medication significantly reduced proteinuria. This indicates that the beneficial effects were not due to an indirect effect of reduced tubular traffic of FA and protein, but reflected specific drug action on oxidative stress, and also underscores that oxidant stress is a key mediator of proteinuria-induced inflammatory effects. As neither drug achieved total prevention of macrophage influx, alternative pro-inflammatory pathways apart from oxidant stress are operational in the pathophysiologic process. Even so, the present observations raise the possibility that during proteinuric diseases, targeted measures to strengthen oxygen radical dismutation capacity, and to counter specific sources of oxidant stress, may provide significant anti-inflammatory benefits, adding to and complementing the advantages of proteinuria reduction therapy.

To determine potential treatment targets, it is vital to identify the source of increased oxidative stress. Previous studies have focused on NADPH oxidase, which has been implicated as a major source of renal oxidant stress in models like nitric oxide synthase (NOS) inhibition [31], high salt intake [30], and renal ablation [29]. However, *in vitro*, ABFA provoked increased ROS from mitochondria, and not via NADPH oxidase, xanthine oxidase, or uncoupled NOS [19]. In the present study rotenone reduced ABFA-induced renal macrophage influx without significantly affecting proteinuria, supporting that increased mitochondrial ROS per se plays a pathophysiologic role in the inflammatory process. Increased mitochondrial oxidant stress has been identified as a key element in the

pathogenesis of systemic complications in diabetes [32], and mutant mice lacking mitochondrial SOD suffer devastating consequences including increased oxidant damage and severely shortened life span [33, 34]. Conversely, genetically modified mice that are specifically highly resistant to mitochondrial oxidant stress have significantly longer life span, less oxidant damage, and delayed cardiac lesions when compared with wild type mice [35]. Thus, strategies to reduce mitochondrial oxidant stress may prove valuable in proteinuric disease.

In general, outcomes of antioxidant therapy in relieving renal injury have been mixed. For example, probucol and vitamin E failed to relieve interstitial inflammation in puromycin aminonucleoside nephrosis [36], but a combination of the two drugs in hypercholesterolaemic rats was partly successful, alleviating renal fibrosis but not interstitial inflammation [37]. Beneficial effects of antioxidants were observed using the ROS scavenger edaravone in renal ischaemia [38], and tempol in a renal ablation model [29] and in aldosterone/salt-treated rats [27], although specific impact on tubulointerstitial inflammation were not assessed in these reports. In the present study, SOD supplementation with tempol was more effective in reducing inflammation than mitochondrial blockade, highlighting that success depends on specific understanding of the most appropriate points of intervention within the redox signalling networks in each disease model.

We examined plasma lipid profiles following OA-BSA treatment, in the presence or absence of intervention agents. OA-BSA exposure led to an expected increase in plasma NEFA. However, OA-BSA also reduced plasma cholesterol. This might have been due to reduced hepatic cholesterol synthesis, and probably reflects an unfavourable fall in HDL cholesterol. Kaysen and colleagues have demonstrated that increasing albumin levels in nephrotic rats (by infusing albumin) reduces hepatic apolipoprotein synthesis [39]. Tempol, administered alone or along with OA-BSA, had no significant effect on plasma lipids. With rotenone, the most notable effect was that it increased plasma triglycerides independent of OA-BSA treatment. The reason for this is not clear, but it is conceivably related to inhibition of mitochondrial fatty acid oxidation. Although rotenone was otherwise well tolerated by mice in this study, this finding highlights the point that strategies to reduce mitochondrial oxidant stress require careful balancing of potential benefits against the possible risks of suppressing essential mitochondrial functions.

There is on-going discourse about the merits of hypolipidaemic treatment in progressive renal disease [40-42]. In this study, we used a sustained-release nicotinic acid (NA) derivative in an attempt to selectively focus on the specific impact of NEFA-lowering. NA is an established anti-lipolytic agent [43, 44] that is now thought to work via a G-protein-coupled receptor [43]. As expected, NA ameliorated the increase in NEFA induced by OA-BSA treatment. This effect was accompanied by a significant reduction in the renal inflammatory cell influx provoked by OA-BSA. Unexpectedly but interestingly, NA also powerfully reduced proteinuria. Thus, it is not clear whether the anti-inflammatory effect of NA was due to plasma NEFA reduction or to the suppression of proteinuria. Anti-proteinuric effects were previously observed following the use of a different NA formulation in human subjects [45]. In terms of plasma lipids, apart from reducing NEFA, NA also induced a rise in plasma cholesterol that was probably due to an increase in HDL cholesterol [46], and thus a beneficial influence. Together, the observed effects raise the possibility that sustained-release NA may be of therapeutic value in proteinuric models. Further studies will be needed to clarify its effects on proteinuria vis-à-vis the anti-inflammatory impact.

In conclusion, the present study adds to the data demonstrating the renal inflammatory impact of albumin-bound fatty acids, independent of the effect of albumin itself. Our findings indicate that ABFA-induced inflammation is highly dependent on oxidative stress that is partly of mitochondrial origin. SOD supplementation powerfully suppressed inflammation provoked by ABFA. Blockade of mitochondrial ROS production produced a similar, although less powerful effect. Beneficial effect of nicotinic acid, a lipolytic agent, included reduction of both proteinuria and renal inflammation.

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Chapter 5

Resistance to oxidative stress by chronic infusion of angiotensin II in mouse kidney is not mediated by the AT_2 receptor

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Abstract

Wild-type mice are resistant to ANG II-induced renal injury and hence form an attractive model to study renal defense against ANG II. The present study tested whether ANG II induces expression of antioxidative genes via the AT_2 receptor in renal cortex and thereby counteracts prooxidative forces. ANG II was infused in female C57BL/6J mice for 28 days and a subgroup received AT_2 receptor antagonist (PD-123,319) for the last 3 days. ANG II induced hypertension and aortic hypertrophy; proteinuria and renal injury were absent. Urinary nitric oxide metabolites (NO_x) were decreased, and lipid peroxide (TBARS) excretion remained unchanged. Expression of NADPH oxidase components was decreased in renal cortex but induced in aorta. Heme oxygenase-1 (HO-1) was induced in both renal cortex and aorta. In contrast, ANG II suggestively increased AT_2 receptor expression in kidney but not in aorta. AT_2 receptor blockade enhanced hypertension in ANG II-infused mice, reversed ANG II effects on NO_x excretion, but did not affect TBARS. Despite its prohypertensive effect, expression of prooxidative genes in the renal cortex decreased rather than increased after short-term AT_2 receptor blockade and renal HO-1 induction after ANG II was normalized. Thus chronic ANG II infusion in mice induces hypertension but not oxidative stress. In contrast to the response in aorta, gene expression of components of NADPH-oxidase was not enhanced in renal cortex. Although ANG II administration induced renal cortical AT_2 receptor expression, blockade of that receptor did not unveil the AT_2 receptor as intrarenal dampening factor of prooxidative forces.

Introduction

Angiotensin II (ANG II) is a pivotal physiological regulator of blood pressure and cardiovascular and renal cell growth [1]. An excess of ANG II or unopposed actions of ANG II [2], however, can induce hypertension, cardiac hypertrophy and remodeling, heart failure, vascular hypertrophy, atherosclerosis, and profound renal damage [3]. Most of these actions are mediated via the AT_1 receptor [4], whereas the AT_2 receptor seems to oppose many of these processes. There is now strong evidence that actions of ANG II are exerted via generation of superoxide ($O_2^{-}\cdot$) and are opposed by nitric oxide (NO) [2, 5]. Several of the actions of the AT_2 receptor are mediated by stimulating NO synthesis [6].

Prooxidative actions of ANG II have been extensively studied in endothelial cells and vascular smooth muscle cells. Both cell culture studies and animal experiments indicate that ANG II induces $O_2^{-}\cdot$ by enhancement of NADPH-oxidase [7-9]. The kidney clearly is a target organ for the physiological actions of ANG II (i.e., blood pressure regulation) and for ANG II-mediated inflammation and cell damage. Nevertheless, pro- and antioxidant responses to ANG II in the renal cortex are less clear. Induction of components of NADPH-oxidase was found after 1 wk of ANG II infusion in rats [10]. Renal tubular cells exposed to ANG II can modulate the cell cycle via NADPH-oxidase generated $O_2^{-}\cdot$ [11]. Information about antioxidant forces opposing ANG II-induced oxidative stress, however, is limited.

Chronic NO synthase (NOS) inhibition leads to profound renal damage in rats [12-14] but not in mice [15]. The effects of NOS inhibition in rats can be completely prevented by AT_1 receptor blockade, indicating that damage is caused by unopposed actions of ANG II [2]. We therefore reasoned that mice might well be relatively resistant to ANG II. The hypothesis of the present study is that elevated ANG II levels induce expression of antioxidative genes via the AT_2 receptor in the mouse renal cortex and thereby counteract prooxidative forces. To address this, we first studied renal cortical and aortic expression of prooxidative genes, the AT_2 receptor, heme oxygenase-1 (HO-1) [16], and NOS isoforms during chronic ANG II infusion in female mice. ANG II induced expression of components of NADPH oxidase in the aorta, but failed to do so in the kidney. HO-1 and the AT_2 receptor were both induced in kidney cortex by ANG II infusion; however, only HO-1 was induced in the aorta. These findings suggested that in the kidney, the angiotensin AT_2 receptor mediated the defense against prooxidative gene expression. Therefore, we compared the impact of AT_2 receptor blockade on pro- and antioxidative genes in renal cortex and aorta in a second protocol.

Methods

Animals. Female C57BL/6J mice (12 wk of age, 18 to 22 g; Charles River Laboratories, Maastricht, The Netherlands) received a standard diet containing 0.6% NaCl (wt/wt; RMH-TM; Hope Farms, Woerden, The Netherlands) and had free access to acidified tap water. Mice were housed in groups at 22°C, 60% humidity with a 12:12-h light-dark cycle. During the experiment, sentinel mice were regularly monitored for infection by nematodes and pathogenic bacteria, as well as antibodies to rodent viral pathogens. The Animal Ethical Committee of the University of Utrecht approved the protocols.

Infusion protocols. At 12 wk of age, the mice were trained for the tail cuff procedure. At 14 wk, all mice had reached a body weight of 20 g, the minimum requirement for subcutaneous implantation of osmotic minipumps (Alzet 2004; Charles River Laboratories). Mice were anesthetized with isoflurane and the minipumps were implanted subcutaneously in the nape of the neck and housed individually for 3 days after the operation. In the first experiment, mice were continually infused with saline (control; $n = 6$) or a low (Low-ANG II; $n = 5$), medium (Med-ANG II; $n = 5$), or high (High-ANG II; $n = 5$) dose of ANG II (0.5, 1.0, and 1.5 mg·kg body wt^{-1}·day^{-1}, respectively; Sigma, Zwijndrecht, The Netherlands) for 28 days. Separate groups were also infused with Med-ANG II for 1 and 9 days (both $n = 5$). Osmotic minipump Alzet 1003D and Alzet 1002 were used for 1- and 9-day infusions, respectively. In the second experiment, ANG II was infused at the medium dose (1.0 mg·kg body wt^{-1}·day^{-1}) for 28 days. Control mice were infused with saline. Half of the control and ANG II-infused mice were treated with PD-123,319 (PD; an AT_2 receptor antagonist; Parke-Davis, Ann Arbor, MI) twice a day (50 mg·kg body wt^{-1}·12 h^{-1}), beginning at *day 25*. Each treatment group had five mice. ANG II and PD-123,139 were both dissolved in a sterile solution of 0.9% (wt/vol) NaCl with 0.01 N AcOH. The final concentration of PD-123,319 was 10 mg/ml.

Systolic blood pressure. Systolic blood pressure (SBP) was measured using the tail cuff method (IITC, San Diego, CA) before the implantation procedure and at regular intervals during the rest of the experiment. Urine collections were obtained at regular intervals by placing the mice in metabolic cages for 14 h. During urine collection, mice had access to water containing 2% D-glucose. To prevent degradation of NO metabolites, urine was collected in tubes containing Antibiotic Antimycotic Solution (Sigma).

Tissue collection. At the end of the infusion period, mice were anesthetized with an intraperitoneal injection of 0.1 ml KXA (46.7 mg/ml ketamine, 8 mg/ml xylazine, and 0.067 mg/ml atropine) and blood was collected from the retroorbital plexus for plasma isolation and hematocrit measurement in triplicate per individual. Kidneys and aorta were excised and weighed. Renal cortex from both kidneys was sliced off the poles with a scalpel. The middle part of one kidney was stored in 4% formaldehyde for histological evaluation, and the middle part of the other kidney was processed for BoC11 measurements (lipid peroxidation; see below). Tissues not used for histology or lipid peroxidation were immediately snap-frozen and stored at –80°C.

RNA isolation from renal cortex. Frozen renal cortex (20–40 mg) was homogenized in 1 ml of TRIzol reagent (Invitrogen Life Technologies, Breda, The Netherlands) using 1-mm glass beads in a Mini Bead Beater (Merlin Diagnostic Systems, Breda, The Netherlands) for 20 to 30 s. Total RNA was extracted from the TRIzol solution using the procedure recommended by the supplier. Total RNA was finally dissolved in 50 µl of distilled H_2O and stored at –80°C. Total RNA yields were determined on a UV Mini-1240 spectrophotometer (Shimadzu Deutschland, Duisburgh Germany). The quality of the RNA samples was determined with the BioAnalyzer (Agilent, Amstelveen, The Netherlands) using the Eukaryote Total RNA Nano Assay.

RNA isolation from aorta. Frozen aortas (3–9 mg) were embedded in Tissue-Tek (Sakura Finetek Europe, Zoeterwoude, The Netherlands) and quickly frozen in liquid nitrogen. Cryo-static 5-µm slices were dissolved in 1 ml of TRIzol. Further procedures were as mentioned above. Total RNA was dissolved in 15 µl of ddH_2O.

Semiquantitative and real-time quantitative PCR. Total RNA isolated from the samples was pooled per group in equal amounts per subject. RT was done in batches of 5 µg total RNA for renal cortex and 1 µg for aorta; 1 µg of random hexanucleotide was added to total RNA in an end-volume of 11 µl. The solution was heated to 70°C for 10 min. Then, the RT was performed in 30 µl of reaction volume containing 500 µM dNTP (Ambion Europe, Huntingdon, UK), 20 U RNaseOUT recombinant ribonuclease inhibitor, 1x 1st-strand buffer, 10 mM DTT, 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 cooled on ice. The cDNA samples were stored at –20°C. PCR was performed in a 50-µl reaction volume containing cDNA, 10 mM Tris·HCl (pH 8.3), 50 mM KCl, 200 µM dNTP, 1.5 mM $MgCl_2$, 1.25 U *Taq* DNA

Polymerase (Invitrogen), and 125 ng sense and antisense primers (Sigma-Genosys, Haverhill, UK). Negative controls contained a PCR mix with cDNA but without polymerase or a PCR mix with polymerase but without cDNA. The primers used for each gene are listed in Table 1.

The PCR reaction was performed with a hot start at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s using 35 or 40 PCR cycles. PCR samples were run on a 2% agarose gel containing edithium bromide (17 μl/l agarose; MP Biomedicals, Irvine, CA). Bio-Rad Chemidoc XRS photographed the gel, and the intensities of the bands were measured and quantified using the program Bio-Rad Quantity One (Bio-Rad Laboratories, Veenendaal, The Netherlands). Results were described as 18S-corrected mean intensities. None of the negative controls showed bands.

Real-time quantitative PCR (qPCR) was performed on aortic and renal cortical samples. *Taq*man Gene Expression Assays (Applied Biosystems, Foster City, CA) were used. The genes studied were the AT_2 receptor (Agtr2, Mm00431727), p47phox (Ncf1, Mm00447921), heme oxygenase-1 (HO-1, Mm00516004), and 18S (18S, Hs99999901). qPCR was performed as recommended by the supplier. In short, duplicate samples of cDNA per individual per gene (50 ng of starting material total for AT_2 receptor, HO-1 and p47 or 80 pg for 18S) were mixed with *Taq*man Universal PCR Mastermix with AmpErase UNG and *Taq*man Gene Expression Assays in an end-volume of 25 μl. qPCR was performed using an ABI 7700 Single Reporter system. The gene threshold was determined by SDS 1.91 (Applied Biosystems).

Lipid peroxidation. Measurement of lipid peroxidation in mouse kidney was performed as described previously in rat kidney [13]. In short, slices of kidney were placed in Krebs-HEPES buffer and the probe BoC11 was added to the buffer in final concentration of 10 μM. After 30 min of incubation, the buffer was refreshed and the BoC11 incorporation and oxidation in the kidney were observed using confocal laser-scanning microscopy (CLSM; Bio-Rad Laboratories, Hercules, CA) equipped with a krypton/argon laser. Slices were imaged in the red and green spectrum (nonoxidized and oxidized probe, respectively). Green and red fluorescence excitations are 488 and 568 nm, respectively, and green and red fluorescence emissions are 530 and 590 nm, respectively. Images were analyzed using Scion imaging software (Scion, Frederick, MD). Lipid peroxidation in each mouse was measured in duplicate.

