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).

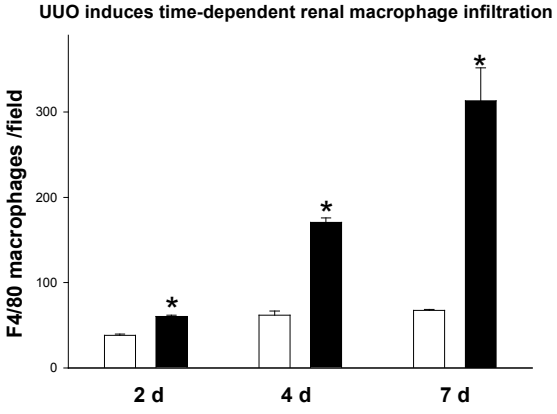

Figure 1. Renal interstitial macrophage population after 2, 4 or 7 days of UUO. White bars: contralateral kidneys, black bars: obstructed kidneys. Abbreviation: d=day (duration of UUO). *p<0.01, obstructed vs. contralateral kidney. Microscopic field area: 0.245 mm^2

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.

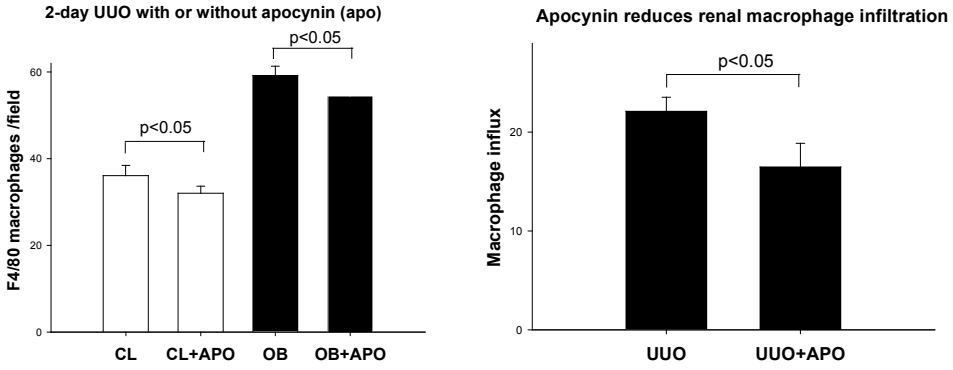

Figure 2. A (left panel). Renal interstitial macrophage population after 2 days of UUO, with or without apocynin treatment. **B (right panel).** Net macrophage influx into obstructed kidneys after 2 days of UUO, with or without apocynin treatment. White bars: contralateral kidneys (CL), black bars: obstructed 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.

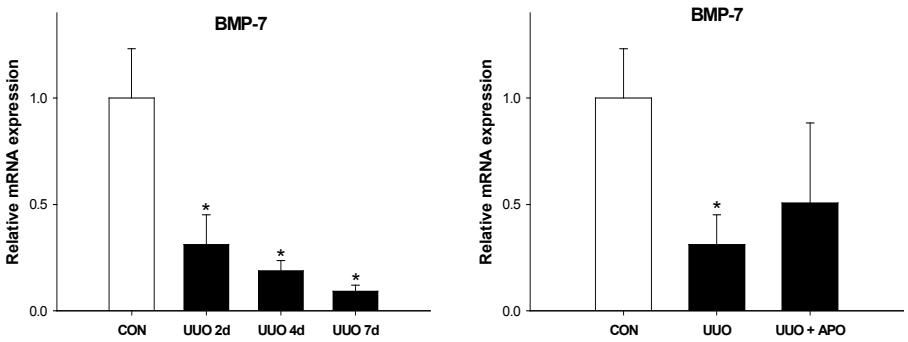

Figure 3. A (left panel). Kidney mRNA expression of BMP-7 after 2, 4, or 7 days (d) of UUO. **B (right panel).** Effect of apocynin (APO) on kidney mRNA expression of BMP-7 after 2d of UUO. mRNA in obstructed kidneys relative to contralateral. White bar: contralateral kidneys used as control (CON), black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

