Anti-LOX-1 therapy in rats with diabetes and dyslipidemia: ablation of renal vascular and epithelial manifestations

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Dominguez JH, Mehta JL, Li D, Wu P, Kelly KJ, Packer CS, Temm C, Goss E, Cheng L, Zhang S, Patterson CE, Hawes JW, Peterson R. Anti-LOX-1 therapy in rats with diabetes and dyslipidemia: ablation of renal vascular and epithelial manifestations. Am J Physiol Renal Physiol 294: F110–F119, 2008. First published November 7, 2007; doi:10.1152/ajprenal.00013.2007.—LOX-1 is a multifunctional membrane receptor that binds and internalizes oxidized LDL (oxLDL). We tested the hypothesis that blockade of LOX-1 with an anti-LOX-1 antibody limits nephropathy in male rats with diabetes and dyslipidemia (ZS rats; F1 hybrid product of Zucker fatty diabetic rats and spontaneous hypertensive heart failure rats). Lean ZS rats were controls, while untreated obese ZS (OM) rats expressed LOX-1 in capillaries and tubules, were larger, accumulated lipid, had intense oxidative stress, leukocyte infiltration, depressed mitochondrial enzyme level and function, and peritubular fibrosis (all P < 0.05 vs. lean ZS rats). Injections with LOX-1 antibody limited these abnormalities (P < 0.01 vs. data in OM or OM-IgG rats). In vitro, renal epithelial LOX-1 expression was verified in a cultured proximal tubule cell line. Our study indicates that anti-LOX-1 (vascular and epithelial) therapy may effectively reverse critical pathogenic elements of nephropathy in diabetes and dyslipidemia.

renal failure; diabetic nephropathies; atherosclerosis

OXIDIZED LOW-DENSITY LIPOPROTEINS (oxLDL) cause endothelial injury and play a significant role in the pathogenesis of atherosclerosis and tissue ischemia (23). Entry of oxLDL, and perhaps other oxidized lipids, modifies cellular function, causing vascular dysfunction and/or insufficiency. The endothelial injury requires binding oxLDL to the surface membrane, and its subsequent entry into the cell (20), and a search for a putative cellular binding site yielded the endothelial oxLDL receptor 1 (LOX-1), a 52-kDa lectin-like molecule now recognized as an important portal for oxidized lipids (37).

LOX-1 is a multifunctional membrane receptor that binds oxLDL (45), other oxidized lipids (2, 45), platelets (28), leukocytes (24), and aged cells (43), all of which participate in oxidative stress and inflammatory responses (37). While basal endothelial LOX-1 expression is generally low, LOX-1 levels are increased by a feed-forward system, triggered by oxidant stress (2, 12, 32), cytokines (18), and angiotensin II (34). Accordingly, LOX-1 expression is closely linked to endothelial dysfunction (28) and the expression of adhesion molecules, which anchor leukocytes in addition to LOX-1 itself (24, 32, 43). Thus it is not surprising that expression of LOX-1 results in impaired endothelial function (3, 13) and vasculopathy (25).

Vascular LOX-1 expression is upregulated in conditions characterized by vascular injury, such as atherosclerosis, hypertrophy, and diabetes, as well as in rat carotid arteries subjected to balloon injury (37). Moreover, ischemic-reperfused myocardial tissues also express LOX-1, which may underlie its relationship to cellular stress (33). It is also noteworthy that rat cardiac fibroblasts, when manipulated to express LOX-1, acquire an endothelial phenotype in the presence of oxLDL, which suggests a broader regulatory role for LOX-1 in cell function (7). Renal LOX-1 expression has been demonstrated in rats with obesity, diabetes, and dyslipidemia (17) and in other situations of oxidant injury (4, 40).

There is now abundant evidence for intense oxidative stress in diabetes and the metabolic syndrome (38). Hence, we tested the hypothesis that in obese rats with uncontrolled diabetes and dyslipidemia, renal LOX-1 upregulation promotes lipid peroxidation stress, inflammation, and fibrosis and that blockade of LOX-1 limits lipotoxicity, renal inflammation, and fibrosis.

MATERIALS AND METHODS

Animals. The research involving animals adhered to 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 Genetic Models (Gmi, Indianapolis, IN). We studied first generation (F1) male hybrid rats derived from a well-characterized parental strain: the Zucker fatty diabetic (fa/fa) (50) and the spontaneous hypertensive heart failure rat (SHHF/ Gmi-fa) (1). These hybrid rats develop obesity, diabetes, and dyslipidemia (16, 17). The rats were studied from 6–21 wk of age while they were housed in steel cages and acclimatized to 12-h cycles of light and darkness (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 libitum Purina diet 5008, which contains 27% protein, 17% animal fat, and 56% carbohydrate (15–17).