Table 1. Primer sequences for semiquantitative PCR

Gene	Synonym	Sequence Primer		Mass
		Sense	Antisense	
18S	18S	AGTTGGTGGAGCGATTTGTC	TATTGCTCAATCTCGGGTGG	143
p22phox	p22	TTTCACACAGTGGTATTTCG	CGTAGTAATTCCTGGTGAGG	170
gp91phox	gp91	TCACATCCTCTACCAAAACC	CCTTTATTTTTCCCCATTCT	198
Kidney gp91 isoform	NOX4	GTTTTGGCAAGAAAACAGAC	GAAATAGAACTGGGTCCACA	213
p47phox	p47	AGAACAGAGTCATCCCACAC	GCTACGTTATTCTTGCCATC	247
p40phox	p40	TGGAGATGTGATCTTCCTTC	CTAGCAGGTCTTTGAACAGG	236
p67phox	p67	GGCCAAGTGAAAAACTACTG	GCCTCATAACTGAAGATTGC	167
Rac-2	Rac2	CTCATCTGCTTCTCGCTAGT	AGCTTCTTCTCCTTCAGCTT	167
Xanthine oxidase	XO	TGAGGATCTCTCTCGGAGTA	CGACCTCACTCATCCAGTAT	182
Heme oxygenase 1	HO-1	AACAAGCAGAACCCAGTCTA	GGAGACGCTTTACATAGTGC	154
Heme oxygenase 2	HO-2	CAGGTACTGAAGAAGGTTGC	TCCTCTCTTTGGTCTTCAAA	163
Catalase	CAT	TCCTTTATCCATAGCCAGAA	AACTTGAAGGTGTGTGATCC	179
Nitric oxide synthase 1	NNOS	ACCAATGAGAAAGAGAAGCA	TGGAATAGTAGCGAGGTTGT	181
Nitric oxide synthase 2	INOS	GTCTTGGTGAAAGTGGTGTT	GTGCTTGCCTTATACTGGTC	188
Nitric oxide synthase 3	eNOS	TGGATGAGTATGATGTGGTG	GGATTTTGTAGCTCTTGTGC	177
Superoxide dismutase 1	SOD1	TGCAGGACCTCATTTTAATC	TGCTCTCCTGAGAGTGAGAT	153
Superoxide dismutase 2	SOD2	TTACAACTCAGGTCGCTCTT	GCTGTCAGCTTCTCCTTAAA	178
Superoxide dismutase 3	SOD3	ATGTTGGCCTTCTTGTTCTA	GTGTCGCCTATCTTCTCAAC	152
ANG II receptor type 1A	AT1A-R	GAAGAACAAGCCAAGAAATG	AATACGCTATGCAGATGGTT	191
ANG II receptor type 1B	AT1B-R	TCGAGAACACCAATATCACA	AAGATGTCATCATTCCTTGG	202
ANG II receptor type 2	AT2-R	AGGTGTCCAGCATTTACATC	AGGGATTCCTTCTTTGAGAC	243
Angiotensinogen	AGT	TTTATCCACTGACCCAGTTC	CTGAGAGAAACCTCTCATCG	163
Renin [preprorenin]	REN	GGACACTGGTTCATCCTTTA	TGTCTCTCCTGTTGGGATAC	209
AngII-converting enzyme	ACE	AGCATCACCAAGGAGAACTA	ACTGGAACTGGATGATGAAG	175

Determination of NO_x, thiobarbituric acid-reactive substances, proteins, and creatinine in urine. Urine protein was determined with a Bio-Rad Protein Assay Kit based on the Bradford method (Bio-Rad Laboratories). Creatinine was enzymatically determined with Creatinine F L-Type R1 and R2 (Wako Chemicals, Neuss, Germany). Total NO_x was determined using the Nitrate/Nitrite Colorimetric Assay Kit of Cayman Chemical (ITK Diagnostics, Uithoorn, The Netherlands). For determination of thiobarbituric acid-reactive substances (TBARS), thiobarbituric acid was added to urine samples supplemented with butylhydroxytoluene to prevent auto-oxidation. Samples were boiled for 75 min, followed by measurement at 540 nm at room temperature [17].

Statistics. Results are expressed as means ± SE. Data were compared using one-way ANOVA or two-way repeated-measures ANOVA where appropriate followed by a Student-Newman-Keuls post hoc test. $P < 0.05$ was considered significant. In semiquantitative PCR, the bands of the PCR products were photographed and a program calculated the intensities of the bands. This semiquantitative approach does not allow statistical analysis. Because of this reason, variation in replicates is not included in graphs.

Results

ANG II increased blood pressure and hematocrit and induced hypertrophy of aorta. ANG II infusion dose dependently increased blood pressure (Fig. 1*A*). On *day 10* the SBP of High-ANG II was increased compared with control and Low-ANG II ($P < 0.01$). For control, Low-ANG II, Med-ANG II, and High-ANG II the SBP at *day 26* was 99 ± 3, 103 ± 4, 120 ± 2 ($P < 0.01$ Med-ANG II vs. both control and Low-ANG II), and 140 ± 4 mmHg ($P < 0.01$ vs. all other groups), respectively. No significant elevation of tail cuff pressure was found before *day 10* in any of the ANG II-infused groups. At 1 and 9 days, there was no effect of Med-ANG II on aorta weight. However, at 28 days aorta weight was dose dependently increased ($P < 0.005$ Med-ANG II vs. control and Low-ANG II and $P < 0.005$ High-ANG II vs. control and Low-ANG II and $P < 0.05$ vs. Med-ANG II; Fig. 1*B*). There were no effects on body or kidney weight. Hematocrit was increased in all ANG II-infused mice, even at *day 1*. Hematocrit for Med-ANG II vs. control at *days 1*, *9*, and *28* was 51.1 ± 0.3 vs. 48.0 ± 0.3% ($P < 0.001$), 50.8 ± 0.4 vs. 48.5 ± 0.2% ($P < 0.001$), and 53.1 ± 1.3 vs. 48.6 ± 0.2% ($P < 0.001$), respectively.

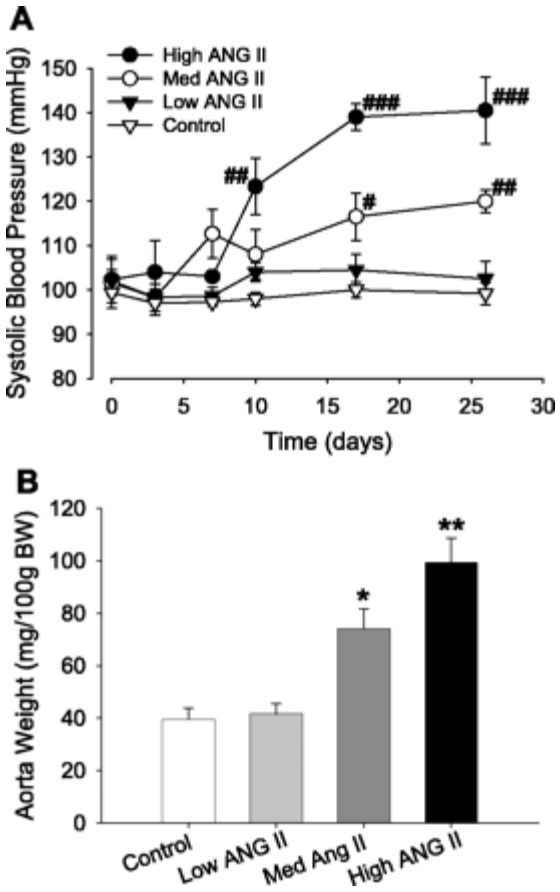

Fig. 1. Systolic blood pressure (***A***) and aortic weight (***B***) of mice infused with saline (n = 6; △), low dose of ANG II (0.5 mg·kg^{-1}·day^{-1}; Low-ANG II; n = 4; ▲), medium dose of ANG II (1.0 mg·kg^{-1}·day^{-1}; n = 5; Med-ANG II; ○), and high dose of ANG II (1.5 mg·kg^{-1}·day^{-1}; High-ANG II; n = 4; ●).

#P < 0.01 vs. control. ##P < 0.01 vs. Low-ANG II and control. ###P < 0.01 vs. all.

*P < 0.005 vs. control and Low-ANG II. **P < 0.005 vs. control and Low-ANG II and P < 0.05 vs. Med-ANG II.

BW, body weight.

Chronic ANG II infusion dose independently decreased urinary NO_x without renal damage. Urine NO_x, TBARS, and protein were determined (Table 2). There were no differences in plasma creatinine and there were no changes in creatinine clearance (data not shown). Urinary excretions were corrected for urinary creatinine excretion. No significant differences were found after 1 and 9 days of Med-ANG II infusion (data not shown). Urinary NO_x were decreased in all doses of ANG II-infused mice at end of infusion period (P < 0.05 vs. control); there were no difference between doses of ANG II. There were no increases in protein or TBARS excretion, and none of the ANG II-infused mice showed histological evidence of renal damage (Fig. 2). There was no change in renal cortical lipid peroxidation in 28-day Med-ANG II mice (n = 4). However, 9-day Med-ANG II mice (n = 4) showed ANG II-induced lipid peroxidation (P < 0.05) compared with control (n = 7) and 1-day Med-ANG II (n = 5; Fig. 3).

Table 2. Urinary NO_x, TBARS, and protein

Treatment	TBARS/Creatinine, µmol/mmol		NO_x/Creatinine, µmol/mmol		Protein/Creatinine, mg/mmol	
	Before	End	Before	End	Before	End
First experiment						
Control (*n* = 6)	1.72±0.24	3.52±0.90	297±62	368±77	0.32±0.06	0.30±0.05
Low-ANG II (*n* = 5)	1.88±0.41	3.64±0.45	274±71	158±60	0.28±0.06	0.34±0.05
Med-ANG II (*n* = 5)	1.94±0.40	3.67±0.36	325±35	183±60	0.39±0.05	0.27±0.03
High-ANG II (*n* = 5)	1.73±0.36	3.96±0.63	404±40	98±70*	0.30±0.02	0.33±0.07

	Control (*n* = 5)	Control + PD (*n* = 5)	ANG II (*n* = 5)	ANG II + PD (*n* = 5)
Second experiment				
NO_x/creatinine, µmol/mmol	444±137	268±31	114±24*	261±64
TBARS/creatinine, µmol/mmol	6.17±1.24	5.12±0.53	6.26±1.52	3.75±0.93
Protein/creatinine, mg/mmol	0.64±0.14	0.50±0.03	0.50±0.07	0.47±0.06

Urinary data before and at the end of infusion period are shown. In the second experiment, urinary data at the end of infusion period are shown. NOx, nitric oxide metabolites; TBARS, thiobarbituric acid-reactive substances; PD, PD-123, 319, the AT2 receptor antagonist.

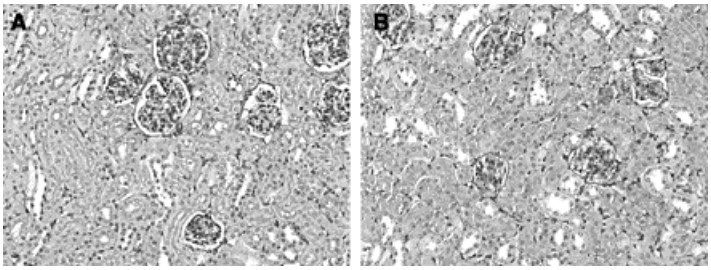

Fig. 2. Renal histology of a control mouse (***A***) and a High-ANG II mouse (***B***). None of the ANG II-infused mice showed any change in histology (PAS stain). Magnification: x100.

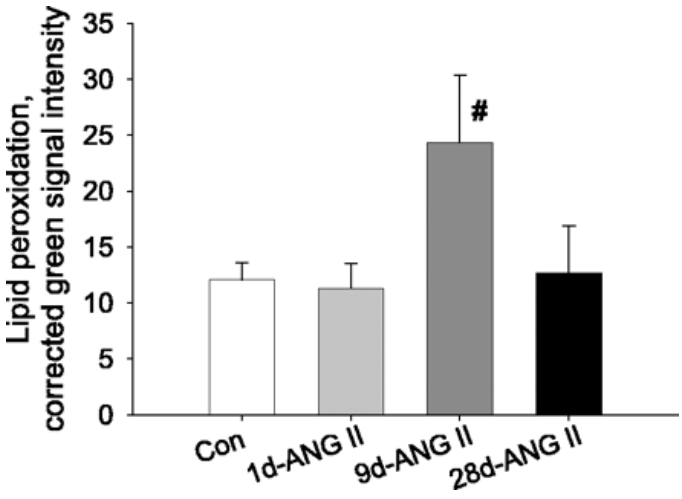

Fig. 3. Lipid peroxidation in renal cortex measured using BoC11 in 1 and 9 days control together (n = 7), 1-day ANG II (1d-ANG II, n = 5), 9 days ANG II (n = 4), and 28 days ANG II (n = 4). Mice infused with ANG II (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$) for 9 days showed increased renal cortical lipid peroxidation. #P < 0.05 vs. all other groups.

Renal cortical and aorta gene expression in chronic ANG II infusion. In Fig. 4, gene expression is shown for pro- and antioxidative genes and for components of the renin-angiotensin system after 28 days of ANG II infusion. The semiquantitative PCR (RT) was performed in triplicate on pooled samples and was corrected using 18S gene expression. Because there was no dose dependency for any of the tested genes, data were pooled for all ANG II doses (n = 11). Prooxidative genes were clearly not induced, the NADPH oxidase components p22 and p47 were decreased, and Rac2 tended to decrease. Expression of HO-1 was increased in this first experiment. Expression of the AT_2 receptor in renal cortex was markedly increased by 50% (Fig. 4*A*) but this was independent of ANG II dose (Fig. 5*A*). AT_2 receptor expression was not induced by the medium ANG II dose up to 9 days, but again was induced at *day 28* (Fig. 5*B*). Quantitative real-time PCR was performed on renal cortex for the AT_2 receptor, HO-1, p47, and 18S (Fig. 5*C*). No significant changes were observed due to great variation between individuals and small sample size per treatment group. When all ANG II data were pooled and compared with control data, the AT_2 receptor or HO-1 was significantly changed (P < 0.05 and P < 0.005, respectively). In the aorta of Med-ANG II mice several prooxidative genes were induced, in particular p47 (Fig. 6, *A* and *B*). HO-1 was also induced during ANG II infusion. In contrast to the kidney cortex, the AT_2 receptor was not induced in the aorta following 28 days of ANG II infusion. The induction of aortic p47 and HO-1 in ANG II-infused mice was confirmed by qPCR (P < 0.05; Fig. 6*C*).

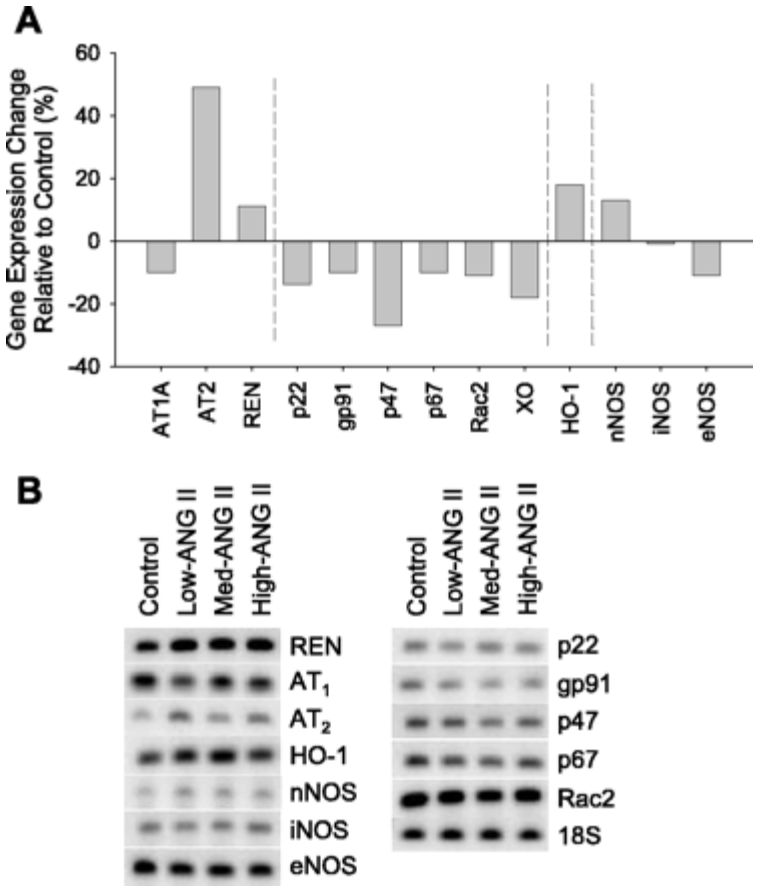

Fig. 4. Gene expression changes in renal cortex at 28 days. Semiquantitative PCR on pooled samples (***A*****;** *n* = 11 for ANG II and *n* = 6 for control), related to 18S gene expression, and compared with control. ***B*****:** representative PCR. Gene synonyms are shown in Table 1.

