ABSTRACT

Kidney is one of the target organs for various metabolic diseases, including diabetes, metabolic syndrome and obesity. Most of the metabolic studies underscore glomerular pathobiology, while the tubulo-interstitial compartment has been underemphasized. Current study highlights mechanisms concerning pathobiology of tubular injury in the context of myo-inositol oxygenase (MIOX), a tubular enzyme. Kidneys of mice fed with a high fat diet (HFD) had increased MIOX expression and activity, the latter was related to phosphorylation of serine/threonine residues. Also, expression of sterol regulatory element-binding protein1 (SREBP1) and markers of cellular/nuclear damage was increased along with accentuated apoptosis and loss of tubular brush border. Similar results were observed in cells treated with palmitate:BSA. Multiple sterol response elements and E-box motifs were found in the MIOX promoter, and its activity was modulated by palmitate:BSA. Electrophoretic mobility and Chip assays confirmed binding of SREBP to consensus sequences of MIOX promoter. Exposure of palmitate:BSA-treated cells to rapamycin normalized MIOX expression and prevented SREBP1 nuclear translocation. In addition, rapamycin treatment reduced p53 expression and apoptosis. Like rapamycin, SREBP siRNA reduced MIOX expression. Increased expression of MIOX was associated with generation of reactive oxygen species (ROS) in kidney tubules of mice fed HFD and cells exposed to palmitate:BSA. Both MIOX- and SREBP1-siRNAs reduced generation of ROS. Collectively, these findings suggest that HFD- or fatty acids modulate transcriptional, translational and post-translational regulation of MIOX expression/activity and underscore MIOX being a novel target of transcription factor SREBP1. Conceivably, activation of mTORC1/SREBP1/MIOX pathway leads to the generation of ROS culminating into tubulo-interstitial injury in states of obesity.

INTRODUCTION

Various metabolic diseases, including diabetes, obesity and metabolic syndrome (MetS), adversely affect different compartments of the kidney to a varying degree culminating into chronic kidney disease (CKD) and renal failure (1-4). For instance, in diabetic nephropathy (DN) all the renal compartments, i.e., glomeruli, tubules, interstitium and vasculature, are affected; however, the most notable lesions are confined to the glomerular compartment (5). Typical glomerular lesions of advanced DN are characterized by formation of Kimmelstiel-Wilson mesangial nodules (5). Like DN, obesity also affects the glomerular compartment and advanced pathologic lesions seen often are reminiscent of focal segmental glomerulosclerosis (FSGS) (1,6). The shared pathogenetic events between DN and obesity that
lead to renal glomerular damage include glomerular hyperfiltration, albuminuria or proteinuria and oxidant stress in the form of increased expression of NADPH oxidase 4 (Nox4), although upregulation of Nox4 may be related to decreased fatty acid oxidation in obesity (1,5,6). Interestingly, oxidant stress is regarded as the common denominator of various metabolic disturbances that perturb several signaling pathways and lead to renal damage in DN, and this situation is further complicated by activation of renin-angiotensin system (RAS) (1,2,5). In states of obesity there is stimulation of the sympathetic nervous system which may very well also activate RAS along with increased insulin resistance and hyperinsulinemia (1,2). In addition to insulin resistance and hyper-insulinemia aberrant levels of various adipokines, including those of leptin and adiponectin, have been observed, which apparently are directly related to the pathobiologic effects of fatty acids (7,8). Fatty acid disturbances leading to obesity are well-exemplified in various animal models, i.e., ob/ob and db/db mice with defective leptin gene or its receptor, respectively (9,10). One of the long term effects of obesity with coexistence of high fasting glucose includes development of MetS, which is characterized by elevated lipid levels, low high density lipoproteins, hypertension and associated insulin resistance (11).

