**CD36 and Na/K-ATPase-α1 Form a Pro-inflammatory Signaling Loop in Kidney**

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***ONLINE SUPPLEMENT***

## SUPPLEMENTAL METHODS

*Assessment of blood pressure and renal function*

Conscious blood pressure was monitored at the indicated times using the tail-cuff method (IITC Life Science)[1, 2]. Prior to sacrifice, urine was obtained from individual mice housed in metabolic cages for 24 hours. Blood samples were collected via cardiac puncture. Urine and plasma creatinine concentrations were determined by a modification of the Jaffé's reaction method with use of the Abbott Architect platform (Abbott Architect ci8200, Abbott Park IL). Plasma levels of the cardiotonic steroid (CTS) marinobufagenin (MBG) were determined using a competitive ELISA based on 4G4 anti-MBG murine monoclonal antibody[3].

*Reagents and Cell Culture*

Tissue culture media and supplements were from Life Technologies. All other chemicals and reagents, including ouabain, were from Sigma. Peritoneal macrophages were obtained by lavage 4 days after injection with thioglycollate and adherent cells maintained in culture as described[4]. The human HK-2 and porcine LLC-PK1 renal proximal tubule cell lines were obtained from American Tissue Type Culture Collection (Manassas, VA). Sublines of LLC-PK1 cells expressing Na/K-ATPase-$\alpha$1 small interfering RNA to knock down expression by 40% (A411 cells) or 90% (PY-17 cells), or control transfected cells (P-11) were cultured in the same manner as the parent cells[5]. A form of oxLDL (referred to as $NO_2$LDL) specific for CD36 was prepared by exposure of human LDL to a myeloperoxidase (MPO)/$H_2O_2$/$NO_2^-$ generating system as previously described[6].

HK-2 cells were cultured in Dulbecco's modified Eagle's medium/F-12 mixed medium (1:1, vol/vol), with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. LLC-PK1 P11, A411, and PY-17 cells were serum-starved for 16-18 h before treatment, and HK-2 cells were changed to medium containing 1% FBS for 16-18 h before treatment. A form of oxLDL (referred to as $NO_2$LDL) specific for CD36 was prepared by exposure of human LDL to a myeloperoxidase (MPO)/$H_2O_2$/$NO_2^-$ generating system as previously described[6]. Native LDL and LDL exposed to MPO and $H_2O_2$ in the absence of $NO_2^-$ were used as controls. After oxidation, LDL was stored under nitrogen gas in buffer containing EDTA, catalase, and butylated hydroxytoluene, and used within 2 weeks.

*Preparation of tissue homogenates, cell lysates, and immunoblotting*

Tissues were homogenized in a mortar with pestle under liquid nitrogen prior to transfer to homogenization buffer. Cells were washed with ice cold PBS and then homogenized on ice directly in culture plates. The homogenization buffer contained 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1mM EDTA, 1mM EGTA, 1% Triton X-100, 2.5mM sodium pyrophosphate, 1mM sodium orthovanadate, 1mM beta-glycerophosphate and protease inhibitor cocktail (complete Mini, EDTA-free; Roche). Samples were rotated at 4º C for 30 minutes prior to centrifugation for 15 minutes. Supernatant was collected and total protein of the whole cell lysate was determined using the detergent-compatible modified Lowry assay (BioRad).

Equal amounts of protein were prepared using standard biochemical methods and subjected to SDS-PAGE and electrotransfer to Immobilon-P membranes (Millipore). Membranes were incubated with antibodies to: phospho-Lyn($Tyr^{386}$) (Abcam); Src and phospho-Src($Tyr^{416}$) (Cell Signaling Technology); $\beta$-actin (Santa Cruz Biotechnology); CD36 (Novus Biologicals); Na/K-ATPase $\alpha$-1 (clone a6F, Developmental Studies Hybridoma Bank). Immunoprecipitations were performed as described previously[6, 7] and the precipitated protein complexes were subjected

to immunoblot as described above. Immunoreactive bands were detected using the SNAP i.d.™ Protein Detection System (Millipore) and Super Signal Chemiluminescent Substrate Products (Pierce). Band intensities were determined by densitometry using the ImageQuant™ system and software (GE Healthcare). In some studies cell surface proteins were labeled by biotinylation as previously described[7]. Biotinylated proteins were purified on immobilized streptavidin-agarose beads and then analyzed by immunoblot as above.