AT_2 receptor activation antagonizes ANG II-induced hypertension. In the second experiment, we attempted to dissect the function of secondary AT_2 receptor expression in the renal cortex. PD was administered during the final 3 days of ANG II infusion when blood pressure had reached a plateau. Once again, ANG II infusion significantly increased SBP (Fig. 7) at *day 25* compared with control (105 ± 5 vs. 91 ± 3 mmHg, $P < 0.01$). At *day 28* of continued ANG II infusion, SBP had not increased further (106 ± 4 mmHg). PD treatment significantly increased blood pressure in ANG II-infused mice, but not in control mice. ANG II mice treated with PD had SBP of 104 ± 3 mmHg at *day 25*, before initiation of PD treatment and 117 ± 4 mmHg ($P < 0.01$ vs. *day 25* and $P < 0.05$ vs. ANG II alone *day 28*) after 3 days of PD treatment. Hematocrit was elevated in ANG II-infused mice (53.1 ± 1.3 vs. 48.6 ± 0.2; $P < 0.001$) and was not affected by AT_2 receptor blockade (52.7 ± 0.6 vs. 45.8 ± 1.0; $P < 0.001$).

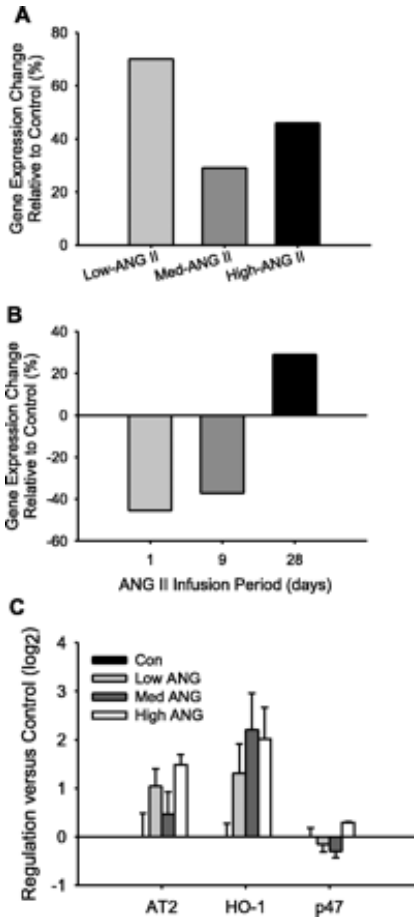

Fig. 5. Dose- (***A***) and time-dependent (***B***) effects of ANG II on expression of the AT_2 receptor gene in the renal cortex. Time-dependent effects were measured in response to Med-ANG II. ***C*****:** quantitative PCR was performed for renal cortical genes of AT_2 receptor, HO-1, p47, and 18S.

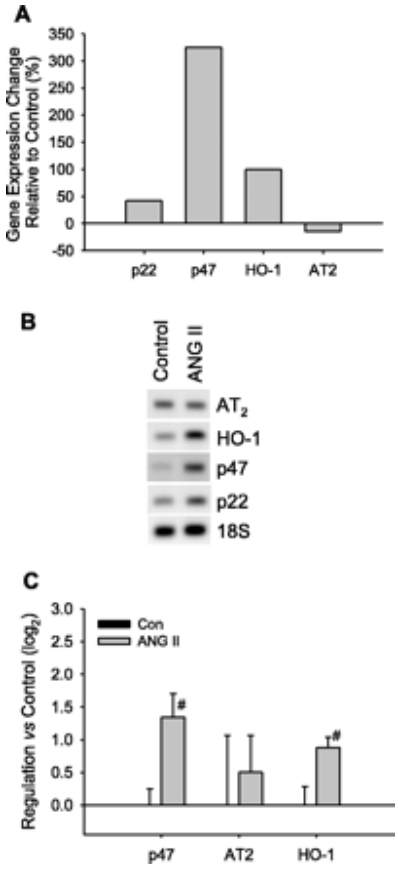

Fig. 6. Aortic gene expression at 28 days in ANG II-infused (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$) mice ($n$ = 5, control n = 6; ***A***). Note that, in contrast to enhanced expression in renal cortex following 28 days of ANG II infusion, the AT_2 receptor was not induced in the aorta. ***B*****:** representative PCR of these genes. *C*: quantitative PCR confirmed the induced gene expression of p47 and HO-1 in ANG II infused mice. #P < 0.05 vs. control.

Urinary NO_x was not significantly affected by AT_2 receptor antagonist. NO_x, TBARS, and protein measured in urine in the second experiment are listed in Table 2.

Similar to the first experiment, NO_x was decreased in ANG II-infused mice vs. control on *day 28* ($P < 0.05$), and this was partially corrected by PD (not significant vs. control). PD did not enhance TBARS in either the ANG II or the control group.

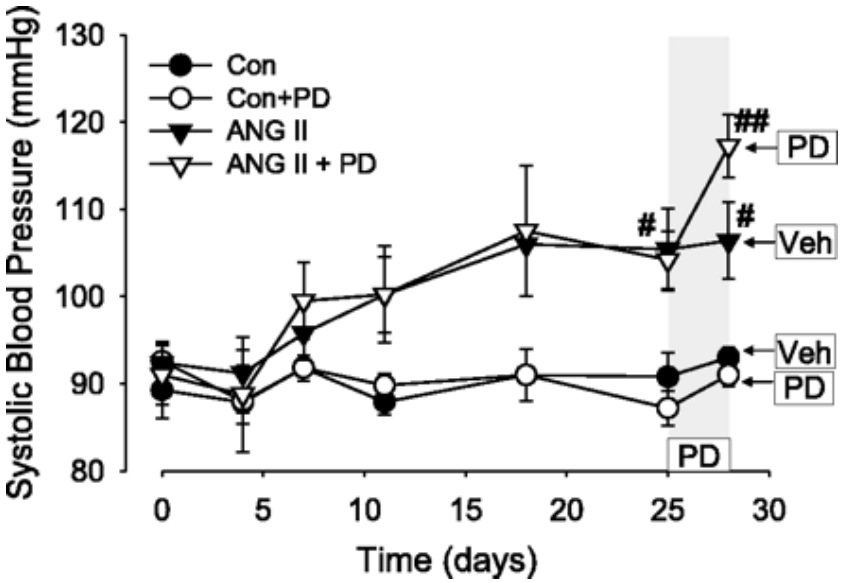

Fig. 7. Systolic blood pressure in ANG II-infused mice (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$ for 28 days) with (open symbols) and without (filled symbols) AT_2 receptor blockade (PD; 100 $mg \cdot kg^{-1} \cdot day^{-1}$) for the last 3 days of the experiment. Mice were infused with saline (circles, both $n = 5$) or ANG II (triangles, both $n = 5$). #$P < 0.01$ vs. Con±PD on same day. ##$P < 0.001$ vs. 28d-Con±PD (same day) and $P < 0.05$ vs. 28d-ANG II and 25d-ANG II±PD.

Responses of renal cortical and aortic gene expression to AT_2 receptor antagonist. In the second experiment, gene expression of components of the renin-angiotensin system, of NADPH oxidase, and of HO-1 was evaluated in control mice ($n = 10$) and in mice infused with Med-ANG II ($n = 10$), with and without concomitant treatment with PD from *day 25* to *day 28* ($n = 5$ for each group). Gene expression in renal cortex was performed in duplicate on pooled samples, corrected by 18S, and compared with control. Similar to the first experiment, chronic ANG II infusion induced expression of HO-1 and nNOS (Fig. 8*A*) and depressed expression of p22 and Rac2 (Fig. 8*B*). Remarkably, inhibition of the AT_2 receptor led to decreased expression of all tested prooxidative genes except XO in controls and accentuated decreased expression of many prooxidative genes (gp91,

NOX4, p47, and XO) in ANG II-infused mice (Fig. 8*B*). Thus antagonizing the AT_2 receptor did not induce but decreased the expression of prooxidative genes. In control mice, AT_2 receptor blockade led to depressed inducible NOS (Fig. 8*A*). PD cotreatment in ANG II-infused mice normalized HO-1 expression and decreased inducible NOS and endothelial NOS, but not neuronal NOS. Representative PCR of some of these genes is shown in Fig. 8*C*. Quantitative real-time PCR showed gene induction of HO-1 by ANG II ($P < 0.001$ vs. control), which was normalized by AT_2 receptor blockade ($P < 0.05$; Fig. 8*D*).

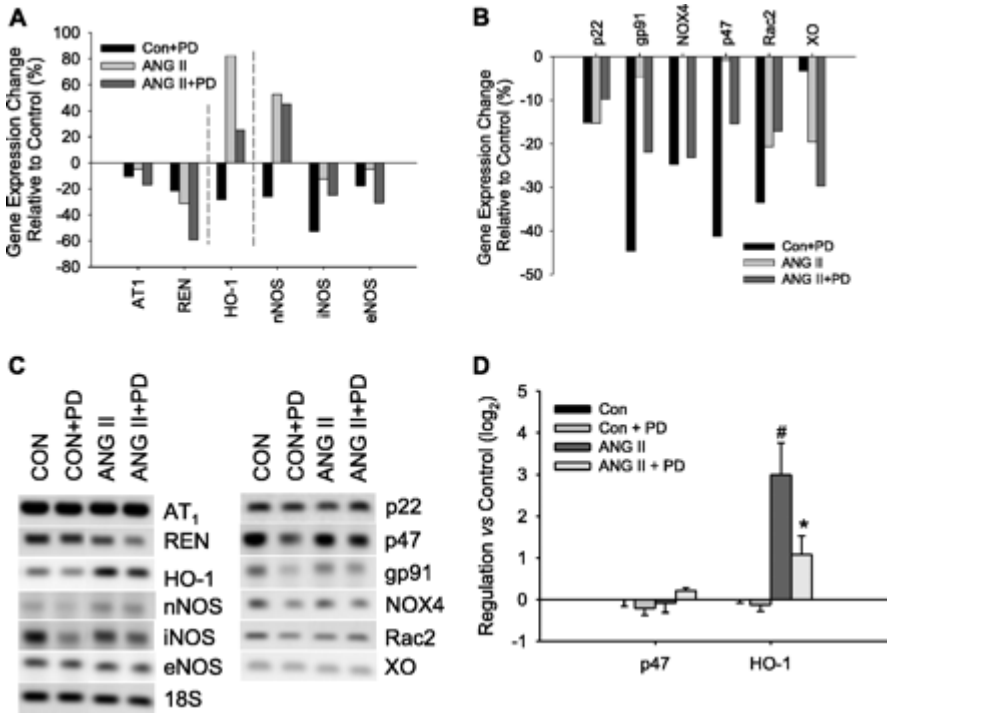

Fig. 8. Renal cortical gene expression of components of the renin-angiotensin system, NADPH oxidase, and HO-1 in control mice (Con; $n = 10$) and in mice infused with ANG II (1.0 $mg \cdot kg^{-1} \cdot day^{-1}$; $n = 10$), with and without treatment with the AT_2 receptor antagonist PD (100 $mg \cdot kg^{-1} \cdot day^{-1}$; $n = 5$ for each group) corrected by expression of 18S and compared with 28 days control ($n = 5$; ***A***). Of the 6 prooxidative genes, 3 (p22, Rac2, and XO) were decreased in ANG II-infused mice. However, after concomitant AT_2 receptor blockade in ANG II-infused mice, all 6 prooxidative genes decreased (***B***). The impact of AT_2 receptor blockade on the reduction of prooxidative genes was much greater in control than in ANG II mice. ***C*:** representative PCR. Quantitative PCR showed gene induction of HO-1 in ANG II-infused mice and was normalized by AT_2 receptor blockade. #$P < 0.001$ vs. control. *$P < 0.05$ vs. ANG II (***D***).

As mentioned, in the aorta prooxidative genes were induced in ANG II-infused mice (Fig. 9, *A* and *B*). Interestingly, expression of these genes decreased during AT_2 receptor blockade (ANG II+PD), being that expression remained higher than in mice treated with AT_2 receptor antagonist only (control+PD). Aortic HO-1 induction by ANG II infusion was not affected by AT_2 receptor blockade.

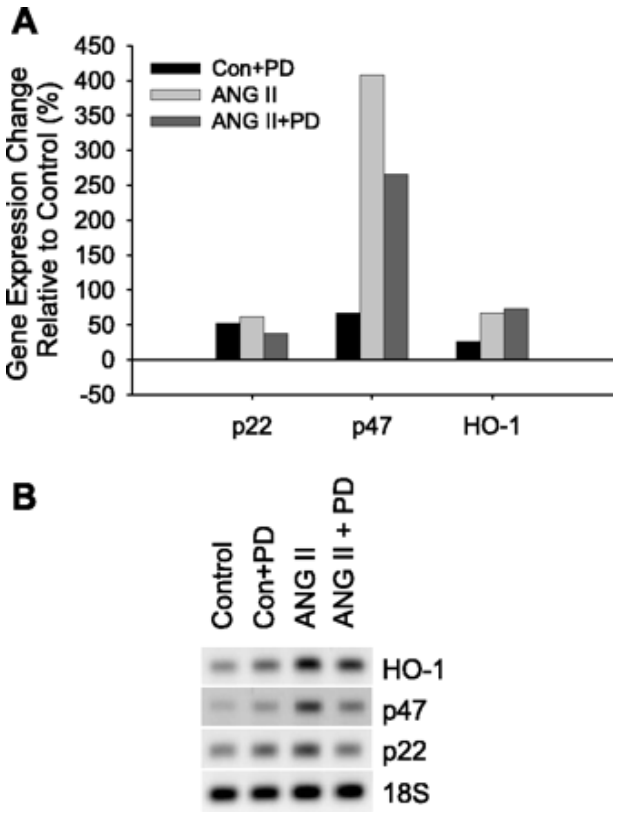

Fig. 9. Gene expression changes in aorta (semiquantitative PCR). The genes p22, p47, HO-1, and AT_2 receptor in control+PD (*n* = 3), 28 days ANG II (*n* = 3) and ANG II+PD (*n* = 3), corrected by 18S, compared with 28 days control (*n* = 3; ***A***). Chronic ANG II infusion markedly increased p47. AT_2 blockade in control induced p22 and p47, but in ANG II mice these genes were decreased (vs. ANG II only). HO-1 was induced in ANG II-infused mice and did not change during AT_2 receptor blockade. AT_2 receptor was induced slightly in AT_2 receptor blocked ANG II but not control mice. ***B*****:** representative PCR.

Discussion

Knowledge about the protection of the kidney from hypertensive damage is limited. While glomerular hypertension has been recognized to be pivotal for the development of damage, prooxidative forces are also becoming recognized as relevant players. Interestingly, mice seem to be relatively protected from damage caused by hypertension [15] or renal ablation [18, 19]. In the present study, mice chronically infused with different doses of ANG II clearly show dose-dependent changes in SBP and hypertrophy of the aorta. Yet,

creatinine clearance was normal, proteinuria was absent, and there was no detectable histological damage in the kidney, even at the highest dose of ANG II. Gene expression of NADPH-oxidase components and xanthine oxidase (XO) was not induced in the kidney during ANG II infusion, but upregulation of the AT_2 receptor, HO-1 and nNOS was observed. In contrast, expression of NADPH-oxidase components p22 and p47 was induced in the aorta. Thus the mouse kidney seems to be strongly protected against elevated ANG II, and hypertension and damage are dissociated. This is quite different from the rat where ANG II infusion induces pressure-dependent injury [20, 21]. To dissect the direct effects of ANG II and systemic hypertension, an angiotensin-independent hypertension model (e.g., norepinephrine) would be required, but that was not necessary in the present study as low dose of ANG II did not result in hypertension or any damage but resulted in the same gene responses and urinary NO_x excretion as higher doses of ANG II.

Initiation of oxidative stress under conditions where ANG II is increased appears to involve activation of NAPDH oxidase [22]. Components of this $O_2^{-}\cdot$-generating complex are transcriptionally induced, in particular p22 in vascular smooth muscle cells [23], and p47 in vascular smooth muscle cells [24] and endothelial cells [25, 26]. Evidence for this mechanism is particularly solid in (components of) the vascular wall, and data are now available that ANG II may induce NADPH-oxidase in the kidney as well [11]. The first step in the present study was to evaluate the transcriptional induction of components of the NADPH-oxidase in the kidney and vasculature. In line with previous observations, expression of the p22 and p47 components of NADPH-oxidase was increased in the mouse aorta after ANG II infusion. In contrast, ANG II did not regulate p22 and p47 in the renal cortex. Oxidative stress, assessed by TBARS excretion in the urine, was not altered after 28 days of ANG II infusion, despite persistent increases in blood pressure. Closer evaluation of oxidative stress by use of the probe BoC11, which is sensitive for lipid peroxidation [13], revealed only slight increased oxidative stress at *day 9* that was not sustained. Thus blood pressure on the one hand, and damage and oxidative stress on the other, was dissociated. This seems to corroborate the view that oxidative stress is an essential key pathogenic factor for the establishment of hypertension-related renal injury. This should not be overinterpreted in the sense that renal injury cannot occur in the absence of oxidative stress during ANG II-induced hypertension. Testing this would require, for instance, a p47phox knockout mouse.

