LOX-1 and inflammation: a new mechanism for renal injury in obesity and diabetes

Katherine J. Kelly,1 Pengfei Wu,1 Carolyn E. Patterson,1 Constance Temm,1 and Jesus H. Dominguez1,2

1Department of Medicine, Indiana University School of Medicine and 2Veterans Administration Medical Center, Indianapolis, Indiana

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Kelly KJ, Wu P, Patterson CE, Temm C, Dominguez JH. LOX-1 and inflammation: a new mechanism for renal injury in obesity and diabetes. Am J Physiol Renal Physiol 294: F1136–F1145, 2008. First published March 5, 2008; doi:10.1152/ajprenal.00396.2007.—The early nephropathy in obese, diabetic, dyslipidemic (ZS) rats is characterized by tubular lipid accumulation and pervasive inflammation, two critically interrelated events. We now tested the hypothesis that proximal tubules from ZS obese diabetic rats in vivo, and proximal tubule cells (NRK52E) exposed to oxidized LDL (oxLDL) in vitro, change their normally quiescent epithelial phenotype into a proinflammatory phenotype. Urine of obese diabetic rats contained more lipid peroxides, and LOX-1, a membrane receptor that internalizes oxidized lipids, was mobilized to luminal sites. Levels of ICAM-1 and focal adhesion kinase, which participate in leukocyte migration and epithelial dedifferentiation, respectively, were also upregulated in tubules. NRK52E cells exposed to oxLDL showed similar modifications, plus suppression of anti-inflammatory transcription factor peroxisome proliferator-activated receptor-γ. In addition, oxLDL impaired epithelial barrier function. These alterations were prevented by an anti-LOX-1 antibody. The data support the concept that tubular LOX-1 activation driven by lipid oxidants in the preurine fluid is critical in the inflammatory changes. We suggest that luminal lipid oxidants and abnormal tubular permeability may be partly responsible for the renal tubulointerstitial injury of obesity, diabetes, and dyslipidemia.

Nephropathy in obese diabetics is common, complex, and very poorly understood. Hence, we study obese dyslipidemic rats with diabetes to find mechanisms of renal failure in the metabolic syndrome (17–20). Rat nephropathy is characterized by lipid loading of renal tubules (17, 18) and progressive damage to glomerular and peritubular capillaries (54). The evolving vasculopathy causes abnormal permeability of damaged glomerular capillaries, exposing tubular lumens to unanticipated plasma macromolecules, generically reported as proteinuria (6, 26) and lipuria (31, 36, 43). On the basolateral side, greater peritubular capillary permeability also brings albumin and lipid peroxides in direct contact with tubular cells (54). Moreover, clusters of neutrophils and macrophages are attracted to the areas of tubular injury, which become foci of tubular decay and expanding fibrosis (19, 20). This blend of inflammation and fibrosis is a key element in the renal injury of the metabolic syndrome (19, 20).

The potential for renal damage from plasma macromolecules in the urinary space has attracted considerable attention. Several studies assert that proteinuria is a risk factor for the progression of renal disease (6, 15, 48, 50), and, based on these observations, some have shown toxic actions of albumin on renal cells (16, 21, 47, 53). However, albumin may function as a delivery vehicle for bound toxic fatty acids as reported in vitro (3, 29, 45) and in vivo (32, 55). Accordingly, renal effects of fatty acids and complex lipid peroxides filtered with albumin into the preurine (45) cannot be accounted for in epidemiological studies of proteinuria. In any case, toxic urinary lipids can reach tubular lumens (43), and tubule lipid loading can be detected as lipid-laden tubular cells (7) frequently lost in the urine of proteinuric subjects (46). Renal tubular accumulation of unutilized lipid is also ubiquitous in young diabetic proteinuric rats and plays a critical role in their nephropathy (18–20). The toxic role of albumin in tubules was also brought into question by the demonstration that a large fraction of blood albumin is filtered and then reabsorbed by proximal tubules of normal rats (51). In this work, we tested the hypothesis that oxidized luminal lipid activates the expression of the oxidized LDL (oxLDL) transporter LOX-1, promoting a tubular proinflammatory phenotype. The changed phenotype is characterized by upregulation and redistribution of LOX-1, greater epithelial permeability, increased ICAM-1 expression, enhanced leukocyte adherence, and apoptosis.

