Atherogenic scavenger receptor modulation in the tubulointerstitium in response to chronic renal injury

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Abstract

Oxidized lipoproteins (oxLDL) and their scavenger receptor (SR) binding partners play a central role in atherosclerosis and by analogy may play a role in chronic kidney disease pathogenesis. The present study was designed to investigate in C57BL/6 mice the effects of hypercholesterolemia on renal injury severity and oxLDL generation after unilateral ureteral obstruction (UUO). The expression profiles of CD36, SR class AI/II (SR-A), lectin-like receptor for oxidized low-density lipoprotein-1 (Lox-1), and SR that binds phosphatidylserine and oxidized lipoprotein (SR-PSOX/CXCL16) were examined. Four experimental groups were studied: sham and UUO male mice on either a high fat Western diet or a control diet. Significantly more oxLDL accumulated in the tubulointerstitium of hypercholesterolemic mice compared to normocholesterolemic mice after 14 days of UUO ($P<0.01$). Total kidney collagen was significantly higher in the obstructed kidneys of hypercholesterolemic mice compared to normocholesterolemic mice on day 14 ($P<0.01$). After 14 days of obstruction, the number of interstitial F4/80+ macrophages and NF-kappa-B activation increased in hypercholesterolemic mice compared to normocholesterolemic mice ($P<0.01$). In normal kidneys, CD36, SR-A, Lox-1, and CXCL16 were primarily localized to renal tubular epithelia. After ureteral obstruction, CD36 increased at day 7; SR-A and Lox-1 progressively decreased in a time-dependent manner; and CXCL16 increased significantly with the onset of obstruction ($P<0.01$). Strong tubular expression suggests that in addition to inflammatory interstitial cells, renal tubular scavenger receptors may help to orchestrate the inflammatory and fibrogenic pathways that are activated by oxLDL.
Key words: interstitial fibrosis, CD36, SR-A, Lox-1, CXCL16, SR-PSOX, hypercholesterolemia, oxidized lipoprotein
Introduction

The number of patients with chronic and end-stage renal disease (ESRD) is increasing at an alarming rate (12). Several studies have demonstrated that chronic kidney disease increases the risk for cardiovascular morbidity and mortality primarily by accelerating atherosclerotic disease (12, 29). Many parallels have been drawn between atherogenesis and the pathogenetic mechanisms that cause progressive kidney destruction by fibrosis. Can the same pathologic mechanisms that accelerate atherosclerotic disease in chronic kidney disease amplify fibrogenic pathways in the kidney?

Accumulation of oxidized lipoproteins (oxLDL) has been reported in the circulation and renal interstitium in both experimental models and patients with chronic kidney disease and ESRD (7, 40). In vitro and in vivo studies suggest that oxidized low-density lipoprotein (oxLDL) may exert biologically relevant responses in situ in the kidney. In vitro, LDL and oxLDL stimulate mesangial cells to secrete chemoattractant chemokines that may trigger an influx of monocytes (41). Several animal studies have further suggested that chronic exposure to oxLDL promotes collagen synthesis and activates pro-inflammatory pathways (9, 40). Few studies of progressive renal fibrosis have examined the relationship between renal oxidized lipoproteins and their primary binding partner, members of the scavenger receptor superfamily. Scavenger receptors mediate the endocytosis and degradation of oxLDL in vivo, but they may also activate intracellular signaling pathways that contribute to the pro-inflammatory milieu that initiates and propagates atherosclerosis (37). However, little is known about the modulation and function of scavenger receptors in kidneys during normal and
hypercholesterolemic states and how scavenger receptors are regulated in response to acute and chronic injury.

Scavenger receptors are transmembrane receptors that bind several ligands in addition to oxidized lipoproteins. They are expressed by many cell lineages but are best characterized in macrophages and the microvascular endothelium. Epithelial cell expression of several members of the scavenger receptor family has been reported but in general much less is known about their biological effects after ligand binding by this cell type. Nine classes of scavenger receptors (SR) have been identified based on structural similarities: class A (e.g. SR-A); SR-A like SRs; class B (e.g. CD36); class D (e.g. macrosialin); class E (lectin-like oxidized low-density lipoprotein receptor, Lox); class F (SRs expressed by endothelial cells, SREC); class G (scavenger receptor for phosphatidylserine and oxidized lipoprotein, SR-PSOX); fasciclin epidermal growth factor (EGF)-like, laminin-type EGF-like, and link domain (FEL); and CD163. However, SR-A, CD36, Lox-1, and CXCL16 appear to be the primary receptors for oxLDL based on atherogenesis data. Each receptor has been shown to promote or attenuate atherogenic effects in vivo through the endocytosis of oxLDL and activation of pro-inflammatory cascades following ligand binding (4, 18, 52, 54). Many of the other SRs function primarily as receptors in the immune response system (3, 6).