References

1. Schreiner GF, Harris KP, Purkerson ML, Klahr S: Immunological aspects of acute ureteral obstruction: immune cell infiltrate in the kidney. *Kidney Int* 34:487-493, 1988
2. Klahr S, Morrissey J: Obstructive nephropathy and renal fibrosis. *Am J Physiol Renal Physiol* 283:F861-875, 2002
3. Bascands JL, Schanstra JP: Obstructive nephropathy: insights from genetically engineered animals. *Kidney Int* 68:925-937, 2005
4. Vielhauer V, Anders HJ, Mack M, et al.: Obstructive nephropathy in the mouse: progressive fibrosis correlates with tubulointerstitial chemokine expression and accumulation of CC chemokine receptor 2- and 5-positive leukocytes. *J Am Soc Nephrol* 12:1173-1187, 2001
5. Anders HJ, Vielhauer V, Frink M, et al.: A chemokine receptor CCR-1 antagonist reduces renal fibrosis after unilateral ureter ligation. *J Clin Invest* 109:251-259, 2002
6. Ophascharoensuk V, Giachelli CM, Gordon K, et al.: Obstructive uropathy in the mouse: role of osteopontin in interstitial fibrosis and apoptosis. *Kidney Int* 56:571-580, 1999
7. Taneda S, Hudkins KL, Topouzis S, et al.: Obstructive uropathy in mice and humans: potential role for PDGF-D in the progression of tubulointerstitial injury. *J Am Soc Nephrol* 14:2544-2555, 2003
8. Le Meur Y, Tesch GH, Hill PA, et al.: Macrophage accumulation at a site of renal inflammation is dependent on the M-CSF/c-fms pathway. *J Leukoc Biol* 72:530-537, 2002
9. Chevalier RL: Molecular and cellular pathophysiology of obstructive nephropathy. *Pediatr Nephrol* 13:612-619, 1999
10. Kawada N, Moriyama T, Ando A, et al.: Increased oxidative stress in mouse kidneys with unilateral ureteral obstruction. *Kidney Int* 56:1004-1013, 1999
11. Pat B, Yang T, Kong C, Watters D, Johnson DW, Gobe G: Activation of ERK in renal fibrosis after unilateral ureteral obstruction: modulation by antioxidants. *Kidney Int* 67:931-943, 2005
12. Saborio P, Krieg RJ, Jr., Kuemmerle NB, Norkus EP, Schwartz CC, Chan JC: Alpha-tocopherol modulates lipoprotein cytotoxicity in obstructive nephropathy. *Pediatr Nephrol* 14:740-746, 2000
13. Lin KC, Krieg RJ, Jr., Saborio P, Chan JC: Increased heat shock protein-70 in unilateral ureteral obstruction in rats. *Mol Genet Metab* 65:303-310, 1998
14. Moriyama T, Kawada N, Nagatoya K, Horio M, Imai E, Hori M: Oxidative stress in tubulointerstitial injury: therapeutic potential of antioxidants towards interstitial fibrosis. *Nephrol Dial Transplant* 15 Suppl 6:47-49, 2000
15. Sugiyama H, Kobayashi M, Wang DH, et al.: Telmisartan inhibits both oxidative stress and renal fibrosis after unilateral ureteral obstruction in acatalasemic mice. *Nephrol Dial Transplant* 20:2670-2680, 2005
16. Sunami R, Sugiyama H, Wang DH, et al.: Acatalasemia sensitizes renal tubular epithelial cells to apoptosis and exacerbates renal fibrosis after unilateral ureteral obstruction. *Am J Physiol Renal Physiol* 286:F1030-1038, 2004