The rats were divided into four groups at the onset of the study: Eight lean rats were included in the lean control group, four obese rats...
were not treated, four obese rats were injected with normal rabbit IgG, and four obese rats were injected with anti-LOX-1 IgG (see below). Their body weights and blood metabolic profiles were monitored throughout the experiment. Blood samples were obtained sequentially from the tail vein and at the termination of the experiment. Appearance of overt diabetes was verified by serial determinations of blood glucose levels. Twenty-four-hour urine was collected in metabolic cages, and urinary albumin was measured and expressed as described (16), using a commercial kit (Neprat II, Excocell, Philadelphia, PA).

**Blood and tissue chemical analysis.** The blood levels of triglyceride, cholesterol, creatinine, and blood urea nitrogen (BUN) were measured on a Beckman CX4+CE Clinical System. Plasma and tissue levels of GSH were measured by the colorimetric method with a GT 10 assay kit (Oxford Biomedical Research, Oxford, MI). The levels of lipid peroxides were determined colorimetrically with assay kit FR12, which detects malondialdehyde and 4-hydroxy-2-nonenal in the presence of heptanesulfonic acid (Oxford Biomedical Research). Tissue triglycerides were measured in tissue homogenates with kit 334A (Sigma-Aldrich, St Louis, MO).

**Gel filtration chromatography.** Lipoproteins were separated by gel filtration of 100-μl aliquots of rat plasma as described previously (16). The elution buffer was made of 150 mM NaCl, 20 mM HEPES, pH 7.4, and 0.02% NaN3. The column flow rate was 0.25 ml/min, and 0.5-ml fractions were collected sequentially. Human plasma was used to standardize the column. The plasma fractions were assayed for cholesterol utilizing a commercially available kit (Roche Pharmaceuticals).

**Anti-LOX-1 antibody.** Anti-LOX-1 antibody was generated in rabbits against the LOX-1 peptide (residues 188–233 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 (www.covance.com). The anti-LOX-1 antibody was used in Western analysis of cell lysates (32). Proteins in 50 μg of cell lysate were separated by a 10% acrylamide SDS-PAGE gel, electrophoretically transferred to a Bio-Rad Immunoblot polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA) at 15 mA, and then labeled with anti-LOX-1 antibody without and with antigenic peptide in a ratio of 1:10: antibody to peptide. Obese rats were injected in the tail vein with either 2 μg/wk of normal rabbit IgG (Covance) or 2 μg/wk of anti-LOX-1 IgG. The latter anti-LOX-1 IgG dose was based on in vitro titrations of anti-LOX-1 IgG binding to LOX-1 peptide (performed by Covance; not shown).

**oxLDL binding assay.** The blocking activity of the anti-LOX-1 antibody was tested in vitro, using an assay designed to measure human oxLDL binding to HCAECs (34). The studies were conducted at 4°C, with cells prechilled for 30 min in HEPES buffer, pH 7.4, before addition of lipoprotein. 125I-oxLDL was added to each dish in a final concentration of 10 μg/ml. To determine specific binding of 125I-oxLDL, a 100-fold excess of unlabeled oxLDL was added in parallel dishes. Incubation was carried out at 4°C for 2 h, and then cells were washed on ice with 150 mM NaCl, 50 mM Tris, 2 mM EDTA, pH 7.4, containing 2 mg/ml BSA. Cells were washed and then dissolved in 0.5 M NaOH solution at room temperature. An aliquot of the cell lysate was counted to determine the amount of bound 125I-oxLDL. LOX-1 binding activity was expressed as counts per minute per milligram lysate protein.

**Renal histology and immunohistochemistry.** Renal histology and immunohistochemistry were conducted on paraffin-embedded renal sections at termination. Transverse renal sections of 5-μm thickness were stained with hematoxylin and eosin, periodic acid-Schiff, and trichrome dyes. Morphometric analysis was performed at ×200 and ×400 magnification by an examiner blinded to the study groups (15, 16). Sections from the entire kidney were analyzed, and all glomeruli and tubules were included in the calculations. Glomerular areas were calculated from the glomerular perimeters on captured digital images with the aid of Sigmascan v 5.0 algorithms (SPSS, Chicago, IL).