In murine models of atherosclerosis, both scavenger receptor A (SR-A) and CD36 has clearly been shown to play important functional roles in oxLDL processing and in disease progression. SR-A and CD36 account for up to 90 percent of the uptake of oxLDL by macrophages in vitro (28). Both are multi-ligand transmembrane receptors expressed by macrophages. In addition, CD36 is expressed by several other cell types
including microvascular endothelium, platelets, and epithelial cells. Recent evidence suggests that SR-A and CD36 may also serve as signal transduction receptors that modulate the inflammation associated with atherosclerosis (14, 26, 28, 35).

Lox-1 differs from other scavenger receptors in that it is primarily expressed on microvascular endothelial cells with low-level expression on macrophages, platelets, and smooth muscle cells (8). Recent studies suggest that Lox-1 may also mediate endothelial dysfunction by modulating the expression of endothelial constitutive nitric oxide synthase (eNOS) and by up-regulating adhesion molecules (34).

SR-PSOX/CXCL16 is one of the few scavenger receptors that is found in two distinct forms: membrane-bound and soluble. Membrane bound CXCL16 binds and internalizes oxLDL and promotes adhesion of cells expressing its cognate receptor CXCR6 (45, 46). Proteolytic cleavage of membrane bound CXCL16 releases soluble CXCL16 (1, 20) that is a chemoattractant for CXCR6+ cells such as polarized T helper cells (27). CXCL16 is expressed on endothelial cells, macrophages, and smooth muscle cells (22, 45).

In order to gain further insight into the role of renal scavenger receptors in response to intrarenal oxLDL generation, the present study was designed to investigate the expression pattern of the four atherogenic scavenger receptors (CD36, SR-A, Lox-1, and CXCL16) in kidneys chronically damaged by unilateral ureteral obstruction, both in a normal and a hypercholesterolemic environment. It is known from previous studies that oxidant stress and oxLDL production occur within kidneys that are damaged by chronic obstruction. This study was also designed to investigate the hypothesis that
hypercholesterolemia is associated with higher renal oxLDL levels during chronic injury, an effect that leads to worse fibrosis.
METHODS

Experimental Design

Studies were performed on male C57BL/6 mice purchased from Harlan Laboratories (Kent, WA, USA). Male mice were fed either a high fat Western diet (15.8% total fat with 0.5% sodium cholate (Harlan Teklad, Madison, WI), (39)) or a control diet of standard chow supplemented with 0.5% sodium cholate beginning after weaning at 3-4 weeks of age. After a run-in period of 8 weeks on the experimental diets, animals were randomly assigned to one of four experimental groups: sham surgery or unilateral ureteral obstruction (UUO) and either a high fat diet or a control diet. Groups of mice (n =4-10 each) were sacrificed at 3, 7, and 14 days after surgery. These time-points were selected as representative of the early phase when cellular recruitment is just beginning (day 3), a mid-point when growth factor-dependent matrix accumulation has begun (day 7) and a more advanced phase when structural kidney damage is becoming evident (day 14). All surgeries were performed under general anesthesia with isoflurane.

For mice in the UUO group, the left ureter was exposed through a mid-abdominal incision and ligated using 4-0 silk. Sham and UUO kidneys were harvested and processed for RNA and protein extraction and histological studies as previously described (31). Frozen tissue samples were stored at minus 80°C. All procedures were performed in accordance with the guidelines established by National Research Council Guide for the Care and Use of Laboratory Animals and approval of our Institute Animal Care and Use Committee (IACUC). Serum cholesterol levels were measured in blood samples that were obtained at sacrifice using the Total Cholesterol Kit (WAKO Chemicals USA, Inc., Richmond, VA, USA).
**Collagen Content**

Hydroxyproline content of kidney tissue (µg of hydroxyproline per mg of wet weight kidney section) was performed by acid hydrolysis of the tissue section using procedures established in our laboratory (31).

**Histological Examination**

Immunohistochemical staining was performed on sections of paraffin-embedded tissue or cryosections of snap-frozen tissue using procedures established in our laboratory with VECTASTAIN Elite ABC Kits (Vector Laboratories, Inc., Burlingame, CA, USA) and AEC Substrate Chromogen K3464 (DAKO Corp., Carpinteria, CA, USA) as the peroxidase substrate. Sections were blocked with Avidin/Biotin blocking kit (Vector Laboratories, Inc., CA, USA). For CXCL16 immunostaining, the Tyramide Signal Amplification (TSA) Biotin System was used (Perkin Elmer, Boston, MA, USA). Primary antibodies used were reactive with F4/80 (rat anti-mouse F4/80 monoclonal antibody; AbD Serotec, Raleigh, NC, USA), CD36 (rabbit anti-human CD36 polyclonal antibody; Cayman Chemical, Ann Arbor, MI, USA), Lox-1 (goat anti-mouse oxidized low density lipoprotein receptor-1 polyclonal antibody; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), scavenger receptor A (goat anti-mouse macrophage class A scavenger receptor; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), IkB-α and phosphorylated IkB-α (rabbit anti-human IkBα and rabbit anti-human phospho IkB-α; Cell Signaling Technology, Danvers, MA, USA), and oxLDL (rabbit anti-human hypochlorous acid modified LDL polyclonal antibody; Chemicon International, Inc., Temecula, CA, USA). Secondary antibodies were shown to be non-reactive with tissue sections stained without primary antibody. Semi-quantitative computer-assisted image
analysis of tubulointerstitial proteins was performed on six randomly selected 400x magnified images of slides from individual animals with Image-Pro Plus software (Mediatech, CA, USA). The glomerular area and space not occupied by tissue were subtracted in the analysis. Final results were expressed as the mean positive tubulointerstitial area. The investigator was blinded to the experimental groups at the time of analysis.