Besides damage to the glomerular compartment by oxidant stress in various metabolic disturbances, peroxynitrite, generated by interaction of superoxide and nitric oxide, can also cause oxidant damage to proximal tubule which conceivably leads to tubulo-interstitial injury (5,12,13). On a long term basis tubulo-interstitial injury could be reflected by mitochondrial dysfunctions and increased expression of extracellular cellular matrix (ECM) proteins, the changes similar to those seen in the glomerular compartment. In line with the observations that there is hyperlipidemia and high levels of non-esterified fatty acids (NEFA) in type 2 diabetes Zhang et al. reported that a high fat diet induces glomerular as well as tubulo-interstitial damage in db/db mice, which could be alleviated by the inhibition of hsp90 accompanied with reduced levels of renal nitrotyrosine and mitochondrial Ca$^{2+}$ efflux (14). These observations suggest that various metabolic disturbances, whether related to hyperlipidemia or hyperglycemia, induce tubular damage and subsequent tubulo-interstitial injury, although this concept has been sparsely described in the literature. In support of this concept recent investigations suggest that excessive leakage of albumin during glomerular proteinuria in various metabolic disturbances may not necessarily be the culprit in induction of tubulo-interstitial injury rather it is due to the fatty acids bound to albumin (15-17). Albumin filtered across renal glomerulus is known to be largely reabsorbed by proximal tubules by receptor-mediated endocytosis, thus suggesting that this segment of the nephron would be readily vulnerable to fatty acid-induced injury (18). Albumin can bind efficiently with fatty acids (FA) and transport FA to be delivered to tubules, and thus it is conceivable that FA-bound albumin can induce tubulo-interstitial injury. In this regard in vitro studies by Ruggiero et al. suggest that exposure of tubular cells to palmitate bound to albumin, but not albumin itself, induces mitochondrial dysfunctions, redox imbalance and deactivates antioxidant protein peroxiredoxin 2, ultimately leading to peroxide-mediated cellular apoptosis (19). Overall, the above discussion emphasizes the importance of tubulo-interstitial compartment in bearing the brunt of injury in various metabolic disorders, such as, diabetes and obesity.

Several years ago we reported the discovery of one of the metabolic enzymes, i.e., myo-inositol oxygenase (MIOX) that is exclusively expressed in the renal proximal tubular compartment and is upregulated in diabetic state (20). The enzyme metabolizes myo-inositol to glucuronic acid via glucuronate-xylulose (G-X) pathway and its metabolites enter into the pentose pathway (21). The enzyme activity of MIOX is dependent upon phosphorylation of its serine/threonine residues (22). MIOX promoter includes osmotic-, carbohydrate- and both oxidant and antioxidant response elements and its transcription is heavily influenced by organic osmolytes, high glucose ambience and oxidant stress (22-24). Further initial examination of MIOX promoter suggested that it also includes multiple sterol response elements (SRE) and E-Box motifs, which led us to investigate its transcriptional and translational modulation by fatty acids and in states of obesity.
EXPERIMENTAL PROCEDURES

Reagents - HK-2 and LLC-PK1 cells (renal proximal tubular cell lines) were purchased from the American Type Culture Collection (ATCC). Trizol and pcDNA3.1 plasmid vector were obtained from Invitrogen Corporation. Other reagents were purchased from the following vendors: Sigma-Aldrich: Dulbecco’s Modified Eagle’s Medium (DMEM), M199 media, Fatty acid free BSA (A7030), Sodium palmitate (P9767), Insulin and anti-β-actin antibody; Life Technologies: OptiMeM I reduced serum medium, Fetal Bovine Serum (FBS), Antibiotic-antimycotic, Fast SYBRR Green Master Mix and TO-PRO®-3 iodide; Research Diet Inc: High fat diet chow (HFD, 5.24 Kcal/gm, 60% fat, 20% protein, 20% carbohydrate, D12492); SantaCruz: Anti-SREBP1, -Bax, -p53, -Bcl2 and -His probe antibodies; Millipore: Protein G agarose and Insulin ELISA kit; OriGene Technologies: SREBP1 siRNA and MIOX siRNA; Thermo Scientific: Lightshift Chemiluminescent EMSA kit and Infinity Cholesterol Reagent; STRATAGENE: QuikChange Multi Site Directed Mutagenesis kit; Promega: pGL4-Promoter vector, pRL-CMV- Renilla, FuGENE6 and Dual-Luciferase kit; LC Laboratories: Rapamycin; Roche: Accu-Chek glucometer; Cell Signaling Technologies: Anti-pH2AX antibody; -phospho serine/threonine-specific PKA substrate antibody, -phosphoserine-specific PKC substrate antibody, -phosphothreonine antibody and -phosphotyrosine antibody.