Which factors in the kidney could have dampened the prooxidative forces of ANG II, which were clearly observed in the aorta? Several candidates were considered: NOS, HO-

1, or the angiotensin AT_2 receptor. There is ample evidence in rats that the renal vasoconstrictive and sodium-retaining actions of ANG II are opposed by NO [2, 5, 27, 28]. Furthermore, L-arginine supplementation has been shown to completely inhibit the salt loading-induced induction of p47 and gp91 in the kidney of Dahl-sensitive rats [29]. In contrast to these indications that NO could oppose the actions of ANG II, NO_x excretion decreased rather than increased in chronically ANG II-infused mice in the present study. Furthermore, only renal cortical nNOS gene expression seemed increased, which is unlikely a sufficient explanation for a global dampening of NADPH-oxidase, as cortical expression of nNOS is limited to the juxtaglomerular apparatus [30-33] and NADPH-oxidase is expressed widely in the tubular system [34]. Together, these findings suggest that NO is not primarily responsible for renal protection in ANG II-infused mice.

HO-1 and the AT_2 receptor were also considered as suitable candidates for renal protection against ANG II-induced damage. HO-1, which can be induced by hypertension [35], can attenuate ANG II-mediated increase in oxidative injury in the thick ascending loop of Henle [36] and is able to reduce the pressor responsiveness to ANG II [37]. HO-1 is important for protection of renal [38, 39] and vascular systems against hypertensive injury [40, 41]. In line with this, HO-1 was upregulated in the rat by ANG II infusion, inhibition of HO-1-exacerbated injury, while induction of HO-1 was protective [16]. In the present study, ANG II administration seemingly induced HO-1 gene expression in the renal cortex and was also induced in the aorta. Systemic ANG II administration indicated an induction of gene expression of the AT_2 receptor in the renal cortex; however, it did not affect AT_2 receptor gene expression in the aorta. Although to the best of our knowledge aortic AT_2 receptor expression has not been studied during ANG II-induced hypertension, ANG II infusion in rats has been shown to increase AT_2 receptor expression in (mesenteric) resistance arteries [42]. Nevertheless, in the present study, the AT_2 receptor was induced only in renal cortex after 28 days, while HO-1 was upregulated in both aorta and kidney. This differential response suggests that a secondary increase in the AT_2 receptor could be an important component of endogenous renal defense in the mouse, as only the aorta showed injury, namely, hypertrophy, suggesting that the AT_2 receptor perhaps specifically counteracted prooxidative actions of ANG II in the kidney (and hence conferred renal protection).

This led to the second experiment in which AT_2 receptor blockade was applied to observe the functional aspect of the AT_2 receptor. AT_2 receptor blockade was applied at the end of the chronic infusion for several reasons: *(1)* renal cortical AT_2 receptor gene

expression was lower after 1 and 9 days than after 28 days of ANG II infusion (Fig. 4*B*); *(2)* renal lipid peroxidation was increased after 9 days of ANG II infusion (Fig. 2) but was normalized after 28 days in ANG II-infused mice, suggesting the presence of a renal protective force at the end of chronic infusion; and *(3)* elevated SBP at end of the chronic ANG II infusion was stable, suggesting establishment of a new balance between pro- and antioxidative forces, for which the AT_2 receptor might be partly responsible. Administration of the AT_2 receptor antagonist PD-123,319 after 25 days of ANG II infusion exacerbated hypertension, while in control mice blood pressure did not change. This supports the concept that AT_2 receptor activation is a secondary effect because for up to 21 days of administration, effects of ANG II on blood pressure were reported to be unaffected by concomitant PD administration [43-45]. The blood pressure response is a clear indication that, at least for systems controlling blood pressure, induction of gene expression of the AT_2 receptor is accompanied by a functional response.

AT_2 receptor inhibition did not affect the expression of p22 in the renal cortex and the aorta; however, it led to an unexpected decreased expression (by RT-PCR) of p47, gp91, Nox4, and Rac2 in the kidney, in both control and ANG II-infused mice. TBARS excretion displayed a tendency to decrease during concomitant administration of ANG II and AT_2 receptor antagonist. These findings suggest that the AT_2 receptor is not the specific renal protection factor in this model. On the contrary, the observation that AT_2 receptor blockade was followed by a decreased expression of prooxidative genes suggests that in the renal cortex the net effect of the AT_2 receptor may even be prooxidative. This is in discordance with a study in rats showing that administration of a AT_2 receptor antagonist during 1 wk of low-dose ANG II infusion increased urinary excretion of markers of oxidative stress, malonyl-dialdehyde and 8-iso-prostanes, and increased expression of the NADPH-oxidase components p22, p67, and the gp91 isoform NOX1 [10]. Besides methodological differences (species, different dosage, and duration of ANG II administration), blood pressure was strongly increased in the current experiments and the AT_2 receptor was suggestively induced. Another option is that the AT_2 receptor transmitted a prooxidative signal through NF-κB. High-dose ANG II infusion in mice has been shown to induce NF-κB in the renal cortex, a process that could be completely inhibited by concomitant AT_2 receptor blockade [46, 47]. Similarly, NF-κB activation could be inhibited by an AT_2 receptor antagonist in the kidney-derived cell line Cos7 [48]. The other view that can be derived from these experiments is that the AT_2 receptor induced an oxidative force that activated NF-κB, which in turn activated the transcription of HO-1 [49, 50]. It has been shown previously that ANG II

induces HO-1 in the aorta [35] and kidney [16]. The present data support that under particular circumstances, this is mediated, at least in part, via the AT_2 receptor, as AT_2 receptor blockade inhibited the induction of HO-1 in renal cortex. Many questions around AT_1/AT_2 and redox balance remain unanswered. The present data suggest that the scheme wherein the AT_2 receptor simply opposes actions of the AT_1 receptor is too simple [51].

Interesting in the present study is also that hypertension and oxidative stress do not seem to correlate. The report that chronic ANG II infusion in rats induced oxidative stress and that concomitant infusion of recombinant heparin-binding superoxide dismutase ameliorates the increase in blood pressure [23] has introduced the idea that (part of) the actions of ANG II are mediated via the generation of $O_2^{-}\cdot$. Accordingly, inhibition of assembly of p47 with gp91 [52] and deletion of p47 expression [25] both alleviate ANG II-induced hypertension in mice. Moreover, blood pressure was normalized in rats with two-kidney, one-clip Goldblatt hypertension by administration of the SOD mimetic tempol [53]. Our data clearly show that conventional signaling of ANG II may induce hypertension without increased oxidative stress caused by NADPH-oxidase. Others have found hypertension during ANG II infusion much earlier than we did in the present study [54]. These studies used male mice or other strains (e.g., 129/Sv), which were more sensitive to ANG II. Note that in the present study hematocrit was already elevated after 1 day of ANG II infusion, indicating that the osmotic minipumps were effective within 1 day.

Taken together, the present study shows that infusion of ANG II in wild-type female mice induces hypertension, but not oxidative stress. In contrast to the aorta, gene expression of components of NADPH-oxidase was not enhanced in the renal cortex. The angiotensin AT_2 receptor tended to be induced in the kidney; however, blockade of that receptor did not reveal this to be the factor that dampened prooxidative forces. Thus the factor responsible for renal protection during chronic ANG II administration in female mice remains unknown. A possible candidate is HO-1 but this remains to be tested.

Perspectives

Without doubt unopposed actions of ANG II will lead to target organ damage. The mouse model, rather than frustrating us because of lack of renal injury, could form a valuable source of information about mechanisms that can be activated in response to a primary noxious agent. In this light, experiments in mice can lead to insights into protective systems

that potentially can be manipulated in a beneficial direction in other species including humans. Besides, the experiments indicate that the downstream actions of the AT_2 receptor may be highly species dependent; in mice the renal cortical AT_2 receptor is potentially prooxidative, which raises the question whether conditions exist in humans where use of AT_1 receptor antagonists may be disadvantageous.

Grants

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Chapter 6

Oxidant stress in obstructive nephropathy - a literature review and pilot study

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Part I: Literature review

Inflammation in UUO

Unilateral ureteral obstruction (UUO) is an experimental model of renal injury that mimics the complex pathophysiology of chronic obstructive nephropathy in an accelerated manner. Within the first week of induction, a network of inflammatory, vasoactive and apoptotic processes result in the rapid appearance of signs of tubular atrophy and features of tubulointerstitial fibrosis. Tubulointerstitial infiltration of leukocytes, predominantly macrophages [1], is a particularly early, prominent and crucial event at the onset of UUO, helping to lay the foundation for all subsequent developments. Increased numbers of macrophages are observed as early as four hours after UUO in rats [1, 2]. Leukocyte recruitment after UUO is mediated by fast local renal cellular responses of resident kidney cells [3], involving increased expression of chemokines, chemokine receptors [4, 5], and adhesion molecules like osteopontin [3, 6] and selectins [3]. Other induced molecules include platelet-derived growth factor-D (PDGF-D) [7], and macrophage-colony stimulating factor (M-CSF), which supports both systemic macrophage recruitment and local macrophage proliferation [8]. Upon recruitment and stimulation, infiltrating inflammatory cells themselves produce numerous cytokines and vasoactive agents that sustain and enhance the inflammation, and contribute to stimulation of fibrogenic, apoptotic and gene regulatory signalling pathways involving among several other mechanisms, the renin-angiotensin system, transforming growth factor beta (TGF-β), and nuclear factor kappa B (NF-κB) [2, 3]. During obstruction, leukocyte infiltration was found to correlate in time with glomerular filtration functional decline. After up to six days of UUO, relief of obstruction resulted in slow but remarkable resolution of tubulointerstitial infiltration [1]. Without relief of obstruction, fibrotic and atrophic and processes continue to progressive tissue loss, massive deposition of extracellular matrix, and irreversible loss of function.

Oxidant stress in UUO

Oxidative stress is involved in these processes [9]. Various markers of oxidant stress are increased in UUO kidneys, such as the oxidatively damaged protein product N^{ε}-carboxymethyl-lysine (CML) [10]; the marker of DNA oxidant damage, 8-hydroxy-2'-deoxyguanosine (8-OHdG) [11]; and lipid peroxidation markers such as malondialdehyde

(MDA) [12, 13], 8-iso prostaglandin F2α (8-iPGF2α) [14], and 4-HNE or 4-HHE [15]. Oxidant stress response molecules like heat shock protein-70 (HSP-70) [13] and heme oxygenase-1 (HO-1) [11] are also strongly expressed after UUO. Mice that are genetically deficient in the protective endogenous antioxidant enzyme catalase, are more susceptible to UUO-induced renal injury than normal wild type mice [15, 16]. Furthermore, increased renal concentrations of reactive oxygen species (ROS) have been observed in obstructed kidneys [17]; along with decreased activities of all major native protective antioxidant enzymes, superoxide dismutase (SOD) [15, 17], catalase and glutathione peroxidase [15].

Closely related to these data are reports indicating that nitric oxide (NO) plays a protective anti-apoptotic role in UUO. NO acts as a physiological antioxidant counterbalance to ROS. Apoptosis, mediated by caspases [18], is a prominent feature of injury in this model. To study the role of NO in apoptosis during UUO, Felsen and colleagues [19] subjected cultured tubular epithelial cells to mechanical stretch as an *in vitro* replication of UUO-induced tubular cellular stress. Mechanical stretch induced apoptosis, which was aggravated by the non-specific NO synthase (NOS) inhibitor, L-NAME, but inhibited by both the NO precursor L-arginine, and the NO donor agent SNAP. In the *in vivo* component of the study, inducible NOS (iNOS) knockout mice (iNOS-/-) were compared with wild type mice. iNOS-/- mice expressed significantly less NOS activity, and demonstrated more severe tubular apoptosis than their wild type counterparts. L-NAME further aggravated apoptosis in iNOS-/- mice, indicating the importance of other NOS isoforms. In other studies, NO has been variously shown to protect against interstitial fibrosis and loss of renal function in UUO, and L-arginine supplementation during UUO helps to preserve renal function after relief of temporary obstruction [20]. Recently, liposome-mediated iNOS gene transfer was proposed as an elegant NO delivery technique into obstructed kidneys [21, 22].

Despite the appreciable evidence of oxidant stress involvement in UUO, little is known about the possible source(s) of such increased stress. Because oxidant stress mechanisms vary between models, it is important to identify specific ROS sources that may be potential treatment targets. Various ROS sources implicated in other renal injury models include the mitochondrial respiratory chain [23, 24], NADPH oxidase [25], and uncoupled NOS [26]. Recently, mRNA and proteins of the NADPH oxidase components p22-, p47-, and p67-phox were all found to be upregulated in UUO kidneys [15], raising the possibility that this enzyme is the oxidant stress source in obstructive nephropathy.

There is currently limited information about whether direct antioxidant therapy can reduce inflammation or ameliorate other nephropathic changes that follow UUO. The general antioxidant agent, α-tocopherol, did not convincingly reduce kidney tissue MDA [12], and neither NAC nor vitamin E substantially relieved renal injury [11] induced by UUO. However, statins have demonstrated benefits that appear to stem from reduction of oxidant stress. Simvastatin reduced markers of renal inflammation and fibrosis [27]. Fluvastatin attenuated 8-OHdG expression along with fibronectin and α-smooth muscle actin (α-SMA) [11]. In another study, fluvastatin similarly alleviated UUO-induced expression of α-SMA, and significantly reduced interstitial fibrosis based on morphometric indices; these benefits were accompanied by signs of relief of oxidant stress, shown by reduction in UUO-induced expression of advanced glycation end-products (AGE). Because of the pleiotropic effects of statins, though, it is not clear of their beneficial effects were mediated mainly via antioxidant mechanisms. The present study was designed as a preliminary effort to test whether a specifically directed antioxidant intervention would reduce inflammation due to UUO. The reasoning behind the study is that *if NADPH oxidase is a functionally important source of oxidant stress in UUO, then its inhibition would alleviate UUO-induced inflammation.*

NADPH oxidase and apocynin

Nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) catalyses the production of the superoxide anion through the reaction of NADPH and oxygen. In functional terms, there are two forms of the enzyme. The first form is leukocyte NADPH oxidase, which catalyses the production of large amounts of superoxide to facilitate leukocyte phagocytic function. This is the longest-recognised and best-characterised form of the enzyme. The second form of NADPH oxidase is not simply a single entity but a group of closely related oxidases that are found in non-phagocytic cells. The physiologic function of these enzymes is to generate limited amounts of superoxide for normal cellular functions, such as oxygen sensing and signal transduction [28, 29]. Under pathophysiologic circumstances, however, these oxidases are liable to over-stimulation, leading to excessive production of superoxide, with attendant increase in downstream conversion to other ROS. If the tissue antioxidant defensive mechanisms are overwhelmed by excess radicals, a state of oxidant stress ensues, with numerous possible deleterious effects. Non-phagocyte forms of NADPH oxidase occur in a variety of tissue types, including the kidney. NADPH oxidases have a complex structure, comprising multiple sub-units that are either membrane-bound or located in the cytoplasm in the resting state. Upon stimulation, various cytosolic components

migrate to link up with the membrane-bound subunits, resulting in the fully assembled, biologically active enzyme. To add to the complexity, between different cell and tissue types, non-phagocyte NADPH oxidases differ in the details of sub-unit expression [30].

Various NADPH oxidase subunits have been found expressed in the kidney [31], including Renox (renal NADPH oxidase) [32], a component that seems to be uniquely expressed in renal tissue, and both tubular and glomerular NADPH oxidase activities have been demonstrated [33]. Therefore, in renal pathophysiologic conditions that involve increased oxidant stress, NADPH oxidase is always considered a possible source of oxidant stress. It has been shown to contribute to stress in key cardiovascular events that are of renal interest, such as atherosclerosis [30, 34] and hypertension [30], and is implicated in a range of specific renal disease models, such as 5/6 subtotal nephrectomy [25], anti-Thy 1.1 nephritis [35], diabetic nephropathy [26], and NOS inhibition [36]. NADPH oxidase may account for increased ROS production in the aging kidney [37]. It also seems to be the source of oxidant stress in UUO, based on increased expression of major subunits [15]. Further evidence of NADPH oxidase contribution to tissue injury is derived from observations in NADPH oxidase-deficient mice, developed by genetic knockout of crucial enzyme subunits such as gp91phox or p47phox. For example, the gp91phox-/- mice are protected against oxidant stress and injury in hypoxic pulmonary hypertension [38], revealing the enzyme's crucial role in the pathogenesis of that condition. The drawback with these genetically modified animals is that they are immunodeficient due to severe loss of normal leukocyte function [39, 40].

One of the major difficulties with assessing the functional role of NADPH oxidase is that specific blockers are relatively scarce. The iodonium compound, diphenylene iodonium (DPI) has been widely applied in the role of NADPH oxidase inhibitor [28, 30]. Of importance, however, DPI is also an efficient inhibitor of other ROS-producing enzymes, including xanthine oxidase, NOS, and other flavin-containing oxidases [41]. Furthermore, DPI is also recognised as a powerful mitochondrial ROS inhibitor [42]. Because of this non-specificity, data derived from experiments based on the use of DPI can only yield cautious conclusions about the role of NADPH oxidase. Thus, in the present study we have utilised apocynin, a specific, well-characterised NADPH oxidase inhibitor that acts by preventing the assembly of enzyme subunits at the membrane, thereby blocking enzyme activity [43]. Moreover, by curtailing the amounts of superoxide available for reacting with NO, apocynin indirectly provides the additional benefit of also suppressing peroxynitrite formation [44].