**Northern blotting**

Total kidney RNA was extracted using the TRIZOL single-step reagent (GIBCO BRL Life Technologies, Grand Island, NY, USA). Total kidney RNA (20ug) from each individual animal was separated by electrophoresis in a 1.2% agarose formaldehyde gel, transferred to a nylon membrane (Nytran; Schleicher & Schuell Bioscience Inc., Keene, NH, USA) by capillary blotting, and fixed by ultraviolet cross-linking. Northern blotting was performed as previously described (31). After washing, membranes were scanned with Typhoon 9410 PhosphorImager (GE Healthcare, Pittsburgh, PA, USA). The results were adjusted for RNA loading inequality based on the density of the 18s ribosomal RNA band. The cDNA probe used was mouse SR-PSOX/CXCL16 (EST GenBank AI019535 (33); ATCC, Manassas, VA, USA).

**Western blotting**

Pieces of frozen kidney were homogenized in buffer (1% SDS in 50mM Tris pH 7.6), frozen and thawed, and centrifuged at 13,000rpm in a microfuge at 4ºC. The supernatant was removed and protein concentration was determined using the BCA protein assay (Pierce Biotechnology, Rockford, IL, USA), and aliquots were stored at –80ºC prior to analysis. Kidney protein samples (40ug) were separated by 10% SDS-
polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The primary antibodies are described above. The secondary antibodies were HRP-conjugated bovine anti-goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA). Protein bands were visualized using the enhance chemiluminescence detection system (Pierce Biotechnology, Rockford, IL, USA). Band intensities were measured using the Image-Pro Plus software and normalized for protein loading equality as determined by staining membranes with Ponceau red. The major band for each scavenger receptor that was detected by Western blot was analyzed by densiotometry.

Statistical analysis

All data are presented as the mean and standard deviation. A nested analysis of variance (ANOVA) was utilized for all semi-quantitative computer-assisted image analysis. For image analysis data, the arithmetic mean of six randomly selected images of slides for each animal was used to calculate the reported mean of the group and the standard deviation. All other results were analyzed by unpaired Student’s t test. A \( P \) value <0.05 was considered statistically significant.
Results

Normal Renal SR Expression and Effects of Diet-Induced Hypercholesterolemia

Tubular expression of renal scavenger receptors

To elucidate the intrarenal scavenger receptor expression pattern in normal kidneys, protein and/or mRNA expression levels were analyzed in sham-operated kidneys. By immunostaining, CD36, SR-A, Lox-1, and CXCL16 were detected primarily on renal tubular epithelial cells in the sham-operated kidneys of normocholesterolemic and hypercholesterolemic mice (Fig. 1A-D). In normal kidneys, CD36 was detected predominantly in distal renal tubular epithelial cells and peritubular cells, likely microvascular endothelium; CD36 immunolocalized to the basolateral side of distal tubular epithelial cells (Fig. 1A). SR-A and CXCL16 were also expressed in low-levels on normal renal tubular epithelium (Fig. 1C, D).

By Western blotting the CD36 (88kD glycosylated), SR-A (70kD), and Lox-1 (84kD dimer) protein bands were detectable to varying degrees in sham kidneys from normal and hypercholesterolemic mice (Fig. 1E). Two protein bands were detected for Lox-1 and CD36 suggesting either differential glycosylation or alternative splicing (2, 38). Basal SR-A protein densities was relatively low and variable between individual animals compared to the expression levels of the other scavenger receptors. Due to low expression, a specific CXCL16 band was not detected by Western blotting. By Northern blotting low CXCL16 steady-state mRNA levels were detected in normal sham-operated kidneys (Fig. 1G).

Modulation of normal renal scavenger receptor expression with a high fat diet
To determine if levels of renal scavenger receptor expression are modulated in response to hypercholesterolemia, as has been reported for macrophages, protein or mRNA levels were compared between sham-operated kidneys from mice fed a control diet and mice fed a high fat diet. Fourteen days after sham-operation, renal protein or mRNA levels for each of the four scavenger receptors were not significantly different between sham kidneys of normocholesterolemic and hypercholesterolemic mice (Fig. 1F, H). Western blotting for PPAR γ in sham-operated kidneys was also not significantly different between normocholesterolemic and hypercholesterolemic mice (control vs high fat diet, \( n =4-6/\text{group}: 22 \text{ (SD 6)} \text{ vs } 22 \text{ (SD 5)}, \ P = 0.97 \)).