MATERIALS AND METHODS

Cell culture. NRK-52E cells, CRL-1571, were acquired from ATCC (Manassas, VA) and cultured on polystyrene culture dishes in DMEM containing 1.5 g/l sodium bicarbonate and 10% bovine serum in an atmosphere of 5% CO2–95% air at 37°C. When cells became confluent, human oxLDL was added to the medium at indicated concentrations. Human LDL from Sigma (St. Louis, MO) was oxidized in the presence of CuSO4 and oxidation was confirmed by measurements of thiobarbituric acid-reactive substances (TBARS) of dialyzed oxLDL. The levels of oxLDL were expressed as TBARS in nanomoles and protein as micrograms per milliliter (2). TBARS were quantified spectrophotometrically using a malonaldehyde standard curve, as described elsewhere (18). The concentration of oxLDL was chosen to approximate the in vivo exposure [~0.5 μM in urine (below) with a concentrating factor of ~150]. oxLDL was added to the media for the first 24 h, removed, and cells were cultured for an additional 24–48 h. In some cases, cultured cells were immediately lysed after completion of the experiment in preparation for Western blot analysis (below) or fixed with 4% paraformaldehyde in preparation for confocal microscopy (54). LDH was measured with a CytoTox 96 Assay Kit from Promega (Madison, WI) and expressed as percent release of total cell LDH.

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NRK52E cell adherence and apoptosis. Leukocytes were isolated from 5 ml of heparinized whole blood collected from 10 wk-old normal Sprague-Dawley rats. The blood was centrifuged at 3,000 rpm for 15 min, theuffy coat was isolated, red blood cells (RBC) were lysed in an ammonium chloride solution, and leukocytes were recovered from the Buffy coat labeled with acridine orange (30 μg/ml) for 10 min at 37°C in culture medium (35). NRK52E cells were exposed to saline (control), oxLDL (1 nM or 50 μg/ml protein), or oxLDL (2 nM) and anti-LOX-1 blocking antibody (IgG; 10 μg/ml) for 48 h. The specificity and blocking characteristics of the anti-LOX-1 antibody have been described elsewhere (20). The experimental media were all replaced with identical control medium containing rat leukocytes. The pretreated NRK52E cells were cocultured with labeled rat leukocytes overnight (10^7 leukocytes in 0.4 ml of media/well), washed three times in media, stained with Hoescht 33342 nuclear fluorescent dye, and then imaged at ×40 magnification with the Zeiss UV LSM-510-Meta confocal microscope system. Leukocyte adhesion was quantified in 6–18 images/condition (without knowledge of the experimental conditions). Apoptosis was defined as the fraction of stained nuclei with clear nuclear condensation and/or fragmentation per field in five to eight images/condition without knowledge of the experimental condition (34).

Transepithelial electrical resistance. Transepithelial electrical resistance, an index of cell layer barrier function, was determined in real time using the Electric Cell-Substrate Impedance Sensor system (Applied BioPhysics, Troy, NY). The system consists of a large gold-plated electrode (1 cm²), eight smaller gold-plated electrodes (10^-4 cm²), and eight 500-μl wells fitted above each of the small electrodes. The small and large electrodes are connected to a phase-sensitive lock-in amplifier and an alternating current (4,000 MHz at 1 V) is supplied through a 1-MΩ resistor. The system consists of a large gold-plated electrode (1 cm²), eight smaller gold-plated electrodes (10^-4 cm²), and eight 500-μl wells fitted above each of the small electrodes. The small and large electrodes are connected to a phase-sensitive lock-in amplifier and an alternating current (4,000 MHz at 1 V) is supplied through a 1-MΩ resistor. The measured electrical impedance (or calculated resistance) indicates the restriction to current flow through the cell monolayer. For resistance measurement, NRK-52E cells (10^4 cells) were plated into each well, and the eight-well plate was mounted onto the Electric Cell-Substrate Impedance Sensor system housed within an incubator maintained at 37°C, 5% CO₂, and 100% humidity and connected to the lock-in amplifier. The cells were grown to confluency (~2 days) as determined by increased resistance until a plateau was achieved (typically ~14,000 Ω) and confirmed by microscopy. The cells were then challenged with reagents according to the experimental protocol, and resistances were recorded continuously in real time.