These effects make apocynin a reasonable candidate for effective suppression of oxidant stress and inflammation, and there is evidence to show that it does indeed possess the potential for such effects. *In vitro,* apocynin inhibited superoxide production in kidneys of rats exposed to chronic NOS inhibition [45], and suppressed LPS-induced pro-inflammatory activation of cultured cardiomyocytes [46]. Apocynin is easily administered *in vivo* by convenient oral route, and is well tolerated by mice without adversely affecting humoral or cellular immunity [47]. Some reported *in vivo* beneficial benefits of apocynin treatment are included in Table 1.

Table 1. Examples of beneficial effects of *in vivo* apocynin treatment

Organ-system	Animal model	Effect of apocynin	Ref
Cardio-vascular / renal	BMP-4 treatment (mice)	Alleviation of hypertension	[48]
	Dopamine D5 receptor null mice	Alleviation of hypertension	[49]
	Dexamethasone hypertension (rats)	Prevention and reversal of hypertension	[50]
	ACTH hypertension (rats)	Prevention and reversal of hypertension	[51]
	Diabetic nephropathy (rats)	Relief of proteinuria and glomerular injury	[52]
	Hyperhomocysteinaemia (rats)	Relief of proteinuria and glomerular injury	[53]
	Angiotensin II infusion (mice)	Relief of renal NO depletion and sodium retention	[54]
Liver	Remote hepatic injury (mice)	Limitation of hepatic parenchymal damage	[55]
	Portal hypertension (rats)	Reduced portosystemic collaterals, improved splanchnic angiogenetic and circulatory indices	[56]
Joints, soft tissues and skin	Zymosan arthritis / otitis (mice)	Relief of tissue inflammation	[57]
	Collagen-induced arthritis (rats)	Relief of tissue inflammation	[58]
	Tubercle bacteria inoculation (rats)	Prevention of ulcerative skin lesions	[59]
Neural	Sleep apnoea model (mice)	Reduced hypoxia/reoxygenation hypersomnolence	[60]
Eye	Ischaemic retinopathy (mice)	Prevention of retinal neovascularisation	[61]

Abbreviations: BMP, bone morphogenetic protein; ACTH,adrenocorticotrophic hormone; NO, nitric oxide

Older and newer therapies in UUO

Different therapeutic approaches have been investigated in UUO over the years. Indicative of the crucial role of Angiotensin II (Ang II) [2], genetic or pharmacological measures that counteract Ang II, such as angiotensinogen gene knockout [62], angiotensin-converting enzyme (ACE) inhibition [63], Ang II type 1 (AT1) receptor inhibition [15, 17], or AT1a receptor gene knockout [64], have shown considerable benefits in reducing UUO-induced renal fibrosis. In the past few years, a number of innovative therapeutic approaches have been probed, with varying degrees of relief of UUO-induced injury. Such approaches include drugs targeted at injury-mediating signal transduction pathways, like NPC 31169, a specific p38α inhibitor that interrupts the p38 mitogen-activated protein kinase (MAPK) pathway [65]. Similarly, Y-27632 was employed to block the small GTPase Rho effector proteins, ROCK (Rho-associated coiled-coil forming protein kinase) [66]. In another study, the chemokine receptor CCR1 was targeted by a non-peptide antagonist, BX471 [5]. Other workers have explored immunologic techniques, such as the use of the anti-*c-fms* antibody to inhibit macrophage activation by macrophage-colony stimulating factor (M-CSF) [8]. Molecular methods have also been exploited, exemplified by antisense oligonucleotide treatment to reduce connective tissue growth factor (CTGF) [67]. Although potentially promising, information about these approaches remains largely limited for the time being.

TGF-β is one of the cytokines that play a major role in the inflammation and tissue damage that characterise obstructive nephropathy [68]. Biologic actions of TGF-β are effected via activation of their transmembrane receptor serine/threonine kinases, with downstream signal transduction through Smad proteins, which are TGF-β-responsive transcription factors. Smads 1, 2, 3, 4 and and 5 variously work together as transcriptional regulators of target genes to effect TGF-β-mediated actions, while Smads 6 and 7 are regarded as intracellular antagonists of TGF-β signalling [69]. When stimulated during UUO, TGF-β signalling favours fibrosis; thus Smad3 deficiency ameliorates inflammation and fibrosis after UUO [70, 71] while Smad7 downregulation contributes to fibrosis [72]. In UUO research, there is currently much interest in the role of bone morphogenetic proteins (BMPs), a large subgroup of the TGF-β superfamily. Although BMPs have their own distinct receptors, they share broadly similar signalling pathways with TGF-β, including transduction via Smad proteins [73, 74]. BMPs are known to also signal through non-Smad pathways involving JNK and p38 MAP kinase [74, 75]. BMPs are multifunctional proteins that exert complicated biological activity in diverse organ systems. The various BMPs differ in their

receptor binding properties, which dictate their biologic effects. BMP signalling behaviour is complex, and involves cross-talk with TGF-β within the Smad network that is not yet fully elucidated [75]. In the kidney, effects of BMP-7 counteract those of TGF-β. BMP-7 (also known as osteogenic protein-1, OP-1) has shown an impressive ability to inhibit UUO-induced tubulointerstitial fibrosis [76], via inhibition of apoptosis and epithelial-mesenchymal transdifferentiation [68]; and to accelerate the restoration of renal function following relief of obstruction [77]. There is much less information about the renal effects of other BMPs, particularly BMP-6, which bears similarity to BMP-7 in terms of amino acid sequence [73] and ALK2 receptor binding [75].

Part II: Pilot study

Apocynin relieves renal interstitial macrophage influx and protects kidney bone morphogenetic protein-7 (BMP-7) gene expression in murine obstructive nephropathy

Introduction

Oxidant stress is a well documented feature in unilateral ureteral obstruction (UUO) and is believed to contribute to tubulointerstitial macrophage infiltration characterising the early phase of injury. NADPH oxidase subunits are upregulated during UUO, and thus this may be the enzymatic source of oxidant stress. To gain an insight into whether NADPH oxidase-dependent oxidant stress contributes to the renal inflammatory response after UUO, we carried out the UUO procedure in mice, and compared renal interstitial macrophage influx in the presence or absence of the specific NADPH oxidase assembly inhibitor, apocynin.

Recently, bone morphogenetic protein-7 (BMP-7) has shown strong potential as a therapeutic option that may prevent or reverse nephropathic effects induced by UUO [77]. The protective effect of BMP-7 in the kidney is thought to involve an ability to suppress pro-inflammatory behaviour particularly in tubular epithelial cells [78]. Since BMP-7 belongs to the transforming growth factor β (TGF-β) superfamily, there is a strong possibility that its expression and signalling might be redox sensitive, but this is not clear in the context of UUO. Moreover, BMP-7 has also been shown to relieve tubulointerstitial injury in some other renal injury models [78] that are known to be associated with increased oxidant stress. Thus,

we took the opportunity to examine whether apocynin, via NADPH oxidase blockade, might also have a beneficial effect on BMP-7 gene expression.

Methods

We used male C57BL/6J mice, weighing about 25g (Harlan Nederland, Horst, The Netherlands). Mice were maintained on a standard diet (RMH-TM; Hope Farms, Woerden, The Netherlands) and tap water *ad libitum* and housed in cages in a room maintained at 22^{O}C, 60% humidity with a 12/12-hour light/dark cycle. The Utrecht University board for studies on experimental animals approved the protocol.

UUO procedure. Mice were lightly anaesthetised, a small incision made on the left flank, and left ureter ligated at two points in the middle third and cut between the ligatures. The wound was sutured, anaesthaesia withdrawn, and animals returned to their cages on regaining consciousness under monitoring.

At termination, mice were injected with an anaesthetic cocktail (46.7mg/ml ketamine, 8mg/ml xylazine, and 0.067mg/ml atropine) at 0.1 ml/20g IP, and blood samples collected. Samples from both obstructed and contralateral kidneys were obtained under RNAse-free conditions and were either fixed in 4% formaldehyde, or snap-frozen in liquid nitrogen and stored at –80^{o}C until analysed.

Pharmacological treatment. A subset of mice received the NADPH oxidase inhibitor apocynin 10 mmol/L in drinking water (Sigma Aldrich, Zwijndrecht, The Netherlands), commenced 72 hours before the UUO procedure.

Renal histology and immunohistochemistry. Formaldehyde-fixed, paraffin-embedded kidney tissue was stained using standard procedures with haematoxylin and eosin and periodic acid-schiff for light microscopic examination by a renal pathologist in blinded fashion. To assess renal macrophage infiltration, frozen kidney sections were dried, fixed with acetone, blocked and incubated with a rat antibody against the mouse macrophage antigen F4/80 [79] (Serotec Benelux, Oxford, UK). Sections were further incubated with horseradish peroxidase-conjugated rabbit anti-rat and swine anti-rabbit antibodies (DakoCytomation BV, Herverlee, Belgium). They were developed with Nova Red

and counterstained with haematoxylin. F4/80-positive cells per high power field (field area 0.245 mm^2) were counted in blinded fashion.

Quantitative RT-PCR. Total RNA was extracted from 30 mg frozen renal cortex using RNeasy columns (Qiagen, Venlo, The Netherlands). After cDNA synthesis, expression of BMP-7 mRNA was assessed by quantitative real-time PCR using TaqMan Gene Expression Assays with pre-designed probe and primers (Applied Biosystems, Foster City, CA, USA). PCR was carried out in an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) with an initial 10-minute step at 95 °C, followed by 45 cycles of 15 seconds at 95 °C and 1 minute at 60 °C. β-actin mRNA expression was used as an endogenous control.

Statistics. Data is shown as mean (SEM) unless otherwise indicated. Differences among groups were analysed using appropriate forms of analysis of variance. Statistical significance was accepted at the level of $p<0.05$.

Results

Study I: In initial experiments with mice treated with apocynin for five days, apocynin induced no histological changes (not shown). The renal macrophage count in apocynin-treated mice was significantly lower than in controls (32 ± 2 vs. 40 ± 2 cells per field, p=0.025) (n = at least 6 per group).

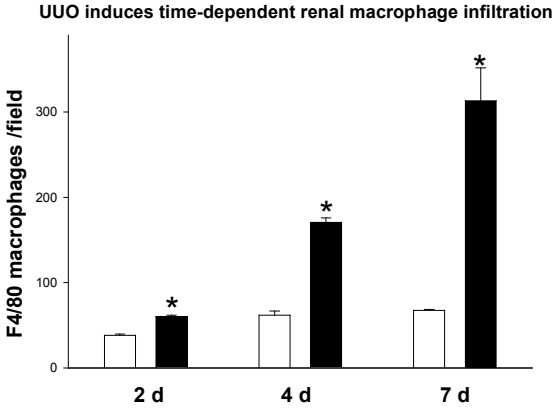

Figure 1. Renal interstitial macrophage population after 2, 4 or 7 days of UUO. White bars: contralateral kidneys, black bars: obstructed kidneys. Abbreviation: d=day (duration of UUO). *p<0.01, obstructed vs. contralateral kidney. Microscopic field area: 0.245 mm^2

Study II: In the second series of experiments, mice underwent UUO without intervention, for 2, 4 or 7 days before termination. UUO induced renal tubulointerstitial injury as expected (not shown). UUO induced renal macrophage infiltration with time-dependent increase over 2, 4, or 7 days (Fig. 1).

Different subsets of mice underwent UUO for 2 or 4 days, along with apocynin treatment. There were 6 mice per group. After 2 days of UUO, apocynin showed a minimal but statistically significant reduction in the total macrophage count (Fig. 2a); upon a closer look at the macrophage influx alone the apocynin effect is clearer (i.e., the differences between macrophage counts in the obstructed and corresponding contralateral kidneys) (Fig 2b). The reduction was not sustained at 4 days (not shown), and apocynin was therefore not continued through to day 7.

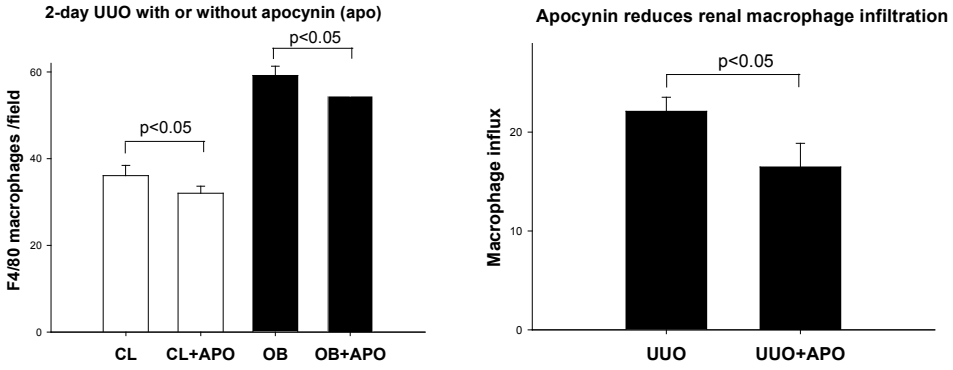

Figure 2. A (left panel). Renal interstitial macrophage population after 2 days of UUO, with or without apocynin treatment. **B (right panel).** Net macrophage influx into obstructed kidneys after 2 days of UUO, with or without apocynin treatment. White bars: contralateral kidneys (CL), black bars: obstructed kidneys (OB). Abbreviation: APO – apocynin. Microscopic field area: 0.245 mm^2

Gene expression studies of BMP-7 revealed a similar temporal pattern of findings. As shown in Figure 3a, there was a drastic, time-dependent decrease of BMP-7 mRNA expression after 2, 4, and 7 days of UUO. To test whether the early anti-inflammatory impact of apocynin might be related to BMP-7, we examined its expression at the 2-day time point.

Figure 3b shows that apocynin treatment of mice during 2-day UUO resulted in significant alleviation of BMP-7 mRNA downregulation.

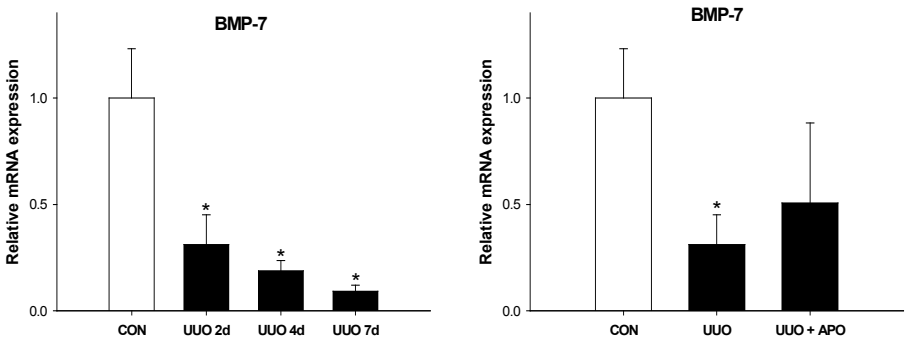

Figure 3. A (left panel). Kidney mRNA expression of BMP-7 after 2, 4, or 7 days (d) of UUO. **B (right panel).** Effect of apocynin (APO) on kidney mRNA expression of BMP-7 after 2d of UUO. mRNA in obstructed kidneys relative to contralateral. White bar: contralateral kidneys used as control (CON), black bars: obstructed kidneys. *p<0.001 vs. contralateral (control) kidney.

Discussion

We conducted a pilot study to probe whether apocynin, a specific inhibitor of the membrane-bound NADPH oxidase enzyme complex, would influence renal interstitial macrophage infiltration in the early phase of unilateral ureteral obstruction (UUO) in mice. Renal inflammation is an early and crucial event in UUO, a model of obstructive nephropathy that can eventually lead to extensive tubulointerstitial destruction. Apocynin achieved a limited, but significant, 25% reduction in the influx of macrophages at 2 days, suggesting NADPH oxidase involvement. The modest effect indicates that UUO-induced macrophage infiltration is not totally oxidant-dependent. Alternatively, UUO may be characterised by multiple sources of oxidant stress. In parallel with its anti-inflammatory effect, apocynin also significantly alleviated UUO-induced downregulation of the reno-protective protein BMP-7.