**Renal SR Expression in response to UUO and Effects of Diet-Induced Hypercholesterolemia**

**Renal fibrosis**

To investigate if the severity of renal fibrosis induced by UUO was accentuated by hypercholesterolemia, total collagen levels measured as hydroxyproline content per wet weight of kidney tissue. Fibrosis was significantly worse in obstructed kidneys from hypercholesterolemic mice compared to normocholesterolemic mice at day 3 and day 14 (control vs high fat diet, \( n =8-10/\text{group}: \text{day 3: } 3.9 \mu g/\text{mg (SD 0.4)} \text{ vs } 4.7 \mu g/\text{mg (SD 0.3)}, \ P =0.002; \text{day 7: } 6.7 \mu g/\text{mg (SD 0.9)} \text{ vs } 6.6 \mu g/\text{mg (SD 0.8)}, \ P =0.91; \text{day 14: } 8.2 \mu g/\text{mg (SD 0.9)} \text{ vs } 10.0 \mu g/\text{mg (SD 1.0)}, \ P =0.001, \text{ Fig. 2} \).  

**Macrophage infiltration**

Since fibrosis severity was increased in hypercholesterolemic mice, the degree of inflammation was compared in obstructed kidneys from normocholesterolemic and
hypercholesterolemic mice. There was significantly a larger F4/80+ interstitial area in obstructed kidneys from hypercholesterolemic mice at day 14 and a non-significant increase at day 3 after UUO (control vs high fat, \( n = 4-7 \) /group: day 3: 0.8% (SD 0.5) vs 1.6% (SD 1.3), \( P = 0.60 \); day 14: 5.2% (SD 2.8) vs 8.2% (SD 4.0), \( P = 0.011 \) by nested ANOVA; Fig. 3).

**Renal OxLDL levels**

To determine if intrarenal oxLDL generation was modulated by diet in normal kidneys, renal oxLDL levels in sham-operated kidneys were first compared between normocholesterolemic and hypercholesterolemic mice. C57BL/6 mice were consistently hypercholesterolemic after 8 weeks on a high fat Western diet (Table 1) without a significant change in bodyweight between the diet groups (Table 2). By immunostaining, HOCl-modified LDL localized predominantly to renal tubules in sham-operated kidneys (Fig. 4A, C). Semi-quantitative analysis of kidney sections stained for HOCl-modified LDL showed a non-significant increase in oxLDL levels in sham-operated kidneys of hypercholesterolemic mice compared to normocholesterolemic mice (percent tubulointerstitial area, control vs high fat diet, \( n = 4 \): 4.9% (SD 2.5) vs 9.8% (SD 4.1), \( P = 0.09 \)).

Since fibrosis was worse when kidneys were obstructed in hypercholesterolemic mice, studies were performed to determine if intrarenal levels of oxidized lipoproteins were modified by serum cholesterol levels. Semi-quantitative analysis of kidney sections immunostained for HOCl-modified LDL identified significantly higher levels in kidneys from hypercholesterolemic mice compared to obstructed kidneys from normocholesterolemic mice at each time point (percent tubulointerstitial area, control vs
high fat diet, \( n = 6 \)/group: day 3: 9% (SD 5) vs 20% (SD 6), \( P = 0.0002 \); day 7: 7% (SD 5) vs 19% (SD 3), \( P = 0.0001 \); day 14: 5% (SD 4) v 24% (SD 8), \( P = 0.0002 \) by nested ANOVA; Fig. 4E). By immunostaining, HOCl-modified LDL appeared \textit{de novo} after the onset of obstruction with the intensity of oxLDL staining increased in both tubular cells and the interstitium of obstructed kidneys (Fig. 4B, D).

\textit{NF kappa B activation}

To investigate if pro-inflammatory pathways that are activated in obstructed kidneys were influenced by hypercholesterolemia, activation of the NF kappa B (NF-\( \kappa \)B) pathway was examined on days 3 and 14. NF-\( \kappa \)B heterodimers are inactive in the cytoplasm due to I\( \kappa \)B-\( \alpha \) binding. Phosphorylation of I\( \kappa \)B-\( \alpha \) results in release and translocation of NF-\( \kappa \)B heterodimers into the nucleus to initiate transcription of several pro-inflammatory genes. Phosphorylated I\( \kappa \)B-\( \alpha \) is then ubiquinated and degraded. Nuclear translocation of NF-\( \kappa \)B was evaluated indirectly by Western blotting measurements of phosphorylated I\( \kappa \)B-\( \alpha \) and I\( \kappa \)B-\( \alpha \). Phosphorylated I\( \kappa \)B-\( \alpha \) -to- total I\( \kappa \)B-\( \alpha \) ratios were significantly higher in obstructed kidneys of hypercholesterolemic mice at both time points (control vs high fat, \( n = 4-6 \)/group: sham: 0.6 (SD 0.1) vs 0.6 (SD 0.1); day 3: 0.9 (SD 0.2) vs 1.5 (SD 0.4), \( P = 0.01 \); day 14: 0.2 (SD 0.1) vs 0.6 (SD 0.1), \( P = 0.0007 \); Fig. 5).