*Detection of reactive oxygen species (ROS), and Cytokine/Chemokines Assays*
Oxidative burst in macrophages was measured using Fc OxyBURST Green assay (Molecular Probes). Pooled peritoneal macrophages were plated at 1.2 x $10^5$ cells/well in 96 well plates, and co-incubated with 50 ug/ml MPO/$H_2O_2$/$NO_2^-$ oxidized LDL ($NO_2$LDL) or ouabain and Fc OxyBURST reagent on a rocking platform at 37º C and 5% $CO_2$, and then analyzed using a fluorescent microplate reader (Gemini EM, Molecular Devices) with 490 nm excitation and 520 nm emission. Cells were collected into 0.2 N NaOH for protein normalization. Similarly, in LLC-PK1 cells, measurement of reactive oxygen species (ROS) was performed using the oxidant sensitive dye, 5-(and-6)-carboxy-2',7'-dichlorodihydrofluorescein diacetate (carboxy-$H_2$DCFDA) (Life Technologies). Cells were grown to confluence in 96 well plates, and co-incubated with $NO_2$LDL or ouabain and carboxy-$H_2$DCFDA reagent on a rocking platform at 37º C and 5% $CO_2$, and then analyzed using a fluorescent microplate reader (Gemini EM, Molecular Devices) with 490 nm excitation and 520 nm emission. Cytokine levels in conditioned media pooled from mouse macrophages was determined using the Proteome Profiler Mouse Cytokine Array, Panel A Array Kit (R&D Systems) and from HK-2 proximal tubule cells using the Human Inflammation Array 3 (RayBio®).

*In vitro adhesion and migration assays*
Macrophage migration was measured with a modified Boyden chamber using Transwell inserts with an 8μM porous membrane (Corning). HK-2 cells were treated for 12hr with combinations of ouabain, $NO_2$LDL, $H_2O_2$, and MCP-1 then washed and incubated with serum-free medium for 8hr. Macrophages (300μL; 0.5x$10^6$/mL) were then loaded into the migration chamber with the conditioned media and after 16hr the cells were removed from the upper side of membranes. Nuclei of migrated cells on the lower side of the membrane were stained with DAPI and visualized by fluorescence microscopy. The number of migrated cells was determined by averaging four fields.

For the adhesion assay, macrophages were plated onto treated tissue-culture plastic (Corning) at 0.5x$10^6$ cells per mL and placed on an orbital shaker at 60rpm for 18hr with vehicle, $NO_2$LDL, or MCP-1. Plates were washed and adherent macrophages counted and expressed as a fraction of bound cells relative to bound control cells.

*Preparations of endosomes*
Endosomes were fractionated on a floating gradient using the technique of Gorvel et al[8]. The early endosomal fraction was collected at the 16% to 10% sucrose interface and the identity of the fractions was determined with antibody against early endosomal antigen 1[7, 9].

*In situ proximity ligation assay (PLA), immunofluorescence staining and confocal microscopy*
This oligonucleotide based cross-linking system detects interactions (denoted by fluorescent dots) when two different protein species are spatially located within 40nm of each other. Macrophages and HK-2 cells were plated on glass chamber slides (Millicell EZ Slide™,

Millipore) and after incubating with CD36 and Na/K-ATPase $\alpha$-1 antibodies for 1hr the oligonucleotide-labelled PLA probes were added. Negative control slides were incubated with CD36 and CD31 antibodies before incubation with PLA probes. Samples were mounted with the Duolink mounting medium and PLA images were acquired using a Leica laser-scanning confocal microscope (Wetzlar, Germany).