Renal polymorphonuclear cells were labeled with napthanal-AS-D chloroacetate esterase (Leder stain, Poly Scientific, R&D Systems, Bay Shore, NY) as reported elsewhere (16, 17, 30). Polymorphonuclear cells, typically found attached to peritubular and glomerular capillaries, were all counted at ×400 magnification.

Immunohistochemistry was carried out on 5-μm thick sections mounted on lysine-coated glass (15, 16). The tissue sections were deparaffinized by sequential exposure to xylene and ethanol. We employed the Vectastain immunostaining protocol as described by the manufacturer (Vector Laboratories, Burlingame, CA). The sections were dehydrated and exposed to H2O2 to eliminate endogenous peroxidase activity. The kidney sections were then blocked with 2% BSA for 30 min and exposed to a primary antibody. The renal microvasculature was labeled with a primary antibody (diluted 1:2,000) specific for factor VIII from Dakocytoimation (Carpinteria, CA). Anti-LOX-1 antibody was applied to the renal sections at a dilution of 1:1,000. Preimmune rabbit serum was included as a negative control. The endothelial labeled areas were measured using color subtraction algorithms contained in Sigmascan Pro, v. 5.0 (SPSS). The glomerular vascular data were expressed as the fraction of factor VIII label present in each glomerulus, and the peritubular capillary data as the fraction of factor VIII label present in the peritubular region.

**Laser capture microdissection.** Isolated glomeruli were collected from paraffin-embedded sections by laser capture microdissection (10). The 5-μm-thick sections were deparaffinized and hydrated, stained with hematoxylin, and then gradually dehydrated in 70–100% ethanol. Glomeruli were identified and removed with a solid-state laser (Pixcell II, Vertias microdissection system, Arcturus Bioscience, Mountain View, CA). For each measurement, 40 glomeruli were pooled in a cup, homogenized in PBS with 0.2 M HCl, 0.1% Tween 20, and 0.2% blotting grade blocker (Bio-Rad), and incubated for 10 min at room temperature. Levels of vascular endothelial growth factor (VEGF) were measured by ELISA in the supernatant (R&D Systems).

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**Fig. 1.** LOX-1 blocking antibody. **Top:** Western blot of LOX-1 protein in human aortic endothelial cell lysates (left and right lanes). LOX-1 detected with untreated antibody (1 ng/ml, left) and with antibody preabsorbed with LOX-1 peptide at 100× concentration (right) is shown. **Bottom:** oxidized LDL (oxLDL) uptake in cultured human coronary endothelial cells. Cells were cultured for 24 h in the presence of oxLDL for 24 h before oxLDL uptake was measured. 125I-oxLDL (10 μg/ml) uptake was measured without additions (open bar) or with 100-fold excess oxLDL (striped bar), with anti-oxLDL antibody (1 μg/ml, black bar), or with normal nonspecific IgG (1 μg/ml, grey bar). Unlabeled oxLDL and anti-oxLDL antibody completely blocked specific oxLDL uptake. *P < 0.001; n = 3, ANOVA.
Isolation of renal mitochondria. Mitochondria were isolated from renal cortices. The kidneys were minced with scissors and homogenized in ice-cold buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, and 1.0 mM EGTA as well as 5% BSA, pH 7.5). The mitochondria were isolated by differential centrifugation and suspended in homogenizing buffer without EGTA. State 3 and 4 mitochondrial respiration was measured in freshly isolated mitochondria as described previously (36).

Activity of 3-hydroxyisobutyrate dehydrogenase. The enzymatic activity of 3-hydroxyisobutyrate dehydrogenase (HIBDH) was measured in renal homogenates by modification of the method previously described (16). S-3-hydroxyisobutyrate was used as a substrate and reported as nmoles NADH per minute per milligram protein (21).

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 was oxidized in the presence of CuSO4, and oxidation was confirmed by measurements of TBARS of dialyzed oxLDL: 0.1 nmol malondialdehyde/mg protein in non-oxLDL and 7 nmol/mg protein in oxLDL (16). oxLDL was added to the media for the first 24 h, removed, and cells were cultured for an additional 24 h and then imaged by confocal microscopy (46). LDH was measured with a CytoTox 96 Assay Kit (Promega, Madison, WI) and is expressed as percent release of total cell LDH.

Confocal microscopy. NRK52E cells cultured on glass coverslips were exposed to either saline (control) or oxLDL (50 µg/ml) for 24 h. The cells were then fixed with 2% paraformaldehyde, permeabilized with 0.2% Triton X-100, and reacted with primary antibodies: rabbit anti-LOX-1 (above) and anti-E-cadherin (murine anti-E-cadherin, 1:25, BD Bioscience, San Jose, CA). The cells were then labeled with secondary antibodies including fluorescein.