Of much interest is that apocynin also significantly reduced baseline macrophage counts in control mice. This may be a further indication of the anti-inflammatory potential of

apocynin. This is to our knowledge the first study suggesting that *in vivo* administration of apocynin can influence either baseline or stimulated renal macrophage infiltration. In the highly inflammatory UUO model, a modest 25% reduction in macrophage influx might represent considerable benefit. Comparable alleviation of macrophage infiltration was achieved by simvastatin [27], and a similar degree of reduction in inflammatory cell influx was observed in CD44 knockout mice [80]. CD44 is a macrophage receptor that plays a key role in macrophage adhesion and transendothelial migration during the process of chemoattraction following UUO. The similarity in degree of relief provided by apocynin and CD44 knockout may indicate that CD44-mediated macrophage recruitment is NADPH oxidase-dependent, while presumably there are other chemotactic pathways that are either not ROS-dependent or are mediated by ROS from other sources. Bascands and Schanstra [3] previously commented that UUO-induced leukocyte infiltration seems to result from additive effects of multiple mechanisms operating in concert. Therefore, multiple interventions would likely be necessary for full inhibition. Potentially, apocynin may be of particular value as an additive treatment in combination with other agents that work through different mechanisms. It would be informative to extend experimentation with apocynin to a multi-dose study, and to test it in other renal injury models with evidence of NADPH oxidase-dependent oxidant stress.

The initial relief of macrophage influx seen with apocynin was not sustained by the fourth day of UUO. This pattern is similar to early but non-sustained reductions in macrophage infiltration were achieved in knockout models of plasminogen activator inhibitor-1 (PAI-1) or osteopontin [2]. These observations indicate that, in the presence of sustained urinary obstruction, when one chemotactic mechanism is inhibited there may be a tendency for other chemoattractant mechanisms to compensate over time. Supporting this notion, it is notable that UUO-induced renal expression of leukocyte attractants, such as M-CSF, increases in a time-dependent fashion [8], which might contribute to later increases in macrophage population. This situation further emphasises that multiple pathways and mediators operate in UUO pathophysiology. To counter such time-based cytokine expression patterns, it may well be that time-dependent dose adjustments in medications will be necessary.

The pronounced downregulation of BMP-7 that we observed in UUO was significantly alleviated by apocynin therapy, suggesting that NADPH-dependent oxidant stress has a role in BMP regulation. Since it has been demonstrated that BMP-7 suppresses

pro-inflammatory effects in the kidney [81], it also seems quite plausible that the beneficial effect of apocynin on macrophage influx is at least partly due to the support of BMP-7 expression. This would be consistent with well-documented renal protective characteristics of BMP-7, and it would be in line with the effect of kielin/chordin-like protein, a molecule that enhances BMP-7 signalling and was recently shown to alleviate renal fibrosis induced by UUO [82]. Thus far, there has been limited information about the relation of oxidant stress and BMP-7, and it has not been previously shown that antioxidant therapy can promote renal BMP-7 expression. Our finding suggests that oxidant stress derived from NADPH oxidase plays a significant role in the transcriptional regulation of BMP-7 during UUO.

It is known that macrophages are present in the normal kidney [6]. We previously observed that for unknown reasons, baseline tubulointerstitial macrophage levels in C57BL/6 mice are relatively high in comparison with another well known mouse strain, the 129S2/Sv [83]. Our present observations now raise the question whether this may be connected with higher basal NADPH oxidase activity. In this study, we found that apocynin reduced baseline macrophage levels. The functional significance of this observation is unclear, but it raises the question whether the relatively high background renal macrophage population in C57BL/6 mice is in some way attributable to a comparatively heightened basal state of renal NADPH oxidase activity. This would be unexpected because we [84, 85] and others [86, 87] have demonstrated that the C57BL/6 strain is relatively resistant to a number of well-known experimental renal injury models. Indeed, we observed that resistance of C57BL/6 to AngII-induced renal injury is based on powerful intrarenal antioxidant status [85]. A high basal NADPH oxidase activity level in the same strain would therefore seem to be a contradiction. However, it is conceivable that a high background oxidant tone may indeed be one of the reasons for the ability of this strain to be relatively resistant to further external injurious stimuli. Could this be speculatively termed a state of natural “oxidative immunisation” or “oxidant pre-conditioning”? It seems an intriguing possibility.

In summary, in this pilot study we have observed that apocynin slightly but significantly reduced renal macrophage influx in mice, 48 hours after ureteral obstruction, suggesting that UUO-induced renal inflammatory cell infiltration is partly NADPH oxidase-dependent. The anti-inflammatory activity of apocynin may be related to its beneficial effect on BMP-7 gene expression. Interestingly, apocynin also diminished basal macrophage count in control mice. Anti-inflammatory potential of apocynin merits further study.

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Chapter 7

General discussion:

Summary of the thesis, Answers to the research questions, and Clinical perspectives

Summary

Oxidant stress in renal inflammation: Mechanisms, and the search for remedies

The research presented in this dissertation was designed to seek increased understanding of oxidant stress mechanisms in renal inflammation, with the aim of identifying and testing potential appropriate remedies. The studies were based on the overall hypothesis that *oxidant stress is a central player in renal inflammation; pharmacological reduction of oxidant stress should therefore relieve renal inflammation.*

Our studies involved three different experimental models of renal inflammation. Our primary focus was on the protein overload model. We used this model to probe the essential underlying disturbances of redox balance during renal exposure to excess proteins, both in the *in vitro* setting (cultured proximal tubular cells exposed to excess protein) (chapter 2) and *in vivo* (mice treated with large doses of exogenous protein) (chapters 3 and 4). Apart from protein overload, we examined two other mouse models: Angiotensin II-induced hypertension (chapter 5); and unilateral ureteral obstruction (UUO), a non-proteinuric, non-hypertensive model of renal inflammation and injury (chapter 6).

Oxidant-dependent renal inflammation in protein overload: Mechanisms and remedies

Greatly increased renal protein trafficking during proteinuric disease is a key precipitant of interstitial inflammation and injury, and there is evidence that oxidant stress contributes to the inflammatory process. The source of oxidant stress is not clear, however. Although the ability of diphenyleneiodonium (DPI) to reduce albumin-induced reactive oxygen species (ROS) production by tubular cells has been taken to mean that NADPH oxidase is a major ROS source in this model, DPI is not a reliable probe for this purpose, as it is a non-specific inhibitor of various possible ROS sources, including other flavin oxidases and mitochondria. Another unclear issue is whether nephropathic effects of excess protein are due to albumin or to other substances that are transported into the renal tubules bound to albumin, such as fatty acids (FA). There is evidence that lipids can directly induce oxidant stress in endothelial cells, but whether they have similar effects on tubular epithelial cells has not been previously determined.

We pursued these lines of enquiry in our *in vitro* protein overload study using HK-2 cells, which are immortalised human proximal tubular epithelial cells (chapter 2). We studied

whether oxidant and inflammatory effects of protein overload are mainly due to excess albumin, or to albumin-bound fatty acids (ABFA). We sought to determine the source(s) of oxidant stress; to identify renal anti-oxidant responses; and through these, to gain an insight into the overall pattern of disturbance of redox balance, i.e. whether oxidant stress in this setting is mainly due to increased pro-oxidant or impaired anti-oxidant capacity, or both. Finally, we investigated links between oxidant stress and cellular inflammatory responses, and explored therapeutic possibilities for relieving inflammation by reducing oxidant stress.

To our knowledge there are no previous studies specifically probing whether ABFA directly stimulate ROS in tubular cells, or whether their pro-inflammatory effects are based on oxidant-dependent mechanisms. We found that FA-rich albumin induced much more ROS than albumin alone, suggesting that in the proteinuric state, ABFA possess an extra oxidant impact, over and above that of albumin alone. The exact mechanism of this added effect is not yet clear. Cellular uptake of FA-rich albumin was similar to that of albumin alone, indicating that the enhanced oxidative capacity was not due to differential uptake. In further experiments to determine the cellular source of oxidant stress, we observed that two mechanistically different inhibitors of the mitochondrial electron transport chain (rotenone, a respiratory chain Complex I inhibitor; and CCCP, an uncoupling agent) significantly ameliorated ABFA-induced ROS. Specific inhibitors of other major possible sources, NADPH oxidase (apocynin), xanthine oxidase (allopurinol), and NOS (L-NAME) all failed to significantly reduce ROS. ABFA also inhibited the defensive antioxidant mitochondrial superoxide dismutase (SOD) mRNA response that was observed in cells treated with albumin alone. These findings revealed that protein overload induces oxidant stress in proximal tubular cells via a dual mechanism, i.e. both by stimulating increased ROS production via mitochondria, and by simultaneously weakening protective antioxidant mitochondrial SOD transcriptional response.

To assess whether the extra oxidant stress induced by ABFA had a functional pro--inflammatory impact, we compared the effects of albumin alone, and FA-rich albumin, on tubular cellular interleukin-6 (IL-6) gene and protein expression. Both treatments similarly provoked increased IL-6 mRNA expression, but FA-rich albumin induced significantly more IL-6 protein expression than albumin alone; suggesting that ABFA drive extra pro-inflammatory responses at the translational, rather than transcriptional level. ABFA-induced IL-6 protein expression was powerfully suppressed by pharmacological augmentation of SOD (using the salen-manganese catalytic mimetic agent, EUK-8), and also by

mitochondrial inhibition with rotenone or CCCP. Thus, ABFA-induced tubular inflammatory cytokine expression is heavily oxidant-dependent.

To extend these lines of investigation into the *in vivo* setting, we needed an experimental model that satisfied two conditions: significant proteinuria (ensuring tubular exposure to excess protein), and tubulointerstitial inflammation (establishing functional significance of the protein leakage). Most previous protein overload models in mice had shown disappointing levels of proteinuria, making them unsuitable for our questions. Therefore in chapter 3, we probed whether the 129 mouse, a strain proven to be sensitive to several other renal injury models, would be suitable. We established that protein overload treatment in 129S2/Sv mice induced marked proteinuria, accompanied by significant renal inflammation demonstrated by tubulointerstitial macrophage influx. By comparison, C57BL/6 mice showed virtually no proteinuria nor renal macrophage in response to albumin treatment. In both strains there was an accompanying mild gender effect; males had more proteinuria.

In chapter 4, using the newly established 129S2/Sv mouse model of protein overload, we conducted further studies to extend the questions earlier addressed in chapter 2 to the *in vivo* setting. Our HK-2 cell studies had shown that ABFA induce increased mitochondrial oxidant stress and hindered SOD2 antioxidant defensive response. We therefore reasoned that if these mechanisms are present in the in vivo setting, then pharmacological blockade of mitochondrial ROS, or supplementation of SOD, should alleviate ABFA-induced renal inflammation. Thus, we treated mice with FA-rich albumin in the presence or absence of tempol (SOD mimetic) or rotenone (mitochondrial inhibitor). Extra SOD markedly reduced tubulointerstitial macrophage infiltration, affirming that ABFA-induced inflammation is ROS-dependent. Mitochondrial inhibition also had a significant anti-inflammatory effect, supporting that mitochondria are a significant ROS source. Neither tempol nor rotenone significantly reduced proteinuria, indicating that their anti-inflammatory effects were due to specific anti-oxidant activity, and not incidental benefits resulting from inhibition of proteinuria. Finally, in mice co-treated with FA-rich albumin and nicotinic acid, both proteinuria and inflammation were significantly reduced, suggesting that lowering free FA may be beneficial in proteinuric states.

Summarising, our *in vitro* findings indicated that tubular cellular oxidant stress in the protein overload model is based on a dual mechanism of increased mitochondrial ROS

and impaired SOD antioxidant defence. Albumin-bound fatty acids play a key pro-oxidant role. The increased oxidant stress serves to aggravate pro-inflammatory cytokine expression in tubular cells. All of these findings were confirmed in proteinuric mice. Pharmacological strategies to provide extra SOD, inhibit mitochondrial ROS, and lower free fatty acid levels, all significantly ameliorated tubulointerstitial macrophage influx. These data highlight the importance of devising antioxidant strategies based on specific understanding of the peculiar redox milieu within a particular model of inflammation.

Angiotensin II infusion in mice: Nature's pointer on renal antioxidant strategy

In rats, Angiotensin II (AngII) infusion induces marked renal inflammation, and injury. Evidence indicates that these effects are mediated via NADPH oxidase-dependent oxidant stress. Mice, in marked contrast, appear to be resistant to hypertensive renal injury. We hypothesised that mice may possess strong intrinsic antioxidant mechanisms that protect them from oxidant-dependent AngII-induced hypertensive renal injury. Therefore, we probed renal pro- and anti-oxidant gene responses to AngII (chapter 5). We employed the C57BL/6 mouse strain, which is particularly known to be resistant to several models of renal injury that even other mouse strains are responsive to. We observed hypertension, but not proteinuria or renal injury. There was no remarkable increase in renal lipid peroxidation. Gene expression analysis revealed that AngII treatment significantly induced renal antioxidant gene expression, while it not only failed to induce, but in fact suppressed the expression of NADPH oxidase components p22phox and Rac2, as well as xanthine oxidase. Gene expression of heme oxygenase-1 (HO-1), an enzyme with protective anti-oxidant and anti-inflammatory properties, was upregulated.

In contrast to the situation in the kidney, AngII induced significant vascular oxidant stress and injury, as shown by markedly upregulated aortic mRNA expression of the NADPH oxidase component, p47phox, accompanied by aortic hypertrophy despite increased HO-1. These data suggested that, in these mice, an organ-specific antioxidant protective mechanism is present in the kidneys. In an attempt to dissect the AngII signalling pathway supporting this antioxidant defensive mechanism, we tested whether AngII AT2 receptor blockade (using PD-123,319) would dampen the protective responses. This manoeuvre was based on the finding that renal, but not aortic, AT2 receptor expression was increased by AngII. However, AT2 receptor blockade had limited impact. Despite enhancing hypertension, the intervention further reduced, rather than increased, cortical expression of pro-oxidant genes. Thus, antioxidant resistance is not mediated by the AT2 receptor. Our data support

the notion that natural resistance to injury in this mouse model results from a heightened endogenous protective capacity against oxidant stress. Via an as yet unidentified pathway in this murine model, renal pro-oxidant factors, particularly NADPH oxidase, and also xanthine oxidase, are downregulated rather than stimulated by AngII treatment. It is especially fascinating that mice of the C57BL/6 strain can so effectively resist renal injury despite the fact that, as we observed in chapter 3, they exhibit a relatively high baseline tubulointerstitial macrophage count (in comparison with the much more sensitive 129S2/Sv mice). It is conceivable that the high basal macrophage population in some way a functional expression of an immunologically tolerant system with reduced sensitivity to exogenous oxidant and inflammatory stimuli.

Unilateral ureteral obstruction: NADPH oxidase, candidate mechanism. Apocynin, candidate remedy

Unilateral ureteral obstruction (UUO) is characterised by early and intense tubulointerstitial inflammatory cell infiltration. Oxidant stress is believed to play a key role in the early initiation and maintenance of inflammation. Without prompt relief of obstruction, oxidant stress and inflammation contribute to other pathological events that rapidly lead to progression of tubulointerstitial injury and onset of fibrosis. The source of oxidant stress is not clear. Upregulation of mRNA and protein of NADPH oxidase components have been observed. We hypothesised that pharmacological blockade of NADPH oxidase would reduce oxidant-dependent renal inflammation. To block NADPH oxidase, we used the specific enzyme complex assembly inhibitor, apocynin.

Apocynin brought moderate but significant benefit with a 25% reduction in the tubulointerstitial macrophage influx observed during early UUO-induced inflammation. The modest impact indicates that other ROS sources than NADPH oxidase are involved; alternatively, there are probably other mechanisms that are not oxidant-dependent. Apocynin may be of particular value in conditions where NADPH oxidase is the chief source of oxidant stress, or as adjunctive therapy in cases of multiple pathophysiologic mechanisms or sources of oxidant stress. In addition, apocynin significantly alleviated UUO-induced downregulation of mRNA expression of bone morphogenetic protein-7 (BMP-7), a molecule that is well recognised to have powerful protective roles against renal injury. This suggests that NADPH oxidase-dependent oxidant stress is involved in BMP-7 gene regulation after UUO.

Answers to the questions of the thesis

Answers to questions outlined at the onset of the studies, as listed in chapter 1

Oxidant stress mechanisms in cultured tubular cells (chapter 2)

In cultured proximal tubular epithelial (HK-2) cells, protein overload induced oxidant stress (gauged by fluorometric assay of intracellular production of reactive oxygen species, ROS). Compared with albumin alone, fatty acid (FA)-loaded albumin induced significantly more ROS, which was mainly of mitochondrial origin. Cellular defensive antioxidant gene responses (superoxide dismutase-2 and heme oxygenase-1) were impaired. FA-loaded albumin also provoked a more pronounced pro-inflammatory cytokine response than albumin alone. The inflammatory response was blocked by both mitochondrial inhibition and antioxidant supplementation, confirming the contribution of mitochondrial ROS.

Developing a suitable in vivo model of renal inflammation due to protein overload (Chapter 3)

In contrast to C57BL/6 mice, male and female 129S2/Sv mice developed marked proteinuria and renal interstitial macrophage infiltration after treatment with bovine serum albumin (BSA). By immunohistochemistry, BSA was visualised in the urinary space and within tubular lumina, confirming exposure of tubular cells to excess injected protein.

Exogenous antioxidants offer protection in vivo (chapter 4)

In this chapter, treatment of mice with fatty acid-rich albumin caused much more renal macrophage influx than albumin alone, showing that ABFA play a key role in protein overload-induced inflammation. This inflammation was powerfully reduced by pharmacological SOD augmentation with tempol, and significantly by the mitochondrial inhibitor rotenone, demonstrating that (i) oxidant stress is pivotal to renal inflammation in this model; (ii) mitochondria contribute significantly to the stress. Reduction of plasma non-esterified fatty acids with the lipolytic agent nicotinic acid resulted in alleviation of both proteinuria and renal inflammation.