*Immunofluorescence staining and confocal microscopy*

Cells were fixed with cold absolute methanol, permeabilized in PBS-Ca-Mg containing 0.3% Triton X-100 and 0.1% BSA, and blocked in buffer containing 0.3% Triton X-100 and 16% (v/v) filtered normal goat serum for 30 min[7]. The cells were then probed with monoclonal Na/K-ATPase $\alpha$-1 (clone 464.6, Millipore) and polyclonal anti-clathrin antibody (BD Transduction Laboratories) overnight at 4°C. Bound antibody was detected with Alexa Fluor®-488 or Alexa Fluor®-546-conjugated anti-mouse or anti-rabbit secondary antibodies. After washing, the specimens were mounted using Vectashield® with DAPI (Vector Labs) and stored at −20°C. Fluorescence intensity was examined by confocal microscopy (Leica) and contrast and brightness were set to ensure that all pixels were within the linear range.

*Histology and Immunohistochemistry*

Deparafinized rehydrated 4µm serial kidney sections were incubated in 3% $H_2O_2$ followed by 5% milk, and then overnight (4°C) with anti-F4/80 (AbD Serotec), anti-MAC-2 (Cedarlane), anti-8-hydroxy-2'deoxyguanosine (Abcam) or isotype matched controls. Histochemical reactions were performed using the EnVision® Doublestain System kit (DakoCytomation) counterstained with hematoxylin. Glomerular basement membranes were detected using the Jones Basement Membrane Reticulum Stain Kit (American MasterTech) and basement membrane width was expressed as the arithmetic mean value of 16 measurements of at least 5 random non-oblique sectioned glomeruli per section (with at least 5 sections per animal from each experimental group)[10, 11]. Morphometric analysis was performed using QCapture Pro 6.0 to quantify the glomeruli area, basement membrane width, and total number of glomeruli present in each kidney section. The kidney sections were also mounted under a Leica DM 2500 microscope equipped with a QImaging MicroPublisher 5.0 RTV camera to perform wide field microscopy to quantify total number of glomeruli. For picrosirius staining of collagen, kidney sections were stained with saturated picric acid containing 0.1% Sirius red (Sigma) for 1hr in the dark. Bright light and polarized images were taken on a Leica DMR upright microscope equipped with a QImaging Retiga EX camera using Image-Pro Plus software (Version 5.1.2.59, MediaCybernetics). For quantitative morphometric analysis, eight randomly chosen cortical fields (at least 6 from each animal from experimental group) lacking major blood vessels were digitized and the collagen volume determined using an Image J software macro (National Institutes of Health) as previously described[2].

*Statistical Analysis*

Data are presented as mean ± standard error of the mean. Data were first tested for normality using the D'Agostino-Pearson omnibus test. If the data did not pass the normality test, the Tukey test (for multiple groups) or the Mann-Whitney Rank Sum test were used. If the data passed the normality test parametric comparisons were performed. If more than two groups were compared, one-way analysis of variance was performed prior to comparison of individual groups with the unpaired Student's t-test with Bonferroni's correction for multiple comparisons. If only two groups of normal data were compared, the Student's t-test was used without correction. Statistical analysis was performed using GraphPad Prism®.