Animal Studies - Eight weeks old male CD1 mice and rats were purchased from Harlan Co. (Indianapolis, IN), and housed in institutional animal facilities. Before initiating the experiments the animals were acclimatized for one week in rooms with a 12 hr light/dark cycle while maintaining a temperature of 22°C with 50% humidity. The mice and rats had free access to food and water. The control animals received a normal mice/rat pelleted chow. The experimental animals received a high fat diet in the form of chow pellets and were sacrificed at 2, 4 and 6 weeks. Likewise, in another set of experiments, mice receiving control or HFD were concomitantly treated with Rapamycin (4 mg/kg, IP, daily). After 6 weeks serum insulin levels were measured using a commercial Rat/Mouse Insulin ELISA Kits (Millipore). Serum cholesterol was measured by using Infinity Cholesterol Reagent (Thermo Scientific). Blood glucose levels were measured by Accu-Chek meter (Roche). Prior to sacrificing the animals 24 hr urine collections were made for determination of protein excretion by SDS-PAGE. All animal studies were approved by the Animal Care and Use Committee of Northwestern University, USA and Tokushima University, Japan.

Morphology studies- Three µm-thick sections were prepared from paraffin embedded tissues and subjected to Periodic Acid Schiff (PAS) staining. For immunohistochemistry to detect the expression of various proteins Avidin-Biotin Complex (ABC) method was employed (20,22,24). The tissue sections were de-paraffinized, rehydrated with PBS and treated with 0.3% H2O2. Following which, the tissue sections were subjected to antigen retrieval procedure in the Histo VT-one by following the vendor’s instructions (Nacalai Tesque, Inc, Japan). After blocking with 5% normal goat serum in PBS, the sections were incubated with various primary antibodies. MIOX expression in the kidneys was gauged by immunofluorescence microscopy, as described previously (20,22,24). Briefly, four m-thick cryostat sections were prepared, air-dried for an hour and fixed with 4% para-formaldehyde for 10 min at 22°C. After equilibrating the sections with PBS, they were incubated with the polyclonal MIOX antibody at 37°C for 1 hr, washed with PBS and re-incubated with goat anti-rabbit IgG antibody conjugated with FITC (Sigma-Aldrich, St. Louis, MO) for another hr. Following a PBS wash, the tissue sections were covered with a drop of buffered glycerol, cover-slip mounted and examined with a Zeiss microscope equipped with UV epi-illumination (Carl Zeiss MicroImaging, Inc.; Thornwood, NY).

Other morphological studies included the assessment of apoptosis in kidney tissues or HK-2 cells by using TdT-mediated dUTP nick-end labeling (TUNEL) method (25). De-paraffinized sections or frozen cryostat sections of the kidneys were digested with Proteinase K (240 unit/ml, Promega, Madison, WI) for 30 min at 22°C. After a rinse with PBS, the sections were incubated with TUNEL reagents (Roche Applied Science, Inc., Indianapolis, IN) for 1 hr. The slides were then washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibody and covered with mounting medium. The sections were then examined under a Zeiss microscope equipped with UV epi-illumination (Carl Zeiss MicroImaging, Inc.; Thornwood, NY).

Animal Studies - Eight weeks old male CD1 mice and rats were purchased from Harlan Co. (Indianapolis, IN), and housed in institutional animal facilities. Before initiating the experiments the animals were acclimatized for one week in rooms with a 12 hr light/dark cycle while maintaining a temperature of 22°C with 50% humidity. The mice and rats had free access to food and water. The control animals received a normal mice/rat pelleted chow. The experimental animals received a high fat diet in the form of chow pellets and were sacrificed at 2, 4 and 6 weeks. Likewise, in another set of experiments, mice receiving control or HFD were concomitantly treated with Rapamycin (4 mg/kg, IP, daily). After 6 weeks serum insulin levels were measured using a commercial Rat/Mouse Insulin ELISA Kits (Millipore). Serum cholesterol was measured by using Infinity Cholesterol Reagent (Thermo Scientific). Blood glucose levels were measured by Accu-Chek meter (Roche). Prior to sacrificing the animals 24 hr urine collections were made for determination of protein excretion by SDS-PAGE. All animal studies were approved by the Animal Care and Use Committee of Northwestern University, USA and Tokushima University, Japan.