Table 1. Organomegaly parameters

<table>
<thead>
<tr>
<th>Group</th>
<th>Kidney Weight, g</th>
<th>Kidney Protein/DNA, µg/µg</th>
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<tbody>
<tr>
<td>LM</td>
<td>1.637±0.034</td>
<td>271±10</td>
</tr>
<tr>
<td>OM</td>
<td>2.760±0.092*</td>
<td>324±8*</td>
</tr>
<tr>
<td>OM-IgG</td>
<td>2.575±0.084*</td>
<td>309±20</td>
</tr>
<tr>
<td>OM-Ab</td>
<td>2.076±0.052†</td>
<td>318±10</td>
</tr>
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Values are means ± SE. LM, lean male rats; OM, obese male rats; OM-IgG, obese male rats injected with nonspecific rabbit IgG; OM-Ab, obese male rats given anti-LOX-1 rabbit antibody. *Significantly different from LM, P < 0.05. †Significantly different from OM and OM-IgG, P < 0.05; n = 8 rats for LM and 4 rats for the 3 remaining obese groups.
isothiocyanate (FITC)-labeled donkey anti-mouse IgG antibody and Texas red-labeled donkey anti-rabbit IgG (Jackson, West Grove, PA). The slips were then 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. All images were processed and merged using MetaMorph (Molecular Devices Universal Imaging, Sunnyvale, CA).

Statistical analysis. The results are expressed as means ± SE. Any differences between groups were evaluated by one-way ANOVA and considered significant if $P < 0.05$.

RESULTS

Verification of blocking LOX-1 antibody. LOX-1 was identified in HCAECs treated with oxLDL (Fig. 1) in accordance with the observation that LOX-1 is expressed in endothelial cells. The expression of LOX-1 in these cells was confirmed by the staining of the endothelial cells with a specific antibody against LOX-1. The staining was observed in the cytoplasm of the cells, indicating the presence of LOX-1 protein.

Fig. 4. Renal vascular parameters. Glomerular surface areas were 60% higher in OM and OM-IgG than in LM rats ($P < 0.001$). Therapy with anti-LOX-1 antibody limited glomerular enlargement in OM-Ab compared with OM and OM-IgG rats ($P < 0.001$). Glomerular vascular density, represented as fractional area of glomerular areas, was not expanded in proportion to glomerular size in untreated and random IgG-injected obese males compared with lean males ($P < 0.001$). Anti-LOX-1 therapy helped support, in part, glomerular vascular density in OM-Ab compared with OM and OM-IgG rats ($P < 0.01$). Peritubular vascular density, represented as fractional area of renal areas, was not expanded in proportion to renal enlargement in OM and OM-IgG compared with LM rats ($P < 0.001$). Anti-LOX-1 therapy supported, in part, peritubular vascular density in OM-Ab compared with OM and OM-IgG rats ($P < 0.01$). Glomerular VEGF levels, measured in 40 glomeruli microdissected from all rats in the groups, were lower in OM and OM-IgG than in LM rats ($P < 0.001$). Therapy with anti-LOX-1 antibody (OM-Ab) prevented in part the fall of VEGF levels compared with OM and OM-IgG rats ($P < 0.001$). The number of dissected glomeruli was 640 in LM and 480 in each of the 3 obese groups.

Fig. 5. Factor VIII immunoreactivity ($×600$ amplification). Renal cortex labeling of factor VIII is outlined in brown for the glomerular and peritubular vasculature. In contrast to lean males (A), density of glomerular and peritubular vascular labels was markedly reduced in obese untreated (B) and normal IgG-injected obese rats (C), whereas injections of anti-LOX1 antibody preserved vascular density in obese males (D).
with previous studies (34). This band was eliminated when the antibody was preabsorbed with LOX-1 peptide at 100× concentration. The capacity of LOX-1 antibody to block oxLDL binding was also examined using the same cells. Data on 125I-oxLDL binding are also shown in Fig. 1, bottom. Labeled oxLDL binding was reduced by 100× excess “cold” oxLDL. Treatment with the LOX-antibody also reduced labeled oxLDL binding, whereas nonspecific IgG had no effect.