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**Table S1.** Comparison of cytokine/chemokine expression in media of HK-2 proximal tubule cells cultured for 24 hours with vehicle (control), 10 nM ouabain, or 50 μg/mL $NO_2$LDL.

| | **Control** | **10nM Ouabain** | **50 μg /mL $NO_2$LDL** |
|---|---|---|---|
| C5a | 3613±93 | 5881±211* | 5533±224* |
| IL-3 | 3511±80 | 3854±20* | 4588±217* |
| IL-4 | 4578±187 | 6161±326* | 6354±79* |
| CXCL10 | 4346±174 | 5599±15* | 5012±39* |
| CXCL11 | 4696±52 | 5462±330* | 5449±193* |
| CCL17 | 4813±163 | 3913±106* | 6925±174* |
| TIMP-1 | 4603±353 | 5590±375* | 5633±298* |

Media pooled from HK2 cells (n≥ 3 separate wells) and assayed with the Human Inflammation Array 3 (RayBio®). Values are the mean ± SEM density of the chemiluminescent array spots that had at least 20% changes in relative expression of the indicated cytokines/chemokines, * p≤0.05 vs control.

**Table S2.** Comparison of cytokine/chemokine expression in media of macrophages isolated from C57/B6 mice and cultured for 24 hours with vehicle (control), 50 μM ouabain, or 50 μg/mL $NO_2$LDL.

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| | **Control** | ***50 μM Ouabain*** | **50 μg /mL $NO_2$LDL** |
|---|---|---|---|
| IL-3 | 78±14 | 1047±151* | 3443±237* |
| IL-5 | 3779±117 | 7463±127* | 4243±88 |
| IL-7 | 9337±224 | 14183±201* | 10744±154* |
| IL-12p70 | 115±36 | 281±21* | 2357±82* |
| IL-16 | 147±28 | 1238±42* | 2958±118* |
| IL-17 | 1455±103 | 3193±97* | 2863±135* |

Media pooled from peritoneal macrophages of C57/B6 mice (n≥5) assayed with the Mouse Cytokine Array Panel A (R&D Systems). Values are the mean ± SEM density of the chemiluminescent array spots that had at least 20% changes in relative expression of the indicated cytokines/chemokines, * p≤0.05 vs control.