17. Manucha W, Carrizo L, Ruete C, Molina H, Valles P: Angiotensin II type I antagonist on oxidative stress and heat shock protein 70 (HSP 70) expression in obstructive nephropathy. *Cell Mol Biol (Noisy-le-grand)* 51:547-555, 2005
18. Truong LD, Choi YJ, Tsao CC, et al.: Renal cell apoptosis in chronic obstructive uropathy: the roles of caspases. *Kidney Int* 60:924-934, 2001
19. Miyajima A, Chen J, Poppas DP, Vaughan ED, Jr., Felsen D: Role of nitric oxide in renal tubular apoptosis of unilateral ureteral obstruction. *Kidney Int* 59:1290-1303, 2001
20. Frokiaer J: Pharmacologic intervention in urinary tract obstruction--is it possible? *Kidney Int* 68:894-895, 2005
21. Ito K, Chen J, Khodadadian JJ, et al.: Liposome-mediated transfer of nitric oxide synthase gene improves renal function in ureteral obstruction in rats. *Kidney Int* 66:1365-1375, 2004
22. Chevalier RL: Promise for gene therapy in obstructive nephropathy. *Kidney Int* 66:1709-1710, 2004
23. Brownlee M: Biochemistry and molecular cell biology of diabetic complications. *Nature* 414:813-820, 2001
24. Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54:1615-1625, 2005
25. Vaziri ND, Dicus M, Ho ND, Boroujerdi-Rad L, Sindhu RK: Oxidative stress and dysregulation of superoxide dismutase and NADPH oxidase in renal insufficiency. *Kidney Int* 63:179-185, 2003
26. Satoh M, Fujimoto S, Haruna Y, et al.: NAD(P)H oxidase and uncoupled nitric oxide synthase are major sources of glomerular superoxide in rats with experimental diabetic nephropathy. *Am J Physiol Renal Physiol* 288:F1144-1152, 2005
27. Vieira JM, Jr., Mantovani E, Rodrigues LT, et al.: Simvastatin attenuates renal inflammation, tubular transdifferentiation and interstitial fibrosis in rats with unilateral ureteral obstruction. *Nephrol Dial Transplant* 20:1582-1591, 2005
28. Babior BM: NADPH oxidase: an update. *Blood* 93:1464-1476, 1999
29. Babior BM: NADPH oxidase. *Curr Opin Immunol* 16:42-47, 2004
30. Griendling KK, Sorescu D, Ushio-Fukai M: NAD(P)H oxidase: role in cardiovascular biology and disease. *Circ Res* 86:494-501, 2000
31. Chabrashvili T, Tojo A, Onozato ML, et al.: Expression and cellular localization of classic NADPH oxidase subunits in the spontaneously hypertensive rat kidney. *Hypertension* 39:269-274, 2002
32. Geiszt M, Kopp JB, Varnai P, Leto TL: Identification of renox, an NAD(P)H oxidase in kidney. *Proc Natl Acad Sci U S A* 97:8010-8014, 2000
33. Gwinner W, Deters-Evers U, Brandes RP, et al.: Antioxidant-oxidant balance in the glomerulus and proximal tubule of the rat kidney. *J Physiol* 509 (Pt 2):599-606, 1998
34. Griendling KK, Harrison DG: Out, damned dot: studies of the NADPH oxidase in atherosclerosis. *J Clin Invest* 108:1423-1424, 2001

35. Gaertner SA, Janssen U, Ostendorf T, Koch KM, Floege J, Gwinner W: Glomerular oxidative and antioxidative systems in experimental mesangioproliferative glomerulonephritis. *J Am Soc Nephrol* 13:2930-2937, 2002
36. Bapat S, Post JA, Braam B, et al.: Visualizing tubular lipid peroxidation in intact renal tissue in hypertensive rats. *J Am Soc Nephrol* 13:2990-2996, 2002
37. Adler S, Huang H, Wolin MS, Kaminski PM: Oxidant stress leads to impaired regulation of renal cortical oxygen consumption by nitric oxide in the aging kidney. *J Am Soc Nephrol* 15:52-60, 2004
38. Liu JQ, Zelko IN, Erbynn EM, Sham JS, Folz RJ: Hypoxic pulmonary hypertension: role of superoxide and NADPH oxidase (gp91phox). *Am J Physiol Lung Cell Mol Physiol* 290:L2-10, 2006
39. Jackson SH, Gallin JI, Holland SM: The p47phox mouse knock-out model of chronic granulomatous disease. *J Exp Med* 182:751-758, 1995
40. Pollock JD, Williams DA, Gifford MA, et al.: Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nat Genet* 9:202-209, 1995
41. Moulton P, Martin H, Ainger A, et al.: The inhibition of flavoproteins by phenoxaiodonium, a new iodonium analogue. *Eur J Pharmacol* 401:115-120, 2000
42. Li Y, Trush MA: Diphenyleneiodonium, an NAD(P)H oxidase inhibitor, also potently inhibits mitochondrial reactive oxygen species production. *Biochem Biophys Res Commun* 253:295-299, 1998
43. Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ: Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* 11:95-102, 1994
44. Muijsers RB, van Den Worm E, Folkerts G, et al.: Apocynin inhibits peroxynitrite formation by murine macrophages. *Br J Pharmacol* 130:932-936, 2000
45. Attia DM, Verhagen AM, Stroes ES, et al.: Vitamin E alleviates renal injury, but not hypertension, during chronic nitric oxide synthase inhibition in rats. *J Am Soc Nephrol* 12:2585-2593, 2001
46. Peng T, Lu X, Feng Q: Pivotal role of gp91phox-containing NADH oxidase in lipopolysaccharide-induced tumor necrosis factor-alpha expression and myocardial depression. *Circulation* 111:1637-1644, 2005
47. Hart BA, Elferink JG, Nibbering PH: Effect of apocynin on the induction of ulcerative lesions in rat skin injected with tubercle bacteria. *Int J Immunopharmacol* 14:953-961, 1992
48. Miriyala S, Gongora Nieto MC, Mingone C, et al.: Bone morphogenic protein-4 induces hypertension in mice: role of noggin, vascular NADPH oxidases, and impaired vasorelaxation. *Circulation* 113:2818-2825, 2006
49. Yang Z, Asico LD, Yu P, et al.: D5 dopamine receptor regulation of reactive oxygen species production, NADPH oxidase, and blood pressure. *Am J Physiol Regul Integr Comp Physiol* 290:R96-R104, 2006