Cell proteins. An anti-LOX-1 antibody was generated in rabbits against the LOX-1 peptide (residues 188 to 233 of the 364-amino acid protein, LOX-1 accession number NP_579840). The 46-amino acid LOX-1 peptide was synthesized by Dr. Suzanna Holgrath (California Institute of Technology, Pasadena, CA). The antibody was manufactured and affinity purified by Covance (Princeton, NJ) (20). The anti-LOX-1 antibody was used in Western blot analysis (IgG, 2 ng/μl) and as a blocking antibody added to cultured cells (5–10 μg/ml).

The levels of unmodified and phosphorylated ERK1, pERK, p38, p-p38, JNK, and p-JNK were also measured on Western blots. These antibodies were also affinity-purified specific rabbit polyclonal antibodies to synthetic peptides (1:1,000 dilution of stock, Santa Cruz Biotechnology, Santa Cruz, CA). Cell proteins were lysed and separated on 12% acrylamide SDS-PAGE gel, electrophoretically transferred to Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad) at 15 mA, and labeled with specific primary antibodies followed by peroxidase-linked secondary anti-rabbit or anti-mouse IgG goat antibody (Pierce, Rockford, IL) (19). Equal protein gel loading was verified with an anti-actin monoclonal antibody on the blots (Actin, C-2, Santa Cruz Biotechnology). The relative intensities of the protein levels were measured on antibody-stained membranes using VitaScan software (ESA, Chelmsford, MA).

Confocal microscopy. NRK52E cells cultured on glass coverslips were exposed to either saline (control) or oxLDL (50 μg/ml) for 24 h.

Fig. 1. Renal oxidized LDL transporter (LOX-1) in lean and obese rats. Kidneys from lean (A–C) and obese (D–F) rats were fixed, sectioned, and stained with primary anti-LOX-1 antibody and secondary anti-rabbit Texas red-labeled mouse IgG. In lean rats, LOX-1 was visible in tubules. In obese rats, LOX-1 was far more strongly expressed in the luminal aspect of proximal tubules (yellow arrows). In obese rats, renal LOX-1 was also visible in peritubular regions (white arrows).
The cells were then fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, and reacted primary antibodies: rabbit anti-LOX-1 (above), anti-E-cadherin (murine anti-E-cadherin, 1:25, BD Bioscience, San Jose CA), anti-focal adhesion kinase (murine anti-FAK, 1:50, Transduction Laboratories, Lexington, KY), and anti-ICAM-1 (murine anti-rat ICAM-1, 1:50, Santa Cruz Biotechnology). The slips were incubated with secondary antibodies including FITC-labeled donkey anti-mouse IgG antibody and Texas red-labeled donkey anti-rabbit IgG (Jackson, West Grove, PA). Slips were mounted on slides with AntiFade-Gold with 4',6-Diamidino-2-phenylindole (Invitrogen Molecular Probes, Carlsbad CA), and viewed on a Zeiss UV LSM-510 Microscope System with an oil objective at ×63 magnification. Images were processed and merged using MetaMorph (Molecular Devices Universal Imaging, Sunnyvale, CA) or for 3D imaging (Voxx and Image J imaging software).

Animals. The research involving animals adhered to The American Physiological Society’s Guiding Principles in the Care and Use of Laboratory Animals. The investigative protocols were approved by the institutional Animal Care and Use Committee at Indiana University. Pathogen-free rats were obtained from Charles River, Wilmington, MA. We studied first generation (F1) male hybrid rats derived from a well-characterized parental strain: the Zucker fatty diabetic (ZDF; fa/fa) (57) and the spontaneous hypertensive heart failure rat (SHHF/Gmi-fa) (1). These hybrid rats develop obesity, diabetes, dyslipidemia, and hypertension (18, 19). Renal immunohistochemistry shown here was performed on kidneys from 21-wk-old lean and obese rats as previously reported (54). Rat leukocytes were obtained from normal 10-wk-old Sprague-Dawley rats (Harlan, Indianapolis, IN). Rats were housed in steel cages and acclimatized to 12:12-h light-dark cycles (7 AM-7 PM). The ambient temperature was kept at 70°F, with food and water available at all times. All rats were fed ad lib Purina diet 5008, which contained 27% protein, 17% animal fat, and 56% carbohydrate (17, 18).