# Figure S1

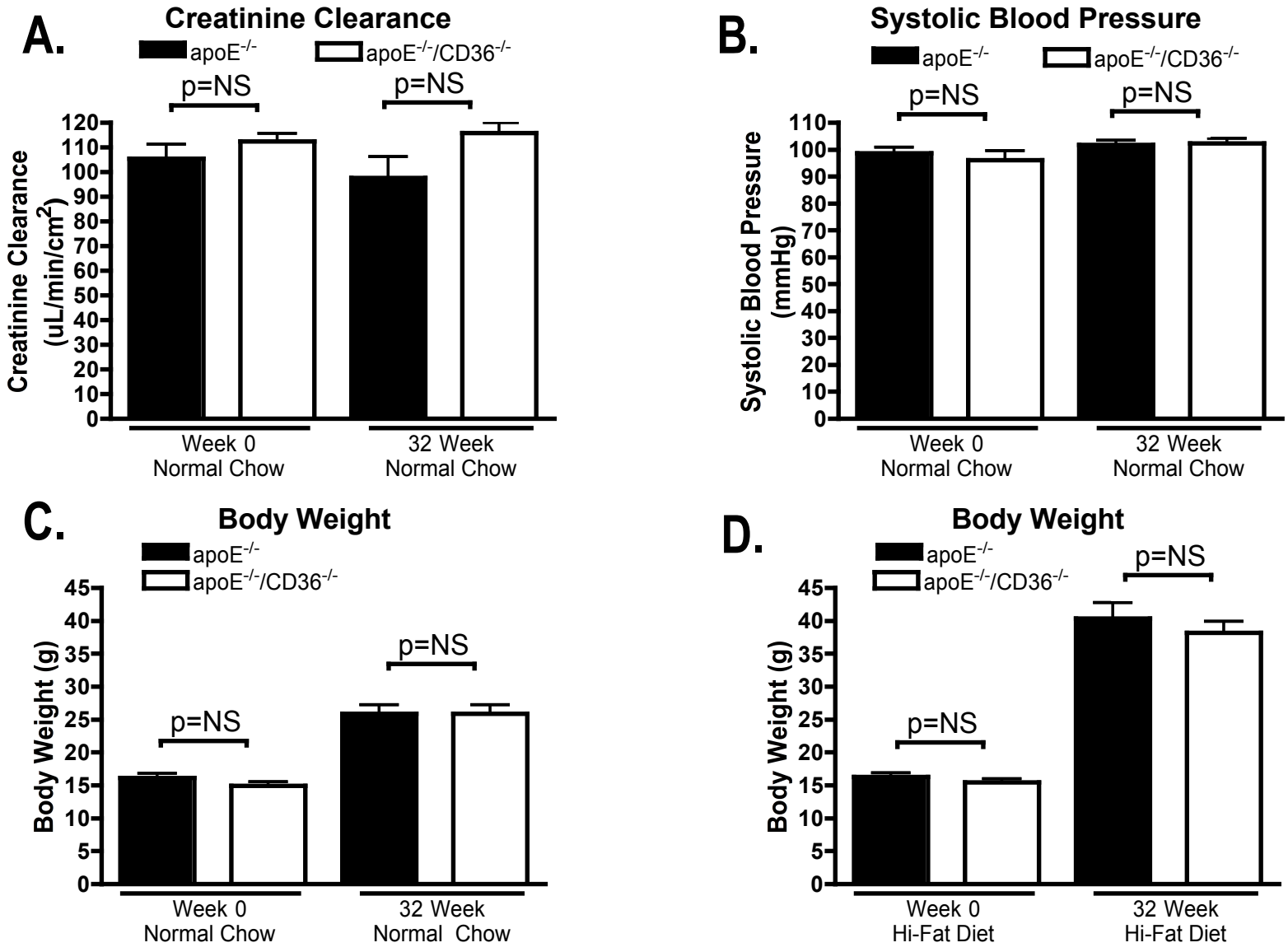


**Figure S1.** (A) 24 hour creatinine clearance adjusted for Body Surface Area and (B) systolic blood pressure in apoE$^{-/-}$ and apoE$^{-/-}$/CD36$^{-/-}$ mice after on normal chow diet for the indicated times, n ≥ 8 mice per group per time point. (C) Body weight apoE$^{-/-}$ and apoE$^{-/-}$/CD36$^{-/-}$ mice on normal chow or 32 week high-fat diet feeding. n ≥ 8 mice/group.

# Figure S2

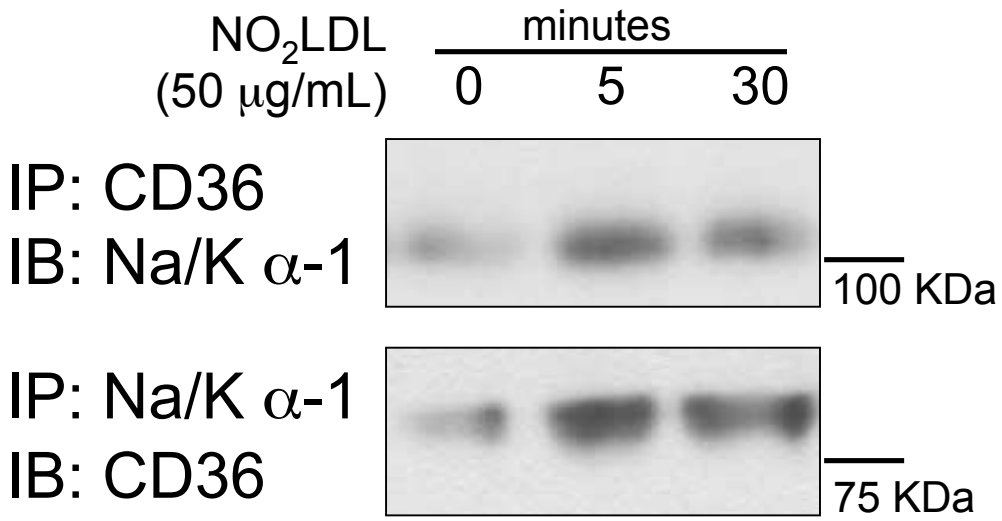


**Figure S2.** CD36 interaction with the Na/K-ATPase. Co-immunoprecipitation of CD36 with the Na/K-ATPase α-1 subunit in LLC-PK1 cells is increased by $NO_2$LDL as early as 5 minutes after treatment.