Morphology studies- Three µm-thick sections were prepared from paraffin embedded tissues and subjected to Periodic Acid Schiff (PAS) staining. For immunohistochemistry to detect the expression of various proteins Avidin-Biotin Complex (ABC) method was employed (20,22,24). The tissue sections were de-paraffinized, rehydrated with PBS and treated with 0.3% H2O2. Following which, the tissue sections were subjected to antigen retrieval procedure in the Histo VT-one by following the vendor’s instructions (Nacalai Tesque, Inc, Japan). After blocking with 5% normal goat serum in PBS, the sections were incubated with various primary antibodies. MIOX expression in the kidneys was gauged by immunofluorescence microscopy, as described previously (20,22,24). Briefly, four m-thick cryostat sections were prepared, air-dried for an hour and fixed with 4% para-formaldehyde for 10 min at 22°C. After equilibrating the sections with PBS, they were incubated with the polyclonal MIOX antibody at 37°C for 1 hr, washed with PBS and re-incubated with goat anti-rabbit IgG antibody conjugated with FITC (Sigma-Aldrich, St. Louis, MO) for another hr. Following a PBS wash, the tissue sections were covered with a drop of buffered glycerol, cover-slip mounted and examined with a Zeiss microscope equipped with UV epi-illumination (Carl Zeiss MicroImaging, Inc.; Thornwood, NY).

Other morphological studies included the assessment of apoptosis in kidney tissues or HK-2 cells by using TdT-mediated dUTP nick-end labeling (TUNEL) method (25). De-paraffinized sections or frozen cryostat sections of the kidneys were digested with Proteinase K (240 unit/ml, Promega, Madison, WI) for 30 min at 22°C. After a rinse with PBS, the sections were incubated with TUNEL reagents (Roche Applied Science, Inc., Indianapolis, IN) for 1 hr. The slides were then washed with PBS and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-digoxigenin antibody and covered with mounting medium. The sections were then examined under a Zeiss microscope equipped with UV epi-illumination (Carl Zeiss MicroImaging, Inc.; Thornwood, NY).
Indianapolis, IN). The cells were processed for evaluation of apoptosis as follows: The HK-2 cells were fixed with 4% fresh paraformaldehyde for 20 min at 22°C. They were permeabilized with 0.1% Triton X-100 at 4°C for 1 min and then incubated with TUNEL reagents at 37°C for 1 hr, as per instructions of the vendor. The nuclei were also stained with TO-PRO-3®-iodide. The cells undergoing apoptosis were identified as fluorescent nuclei with an UV microscope.

For senescence-associated beta-galactosidase (SA-β-gal) staining, 4-µm-thick cryostat kidney sections were prepared from freshly frozen kidneys (26). The sections were immersed in a 0.2% glutaraldehyde fixative solution, containing 0.1M phosphate buffer, pH7.2, 2 mM EGTA, 2 mM MgCl2 and 0.1% NP-40, for 20 min. Sections were washed with PBS and then immersed in SA-β-gal staining solution (0.1M phosphate buffer pH 7.2, 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 20 mM Tris-HCl, pH7.3, and 1 mg/ml X-gal). Sections were incubated in dark at 37°C for 16–18 hrs. They were then washed with PBS, and counterstained for 5 min with 0.1% nuclear fast red.

Preparation of fatty acid conjugates with bovine serum albumin (BSA) - A Preparation of palmitate-conjugated BSA was made by modifying a previously described method (27). A working solution of 5 mM palmitate-BSA was prepared by conjugating BSA with palmitate as follows: One gm of Ultra Fatty Acid Free BSA was mixed with 20 ml of 0.15 M NaCl in a small beaker. This beaker was placed in a larger 1 liter beaker with pre-warmed water and temperature maintained at 37°C while being stirred on a hot plate. The dissolved BSA was filtered through a 0.45 µm filter and the pH was adjusted to pH 7.4. The filtered BSA was constantly stirred at 37°C as indicated above. In another beaker 279 mg of the sodium palmitate was mixed with 10 ml of 0.15 M NaCl. The palmitate solution was placed in a 70°C water bath with frequent gentle shaking till completely dissolved. Then, 0.5 ml of the heated palmitate solution was added to the 9.5 ml of BSA solution while stirring at 37°C for 1 hr. The dissolved palmitate-conjugated BSA was re-filtered through a 0.45 µm filter and used.

**Cell Culture Studies** - HK-2 cells were grown in low glucose DMEM medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. LLC-PK1 cells were grown in low glucose M199 medium supplemented with 3% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin. The cells were maintained in a humidified atmosphere of 5% CO2 - 95% air at 37°C. Approximately 2.2 X 10⁶ cells were seeded on 55 cm² culture dishes and maintained to achieve 80% confluency. The cells were then treated with various concentrations of palmitate-BSA (10 µM - 300 µM) for 24 - 48 hrs. For the inhibition assay, cells were treated with palmitate-BSA in presence/absence of Rapamycin (10 mM), SREBP1 siRNA and MIOIX siRNA.