Renal histology. Kidneys from lean and obese rats were fixed in 4% paraformaldehyde overnight and then sectioned into 100-μm slices with a vibratome (Vibratome, St. Louis, MO). The sections were immersed in PBS with 0.2% Triton X-100 for 5 min, washed three times with PBS, blocked for 15 min in PBS with 0.2% bovine serum albumin, and then incubated with the primary anti-ICAM-1 (1:40) and anti-focal adhesion kinase (1:30) followed by FITC-conjugated secondary antibody, anti-mouse IgG (1:200, Vector Laboratories), 30 min at 37°C each. 4',6-Diamidino-2-phenylindole (Sigma) was added to the secondary incubation for the localization of nuclei. Renal sections were also double labeled with either anti-von Willebrand factor rabbit polyclonal antibody (Dako, Carpinteria CA) plus ICAM-1 or von Willebrand factor plus FAK antibodies (1:50 dilution for all) for 30 min at 37°C. The sections were then washed three times in PBS-0.5% BSA and incubated with a solution containing 1:200 of each goat-anti-mouse-Alexa 488 and goat anti-rabbit-Alexa 633. The wavelength (633 nm) is not visible, and the color purple was assigned using the Zeiss software. Separate sections were incubated with primary and secondary antibody individually to control for nonspecific fluorescence. Sections were washed between and after incubations in PBS and placed in PBS containing 1% DABCO (Sigma). The sections were viewed with the Zeiss LSM510-Meta confocal microscope system (19), and fluorescent intensity was measured with MetaMorph.

Statistical analysis. The results are expressed as means ± SE. Any differences between two groups were evaluated by Student’s t-test for unpaired variables and among three or more groups by one-way ANOVA, and results were considered significant if \( P < 0.05 \).

RESULTS

LOX-1 in renal tubules. Renal LOX-1 localization was investigated in the kidneys of 21-wk-old ZS lean and obese rats

Fig. 2. Renal ICAM-1 in lean and obese rats. Kidneys from lean (A–C) and obese (D–F) rats were fixed, sectioned, and stained with primary anti-ICAM-1 antibody and secondary anti-mouse FITC-conjugated mouse IgG. In lean rats, ICAM-1 was present in peritubular capillaries (white arrows). In contrast, in obese rats, ICAM-1 was strongly expressed in luminal membranes of proximal tubules (yellow arrows). The green arrowheads point to endothelial nuclei of peritubular capillaries.
Fig. 3. Renal focal adhesion kinase (FAK) in lean and obese rats. Kidneys from lean (A–C) and obese (D–F) rats were fixed, sectioned, and stained with primary anti-FAK antibody and secondary anti-mouse FITC-conjugated mouse IgG. In lean rats, FAK was limited to a thin and discrete line in basolateral membranes of tubules (white arrows) and in peritubular capillaries. In contrast, in obese rats, tubular FAK expression increased markedly, and beyond the distinct lines characteristic of lean rats (yellow arrows).

Fig. 4. von Willebrand factor, FAK, and ICAM-1. Double staining with anti-FAK (A and C) or anti-ICAM-1 (B and D, green) and anti-von Willebrand factor (pink) demonstrates linear FAK and ICAM-1 (green, closed arrows) staining of peritubular capillary plasma membrane in lean animals. Immunoreactive von Willebrand factor (pink, arrowheads) in Weibel-Palade bodies delineates endothelial cytoplasm. In kidneys of obese animals, FAK and ICAM-1 are attenuated in their peritubular capillaries while their expression is induced in proximal tubules (open arrows). Asterisks indicate tubular lumina.
as previously reported (54). There were three lean normal ZS rats and three obese diabetic ZS rats with nephropathy and proteinuria. In normal rats, renal LOX-1 was faint but plainly visible when reacted with a specific anti-LOX-1 antibody (Fig. 1). In obese, diabetic, dyslipidemic, and proteinuric rats, LOX-1 was strongly expressed in intraluminal blebs and luminal membranes of proximal tubules. LOX-1 was also distinctly expressed in the peritubular capillaries of the obese rats. Specific fluorescent intensity levels for tubular LOX-1 were 43/1006 and 94/1006 for lean and obese rats, respectively (n = 6–10 determinations; P < 0.001).