Metabolic, structural, and functional parameters. Treatment with nonspecific IgG, or the LOX-1 IgG antibody, did not affect the marked increases in body weight observed in all three groups of obese rats: lean males, 421 ± 7 g; obese males untreated, 634 ± 13 g; obese males injected with normal rabbit IgG, 644 ± 11 g; and obese males injected with anti-LOX-1 IgG, 622 ± 21 g. Serum levels of glucose, cholesterol, and triglycerides increased rapidly after 6 wk of age in all obese rats (designated as time 0) and remained much higher than in the lean rats throughout the study (Fig. 2). The lipid profiles, obtained at the end of the study, showed the main lipoprotein fractions in the HDL region in lean rats. In contrast, very LDL and LDL predominated in obese rats, and treatment with nonspecific IgG, or the specific LOX-1 antibody, did not alter lipoprotein distribution (Fig. 2).

Whereas kidney weights were markedly increased in the control and nonspecific IgG-treated obese rats, treatment with LOX-1 antibody dramatically limited nephromegaly (Table 1). The massive nephromegaly of untreated obese rats was not linked to proportional increases in renal protein/DNA ratios, implicating DNA contributions from renal hyperplasia and/or migratory cells (below). Furthermore, nephromegaly in obese rats was accompanied by substantial and progressive increases in 24-h creatinine clearance, which were significantly blunted by LOX-1 antibody treatment. In contrast, creatinine clearance in lean rats hovered around the basal value during the entire study (Fig. 3).

Table 2. Renal inflammation and fibrosis parameters

<table>
<thead>
<tr>
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<th>LM</th>
<th>OM</th>
<th>OM-IgG</th>
<th>OM-Ab</th>
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<tbody>
<tr>
<td>Glomerular PMN</td>
<td>0.05±0.01</td>
<td>0.74±0.04*</td>
<td>0.70±0.04*</td>
<td>0.17±0.03+†</td>
</tr>
<tr>
<td>Peritubular PMN</td>
<td>0.18±0.03</td>
<td>3.39±0.22*</td>
<td>3.64±0.24*</td>
<td>1.08±0.10+‡</td>
</tr>
<tr>
<td>GFF</td>
<td>0.14±0.07</td>
<td>4.05±0.86*</td>
<td>3.84±0.68*</td>
<td>2.73±0.76*</td>
</tr>
<tr>
<td>PFF</td>
<td>0.71±0.17</td>
<td>11.95±1.33*</td>
<td>11.80±1.26*</td>
<td>3.56±0.66+‡</td>
</tr>
</tbody>
</table>

Values are means ± SE. PMN, polymorphonuclear cells; GFF, glomerular fractional fibrosis; PFF, peritubular fractional fibrosis. Glomerular PMN represents the average number of PMN per glomerulus. Peritubular PMN represents the average number of peritubular PMN per ×600-power field. GFF is the averaged % of the glomerular area covered with fibrosis. PFF is the averaged % of the interstitial area covered with fibrosis. *Significantly higher than control, P < 0.01; n = 8 rats in LM and 4 rats each in OM, OM-IgG, and OM-Ab. †Significantly lower than OM and OM-IgG; n = 8 rats LM and 4 rats each in OM, OM-IgG, and OM-Ab.

Fig. 6. Albuminuria. Progressive albuminuria was apparent after 6 wk in the study (rats were 12 wk old) in all obese groups (OM, OM-Ab, and OM-IgG), while urinary albumin remained low and unchanged in LM rats.

Fig. 7. LOX-1 immunoreactivity in renal cortex (×300 amplification). LOX-1 renal expression, brown stain, was localized preferably in glomerular tufts. LOX-1 was also expressed in peritubular capillaries (red arrowheads) and in tubules (brown arrows). In contrast to lean males (A), abundance of glomerular LOX-1 was markedly increased in untreated (B) and in nonspecific IgG-injected obese rats (C), whereas injections of anti-LOX-1 antibody suppressed LOX-1 reactivity in obese rats (D).
Vasculopathy, inflammation, and fibrosis. The increases in renal mass and creatinine clearance in obese untreated control and nonspecific IgG-treated obese rats were associated with an ~60% increase in the size of their glomeruli, and LOX-1 antibody therapy limited glomerular enlargement (Fig. 4). It was remarkable that the larger glomeruli of obese rats did not have proportional increases in their microvascular density, in that there was >60% attenuation in glomerular capillary networks. Furthermore, the renal microvascular damage was not limited to glomeruli, since there was also a 58% decrease in peritubular capillary density throughout the cortex and medulla of untreated obese rats. Therapy with the LOX-1 antibody protected renal microvascular networks, as shown by preservation of glomerular and peritubular capillary density (Fig. 4) and also illustrated in the light micrographs of Fig. 5. In addition, compared with lean rats, glomerular VEGF levels were significantly reduced in microdissected glomeruli of control and IgG-treated obese rats, whereas treatment with the LOX-1-specific antibody limited the fall in glomerular VEGF levels (Fig. 4). In contrast, total renal cortex VEGF levels (pg/100 μg protein) were similar in all four groups: 13.0 ± 0.8 in lean rats, 16.4 ± 2.7 in...
untreated obese rats, 18.3 ± 2.6 in obese rats injected with normal IgG, and 19.5 ± 3.8 in obese rats injected with anti-LOX-1 antibody.