\textit{Renal scavenger receptor expression}

Since CD36, SR-A, Lox-1, and CXCL16 are the major oxLDL scavenger receptors, their expression was examined in obstructed kidneys harvested from the hypercholesterolemic mice. Changes in SR expression was measured by comparing sham-operated and UUO kidneys on the same Western or Northern blot for each time-
point and analyzed by unpaired Student’s t test. By Western blotting, total kidney CD36 protein levels significantly decreased 3 and 14 days after UUO relative to sham kidneys by 70% and 90%, respectively (mean sham group=1.0, n =4-9/group: day 3: 0.3 (SD 0.2), P =0.01; day 14: 0.1 (SD 0.1), P =0.00001, Fig. 6A). However, seven days after ureteral obstruction, a time-point representing a mid-point between inflammation and fibrosis, CD36 total protein levels returned to baseline levels relative to sham kidneys (mean sham group=1.0, n = 4-10/group: 1.7 (SD 0.9), P =0.18). By immunostaining, tubular CD36 expression initially decreased on day 3 after obstruction, then intensified on day 7 but by day 14 only a few tubules were positive compared to sham-operated kidneys. At the same time, CD36 expression progressively increased in the interstitium beginning on day 3 (data not shown) with notable increases in intensity observed at day 7 and 14 after obstruction (Fig. 7B, C).

By Western blotting, both renal SR-A and Lox-1 total protein levels progressively decreased after UUO; by day 14 levels were reduced by 98% and 85% respectively relative to sham kidney levels (mean sham group=1.0, n =4-10/group: day 14, SR-A: 0.01 (SD 0.02), P =0.0002; day 14, Lox-1: 0.1 (SD 0.1), P =0.00001, Fig. 6B, C). By immunostaining, changes in SR-A and Lox-1 protein levels were confirmed to represent loss of tubular expression (Fig. 7E, F, H, I). Despite the dramatic decline in tubular expression, both Lox-1 and SR-A were detected on some interstitial cells in the UUO kidney (see arrowheads Fig 7E, H).

By Northern blotting, there was a significant increase in CXCL16 steady-state mRNA levels after UUO, by approximately 400% relative to sham kidney levels at 7 and 14 days (mean sham group=1.0, n =3-10/group: day 3: 2.6 (SD 0.5), P =0.0002; day 7:
4.4 (SD 1.7), \( P=0.0007 \); day 14: 4.0 (SD 1.8), \( P=0.0006 \), Fig. 6D). By immunostaining, the changes in CXCL16 levels were confirmed and found to represent increased expression by both injured tubules and interstitial cells (see arrowheads Fig. 7K).
**Discussion**

The present study makes three important new observations in a mouse model of chronic kidney disease. First, it demonstrates that the four known atherogenic scavenger receptors that are important ox LDL receptors are expressed by tubular epithelial cells in normal kidneys, adding to the growing list of cellular lineages that may express this family of receptors. Second, when levels of ox LDL, the major ligands for these scavenger receptors, are enhanced within the kidney during chronic damage by systemic hypercholesterolemia, renal fibrosis is exacerbated. Third, the kinetic expression profiles of the scavenger receptors are altered in receptor and cell-specific manner during chronic kidney injury.

Despite extensive information on the role of scavenger receptors in endothelial and macrophage biological responses, information on epithelial scavenger receptor function is still in its infancy. In fact, previously published studies have reported Lox-1 by endothelial cells and macrophages but not in epithelia. The present findings demonstrate that the dimeric form of Lox-1 (38) is expressed by renal tubular epithelial cells. CD36 has also been identified on epithelial cells in the intestine and retina where it may mediate fatty acid transport and photoreceptor phagocytosis, respectively (15, 42). SR-A and CXCL16 have recently been reported to have low levels of expression in epithelial cells but their function at these sites still remains unclear (21, 25). Several studies have reported that scavenger receptors expressed by macrophages or endothelial cells are up-regulated by ox LDL (8, 19, 32). However, in the present study relatively short-term hypercholesterolemia did not enhance tubular scavenger receptor expression in vivo in normal (sham-operated) kidneys. A recent study demonstrated that the
transcriptional control of CD36 is very complex with at least five alternative transcripts and expression patterns that are cell-type dependent (2). Cell-type specific regulation may infer differences in biological function. In atherosclerosis, macrophage scavenger receptors are primarily responsible for oxLDL clearance by endocytosis but these receptors are also known to trigger intracellular signaling pathways that may lead to the production of chemokines and cytokines (11, 24). Whether renal tubular CD36, SR-A, Lox-1, and CXCL16 are involved in oxLDL endocytosis and/or intracellular signaling is still unclear and requires further investigation.