The potential lipid oxidant stimulant of luminal LOX-1 might be found in the tubular preurine fluid, and thus the final urine was analyzed for its generic presence. Urinary TBARS concentrations, measured in overnight urine and normalized to urine creatinine concentrations, were much higher in obese diabetic rats. Urinary TBARS in lean rats averaged 0.162 ± 0.014 and 0.473 ± 0.101 μmol/mg creatinine in obese rats, P = 0.02.

**ICAM-1 in renal tubules.** ICAM-1 (or CD54) (49) is a specific component of LOX-1-mediated inflammatory vascular responses in vitro (41) and in vivo (28). However, the role of LOX-1 in the expression of ICAM-1 in renal epithelia is unknown. In lean ZS rats, renal ICAM-1 was constitutively expressed in their glomerular and peritubular vessels (Figs. 2 and 4). In contrast, ICAM-1 was radically altered in obese-diabetic ZS rats with nephropathy: ICAM-1 was strongly expressed apically in renal tubules, while peritubular vascular expression was diminished and inconsistent, in accord with previously reported widespread peritubular capillary damage in these obese-diabetic rats (54). In three lean rats, average arbitrary pixel intensity of ICAM-1-specific green fluorescence in peritubular capillaries was higher than in three obese-diabetic rats: 77.5 ± 2.8, and 33.9 ± 6.6, respectively (P < 0.001). In contrast, tubular apical ICAM-1 label was far lower in lean rats than in obese rats: 27.3 ± 5.3 and 63.6 ± 2.1, respectively (ANOVA in the 4 ICAM-1 groups, P < 0.01).

**FAK in renal tubules.** FAK (24) expression was imaged in the same rat kidneys. In lean rats, FAK was expressed in peritubular capillaries and the basolateral membranes of proximal and distal tubules (Figs. 3 and 4). In obese-diabetic rats, FAK was also expressed in capillaries, but its levels were

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**Fig. 5.** e-Cadherin, FAK, and LOX-1 in proximal tubule epithelial cells exposed to oxLDL. I: NRK52E cells were grown to confluence on glass slips and then exposed to 50 μg/ml oxLDL, fixed, and stained as described. In control cells (A), e-cadherin (green) forms a continuous border around the cell edges, consistent with an intact epithelial layer barrier (white arrows), and LOX-1 (red) is faint and viewed as amorphous staining in the perinuclear area (not visible with staining controls). In the oxLDL-treated cells (B), the cadherin stain is fainter and discontinuous (white arrows), while the LOX-1 staining is increased and relocated to aggregates located more toward the lateral side and at outer edge of the cells (yellow arrows). II: in control cells (A), FAK (green) is only occasionally observed and is primarily in the subnuclear location, and LOX-1 (red) is faint and viewed as amorphous staining in the perinuclear area (as also shown in I). In the oxLDL-treated cells (B), FAK staining is brighter and found in typical focal adhesion triangles at the lower cell borders, consistent with the aggregation and relocation observed in activated, motile cells (white arrows). The specific LOX-1 staining is increased and primarily relocated to aggregates located more toward the cytoplasmic side and at outer edge of the cells (yellow arrows). In I and II, the Z-axis are shown directly below each respective X- and Y-axis. III: exposure to oxLDL (25 and 50 μg/ml) for 24 h increased the level of LOX-1 in NRK42E cells. Blotting of actin protein was also performed to verify comparable protein loading (top). The blots are representative of 3 samples/condition. Optical density (OD) for LOX-1, normalized to actin levels, was significantly higher than control in cells exposed to oxLDL (50 μg/ml, a, P < 0.05, ANOVA).
lower. Measurements of FAK fluorescent pixel intensity in peritubular capillaries averaged 136.8 ± 3.6 and 100.2 ± 3.9 for lean and obese rats, respectively (P < 0.05). On the other hand, intracellular FAK expression increased remarkably in tubules of obese rats. Measurements of FAK fluorescent pixel intensity in proximal tubules averaged 87.1 ± 3.2 and 196.2 ± 14.0 for lean and obese rats, respectively (ANOVA in the 4 FAK groups; P < 0.05). We then double labeled the renal sections with the same two primary antibodies and anti-von Willebrand factor antibody to verify the localization of ICAM-1 and FAK (Fig. 4). In lean rats, FAK colocalized with the endothelial marker in peritubular capillaries, whereas in obese rats FAK was very prominent in renal tubules. ICAM-1 also colocalized with the endothelial marker in lean rats, consistent with peritubular capillary expression. However, in obesity ICAM-1 was also robustly expressed in tubules.