Intrinsic renal antioxidant resistance to Angiotensin II-induced injury (chapter 5)

In this chapter we employed a mouse strain that is known to be resistant to several forms of renal injury. We wondered whether such resistance to injury results from an intrinsic capacity to resist oxidant stress, and whether we could gain an insight into the underlying mechanisms by probing the renal pro and oxidant gene responses following Angiotensin II (AngII) infusion. AngII failed to induce renal pro-oxidant gene expression; in fact, the expression of components of NADPH oxidase was downregulated in the kidney. Instead, there was increased expression of the protective enzyme, heme oxygenase-1 (HO-1). In contrast to the kidney, AngII induced pro-oxidant genes in the aorta, accompanied by aortic hypertrophy. Thus, in C57BL/6 mice, there is selective renal protection against well-known pro-oxidant effects of AngII, based on negative transcriptional response of NADPH oxidase components. Pharmacological suppression of pro-oxidant enzymes may be a beneficial option in renal pathophysiologic states that are associated with increased activity of the renin-angiotensin system.

UUO model: Apocynin mollifies interstitial inflammation and supports renoprotective BMP-7 gene expression (chapter 6)

The specific NADPH oxidase assembly inhibitor apocynin significantly reduced renal macrophage count in control mice. In mice with renal inflammation induced by unilateral ureteral obstruction (UUO), it effected a modest but significant reduction in additional macrophage influx after 2 days of UUO. Apocynin also significantly alleviated UUO-induced downregulation of mRNA expression of bone morphogenetic protein-7 (BMP-7), a molecule that is well recognised to have powerful protective roles against renal injury.

Searching for remedies to oxidant stress in renal inflammation: Hope, hurdles, and the road ahead

In 2004, an expert committee of the American Heart Association (AHA) wrote: *"At this time, the scientific data do not justify the use of antioxidant vitamin supplements for CVD risk reduction… no consistent data suggest that consuming (excess) micronutrients… will confer additional benefit with regard to CVD risk reduction"* [1]. This statement is obviously not a ringing endorsement of the clinical utility of antioxidant supplements. However, the very fact that such a body as the AHA had reason to issue such a statement at all, is a testament to how far the concept of "antioxidant treatment" has come since its humble beginnings; at the same time, it is also an indication of yet how far it still has to go before antioxidants become an integrated part of everyday clinical practice.

As recently as fifty years ago, the subject of "oxygen radicals" was more or less restricted to fairly narrow scientific provinces in chemistry, and far from being a topic of remarkable biomedical relevance. But due to a series of pioneering observations over the years, and particularly in the last two to three decades with the aid of explosive advancements in molecular methods, *oxidant stress* has developed into the core pathophysiologic, and increasingly clinical concept that it is today [2]. From the viewpoint of nephrology, the reality of renal oxidant damage in the clinical setting is now widely accepted. A variety of clinical studies, using different approaches, are increasingly adding to the body of evidence indicating that oxidant stress plays a pivotal role in the pathophysiology of chronic renal diseases and the uremic syndrome, including haemodialysis patients [3, 4]. The huge challenge now lies in working out how to combat it.

Hope: Evidence from experimental studies

Promising results were reported in laboratory animal studies investigating whether antioxidants are of benefit in alleviating renal injury. Dietary vitamins were particularly prominent. Combined treatment with vitamin E and probucol successfully prevented early fibrotic changes associated with hypercholesterolaemia [5]. In diabetic rats, vitamin C prevented any increase in proteinuria, and either vitamins C or E significantly moderated glomerular changes and inflammatory cytokine expression [6]. In the setting of nitric oxide

(NO) depletion, α-tocopherol significantly limited renal damage by alleviating proteinuria and preventing renal vascular injury or a decline in glomerular filtration rate [7]. Moreover, α-tocopherol not only inhibited [8], but indeed reversed [9] renal injury in the renal ablation model. In the ischaemia/reperfusion injury model, the catalytic antioxidants tempol [10] and EUK-134 [11] each significantly relieved ROS-mediated renal functional impairment and tubulointerstitial injury. Similarly, the radical scavenger edarabone attenuated renal impairment, apoptosis, tubular and mitochondrial damage induced by the toxin cisplatin [12]. These and similar experimental findings are important among the strands of evidence supporting the paradigm of a central role for oxidant stress in the pathophysiology of chronic kidney disease [13, 14], with the attendant hope that effective antioxidant therapy should provide significant benefits.

Hurdles: How to translate experimental successes into the clinical setting?

In general, the outcomes of clinical trials of antioxidants in renal patients thus far have not quite lived up to the great expectations raised by the remarkable successes of some of the experimental studies [15]. Perhaps the most promising findings yet are that Vitamin E [16] and acetylcysteine [17] significantly reduce cardiovascular events in patients with end-stage renal disease, but neither of these agents has an effect on overall mortality. As highlighted in the AHA statement quoted above, antioxidant therapy has come under serious consideration, but has not yet earned acceptance, as part of the therapeutic arsenal for cardiocirculatory diseases. In nephrological practice, although there is certainly increasing recognition of the clinical relevance of increased oxidant stress, and evidence is accumulating to suggest that such antioxidant therapies might have a place; important questions remain [3, 18].

But why is it that the outcomes of clinical trials have not been as positive as might have been expected? Perhaps the crucial point lies in the very choice of antioxidants that have been studied so far. As reviewed [1, 4], by far the most commonly used medications are vitamin E, vitamin C, and β-carotene, either as single agents or in various combinations. These substances have presumably been employed mainly because they are accepted as possessing broad in vivo antioxidant capacity (general antioxidants), and not necessarily to specifically target particular pathophysiologic pathways. A possible consequence of this somewhat speculative strategy may well be that inappropriate therapeutic options have been studied in at least some of the trials to date. It has been pointed out that the use of

vitamin C in particular [4, 15], and to some extent also vitamin E [15], deserves caution because they are both potentially capable of pro-oxidant effects. The association of vitamin supplements with adverse outcomes in some trials [1] may not be unconnected with such pro-oxidant effects. Apart from the possible doubts about choice of antioxidants, several authors [1, 4, 15, 19] have highlighted a number of other queries regarding clinical antioxidant studies that have been carried out so far. Among others, there have been questions about the appropriateness of dosages of antioxidants given, oxidant stress markers used, study populations, and modalities for monitoring antioxidant effectiveness. In addition, the exact form of antioxidant used may be crucial; for instance vitamin E efficacy may well depend on whether α-tocopherol and/or γ-tocopherol is present [3].

In basic research terms, perhaps the greatest challenge is to develop accurate, reliable and practical measurements for oxidant stress. As discussed in chapter 1, there are numerous ways to monitor oxidant stress; however, all methods have their drawbacks, and considerable technical difficulties remain with most measurements. Making the task more challenging is the fact that even within the same experimental model, the temporal profiles of expression of different oxidant stress markers are distinct. Illustrating this, in mice subjected to obstructive nephropathy, 8-iso prostaglandin F2α (8-iPGF2α) an index of lipid peroxidation, was expressed within the first week and maximally on the fourth day, whereas the oxidatively damaged protein product *N*ε-carboxymethyl-lysine (CML) gained prominence in the second week, peaking about the tenth day [20]. Thus, it will be necessary to establish not only the appropriate markers, but also the optimal time points for testing with each marker. Considering these complications, it is worth bearing in mind the suggestion that adequate monitoring of oxidant stress will inevitably require the deployment of a battery of tests rather than a single measurement [21].

An additional hurdle is that there may be important inter-species differences that could distort the application of research observations to the clinical front. Furthermore, among patients, genetically determined individual differences, or unexplained idiosyncratic variability, are likely to be identified in due course, and inter-racial variations in therapeutic efficacy may exist, as is known with other medications such as ACE inhibitors.

The road ahead

From the foregoing, it would seem appropriate to propose that the quest to identify and evaluate candidate renal antioxidant remedies needs to be guided by specific objectives tailored to suit defined pathophysiologic and clinical contexts. For practical purposes, these objectives may be summarized in the form of four questions: What is(are) the main mechanism(s) supporting oxidant stress within the specified setting; which is(are) therefore the likely appropriate remedy (or combination of remedies); when, and how best, to administer such; and how to measure remedy effectiveness.

It is especially key that the search should be directed by clear understanding of specific patterns of redox disturbance. Our protein overload studies illustrate the operation of this principle. There is a need to shift away from the inefficient paradigm of non-specific "general antioxidants". Successful antioxidant therapy may not necessarily come in the form of simple, inexpensive, multi-purpose medications like, say, "vitamin supplements". A much more painstaking, focused and specific approach in each experimental and clinical model may well be the way forward. Antioxidant treatments will likely have improved chances of success if directed at specifically identified target points within well-elucidated pathophysiologic redox mechanisms. Approaches that are less well targeted may be more liable to result in inconsistent hit-and-miss outcomes.

Powerful new approaches in molecular and cell biology will be invaluable in these "targeting" efforts. As an illustration, the advent of gene knockout or knockdown techniques has brought extensive possibilities for probing genotypic (and phenotypic) characteristics that should help in dissecting the mechanisms of oxidant stress in different disease models. For both experimental and clinical research, the improvement and refinement of reliable oxidant stress measurement modalities will greatly help in the development of appropriate remedies. Advancements in the fields of biochemistry, biophysics, and bio-imaging will have a major impact on these efforts. As antioxidant therapies eventually start to make the move into clinical use, it will become increasingly necessary to be able to accurately determine the "oxidant stress status" of individual patients in order to appropriately direct clinical treatment. Encouragingly, efforts are being made in this direction, evidenced by recent demonstrations of novel biomarkers of oxidant stress [22-24]. Attempts to use urinary markers [25, 26] are of particular interest because they potentially promise a non-invasive, convenient and easily accessible means of testing. However, most of these methods remain largely experimental,

and much remains to be done. In addition to all these, comparative studies in different animal species and strains may prove valuable in evaluating the potentials for variation in treatment responses.

The potential benefits of renal clinical antioxidant therapy are without doubt, too large to ignore, because chronic renal disease is a major worldwide public health problem. The nephrology community needs to continue, and improve on, efforts to bridge the gaps between experimental and clinical findings. Ways of standardising antioxidant clinical trials need to be agreed. Oxidant stress monitoring protocols also urgently need to be standardised and improved. Our studies provide data indicating that in the settings of proteinuric disease, or in conditions associated with renin-angiotensin system hyperactivity, specific antioxidants based on inhibition of ROS sources and support of protective forces may have a place.

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Oxidatieve stress en ontsteking van de nier: mechanismen en de zoektocht naar behandelingen

Het onderzoek gepresenteerd in dit proefschrift is ontworpen om de kennis te vergroten voor mechanismen van oxidatieve stress bij ontsteking van de nier, met als doel het identificeren en toetsen van potentiële, toepasbare behandelingen. De studies zijn gebaseerd op de overkoepelende hypothese dat oxidatieve stress een centrale rol speelt in ontsteking van de nier. Dientengevolge zou farmacologische reductie van oxidatieve stress de ontsteking van de nier moeten verlichten.

Onze studies gaan over drie verschillende experimentele modellen van nierontsteking. De primaire focus was het eiwitoverloopmodel. Dit model is gebruikt om de noodzakelijke onderliggende verstoringen van redoxbalans te onderzoeken tijdens blootstelling van de nier aan overmatig veel eiwitten. Dit is onderzocht *in vitro* met gekweekte proximale tubuluscellen (**hoofdstuk 2**) en *in vivo* met muizen welke behandeld werden met hoge doseringen van exogeen eiwit (**hoofdstukken 3 en 4**). Hiernaast hebben wij twee andere modellen gebruikt: angiotensine II-geïnduceerde hypertensie (**hoofdstuk 5**) en unilaterale ureterobstructie (UUO), een model van nierontsteking en schade zonder eiwitlek of hoge bloeddruk (**hoofdstuk 6**).

Oxidant-afhankelijke ontsteking van de nier bij eiwitoverstroom: mechanismen en behandelingen

Sterk toegenomen transcellulaire eiwitflux tijdens proteïnurische ziekten is een belangrijke oorzaak van ontsteking en schade van het interstitium van de nier. Er zijn aanwijzingen dat oxidatieve stress bijdraagt aan deze ontsteking maar de bron van deze stress is onduidelijk. Diphenyleneiodonium (DPI) kan de productie van albumine-geïnduceerde reactieve zuurstofmoleculen (ROS) door tubuluscellen verminderen. Daaruit is geconcludeerd dat NADPH oxidase een belangrijke bron van ROS in dit model is, maar DPI is niet volledig betrouwbaar. Het is een aspecifieke remmer van verscheidene bronnen van ROS, o.a. flavine oxidase en mitochondriën. Eveneens is onduidelijk of de beschadigende effecten van overmaat albumine het gevolg zijn van albumine zelf of van

andere stoffen die gebonden aan albumine de nierbuisjes bereiken, zoals albumine-gebonden vetzuren (ABFA). Er zijn aanwijzingen dat vetzuren rechtstreeks schade kunnen veroorzaken in endotheelcellen, maar of ze vergelijkbare effecten hebben op tubulusepitheelcellen is niet eerder onderzocht.

Wij hebben de onderzoekslijn opgezet in de vorm van *in vitro* eiwitbelasting studies met HK-2 cellen, onsterfelijke humane proximale tubulusepitheelcellen (**hoofdstuk 2**). Wij onderzochten of ontsteking door eiwitoverbelasting primair het gevolg is van eiwitbelasting, of door eiwitgebonden vetzuren. Wij zochten de bron(nen) van oxidatieve stress en de anti-oxidatieve responsen om hiermee inzicht te verkrijgen in het overkoepelende patroon van de verstoring in redoxbalans. In andere woorden: is oxidatieve stress in dit model primair het gevolg van toegenomen pro-oxidatieve capaciteit, verlies van anti-oxidatief vermogen, of van beide. Tenslotte bestudeerden wij de verbanden tussen oxidatieve stress en de cellulaire ontsteking, en onderzochten therapeutische mogelijkheden om ontsteking te verlichten door oxidatieve stress te verminderen.

Voor zover wij weten zijn er geen eerdere studies die specifiek onderzocht hebben of ABFA direct ROS stimuleren in tubuluscellen, of dat hun stimulerend effect op ontsteking afhankelijk is van oxidatieve mechanismen. Wij vonden dat FA-rijk albumine veel meer ROS induceerde dan FA-arme albumine. Dit suggereert dat tijdens proteïnurie ABFA een extra ontstekingseffect geeft, bovenop dat van albumine alleen. Het precieze mechanisme van dit effect is vooralsnog onbekend. De opname van FA-rijke en FA-arme albumine was niet verschillend, wat aantoont dat het verschil in toegenomen oxidatieve capaciteit niet het gevolg was van een verschil in opname. Vervolgens hebben wij naar de cellulaire bron van oxidatieve stress gezocht. Wij vonden dat twee mechanistisch verschillende remmers van de mitochondriële elektrontransportketen de ABFA-geïnduceerde ROS significant verminderden. Dit was echter niet het geval voor specifieke remmers van andere bronnen van ROS zoals NADPH oxidase, xanthine oxidase of NO synthase. ABFA remde ook significant de toename van mRNA van het mitochondriële antioxidant superoxide dismutase (SOD), welke geassocieerd was met FA-arm albumine. Deze bevindingen tonen aan dat eiwitoverstroom oxidatieve stress in proximale tubuluscellen verhoogt via een tweeledig mechanisme: door het stimuleren van ROS-productie in de mitochondriën enerzijds en door het verzwakken van beschermende antioxidatieve mitochondriële SOD transcriptie anderzijds.

Om te bepalen of deze extra oxidatieve stress geïnduceerd door ABFA en functionele ontstekingsrespons veroorzaakte, vergeleken wij de effecten van FA-arme en FA-rijke albumine op interleukine-6 (IL-6) gen en eiwitexpressie in tubulusepitheelcellen. Beide behandelingen verhoogden de IL-6 genexpressie, maar FA-rijke albumine significant meer IL-6 eiwit dan FA-arme. Dit suggereert dat FA-rijke albumine vooral ontsteking bevorderde op translationeel en in mindere mate op transcriptioneel niveau. ABFA-geïnduceerde IL-6 eiwit expressie werd krachtig onderdrukt met farmacologische verhoging van SOD en ook met mitochondriële inhibitie zoals boven beschreven. Dus ABFA-geïnduceerde cytokine expressie in de tubulus is sterk afhankelijk van oxidatie.