50. Hu L, Zhang Y, Lim PS, et al.: Apocynin but not L-arginine prevents and reverses dexamethasone-induced hypertension in the rat. *Am J Hypertens* 19:413-418, 2006
51. Zhang Y, Chan MM, Andrews MC, et al.: Apocynin but not allopurinol prevents and reverses adrenocorticotropic hormone-induced hypertension in the rat. *Am J Hypertens* 18:910-916, 2005
52. Asaba K, Tojo A, Onozato ML, et al.: Effects of NADPH oxidase inhibitor in diabetic nephropathy. *Kidney Int* 67:1890-1898, 2005
53. Yi F, Zhang AY, Li N, et al.: Inhibition of ceramide-redox signaling pathway blocks glomerular injury in hyperhomocysteinemic rats. *Kidney Int* 70:88-96, 2006
54. Lopez B, Salom MG, Arregui B, Valero F, Fenoy FJ: Role of superoxide in modulating the renal effects of angiotensin II. *Hypertension* 42:1150-1156, 2003
55. Dorman RB, Wunder C, Saba H, Shoemaker JL, MacMillan-Crow LA, Brock RW: NAD(P)H oxidase contributes to the progression of remote hepatic parenchymal injury and endothelial dysfunction, but not microvascular perfusion deficits. *Am J Physiol Gastrointest Liver Physiol* 290:G1025-1032, 2006
56. Angermayr B, Fernandez M, Mejias M, Gracia-Sancho J, Garcia-Pagan JC, Bosch J: NAD(P)H oxidase modulates angiogenesis and the development of portosystemic collaterals and splanchnic hyperaemia in portal hypertensive rats. *Gut*, 2006
57. Hougee S, Hartog A, Sanders A, et al.: Oral administration of the NADPH-oxidase inhibitor apocynin partially restores diminished cartilage proteoglycan synthesis and reduces inflammation in mice. *Eur J Pharmacol* 531:264-269, 2006
58. t Hart BA, Simons JM, Knaan-Shanzer S, Bakker NP, Labadie RP: Antiarthritic activity of the newly developed neutrophil oxidative burst antagonist apocynin. *Free Radic Biol Med* 9:127-131, 1990
59. Hart BA, Simons JM: Metabolic activation of phenols by stimulated neutrophils: a concept for a selective type of anti-inflammatory drug. *Biotechnol Ther* 3:119-135, 1992
60. Zhan G, Serrano F, Fenik P, et al.: NADPH oxidase mediates hypersomnolence and brain oxidative injury in a murine model of sleep apnea. *Am J Respir Crit Care Med* 172:921-929, 2005
61. Al-Shabrawey M, Bartoli M, El-Remessy AB, et al.: Inhibition of NAD(P)H oxidase activity blocks vascular endothelial growth factor overexpression and neovascularization during ischemic retinopathy. *Am J Pathol* 167:599-607, 2005
62. Fern RJ, Yesko CM, Thornhill BA, Kim HS, Smithies O, Chevalier RL: Reduced angiotensinogen expression attenuates renal interstitial fibrosis in obstructive nephropathy in mice. *J Clin Invest* 103:39-46, 1999
63. Kaneto H, Morrissey J, McCracken R, Reyes A, Klahr S: Enalapril reduces collagen type IV synthesis and expansion of the interstitium in the obstructed rat kidney. *Kidney Int* 45:1637-1647, 1994
64. Satoh M, Kashihara N, Yamasaki Y, et al.: Renal interstitial fibrosis is reduced in angiotensin II type 1a receptor-deficient mice. *J Am Soc Nephrol* 12:317-325, 2001