**NRK52E cells.** Renal epithelial luminal expression of LOX-1 is a novel finding. Accordingly, corroboration and evidence for direct effects of oxLDL on the tubular epithelium were sought in cultured NRK52E cells, which are derived from normal rat proximal tubules (14). In control cells, LOX-1 was localized in a fine reticular perinuclear pattern (Fig. 5I). In contrast, when cells were exposed to oxLDL, 50 μg/ml (TBARS = 2 nm) for 24 h, LOX-1 expression increased markedly. The arbitrary pixel intensity of specific red fluorescence increased from 10.2 ± 1.1 in control cells to 19.8 ± 2.3 following exposure to oxLDL (P < 0.001, n = 12). Furthermore, stimulated LOX-1 aggregated in larger clumps, preferentially localized toward the outer membranes of cells exposed to oxLDL. E-cadherin was visualized as a continuous intercellular green label in controls, and it was interrupted by multiple gaps on exposure to oxLDL (Fig. 5j). FAK was barely detectable in control cells, but FAK became very noticeable in the vicinity of basolateral membranes of cells exposed to oxLDL (Fig. 5Ii). The stimulation of LOX-1 protein (~50 kDa) expression by oxLDL in NRK52E cells was also verified in their lysates by Western blotting (Fig. 5II). LOX-1 levels, measured by densitometry, increased from a basal level of 1.00 ± 0.39 in control cells to 1.67 ± 0.26 and 2.64 ± 0.16 in cells exposed to oxLDL (25 and 50 μg/ml; TBARS = 1 and 2 nm, respectively, P < 0.05 for oxLDL 50 μg/ml) (Fig. 5II). The percentage of cellular lactic dehydrogenase (LDH) released to the culture media was 6.1 ± 1.1% in control cells, 4.4 ± 0.2 and 24.6 ± 2.2% in cells exposed to oxLDL, 25 (1 nM) and 5 (2 nM) μg/ml, respectively, P < 0.001 for 50 μg/ml oxLDL.

**LOX-1 expression and epithelial permeability.** We also studied the role of oxLDL and LOX-1 on epithelial barrier function, a defining characteristic of renal tubules (37). Epithelial resistance was significantly impaired by oxLDL compared with the control group. In addition, the epithelial barrier dysfunction could be prevented by treating cells with an anti-LOX-1 antibody in the presence of oxLDL (P < 0.01). The statistically significant differences were found from 1 to 30 h of exposure to oxLDL (Fig. 6).

**ICAM-1 expression, leukocyte cell adherence, and apoptosis.** LOX-1 activation increases the expression of ICAM-1 (28, 41), a recognition protein that augments host cell adherence to inflammatory cells (52). Thus corroboration of ICAM-1 expression was sought in NRK52E cells cultured with and without oxLDL for 48 h. Control cells had minimal levels of ICAM-1, whereas oxLDL (50 μg/ml, 2 nM TBARS) increased ICAM-1 global cell expression (Fig. 7, top). Furthermore, expression of cellular ICAM-1 was appreciably reduced when anti-LOX-1 antibody (10 μg/ml) was added to oxLDL (50 μg/ml, 2 nM TBARS). In parallel experiments, NRK52E cells previously exposed to saline control, oxLDL (50 μg/ml) and oxLDL (50 μg/ml) with anti-LOX-1 antibody (10 μg/ml) for 48 h were washed and then cocultured in control media overnight with fresh normal rat leukocytes. Leukocyte cell adherence was higher to cells pretreated with oxLDL than to control cells, whereas addition of an anti-LOX-1 antibody to oxLDL reduced the number of adhered leukocytes (Fig. 7, bottom, and Table 1). Apoptosis was also estimated in NRK52E cells cocultured with leukocytes by visual inspection of their stained nuclei. The data are expressed as the fraction of condensed or fragmented nuclei stained with Hoechst 33342 nuclear dye (Table 1). Cells previously treated with oxLDL had a higher fraction of apoptotic nuclei, and the addition of an anti-LOX-1 antibody to oxLDL limited apoptosis.