Om het onderzoek voort te zetten in een *in vivo* setting, was een experiment nodig dat voldeed aan twee voorwaarden: voldoende proteïnurie (zodat de tubuli zeker blootgesteld werden aan eiwit), en tubulointerstitiële ontsteking (bevestigend dat de eiwitlekkage functionele gevolgen had in dit compartiment). Eiwitoverloopmodellen in muizen laten teleurstellend weinig proteïnurie zien, waardoor zij ongeschikt zijn voor onze vraagstellingen. Daarom hebben wij in **hoofdstuk 3** onderzocht of de 129 S2/SV muis, in andere situaties gevoelig voor nierschade, wel, geschikt was. We stelden vast dat in de 129 S2/SV muis eiwitoverstroom een aanzienlijke proteïnurie veroorzaakt vergezeld van nierontsteking. Deze ontsteking konden wij aantonen door het tellen van tubulointerstitiële macrofagen. Daarentegen, C57BL/6 muizen vertoonden vrijwel geen proteïnurie of toename in macrofagen tijdens albumineoverstroom. In beide modellen was er een beperkt effect van geslacht: mannen vertoonden meer proteïnurie.

Gebruikmakend van dit nieuwe 129 S2/SV muismodel van eiwitoverloop, zijn er in **hoofdstuk 4** in vivo studies verricht die verder ingaan op de vragen die uit de *in vitro* opstelling (hoofdstuk 2) zijn voortgekomen. De studies uitgevoerd met de HK-2 cellen hadden aangetoond dat ABFA mitochondriële oxidatieve stress veroorzaakten mede door het belemmeren van de antioxidatieve respons. Daarom veronderstelden wij dat als deze mechanismen eveneens aanwezig zijn *in vivo*, farmacologische blokkade van mitochondriële ROS-productie dan wel toevoeging van extra SOD de door ABFA opgeroepen nierontsteking zouden remmen. Muizen werden dan ook met FA-rijk albumine met of zonder rotenone (een mitochondriële remmer) of tempol (een SOD-mimeticum) behandeld. Zowel mitochondriële remming als extra SOD verminderden de tubulointerstitiële macrofaaginflux, waarbij het effect van tempol wat sterker was. Noch rotenone, noch tempol had een significant effect op proteïnurie. Dit toont aan dat de anti-inflammatoire effecten

specifiek waren voor de antioxidatieve effecten van deze stoffen, en niet indirect het voordeel waren van een effect op proteïnurie. Tenslotte zijn muizen tegelijkertijd behandeld met FA-rijk albumine en nicotinezuur, een stof die FA verlaagt. In dit geval werden zowel proteïnurie als inflammatie geremd, hetgeen de suggestie wekt dat het verlagen van FA misschien een voordeel biedt bij proteïnurie.

Samenvattend, zowel *in vitro* als *in vivo* konden wij aantonen dat oxidatieve stress van tubuluscellen in het eiwitoverloop model gebaseerd is op een dubbel mechanisme van toegenomen mitochondriële ROS en afgenomen antioxidatieve verdediging door SOD. Deze gegevens benadrukken het belang van de ontwikkeling van antioxidatieve strategieën gebaseerd op een specifiek inzicht in het bijzondere redoxmilieu bij elk afzonderlijk model van inflammatie.

Angiotensine II infusie in muizen: een voorbeeld van de antioxidatieve strategie van de nier

In ratten veroorzaakt angiotensine II (AngII) infusie uitgesproken ontsteking en schade. Er zijn veel aanwijzingen dat deze effecten worden gemedieerd door oxidatieve stress afhankelijk van NADPH oxidase. Muizen, daarentegen, zijn zeer resistent tegen hypertensieve nierschade. Onze hypothese was dat muizen over intrinsieke antioxidatieve mechanismen beschikken die ze beschermen tegen oxidatie-afhankelijke AngII-geïnduceerde hypertensieve nierschade. Wij onderzochten de pro- en antioxidatieve genenrespons op AngII (hoofdstuk 5). We gebruikten de C57BL/6 muizenstam, waarvan bekend staat dat zij bijzonder resistent is tegen diverse modellen van nierschade waar zelfs sommige andere muizenstammen gevoelig voor zijn. AngII induceerde hypertensie maar geen nierschade. Er was geen sterke toename in lipidperoxidatie. Genexpressieanalyse toonde aan dat AngII een significante toename veroorzaakte in de expressie van renale antioxidantgenen, daarentegen eerder een afname dan een toename in de expressie van de NADPH componenten p22phox en Rac2 en van xanthine oxidase. Genexpressie van hemoxygenase-1 (HO-1), een enzym met antioxidatieve en anti-inflammatoire eigenschappen, was verhoogd.

In tegenstelling tot de nier, veroorzaakte AngII significante vasculaire oxidatieve stress en schade in de aorta, met onder meer sterk verhoogde expressie van de NADPH oxidase component p47phox en aortahypertrofie ondanks toegenomen HO-1. Deze data

suggereren dat orgaan-specifieke antioxidatieve bescherming aanwezig is in de nieren van deze muizen. Om het onderliggende AngII signaleringspad van deze bescherming te ontleden, hebben wij muizen behandeld met een AngII AT2-receptorblokker. Wij gingen er vanuit dat AT2-blokkade de beschermende respons op AngII zou dempen. Echter, AT2-blokkade had weinig effect hierop. Ondanks een verdere toename in bloeddruk, was de expressie van pro-oxidatieve genen in de nierschors eerder afgenomen dan verhoogd. Dus antioxidatieve bescherming wordt niet gemedieerd door de AT2-receptor. Deze data ondersteunen de gedachte dat de natuurlijke weerstand tegen schade in dit muismodel het gevolg is van een versterkte endogene capaciteit tegen oxidatieve stress. Een tot dusver niet geïdentificeerd pad verlaagt pro-oxidatieve factoren zoals NADPH oxidase en xanthine oxidase tijdens AngII-stimulatie. Het is fascinerend dat C57BL/6 muizen zo effectief nierschade weerstaan gezien het feit dat, zoals wij zagen in **hoofdstuk 3,** zij een hogere basale tubulointerstitiële macrofaagaantal vertonen dan de meer gevoelige 129S2/SV muizen. Het is denkbaar dat de hogere macrofaagaantallen in de uitgangssituatie een functionele weergave is van een immunologisch tolerant systeem met verlaagde gevoeligheid voor endogene oxidatieve en inflammatoire stimuli.

Unilaterale ureterobstructie: NaDPH oxidase: kandidaat mechanisme. Apocynine: kandidaat behandeling

Unilaterale ureterobstructie (UUO) wordt gekarakteriseerd door vroege en uitgebreide tubulointerstitiële ontstekingscelinfiltraten. Oxidatieve stress wordt verondersteld een sleutelrol te spelen in het op gang komen en onderhouden van de ontsteking. Zonder decompressie door het opheffen van de obstructie dragen oxidatieve stress en ontsteking bij aan de verdere pathogenese zodat tubulointerstitiële ontsteking voortschrijdt en er een aanzet tot verbindweefseling onstaat. De bron van oxidatieve stress is onduidelijk. Een toename in mRNA en eiwitexpressie van NADPH oxidase componenten is waargenomen. Onze hypothese was dat farmacologische blokkade van NADPH oxidase de oxidant-afhankelijke ontsteking zou verminderen. Hiertoe gebruikten wij apocynine, een specifieke remmer van de assemblage van het NADPH oxidase complex (hoofdstuk 6).

Apocynine veroorzaakte een beperkte maar significante afname in tubulointerstitiële macrofaagaantallen tijdens de vroege UUO-geïnduceerde ontsteking (d.w.z. na 2 dagen UUO). Dit beperkte effect wijst op andere bronnen van oxidatieve stress naast NADPH oxidase; mogelijk zijn er ook andere mechanismen die niet afhankelijk zijn

van oxidatieve stress. Apocynine is wellicht waardevol onder condities waar NADPH oxidase een belangrijke bron is van oxidatieve stress, of als aanvullende therapie in gevallen waar meerdere mechanismen of verschillende bronnen van oxidatie een rol spelen. Hiernaast verminderde apocynine significant de UUO-geïnduceerde afname in bone morphogenetic protein (BMP-7) mRNA expressie. BMP-7 is een endogeen molecuul met een sterk renoprotectief effect. Dit suggereert dat NADPH-oxidase betrokken is bij BMP-7 gen regulatie na UUO. Apocynine bereikt zijn anti-inflammatoire effect wellicht door het ondersteunen van BMP-7 expressie.

Ẹda ni ṣoki l'edee Yoruba
(Samenvatting in het Yoruba)
(A short summary in Yoruba)

Iwadii ọna l'ati gb'ogun ti ap'apọju ẹya-atẹgun al'akan'pa to nṣe ẹdọ-kidinrin l'eṣe

Akọsọ ọrọ

Iwe yii jẹ iroyin iṣẹ iwadii ninu imọ-ijinlẹ sayẹnsi iṣegun, ti onişegun Dapọ Ishọla ṣe gẹgẹ bii iṣẹ l'ati gba oye ọmọwe ninu sayẹnsi iṣegun. Iṣẹ ti a ṣe iroyin rẹ n'inu iwe yii da le l'ori awọn iwadii l'ati fi kun imọ ti o rọ mọ ipa ti awọn ero'ja ara ti a mọ si ẹya-atẹgun akan'pa n ko ninu orişirişi aisan ti o maa nda ẹya ara ti a npe ni ẹdọ-kidinrin l'aamu.

Kini ẹya-atẹgun akanpa?

Ẹya-atẹgun akan'pa je orukọ ti a fun awon ero'ja ara kan. Awon eroja wọnyi jẹ koşee-ma-nii fun orişi iṣẹ ninu ara. Ṣugbọn, şe a kuku mọ pe "ş'oki l'ọbe oge"; ti awọn eroja yii ba ti pọ ju bi o şe yẹ lọ, wahala ati idaamu ni wọn maa nfa. Ninu ara eda, awọn isọri eroja kan tun wa, ti a le pe ni awọn *agb'ogun-t'akan'pa*, t'o jẹ wipe iṣẹ ti'wọn ni l'ati rii daju pe ẹya-atẹgun akan'pa ko pọ ju l'ara. Ṣugbọn l'asiko aisan, awọn eroja agb'ogun wọnyi le ma şişẹ bi o şe yẹ; nitori eyi, ẹya-atẹgun akan'pa a waa di apọju, a si maa fa "gbọnmi-sii, omi-o-too".

Kini eredii iwadii ti a kọ s'inu iwe yii?

Iwadii to s'iwajuu tiwa ti bẹrẹ sii tu aşiri pe, apọju ẹya-atẹgun akan'pa jẹ pataki l'ara ibajẹ t'o wọpọ l'asiko ọpọlọpọ isọri aisan edọ-kidinrin. Ete iwadii tiwa yii pin si ọna meji: (a) l'akọkọ, a şe afikun imọ nipa awọn ọna ti ẹya-atẹgun akan'pa maa ngba l'ati şe okunfa fun *itara-kikan* ninu ẹdọ-kidinrin. (b) eji, a ş'ayẹwo wipe, boya awọn oogun ti o maa n gb'ogun t'akanpa le ni agbara l'ati d'ẹkun aisan edọ-kidinrin.

Alaye ni ṣoki l'orii ọna ti a gba şe iwadii naa

Ọna meji pataki ni a gba şe awọn iwadii wọnyi. (a) L'akọkọ, a şe ayẹwo awọn *ipilẹ-ẹya-ara* ti a mu lati edọ-kidinrin. (b) L'ọna keji, a lo awọn ẹranko-ayẹwo sayẹnsi (paapaa julọ, awọn ekute-ile al'ayẹwo), to nşe idaamu orişi aisan edọ-kidinrin. A şe ayẹwo wipe, boya awọn oogun agb'ogun-t'akanpa pato le şe anfaani fun awọn ekute al'aisan wọnyi. Ireti ni wipe, ti a ba ri oogun ti o ş'iṣẹ daadaa ninu ekute, boya l'ọjọ iwaju, oogun naa yoo şee lo fun awọn eniyan ti wọn ba ni iru idaamu bẹẹ.

Awọn şaraki ayọrisi ninu iwadii naa

Pataki ninu awọn nkan ti a ri gbe ja'de lati inu iwadii yii ni wipe: (a) Ninu ayẹwo ipilẹ-ẹya-ara, oogun orişi mẹta ni a ri t'o şe anfaani. Orukọ wọn ni rotenone ati CCCP, ati isọri miran to njẹ EUK-8. Oogun wọnyi se id'ẹkun akan'pa, bẹẹ ni wọn tun d'ẹkun eroja itara-kikan ti a pe ni interleukin-6 ninu awọn ipilẹ-ẹya-ara. (b) Ninu ayẹwo ekute-ile, ẹya 129S2/Sv, oogun agb'ogun-t'akanpa rotenone, ati omiran ti a mọ si tempol, şe anfaani ninu aisan kidinrin ti o jẹ mọ ap'apọju ero'ja-ounjẹ proteini ninu itọ. Awọn oogun naa şe id'ẹkun itara-kikan ninu ẹdọ-kidinrin awọn ekute. (d) Ninu aisan ti o jẹ mọ ẹjẹ-riru, paapaa isọri ẹjẹ-riru ti ap'apọju Angiotensin-keji şe agb'atẹru rẹ, ayẹwo ninu awọn ekute-ile ẹya C57BL/6, fi han wipe ẹdọ-kidinrin nşe ida-abo-bo t'o l'agbara fun'ra rẹ pelu ọpọlọpọ ero'ja ara t'o nşe işẹ agb'ogun-t'akan'pa. Ifihan yii fi ye wa wipe, ti a ba le wadii ọna l'ati şe afikun awọn ero'ja ara agb'ogun wọnyi, ireti wa daadaa l'ati ki ọwọ ẹya-atẹgun akan'pa bọ'le patapata. (e) Ninu iwadii aisan ti o rọ mọ idi'na ọna ti itọ ngba jade, oogun agb'ogun-t'akan'pa ti a pe ni apocynin şe id'ẹkun pataki fun itara-kikan ninu edọ-kidinrin ekute-ile ẹya C57BL/6.

Ireti iwulo awọn ayọrisi iwadii yii l'ọjọ iwaju

Ireti wa wipe l'ojọ iwaju, awọn ayọrisi iwadi wọnyi yoo wulo fun itọju awọn eniyan t'o nşe aisan ẹdọ-kidinrin. Awon iwadii wa ninu ipilẹ-ẹya-ara, ati iwadi ninu ekute-ile, ti a kọ l'ẹkun-rẹrẹ s'inu iwe yii l'edee Gẹẹsi, fi han gbanbga pe awọn oogun agb'ogun-t'akan'pa le şe id'ekun pataki fun itara-kikan ninu aisan edọ-kidinrin. Pato awọn oogun ti a ri pe wọn şe anfaani ni tempol, apocynin, EUK-8, rotenone, ati CCCP. Pẹlu ọpọlọpọ iwadii s'iwaju sii, o şeeşe pe n'igba to ba ya, ọkan tabi eji ninuu awọn wọnyi yoo di lilo l'ọwọ awọn onişegun n'ile iwosan.

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Ẹya-atẹgun akan'pa – reactive oxygen species (ROS)
Agb'ogun-t'akan'pa – antioxidant
Itara-kikan - inflammation
Ipilẹ-ẹya-ara - cells

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Curriculum vitae

David Adedapọ Ishọla was born in Accra, Ghana, on 8th November, 1970, to Rachel Aderonkẹ (nee Ajao) and David Adebọwale Ishọla (1939-2001), the first of five children. In 1976 the family moved to their home country of Nigeria, where David attended primary and secondary schools in the western states of Ọyọ and Ọşun. After obtaining his School Certificate in 1985 as the recipient of the Excellence Award of the West African Examinations Council (WAEC), he proceeded to study at the medical school of the Ọbafẹmi Awolọwọ University (OAU), in Ile-Ifẹ, qualifying as a doctor in October 1991. He then worked as a House Officer at the OAU Teaching Hospital (OAUTHC), undertook Nigerian National Service (NYSC) duties in Calabar, and served as a general/primary care practitioner in Ibadan. In October 1995, he returned to the OAUTHC for postgraduate clinical specialty training. He became a junior specialist (senior registrar) in Internal Medicine in October 1998, and a full specialist (consultant) Physician and Nephrologist in October 2001, upon attaining the Fellowship of the West African College of Physicians (FWACP).

Having developed a keen interest in research by this point, David obtained a 12-month clinical and research fellowship award (the MRC-WACP Training Fellowship) at the UK Medical Research Council (MRC) Laboratories in Fajara, The Gambia, where he made observations on the clinical patterns of proteinuric syndromes among Gambian children. Seeking training and experience in experimental medicine, in November 2002 he was accepted for PhD studies in the Department of Nephrology and Hypertension at Utrecht University, under Prof. dr. Hein Koomans, Dr. Jaap Joles, and Dr. Branko Braam. The experimental work was carried out in various laboratories within the Gemeenschappelijk Dierenlaboratorium (GDL), the University Medical Centre (UMC) Utrecht, and the Faculty of Biology. The findings are presented in this thesis.

David has recently taken up a post in Public Health Medicine with the UK National Health Service (NHS) in North West England. He uses the suffix “Jr” after his name, reflecting the shared first name with his Dad. In Nigeria he is commonly called Dapọ, a short form of his middle name. Dapọ takes pleasure and pride in his Yoruba nationality and Nigerian and African identity, and among his many interests are sports, writing, family matters, Christian theology, development issues, and travelling.