**Enzymatic Activity of myo-inositol (MIOX) in kidneys and LLCPK-1 Cells** - MIOX activity was determined, as previously described (24). Briefly, kidney cortices of mice receiving normal and high fat diet or cells undergone various treatments were homogenized in a sodium acetate or phosphatase buffer. The protein concentration of the homogenate was adjusted to 100 µg/ml, and 50 µl of homogenate used from each variable for determining the MIOX activity. The reaction was carried out at 30°C for 30 min in a volume of 500 µl containing 50 mM sodium acetate, 1 mM ferrous ammonium sulfate, 2 mM L-cysteine, and 60 mM myo-inositol. The reaction was terminated by boiling followed by precipitation with 3% TCA. Following centrifugation at 1,000 X g for 5 min, the D-glucuronate concentration was determined in the supernatant with the addition of double the volume of freshly prepared Orcinol reagent (40 mg of Orcinol and 9 mg of FeCl₃.6H₂O dissolved in 10 ml of concentrated HCl) (28). Spectrometric readings were made at A₆₆₀ nm. MIOX activity was averaged from four different experiments or animals per variable. For phosphatase treatment, the kidney homogenates or cellular lysates from various experimental variables were prepared and treated with Protein λ-phosphatase (λ-PPase; Biolabs, New England) for 1 hr at 30°C. The phosphatase reaction was carried out in a mixture containing 50 mM Tris-HCl, pH 7.8, 5 mM DTT, 2 mM MnCl₂, 100 µg/ml BSA, 100 units of λ-PPase, and 50 µl of homogenate protein solution in a total volume of 500 µl, following which the samples...
were processed for determination of MIOX activity. In another set of experiments effect of various kinase activators and inhibitors on the potentiation or dampening of the MIOX activity was carried out following 24 hrs of palmitate: BSA treatment. Various activators and inhibitors used were as follows: PKA activator Forskolin (0.2 µM) and inhibitor H89 (0.2 µM); phosphoinositide-dependent protein kinase-1 (PDK1)/PI3K inhibitor Wortmannin (0.5 µM) and activator insulin (100 nM); PKC activator 12-O-tetradecanoyl phorbol-13-acetate (TPA, 0.2 µM) and inhibitor Calphostin (0.05 µM).

**Immunoblot Analyses** - Cytoplasmic and Nuclear extracts were prepared from HK-2 or LLCCK-1 cells, as detailed previously (22). Protein concentrations in the extracts were measured by Bradford’s method and equal amounts of protein were loaded in each lane of the gels subjected to SDS-PAGE and blotted onto nitrocellulose membrane. Membranes were incubated with 1 mg/ml diluted each of the primary antibodies for 15 hr at 4°C, in Tris-buffered Saline + Tween 20 (TBS-T) supplemented with 5% nonfat dried milk. After a brief wash with TBST the membranes were then incubated with 1:4,000 diluted goat anti rabbit IgG (SantaCruz) or 1:10,000 diluted goat anti mouse IgG (Sigma) as secondary antibodies conjugated with horseradish peroxidase (HRP). Autoradiograms were prepared using Enhanced Chemiluminescence (ECL) detection system (Thermo Scientific). Equal loading of the samples was confirmed by probing the immunoblots with mouse β-actin or LaminB1 antibodies at a dilution of 1:10,000.

**Immunoprecipitation and Western blot analyses with phosphoaminoacid-specific antibodies** - To assess if the high activity of MIOX by HFD administration and palmitate-BSA treatment is due to phosphorylation of given specific amino acid residue by different kinases, the treated cells and kidney homogenates were lysed in a radio-immuno-precipitation (RIPA) assay buffer containing protease and phosphatase inhibitors, as previously described (22,24). Equal amounts (50 µg protein) of the cell lysates/kidney homogenates were used for immunoprecipitation with the following antibodies: anti-phosphoserine/threonine-specific PKA substrate antibody, anti-phosphoserine/threonine-specific PKC substrate antibody, anti-phosphothreonine antibody and anti-phosphotyrosine antibody (Cell Signaling Technology). The immunoprecipitates were subjected to 10% SDS-PAGE. The proteins were then transferred onto PVDF membranes and blots prepared. They were then probed with polyclonal anti-MIOX antibody.