65. Stambe C, Atkins RC, Tesch GH, Masaki T, Schreiner GF, Nikolic-Paterson DJ: The role of p38alpha mitogen-activated protein kinase activation in renal fibrosis. *J Am Soc Nephrol* 15:370-379, 2004
66. Nagatoya K, Moriyama T, Kawada N, et al.: Y-27632 prevents tubulointerstitial fibrosis in mouse kidneys with unilateral ureteral obstruction. *Kidney Int* 61:1684-1695, 2002
67. Yokoi H, Mukoyama M, Nagae T, et al.: Reduction in connective tissue growth factor by antisense treatment ameliorates renal tubulointerstitial fibrosis. *J Am Soc Nephrol* 15:1430-1440, 2004
68. Klahr S, Morrissey J: Obstructive nephropathy and renal fibrosis: The role of bone morphogenic protein-7 and hepatocyte growth factor. *Kidney Int Suppl*:S105-112, 2003
69. Schiller M, Javelaud D, Mauviel A: TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. *J Dermatol Sci* 35:83-92, 2004
70. Inazaki K, Kanamaru Y, Kojima Y, et al.: Smad3 deficiency attenuates renal fibrosis, inflammation,and apoptosis after unilateral ureteral obstruction. *Kidney Int* 66:597-604, 2004
71. Sato M, Muragaki Y, Saika S, Roberts AB, Ooshima A: Targeted disruption of TGF-beta1/Smad3 signaling protects against renal tubulointerstitial fibrosis induced by unilateral ureteral obstruction. *J Clin Invest* 112:1486-1494, 2003
72. Fukasawa H, Yamamoto T, Togawa A, et al.: Down-regulation of Smad7 expression by ubiquitin-dependent degradation contributes to renal fibrosis in obstructive nephropathy in mice. *Proc Natl Acad Sci U S A* 101:8687-8692, 2004
73. Kawabata M, Imamura T, Miyazono K: Signal transduction by bone morphogenetic proteins. *Cytokine Growth Factor Rev* 9:49-61, 1998
74. Miyazono K, Maeda S, Imamura T: BMP receptor signaling: transcriptional targets, regulation of signals, and signaling cross-talk. *Cytokine Growth Factor Rev* 16:251-263, 2005
75. Zwijsen A, Verschueren K, Huylebroeck D: New intracellular components of bone morphogenetic protein/Smad signaling cascades. *FEBS Lett* 546:133-139, 2003
76. Hruska KA, Guo G, Wozniak M, et al.: Osteogenic protein-1 prevents renal fibrogenesis associated with ureteral obstruction. *Am J Physiol Renal Physiol* 279:F130-143, 2000
77. Klahr S: The bone morphogenetic proteins (BMPs). Their role in renal fibrosis and renal function. *J Nephrol* 16:179-185, 2003
78. Zeisberg M: Bone morphogenic protein-7 and the kidney: current concepts and open questions. *Nephrol Dial Transplant* 21:568-573, 2006
79. Inoue T, Plieth D, Venkov CD, Xu C, Neilson EG: Antibodies against macrophages that overlap in specificity with fibroblasts. *Kidney Int* 67:2488-2493, 2005
80. Rouschop KM, Sewnath ME, Claessen N, et al.: CD44 deficiency increases tubular damage but reduces renal fibrosis in obstructive nephropathy. *J Am Soc Nephrol* 15:674-686, 2004
81. Gould SE, Day M, Jones SS, Dorai H: BMP-7 regulates chemokine, cytokine, and hemodynamic gene expression in proximal tubule cells. *Kidney Int* 61:51-60, 2002

82. Lin J, Patel SR, Cheng X, et al.: Kielin/chordin-like protein, a novel enhancer of BMP signaling, attenuates renal fibrotic disease. *Nat Med* 11:387-393, 2005

83. Ishola DA, Jr., van der Giezen DM, Hahnel B, et al.: In mice, proteinuria and renal inflammatory responses to albumin overload are strain-dependent. *Nephrol Dial Transplant* 21:591-597, 2006

84. Ishola DA, Post JA, van Timmeren MM, et al.: Albumin-bound fatty acids induce mitochondrial oxidant stress and impair antioxidant responses in proximal tubular cells. *Kidney Int*, 2006

85. Wesseling S, Ishola DA, Jr., Joles JA, Bluyssen HA, Koomans HA, Braam B: Resistance to oxidative stress by chronic infusion of angiotensin II in mouse kidney is not mediated by the AT2 receptor. *Am J Physiol Renal Physiol* 288:F1191-1200, 2005

86. Ma LJ, Fogo AB: Model of robust induction of glomerulosclerosis in mice: importance of genetic background. *Kidney Int* 64:350-355, 2003

87. Hartner A, Cordasic N, Klanke B, Veelken R, Hilgers KF: Strain differences in the development of hypertension and glomerular lesions induced by deoxycorticosterone acetate salt in mice. *Nephrol Dial Transplant* 18:1999-2004, 2003