In the present study hypercholesterolemia significantly increased renal oxLDL tubulointerstitial deposition in chronically damaged kidneys. After 14 days of UUO, renal oxLDL levels were significantly increased more than 450 percent in hypercholesterolemic mice compared to normocholeserolemic mice. OxLDL may be generated within the kidney by one or more pathways that include copper-oxidation, myeloperoxidase (MPO)-generated reactive nitrogen species, and hypochlorous acid (HOCl)-mediated oxidation (10). Data from the current study provide further evidence that, similar to atherogenesis, oxLDL generated in the kidney are pathologic and promote renal fibrogenesis. Previous studies from our own and other laboratories demonstrated that long-term hypercholesterolemia (12 weeks) alone may increase intrarenal oxidative stress and promote fibrosis in otherwise normal kidneys (9, 40). The present study suggests that hypercholesterolemia, and more specifically oxidized lipoproteins, leads to a progressive increase in interstitial macrophage numbers after the onset of obstruction and results in more severe tissue injury. After 14 days of obstruction, interstitial macrophages increased approximately 160 percent in hypercholesterolemic mice.
compared to normocholesterolemic mice. These changes were associated with greater NF-κB activation that may have primed the kidney for a more severe injury and fibrosis when it encountered a second insult such as ureteral obstruction. Anti-oxidant therapy has been shown to decrease oxLDL generation and to reduce renal injury (8, 16, 43). Perhaps limiting the downstream inflammatory and pro-fibrotic effects of oxLDL by blocking their binding to scavenger receptors will prove to be an effective strategy for reducing renal fibrosis.

In the present study, both oxLDL and its scavenger receptors CD36, SR-A, Lox-1, and CXCL16 immunolocalized to the tubulointerstitial compartment after chronic obstruction suggesting that they may be important in activating the pro-fibrotic pathways activated by oxLDL. It has previously been reported that oxLDL can interact with CD36, CXCL16, and Lox-1–dependent NF-κB activation in both macrophages and endothelial cells (13, 30). The present study demonstrated that increased NF-κB activation correlated with time points of increased fibrosis severity in hypercholesterolemic mice. After three and fourteen days of obstruction, NF-κB activation levels increased 160 percent and 240 percent, respectively, in hypercholesterolemic mice compared to normocholesterolemic mice. Based on the findings in the present study, oxLDL-scavenger receptor interactions leading to NF-κB activation is one potentially relevant pathophysiological mechanism in progressive renal fibrosis.

The present study also demonstrates that the renal expression profile of each scavenger receptor examined was altered in a receptor-specific pattern during chronic injury. After UUO, two convergent events appear to influence overall scavenger receptor expression: infiltration of scavenger receptor-bearing macrophages and changes in renal
tubular epithelial cell expression with progressive renal injury. Lox-1 and SR-A protein levels decreased in a time-dependent manner after UUO despite an increase in levels of oxLDL, likely as a consequence of direct tubular injury. By contrast, Lox-1 expression has been reported to increase after hypertensive and vascular models of renal endothelial cell injury in rats (34, 50). Hypoxic injury is recognized as an integral component of progressive renal damage after ureteral obstruction. Despite the progressive loss of tubular Lox-1 after UUO, increased Lox-1 expression was noted in the interstitium likely due to increased expression by peritubular capillaries (see arrows Fig 1H) (47).

The decline in tubular SR-A expression and the early appearance of small numbers of SR-A+ interstitial cells after UUO suggest that SR-A may play a role in the inflammatory segment of renal fibrogenesis. SR-A+ interstitial cells were detected on day 3 (data not shown) and day 7 but not on day 14 when fibrosis is well-established. Other studies report that SR-A expression is up-regulated during macrophage activation suggesting that it may play a role in acute inflammation (36). In addition, Beamer et al demonstrated that following bleomycin administration, SR-A null mice developed more extensive inflammation but less collagen deposition in the lung suggesting that SR-A may be an important switch in the complex process of inflammatory fibrosis (5). A recent study further suggests that SR-A may promote macrophage infiltration in a model of diabetic nephropathy (51). However, in the present study the overall levels of SR-A protein expression levels were low relative to the other scavenger receptors studied, perhaps suggesting that its role in progression of renal fibrosis is limited.