**Isolation of MIOX Transcripts and Generation of Reporter Constructs** - A DNA fragment was isolated from the human genomic DNA (Promega) and used as a template for generation of PCR products spanning the MIOX promoter region by employing a common antisense primer 5’- GTACCCAGACCAGATTTG TGGCAGTGGAAGTA-3’ and the following sense primers: 5’- GGGGTGACC/GTCCCCACCTTCT CTGACCTATC-3’ (-1499 to +30), 5’- GGGGACC/TGGGGCAGAATCTAGGGC-3’ (-828 to +30), 5’- GGGGTGACC/CAAGTG AGGGC TGG-3’ (-261 to +30). The DNA fragments were cloned into pGL4.16 Promoter vector (Promega). A KpnI site, GTGACC (underlined), was included in the primers. The inserts were sequenced and their orientation confirmed by using vector-specific primer: 5’- CTA GCAAAATAGGCT GTCCC-3’. The upstream 5’ sequence of human MIOX and its homologues were retrieved from NCBI. Transcription-factor-binding motifs and promoter predication were searched at the following web sites: http://dbtss.hgc.jp/. For creating pGL4 mutation constructs, the pGL4 (-1499 to +30) construct vector was modified using QuikChange multi site-directed mutagenesis kit per manufacturer’s instructions. Mutagenic primer 5’-GATACAGCCTTGACCCCCAGG TGCTGACATTCTAGGGGGAGA-3’ (mutated SRE site is in underlined) was used to mutate the SRE site (GACCCCCAGGG GCTGACATT) in the pGL4 inclusive of MIOX promoter (-1499 to +30). The mutation in the modified construct was confirmed by nucleotide sequencing.

**Transfection of Cells and Luciferase Assays** - The reporter plasmid constructs (pGL4-1499 to +30, -828 to +30 and -261 to +30) were transfected into exponentially growing HK-2 cells (1 x 10⁵ cells) seeded onto 24 wells culture plates in Opti-MEM I medium and allowed to grow for 12 hrs. The transfection was carried out by using 1.5 µL of
FuGENE6 and 1 µg of reporter plasmid constructs. Luciferase activity was normalized to Renilla luciferase activity following co-transfection with pRL-CMV-Renilla (100 ng), which served as the optimized equalization control. Assays for both firefly and Renilla luciferase activity was carried out 24 hrs post transfection using a commercial Dual-Luciferase Kit and a TD 20/20 luminometer according to the instructions provided by the manufacturer. Basal promoter activity was determined in cells transfected with reporter construct pGL4.16 only. For comparison between fatty acid free BSA and palmitate-BSA, 24 hr post-transfection of the reporter plasmid-nonliposomal complex, the culture medium was replaced with Opti-MEM I containing 100 µM of BSA or palmitate-BSA.

**Electrophoretic Mobility Shift Assays (EMSA)** - Nuclear extracts were prepared from HK-2 cells, as described previously (22). EMSA were performed using a Thermo Fisher Lightshift Chemiluminescent EMSA kit per instructions provided by the manufacturer. Briefly, single stranded oligomers were custom synthesized by the Integrated DNA Technologies. Their sequences were as follows: -1048 to -1017: 5' GCACCCCACTGCCCTCTCTCCTGCCCCAGCG 3', which corresponded to nucleotide stretches spanning human sterol response elements (SRE). Both sense and complementary oligomers were labeled using a Biotin 3' End DNA Labeling kit and by following the manufacturer's instructions. For EMSA, the binding reactions were carried out using 10 µg of nuclear protein and 20 fmol of biotin end-labeled DNA in a 20 µl volume containing 2.5% glycerol, 5 mM MgCl2, 50 ng/µl of poly(dI·dC) and 0.05% Nonidet P-40. The reaction mixtures were incubated at 22°C for 30 min. The samples were then loaded onto 4% polyacrylamide non-denaturing minigel, and electrophoresis was carried out in a 0.5X running TBE buffer. DNA-protein complexes in the gels were then transferred onto a Hybond-N+ membrane by wet transfer in 0.5X TBE buffer and immobilized by UV cross-linking. For specificity, the blots were probed with SREBP1 antibody. The biotin-labeled reaction products were visualized by treating the blots with Streptavidin horseradish peroxidase conjugate followed by incubation with ECL Chemiluminescent reagents.