# Figure S3

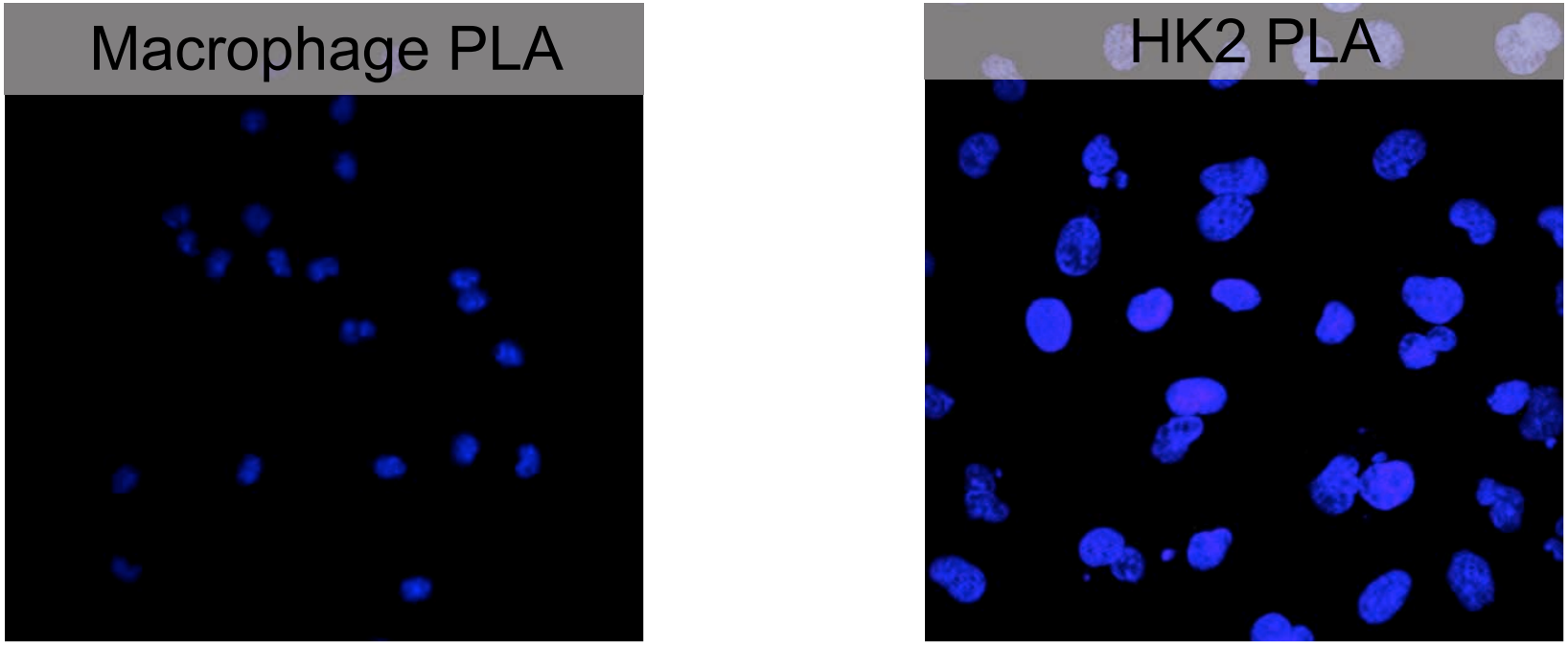


**Figure S3.** Proximity Ligation Cross-linking Assay(PLA). Wild type macrophages (left panel) and HK2 proximal tubule cells (right panel) were incubated with rabbit anti-CD36 IgG, but with anti-CD31 as negative controls for the PLA experiments in Figure 3D and E.