In contrast to declining SR-A and Lox-1 levels after UUO, CD36 positive cells infiltrated the interstitium during chronic obstruction and tubular CD36 expression
intensified leading to a relative increase in total kidney CD36 protein levels at day 7 after UUO. In agreement with the lack of studies reporting CD36 expression by fibroblasts, dual staining for F4/80 and CD36 indicated that most of the CD36+ renal interstitial cells in the present study were macrophages (data not shown). As monocytes differentiate into macrophages in vitro, CD36 expression has been reported to increase (23). Phorbol esters (PMA), macrophage colony stimulating factor (M-CSF), and IL-4 have also been shown to increase monocyte/macrophage CD36 expression (53). Therefore, the results of the present study suggest that CD36+ macrophages infiltrating the interstitium in response to persistent kidney damage may promote renal fibrogenesis, and in particular they may mediate pro-fibrotic effects of oxLDL. Consistent with this hypothesis, a recent study in humans with pulmonary fibrosis reported increased levels of oxidized phosphatidylcholine (oxPC) co-localizing with CD36+ macrophages in fibrotic areas (54). Oxidized phosphatidylcholine is a specific binding moiety for CD36 (28).

Because phosphatidylcholine is a primary component of plasma membranes, it is possible that the part of the oxidized lipoproteins generated during chronic injury represent apoptotic cells which are known CD36 ligands. A recent study suggests that in diabetic nephropathy, CD36 may induce proximal tubular epithelial cell apoptosis through src kinase activation of caspase 3 (48). In addition to oxLDL, other CD36 ligands that have been implicated in the pathogenesis of chronic kidney disease are thrombospondin and advanced glycated end-products (AGEs) (17). Together these data suggest that both CD36+ macrophages and CD36+ renal tubular cells may play important roles in the progression of renal fibrosis.
The CXCL16 expression pattern was unique amongst the oxLDL-binding scavenger receptors examined in that its basal expression level was low and increased by 400 percent with the onset of chronic kidney injury caused by obstruction. In intestinal epithelial cells, membrane-bound CXCL16 may recruit activated T cells (21) and it may play a similar role during chronic injury in the kidney by directing lymphocyte chemotaxis towards injured tubules. However, unlike the protective effects of genetic CD36 or SR-A deficiency in mouse models of atherosclerosis(18, 44, 49), CXCL16 deficient mice develop worse disease (4). The role of CXCL16 as a possible modulator of progressive renal fibrosis deserves further investigation.

**Conclusions**

Systemic hypercholesterolemia was confirmed to enhance intrarenal oxLDL generation and worsen fibrosis severity during chronic kidney injury by obstruction. The results of the present study begin to elucidate the cellular pathways that may be involved. Normal renal tubular epithelial cells were shown to express CD36, SR-A, Lox-1, and CXCL16 in vivo, thus adding renal tubular epithelial cells to the growing list of cells that express scavenger receptors. As receptors for oxLDL, any of these receptors may serve as the bridge between hyperlipidemia, oxidative stress, and more aggressive renal fibrosis. However, based on the observed changes in expression after UUO-induced renal damage, CD36 and CXCL16 appear to be the best candidates and both renal tubules and inflammatory interstitial cells may participate. Further studies should determine their functional role and potential as therapeutic targets in chronic kidney disease.
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Figure Legends

Figure 1. Scavenger receptor expression in sham-operated kidneys: Effect of hypercholesterolemia.

Representative photomicrographs of (A) CD36, (B) SR-A, (C) Lox-1, and (D) CXCL16 tubular immunostaining in sham-operated kidneys. (E) Representative Western blots of day 14 sham-operated kidneys from mice on a control and high fat Western diet with protein loading measured by Ponceau S staining shown below each immunoblot. Lox-1 protein was expressed in the kidney predominantly as a homodimer at approximately 84kD. (G) Representative Northern blot of day 14 sham-operated kidneys. RNA loading for CXCL16 Northern blot indicated by the 18s band. Graphs (F) and (H) summarize the results of the Western blot or Northern blot major band density measurements. Differences in renal scavenger receptor expression from sham-operated kidneys were not significant between normocholesterolemic and hypercholesterolemic mice. All results are expressed as mean (1 SD), n =4-6.

Figure 2. Collagen accumulation in obstructed kidneys: Effect of hypercholesterolemia.

Total collagen content determined by the hydroxyproline assay significantly increased in obstructed kidneys from hypercholesterolemic mice compared to normocholesterolemic mice. The symbols are (□) Control diet, n =9-10; (■) High fat diet, n =6-7. All results are expressed as mean (1 SD). † P < 0.05, control versus high fat diet.
Figure 3. Interstitial macrophage number after obstruction: Effect of hypercholesterolemia.

(A) Representative photomicrographs of kidney F4/80+ immunostaining on day 14 UUO cryosections from mice on control and high fat diets. Original magnification x400. Graph (B) summarizes the results of computer-assisted image analysis of the interstitial area (%) staining positive for F4/80 (n =4-7/group). The symbols are (□) Control diet, n =4; (■) High fat diet, n =6-7. All results are expressed as mean (1 SD). † P < 0.01, control versus high fat diet.

Figure 4. Renal oxLDL levels after obstruction: Effect of hypercholesterolemia.