**Chromatin Immunoprecipitation (ChIP) Assays** - ChIP assays were also carried out to assess the binding of SREBP1 to the promoter region of human MIOX. The HK2 cells were cultured in 100 mm petri dishes and treated with 10 - 100 µM palmitate-BSA for 24 hrs. The ChIP assay was performed, as described previously (22). Cross-linking of DNA and protein was carried out by addition of 1% formaldehyde into the cell medium and incubated for 15 min. The formaldehyde was quenched by the addition of 0.125 M glycine for 5 min. The cells were then washed with cold PBS, scraped from the dishes and centrifuged at 500 x g for 5 min. The pellet was resuspended in 300 µl of swelling buffer (25 mM HEPES pH 7.8, 1.5 mM MgCl2, 10 mM KCl, 0.1% NP40, 1 mM DTT, 0.5 mM PMSF) and incubated on ice for 10 min. The DNA fragmentation for preparation of oligonucleotides was achieved with the treatment of Micrococcal Nuclease (MNase) for 20 min at 37°C. The reaction was terminated by adding 10 µl of 0.5 M EDTA (pH 8.0). This was followed by a brief centrifugation at 1,000 x g for 3 min. The pellet was resuspended in a 250 µl µl of sonication buffer (50 mM HEPES, pH 7.9, 140 mM NaCl, 1% TritonX100, 0.1% sodium deoxycholate, 0.1% SDS and 0.5 mM PMSF) and incubated on ice for 10 min and then sonicated to generate 200 -1,000 bp DNA fragments. The sonicated suspension was centrifuged at 15,000 x g for 15 min to remove the insoluble debris. The soluble chromatin was diluted to 1:10 with ChIP dilution buffer (16.7 mM Tris-HCl pH 8.1, 150 mM NaCl, 1 mM EDTA, 1% TritonX100 and 0.01% SDS). It was pre-cleaned by incubation with Protein G-Sepharose for 2 hr at 4°C. The samples were then re-centrifuged, and an aliquot (800 µl) was saved as input DNA. The remainder was processed for immunoprecipitation with SREBP1 antibody followed by incubation with Protein G-Sepharose beads. Samples with normal IgG were also used as a negative control. The Sepharose beads were centrifuged and washed twice, first with 1 ml of low salt washing solution (20 mM Tris-HCl, pH 8.1, 150 mM NaCl, 2 mM EDTA, 1% TritonX100 and 0.1% SDS) and then with 1 ml of high salt washing solution (20 mM Tris-HCl, pH8.1, 500 mM NaCl, 2 mM EDTA, 1% TritonX100 and 0.1% SDS). The beads were then washed with a LiCl washing solution (10mM Tris-
HCl, pH 8.1, 1mM EDTA, 1% deoxycholic acid, 1% NP40 and 0.25M LiCl) and then twice with 1 ml of Tris-EDTA buffer. DNA fragments from the samples were eluted by adding 400 µl of elution buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaHCO3, 1 mM EDTA and 1% SDS) and then incubated at 65°C for 10 min. After addition of 16 µl of 5 M NaCl, the samples were further incubated at 55°C for 4 hrs for de-crosslinking. Similarly, samples of input DNA were de-crosslinked. One µl of DNase-free RNase A (10 mg/ml) was added and incubated at 37°C for 30 min. This was followed by addition of 10 µl of Proteinase K (2 mg/ml), and then the samples were incubated at 55°C for 1 hr. After a phenol/chloroform extraction and addition of 1 µl of glycogen (20 mg/ml), the DNA was precipitated with 40 µl of 3 M sodium acetate and 1 ml of ethanol. The precipitates of ChIP and input samples were resuspended in 20 µl of 10 mM Tris-EDTA buffer and used for PCR analyses. The primers used for ChIP-PCR included regions spanning SRE motifs included in the MIOX promoter from -1499 to -1229 bp. The primer sequences were 5’-GTCCCACCTCCTGAACCTATCCAG-3’ (sense) and 5’-TGACTTCCTGTGGCACTCAGCA-3’ (antisense), with an expected size of an amplified product of 271 bp.

**Generation of Eukaryotic Expression Constructs and Stable Transfectents -** A SREBP1 cDNA was generated by RT-PCR with SREBP1 specific primers, and it was cloned into pcDNA3.1/V5-His TOPO Vector (Invitrogen) and amplified in an E. coli system, as described previously (29). For cloning of mouse cDNA, SREBP1 was amplified using the mouse kidney cDNA and the respective sense and antisense primers: 5’-GCCACCATGGACGAGCTGGCTTCG-3’ with Kozak sequence and 5’TGACTTCTCTGGTGATCC-3’. The plasmid constructs were then transfected into LLCPK1 cells, and stable transfectents were selected by growing cells in the presence of G418.