**Peroxisome proliferator-activated receptor-δ.** In NRK52E cells, oxLDL also caused a severe reduction in the levels of the anti-inflammatory transcription factor peroxisome proliferator-activated receptor-δ (PPARδ) (4) (Fig. 8). The depressive effect of 2 nM oxLDL (50 μg/ml) was very likely dependent on LOX-1 function, since cell PPARδ suppression was prevented by the concurrent addition of an anti-LOX-1 antibody (5 μg/ml) to the culture medium. PPARδ protein levels, shown as optical density normalized to actin content, were significantly depressed by addition of oxLDL without antibody protection relative to the other three groups: 1.00 ± 0.08 for control; 0.92 ± 0.03 with anti-LOX-1 antibody alone; 0.61 ±
0.01 with oxLDL; and 0.92 0.03 with oxLDL and anti-LOX-1 antibody (\(P < 0.01\), ANOVA).

**oxLDL and the MAPK program.** Some members of the MAPK family of proteins are activated by stimulants of LOX-1 and ICAM-1 (30, 42), and MAPK is inhibited by PPAR\(\gamma\) activation (8). Hence, it was logical to test the potential for oxLDL to activate/phosphorylate the MAPK proteins p38, ERK1–2, and JNK in kidney cells. Exposure to progressively higher concentrations of oxLDL (0, 25, 50, and 75 \(\mu\)g/ml of protein, equivalent to 0–3 nmol of TBARS in oxLDL) increased the p-p38/p38, pERK1–2/ERK1–2, and pJNK/JNK ratios in NRK52E cells (Fig. 9A). The phosphorylated/intact protein ratios, calculated from the measured optical density and normalized to actin levels, are shown in Fig. 9B, along with the percentage of released cell LDH. The maximal dose of oxLDL, 75 \(\mu\)g/ml, or 3 nM TBARS, increased the normalized p-p38/p38 ratio to 2.1 \(\pm\) 1, \(P < 0.04\); pERK1–2/ERK1–2 to 7.0 \(\pm\) 0.7, \(P < 0.001\); pJNK/JNK 7.4 \(\pm\) 1.2, \(P < 0.001\); and cell LDH release increased to 70 \(\pm\) 8%, \(P < 0.001\). The latter reflected concurrent oxLDL-mediated cytotoxicity, and a likely irreversible experimental situation.

**DISCUSSION**

LOX-1 expression was increased in tubules of obese-diabetic ZS rats compared with lean ZS rats. In obese rats, LOX-1 was localized to peritubular capillaries and in apical membranes of renal proximal tubules. LOX-1 expression is regulated by a feed-forward system stimulated by oxLDL (2, 12), which promotes its own uptake via LOX-1 (40). These conditions enhance oxidant stress, alter cell function, and activate apoptosis (40). The uniquely apical distribution of LOX-1 is compatible with luminal activation of LOX-1 by potential oxidants factors (11, 12, 40–42) in the tubular preurine fluid.