Representative photomicrographs of HOCl-LDL immunostaining from sham-operated (A, C) and day 14 UUO kidneys (B, D). Similar to scavenger receptor expression patterns, oxLDL were identified in tubules of sham kidneys from normocholesterolemic and hypercholesterolemic mice. With the onset of obstruction, oxLDL were also present in the interstitium. Original magnification x400. Graph (E) summarizes the quantification by computer-assisted image analysis of the tubulointerstitial area (%) staining positive for HOCl-LDL (n =6/group). The symbols are (□) Control diet, n =6; (■) High fat diet, n =6. All results are expressed as mean (1 SD). † P < 0.01, control versus high fat diet.

Figure 5. NF kappa B activation after obstruction: Effect of hypercholesterolemia.

NF kappa B activation was determined by Western blot analysis of total and phosphorylated IκB-α (phos IκB-α). (A) Representative total and phos IκB-α Western
blots of kidney tissue homogenate from sham and obstructed kidneys of normocholesterolemic and hypercholesterolemic mice. The ratio of phos IκB-α to IκB-α band densities are summarized in graph (B) (n = 4-6/group). Equality of protein loading was determined by Ponceau S staining (data not shown). The symbols are (□) Control diet; (■) High fat diet. All results are expressed as mean (± SD). † P < 0.01, control versus high fat diet.

**Figure 6. Scavenger receptor expression after unilateral ureteral obstruction in hypercholesterolemic mice.**

Representative scavenger receptor Western blots (A-C) or Northern blots (D) of sham-operated and obstructed kidneys. Protein and RNA loading was determined by Ponceau S staining and 18s band densities, respectively. The major scavenger receptor band density measurements are summarized in graphs, expressed as the relative fold expression of UUO to sham kidney protein or mRNA levels, corrected for loading inequalities. Results are expressed as mean (± SD). The symbols are NS, not significant; † P <0.05; ‡ P <0.01. n= 4-6 (sham), n= 6-10 (UUO).

**Figure 7. Scavenger receptor immunolocalization after UUO.**

(A-L) Representative photomicrographs of kidney CD36, SR-A, Lox-1, and CXCL16 immunostaining after sham surgery, day 7 UUO, and day 14 UUO in hypercholesterolemic mice. (A, D, G, J) Scavenger receptors were expressed predominantly by tubular epithelial cells in sham-operated kidneys. After unilateral ureteral obstruction, Lox-1 and SR-A expression decreased on tubular cells. Lox-1+ and SR-A+ interstitial cells noted, as shown for day 7 (see arrowheads: E, H). CD36+
interstitial cells increased at day 7 (see arrows: B) and day 14 after obstruction. CXCL16 increased in injured tubules and interstitial cells at day 7 (see arrowheads: K). Original magnification x400.
Table 1: Mean cholesterol levels on control and high fat diet

<table>
<thead>
<tr>
<th>Time-point</th>
<th>Surgery</th>
<th>Control (mg/dl) $(n=4-6)$</th>
<th>High Fat (mg/dl) $(n=6-10)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 14</td>
<td>Sham</td>
<td>102 (SD 2)</td>
<td>167 (SD 18)</td>
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<tr>
<td>Day 3</td>
<td>UUO</td>
<td>103 (SD 9)</td>
<td>140 (SD 16)</td>
</tr>
<tr>
<td>Day 7</td>
<td>UUO</td>
<td>80 (SD 4)</td>
<td>193 (SD 71)</td>
</tr>
<tr>
<td>Day 14</td>
<td>UUO</td>
<td>86 (SD 11)</td>
<td>191 (SD 39)</td>
</tr>
</tbody>
</table>
Table 2: Percent weight increase on control and high fat diet

<table>
<thead>
<tr>
<th>Time-point</th>
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<th>Control ((n=4-6))</th>
<th>High Fat ((n=6-10))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 14</td>
<td>Sham</td>
<td>80% (SD 30)</td>
<td>87% (SD 26)</td>
</tr>
<tr>
<td>Day 3</td>
<td>UUO</td>
<td>80% (SD 30)</td>
<td>87% (SD 26)</td>
</tr>
<tr>
<td>Day 7</td>
<td>UUO</td>
<td>60% (SD 14)</td>
<td>61% (SD 31)</td>
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<tr>
<td>Day 14</td>
<td>UUO</td>
<td>67% (SD 17)</td>
<td>66% (SD 16)</td>
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A

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>High fat</th>
</tr>
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<tbody>
<tr>
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<td><img src="image" alt="Sham LKBβ, IKBα bands" /></td>
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<td>Day 14 UUO</td>
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<td><img src="image" alt="Day 14 UUO LKBβ, IKBα bands" /></td>
</tr>
</tbody>
</table>

B

![Graph showing band intensity (pLKBβ/IKB) ratio](image)

- Control diet (n=4-6)
- High fat diet (n=6)

- NS
- †
Figure A: Unamplified CXCL16 immunostaining