The glomerular abnormalities observed in obesity were accompanied by glomerular capillary leakage, as indicated by progressive microalbuminuria, which was lower in the obese group treated with the LOX-1 antibody. However, the effect was minimal and variability great, so that it was not statistically significant (Fig. 6).

Glomerular vascular density attenuation in untreated obese rats was accompanied by increased glomerular LOX-1 immunoreactivity (Fig. 7). The data were calculated as the number of LOX-1-labeled pixels per glomerulus, and in parentheses we also include the fraction of LOX-1-positive pixels per each respective glomerulus. Glomerular pixel number was 46,764 ± 1,861 in lean rats (0.15 ± 0.01), and it was significantly higher in untreated obese and normal IgG-injected obese rats, 75,081 ± 3,191 (0.26 ± 0.01), and 83,782 ± 4,390 (0.25 ± 0.05), P < 0.001, respectively. In contrast, anti-LOX-1 therapy to obese rats limited their accumulation (Table 2). The predominant renal peritubular capillary location of leukocytes is illustrated in Fig. 8. While kidneys of lean rats lacked neutrophils, kidneys from untreated and normal IgG-injected obese rats had abundant clusters of neutrophils along capillary lumens, which were absent in obese rats treated with the anti-LOX-1 antibody.

Renal fibrosis was measured in sections stained with Masson's trichrome and expressed as percent fibrosis of total glomerular and interstitial areas (Fig. 9 and Table 2). Lean rats had nearly undetectable glomerular and interstitial fibrosis. The glomeruli of the three sets of obese young rats also showed very minimal and occasional fibrosis, higher than in lean rats (P < 0.05), but not different from each other. On the other hand, renal interstitial (peritubular) fibrosis was more extensive and frequent in untreated obese rats and in normal IgG-injected obese rats than in lean rats. In contrast, interstitial fibrosis was significantly reduced in obese rats treated with the anti-LOX-1 antibody.

Tissue lipid and oxidant loads. The enlarged kidneys of untreated obese rats were overloaded with unutilized lipid, as indicated by their higher levels of renal triglycerides, and the anti-LOX-1 antibody limited triglyceride accumulation (Table 3). In addition, renal levels of 4-hydroxynonenal adducts, presumably derived from oxLDL and other lipid peroxides (19), were markedly increased in control obese rats and in normal IgG-treated obese rats, whereas anti-LOX-1 therapy limited the renal deposition of lipid peroxides. Furthermore, accumulation of oxidants was accompanied by proportional decreases in kidney levels of reduced glutathione. These effects were completely blocked by treatment of obese rats with the anti-LOX-1 antibody (Table 3).

Mitochondrial HIBDH and respiration. Renal HIBDH is a mitochondrial enzyme expressed at high levels (16, 21, 44), and its levels are severely depressed in kidneys of obese rats, consistent with its redox sensitivity (16). Anti-LOX-1 therapy preserved HIBDH enzyme activity in obesity (Table 4). Notwithstanding the suppression of renal HIBDH activity in obesity, renal mitochondrial respiration, represented by measurements of state 3 and 4 respiration, remained largely unchanged in lean and obese rats regardless of treatment (Table 4).

LOX-1 in renal epithelial cells. LOX-1 expression in renal tubules is an entirely novel observation, and its significance may lie in the potential for LOX-1 to promote tubular injury in diabetes and dyslipidemia. Therefore, independent validation of LOX-1 expression was sought in rat renal tubular cells cultured without and with oxLDL (50 μg/ml) for 24 h. Control NRK52E cells had noticeable LOX-1 expression mainly in their perinuclear regions (Fig. 10). oxLDL treatment not only increased LOX-1 intensity, but it also altered the distribution of LOX-1, which now appeared as extensive and larger aggregates. NRK52E cells were also exposed for 24 h to saline control, the anti-LOX1 antibody (2 μg/ml), oxLDL (70 μg/ml), or the anti-LOX1 antibody and oxLDL. Cell lysates were obtained and probed with the anti-LOX-1 antibody on Western blots (Fig. 11). oxLDL increased cellular LOX-1 expression and was cytotoxic, while addition of the anti-LOX-1 antibody