**Figure S4**

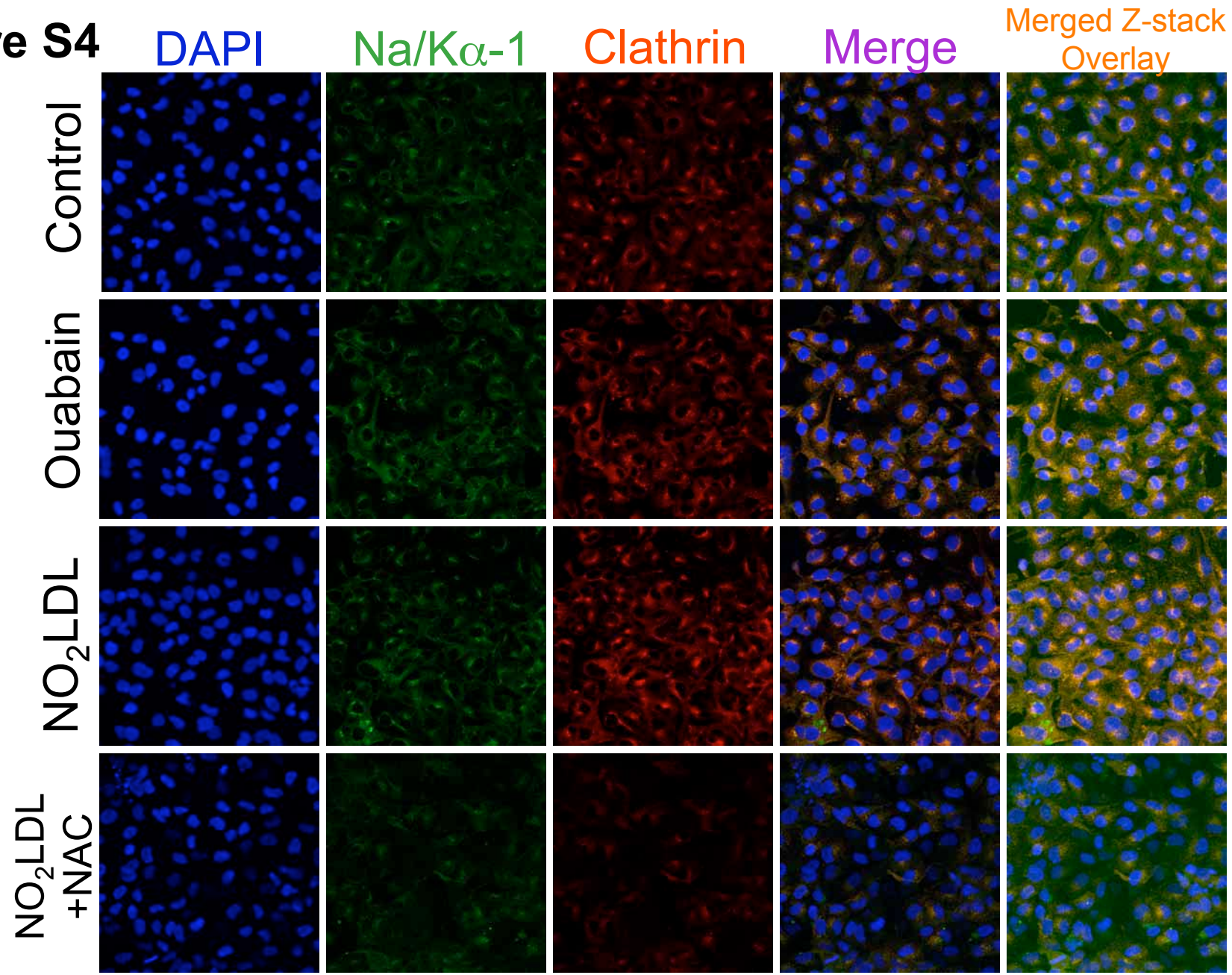


**Figure S4.** Confocal images demonstrating colocalization of Na/K-ATPase α-1 and clathrin in HK-2 cells is increased with both ouabain and $NO_2$LDL and diminished by 30 minute antioxidant pretreatment with 20 mM n-acetyl-cysteine(NAC).