**Table 1. Renal tubular cell proinflammatory phenotype**

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<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>oxLDL</th>
<th>oxLDL + anti-LOX-1 Antibody</th>
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<tr>
<td>ICAM-1, specific fluorescence intensity in counts (\times 1,000)</td>
<td>4,407(\pm)125</td>
<td>6,084(\pm)125*</td>
<td>4,829(\pm)192</td>
</tr>
<tr>
<td>WBC adherence, no. of leukocytes adhered to tubular cells/field</td>
<td>2.94(\pm)0.61</td>
<td>5.29(\pm)0.64*</td>
<td>2.33(\pm)0.56</td>
</tr>
<tr>
<td>Cell apoptosis, no. of apoptotic cells/field</td>
<td>0.13(\pm)0.09</td>
<td>4.10(\pm)0.45*</td>
<td>0.18(\pm)0.18</td>
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Values are means \(\pm\) SE; \(n = 8\) for ICAM-1, \(n = 6–18\) for white blood cell (WBC) adherence, and \(n = 5–8\) for cell apoptosis. oxLDL, oxidized LDL; LOX-1, oxLDL transporter. *Significantly different from control and oxLDL+anti-LOX-1 antibody groups, \(P < 0.02\).
There is evidence for such urinary factors. For example, the urine of proteinuric subjects contains complex lipids with readily oxidizable urinary lipoproteins (31, 36), and diabetic human (45) and rat (38) urine also contains oxidized macromolecules. Hence, we looked for a generic urinary stimulant of LOX-1 expression and found that urinary TBARS were much higher in obese-diabetic rats, consistent with high levels of urinary lipid peroxides. Since clusters of renal neutrophils are common in peritubular and tubular lumens of obese-diabetic ZS rats (18, 19), we searched and found proximal tubule luminal upregulation of the leukocyte adhesion receptor ICAM-1 (49), a protein presumably activated by LOX-1 (41), as indicated by the attenuating effect of the anti-LOX-1 antibody on ICAM-1 expression in NRK52E cells cultured with oxidized lipid. Furthermore, leukocyte luminal binding to NRK52E cells was enhanced by prior exposure to oxLDL, similarly to arterial endothelium (44). This effect was also dependent on LOX-1 activity, since it was prevented by the LOX-1 antibody. The same could be said of cell apoptosis, which was enhanced by oxLDL (33) and prevented by blocking LOX-1.

LOX-1 expression in renal epithelial cells was confirmed in the rat renal epithelial cell line NRK52E. These cells are derived from normal rat proximal tubules (14) and express a consistent polarized proximal tubule cell phenotype (21). Exposure to oxLDL increased levels of LOX-1, which translocated from a perinuclear location in controls to large conglomerates in outer membranes of stimulated cells. oxLDL also changed the robust epithelial phenotype of NRK52E cells, as indicated by the gaps in expressed E-cadherin (21) and the fall in barrier function. The latter action was also dependent on LOX-1 activity, as it was partly prevented with an anti-LOX-1 blocking antibody.

ICAM-1 is a cell surface protein typically stimulated by cytokines, cell stress, and oxidants (9, 10, 49). We were able to identify a remarkable state of ICAM-1 dysregulation in obese rat tubules. In lean rats, ICAM-1 was mostly in peritubular capillaries. However, in obese rats, ICAM-1 was expressed at high levels in proximal tubules. This remarkable finding was also verified in NRK52E cells, which manifested robust ICAM-1 expression on exposure to oxLDL. ICAM-1 expression is upregulated by oxidant loads acting on MAPK signaling (56), and it is best known for promoting neutrophil traffic across the endothelial barrier (9, 13). Others have reported...
renal epithelial ICAM-1 activation and leukocyte binding during cytokine stimulation (5), an expected event in obese/diabetic rats with severe capillary vasculopathy (19). We can only presume that ICAM-1 upregulation was promoted in part by oxidant stress (56) as a direct consequence of LOX-1 activation (28, 41). In any case, to our knowledge this is the first demonstration of ICAM-1 apical expression in tubules from obese/diabetic rats and in renal epithelial cells exposed to oxLDL in vitro. It is also noteworthy that FAK was upregulated in conjunction with ICAM-1 (22).

FAK is a member of the focal adhesion molecular complex, and its activation leads to increase cell focal adhesion turnover, motility, and invasion (23, 24), which can lead to a more permeable epithelium. FAK also enhances cell survival by binding to death domain kinase receptor-interacting protein (RIP) blocking its proapoptotic signals (39), and FAK supports peroxidation of low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells (56). We interpret our data as supportive of the hypothesis that obesity/diabetes modulates oxidatively modified LDL-induced expression of adhesion molecules; role of LOX-1. Circ Res 89: 1155–1160, 2001.

We propose that nephropathy in obesity and diabetes is amplified by a prurine fluid rich in oxidized lipids that activate tubular LOX-1 expression. This critical response enhances oxidized lipid uptake and changes the epithelial phenotype to a more proinflammatory phenotype. It is also remarkable that cell signals involved in these portentous changes also include suppression of PPARδ, a key anti-inflammatory transcription factor (4). However, it is not clear from our data how suppression of PPARδ promoted inflammation.

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