Table 3. Renal unutilized lipid and redox parameters

<table>
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<th>LM</th>
<th>OM</th>
<th>OM-IgG</th>
<th>OM-Ab</th>
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<tr>
<td>Kidney triglycerides, μg/μg DNA</td>
<td>39±7</td>
<td>189±20*</td>
<td>186±10*</td>
<td>128±7†</td>
</tr>
<tr>
<td>Kidney 4-hydroxynonenal, nmol/mg protein</td>
<td>0.67±0.019</td>
<td>1.15±0.127*</td>
<td>1.089±0.098*</td>
<td>0.842±0.017†</td>
</tr>
<tr>
<td>Kidney GSH, nmol/mg protein</td>
<td>0.309±0.01</td>
<td>0.202±0.019*</td>
<td>0.175±0.010*</td>
<td>0.273±0.008†</td>
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</tbody>
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Values are means ± SE. *Significantly different from LM, P < 0.05; †Significantly different from OM and OM-IgG, P < 0.05; n = 8 rats for LM and 4 rats for the remaining obese groups.

Table 4. Mitochondrial parameters

<table>
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<th>LM</th>
<th>OM</th>
<th>OM-IgG</th>
<th>OM-Ab</th>
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<tbody>
<tr>
<td>Kidney HIBDH activity, nmol·mg protein⁻¹·min⁻¹</td>
<td>8.50±0.36</td>
<td>4.92±0.40*</td>
<td>5.63±0.60*</td>
<td>8.66±1.17</td>
</tr>
<tr>
<td>Kidney mitochondrial respiratory state 3, natoms·mg protein⁻¹·min⁻¹</td>
<td>84±7</td>
<td>78±11</td>
<td>95±3</td>
<td>90±5</td>
</tr>
<tr>
<td>Kidney mitochondrial respiratory state 4, natoms·mg protein⁻¹·min⁻¹</td>
<td>34±2</td>
<td>51±7</td>
<td>34±1</td>
<td>32±2</td>
</tr>
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</table>

Values are means ± SE. HIBDH, 3-hydroxyisobutyrate dehydrogenase. *Significantly different from LM, P < 0.05; n = 8 rats for LM and 4 rats for the 3 remaining obese groups.
to the cultures significantly lowered LOX-1 levels and cytotoxicity.

**DISCUSSION**

The role of anti-LOX-1 therapy on renal structure and function was investigated in obese, diabetic, and dyslipidemic rats. This effect was probed with an anti-LOX-1 blocking polyclonal antibody, which was designed to impede oxLDL binding to LOX-1 by blocking the extracellular binding domain of rat LOX-1. This particular peptide site was targeted because it is a component of the unique lectin-binding domain required for LOX-1 binding activity (8, 48). The binding portion of LOX-1 is homologous to the antigen recognition site of NK cells (9), and it may have originated from duplication of an ancestral gene (49). We demonstrated near complete blocking by the anti-LOX-1 antibody of oxLDL binding activity (34).

The administration of the anti-LOX-1 antibody, or normal rabbit IgG, did not alter weight gain or metabolic profiles in obese rats. However, anti-LOX-1 therapy limited kidney enlargement and blunted the rise in creatinine clearance. Anti-LOX-1 therapy also prevented polymorphonuclear neutrophils from gathering on endoluminal surfaces of peritubular capillaries of obese rats. In these young obese rats, emerging foci of peritubular fibrosis were also controlled by anti-LOX-1 therapy. We previously reported that these discrete microdomains of renal fibrosis in obese rats originate in regions of peritubular vasculopathy characterized by capillary leaks and rarefaction (46).

Whereas anti-LOX-1 antibody therapy did not prevent albuminuria, it improved glomerular VEGF levels and helped preserve renal microvascular beds. We favor measurements of VEGF in unambiguous microdissected samples, as total renal cortex VEGF levels were not informative. Indirect or direct results of therapy also included attenuation of renal lipotoxicity, as shown by lower accumulation of lipid and peroxidation products and preservation of the renal redox state. Indeed, the activity of the redox-sensitive renal mitochondrial enzyme HIBDH (16, 21) was severely depressed in obesity, and anti-LOX-1 therapy was protective. However, in this age group renal mitochondrial injury was limited, as indicated by preserved mitochondrial respiration in all obese groups.