# Figure S5

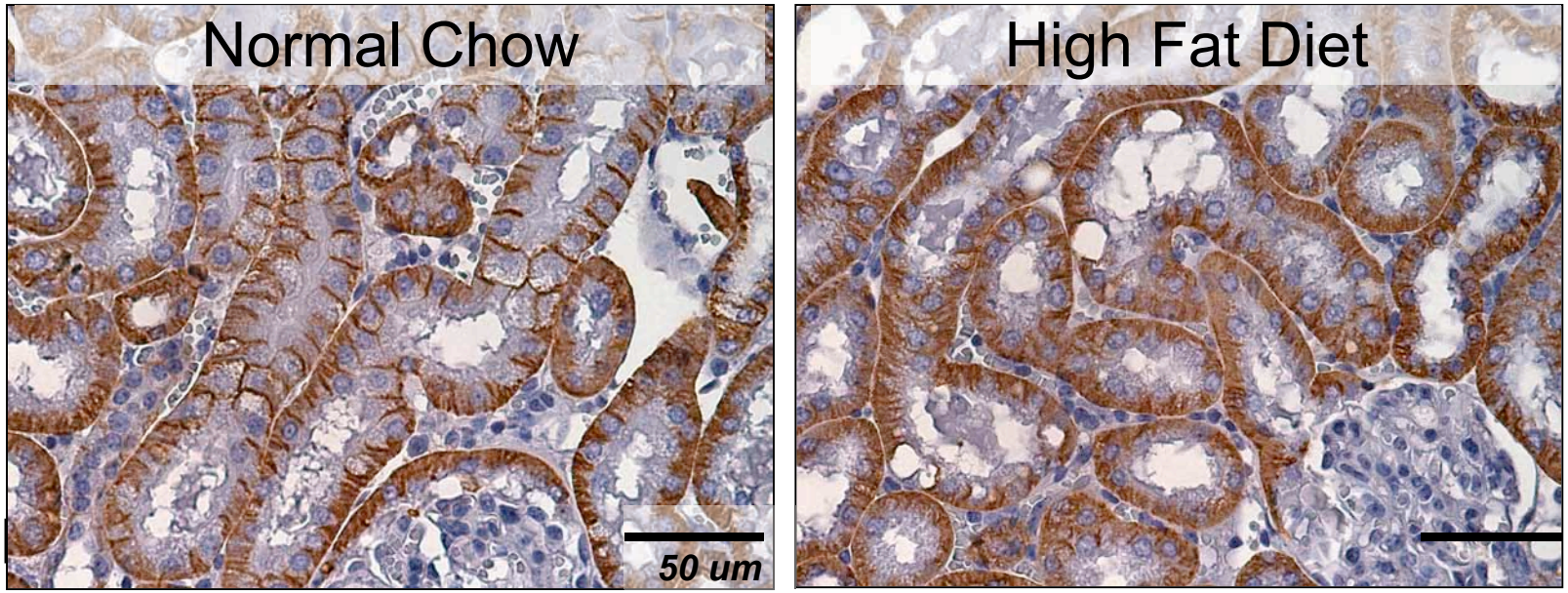


**Figure S5.** Na/K-ATPase $\alpha$-1 immunohistochemistry demonstrates distinct tubular basolateral membrane staining in normal chow fed mouse kidney (left) with a more diffuse pattern in high fat diet fed mice (right).