In summary, targeting LOX-1 protects kidneys of obese rats with diabetes and dyslipidemia. This novel finding is consistent with the hypothesis that targeting LOX-1 may offer a therapeutic benefit in renal diseases associated with obesity and metabolic syndrome.

**Fig. 10.** LOX-1 expression in NRK52E cells by fluorescent microscopy. Confluent NRK cells were treated for 24 h with: saline (vehicle control, A) or (B) oxLDL (50 µg/ml, B). Bar = 25 µM. The cells were stained with antibodies to LOX-1 (red, Texas red), E-cadherin (green, FITC), and 4',6-diamidino-2-phenylindole (blue nuclear stain). As shown in the representative image (n = 6), LOX-1 was expressed in the perinuclear (Golgi) region in control cells. Treatment with oxLDL increased LOX-1 expression with translocation to regions of aggregation (arrows).

**Fig. 11.** LOX-1 levels and LDH release in NRK52E cells. Confluent cells were cultured with oxLDL (70 µg/ml) without and with anti-LOX-1 antibody (5 µg/ml) for 24 h and then for an additional 24 h without oxLDL. LDH was measured in cells and media, and LOX-1 protein by Western blots in cell lysates [data expressed as ratio of LOX-1 optical density (O.D.) over corresponding actin O.D]. The significant increases in LOX-1 protein and LDH release evoked by oxLDL (*P < 0.05) were blunted by anti-LOX-1 antibody (*P < 0.05).
with reports that interfering with LOX-1 function protects discrete cell systems in vitro isolation (4, 6, 13, 29, 32). We now report that anti-LOX-1 therapy limited renal enlargement and lipotoxicity, as well as renal lipotoxicity-derived inflammation, which we have shown elsewhere to be critical for the spread of renal fibrosis later in life (17). We just do not know whether the effects of anti-LOX-1 therapy were derived from improved microvascular function, from direct protection of renal vascular and epithelial cells, or from reduced inflammation. Nevertheless, some deductions may be made from these and from other previously published in vivo data (33). For example, in diabetes the observed renal microvascular drop out (46) would likely prevent any scaling of capillary networks, which must expand to sustain metabolic functions of larger kidneys (47). In this scenario, blockade of endothelial LOX-1 (2, 37, 45) might protect vessels expressing LOX-1, which could otherwise be severely damaged. It is equally noteworthy that renal LOX-1 expression was also tubular and consistent with a far more common expression of LOX-1 (5, 7, 26, 27, 33, 35, 39). Hence, anti-LOX-1 therapy could possibly have afforded direct protection to LOX-1-expressing renal tubules, which comprise the bulk of the affected renal mass. In further support of this notion was our finding that oxLDL induced robust LOX-1 expression and cell stress in NRK52E cultured tubular cells.

Renal protection by the antibody was not complete, as indicated by persistent proteinuria. We reasoned that proteinuria reflects persistent podocyte damage (11), not entirely corrected by anti-LOX-1 therapy. In support of this notion is the fact that depressed glomerular VEGF levels were only partly restored by anti-LOX-1 therapy. It is also remarkable that polymorphonuclear leukocytes were found in large numbers on surfaces of peri-tubular capillaries of obese rats. This location is consistent with leukocyte binding to endothelial LOX-1, as documented in other models (22, 24), or with enhanced leukocyte vascular adherence directed by LOX-1 (4, 31). Indeed, one of those mechanisms may be at play during inflammation (41). Accordingly, we propose that renal protection from anti-LOX-1 therapy might result, at least in part, from attenuated inflammatory responses, as indicated by significantly reduced numbers of renal neutrophils in treated rats. This proposal is supported by our finding that pharmacological reduction of circulating neutrophils also lowers renal fibrosis in diabetes (17). Last, kidneys from treated obese rats had reduced levels of unutilized lipids and related lipid peroxidation products, which are typically found in obese rats with diabetes and dyslipidemia (15, 16). Thus anti-LOX-1 therapy, by preventing accumulation of unutilized tissue lipid and lipid peroxides, may also curtail a well-recognized homing signal for polymorphonuclear leukocytes (14) such as CINC1 (17) and thus limit renal inflammation, a potential protective mechanism for renal vessels and tubules alike. In closing, we emphasize that our conclusions need to be tempered by the relatively low number of study rats. Nonetheless, it appears that blocking LOX-1 is a novel therapeutic option that limits nephropathy in obesity and diabetes.

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