**RNA extraction and quantitative real-time PCR-** Total RNA was isolated from the kidney using TRIzol reagent (Invitrogen) and cDNA was synthesized using Go Script reverse transcription system (Promega). The mRNA level was quantified using Step One Plus System Real Time PCR (Applied Biosystems). A 20 µl total reaction mix included 100 ng of cDNA, 50 nmol/L forward/reverse primers and 1X Fast SYBR Green Master Mix (Life technologies). 18S rRNA was used as an internal control and the amount of mRNA was calculated by the comparative C(T method. All the data was derived from quadruplicate PCR reactions. The levels of MIOX, SREBP1 mRNA and 18SrRNA were determined. The primers used were: MIOX: forward, 5’TGTCTTACCATCTACAAGCTCT3’ and reverse, 5’-GGCTCTCACGACTGCTATTTT-3’; and SREBP1 forward, 5’-AGTGACTTCTCTGGGCCTATTG-3’ and reverse, 5’-TCAAGAGAGGAGCTCATGTGG-3’; and 18S rRNA forward, 5’-CGAGCCGCCTGGATACCC3’ and reverse, 5’-CAGTTCCGAAAACCAACAAATAGA-3’.

**Determination of intracellular reactive oxygen species (ROS) -** Status of cellular ROS was evaluated both by fluorescent microscopy and flow cytometry, using 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCF-DA) dye. The dye following its diffusion into the cell gets deactetylated by cellular esterases and then oxidized by intracellular ROS into a highly fluorescent compound DCF, which can be detected by fluorescent microscopy or flow cytometry with excitation at 488 and emission at 520 nm. About 5×10⁵ HK-2 cells were seeded (on cover-slips for microscopy) in a 6 well plate and treated with various concentrations of palmitate-BSA (30-300 µM) for 24 hrs. In another set of experiments the palmitate-BSA-treated cells were also subjected to SREBP1 or MIOX siRNA treatment for their respective gene disruption. Cells were then washed twice with phosphate buffered saline (PBS), each wash of 5 min. 5 mM stock of CM-H₂DCFDA in DMSO was diluted in PBS to make a final concentration of 5 µM. Cells were then incubated with 5 µM of CM-H₂DCFDA probe for 15 min at 37°C in dark. They were re-washed three times with PBS, each wash of 5 min each, and processed for microscopic examination. For flow cytometry based detection of ROS, cells were gently scraped off from the plates with a rubber policeman and transferred into fluorescence-activated cell sorting tubes for staining. Cells were stained with 5 µM of CM-H₂DCFDA for 15 min at 37°C in dark. They were then washed twice with PBS and re-suspended in 300 µL of PBS and processed for acquisition of fluorescence with a
flow cytometer. Following which, mean fluorescent intensity of the CM-H$_2$DCFDA was measured using standard operational procedures of flow cytometry and employing FACSDiva software (Becton Dickinson).

**MitoSox staining of cells** - Mitochondrial superoxide (O$_{2}^{-}$) generation was detected by using a specific mitochondrial superoxide indicator, MitoSOX red (Molecular Probes). HK-2 cells were seeded in a 6 well plates and treated with various concentrations of palmitate-BSA (30-300 µM) for 24 hrs, as described above. After transferring the cells into the FACS tubes they were incubated with 5 µM MitoSox for 15 min at 37°C in dark. Following two PBS washes the cells were subjected to flow cytometric measurements, and mean fluorescence intensity determined.

**Determination of ROS in kidney tissues** - For detection of ROS in kidney tissues transverse renal slices were prepared from mice fed with control and high fat diet. They were embedded in OCT compound in a plastic mold and immediately frozen in liquid nitrogen. The embedded tissues were mounted on metallic stubs. About 8 µM-thick frozen sections of the kidney were prepared using Leica cryostat and transferred onto glass slides. The tissue sections were then air dried for 5 min at 22°C. To remove OCT compound from tissues the glass slides were rinsed twice with PBS. The sections were then incubated with 5 µM of CM-H$_2$DCFDA or 10 µM of dihydroethidium (DHE) for 15 min at 37°C. They were washed with PBS twice for 5 min each. A drop of mounting media was placed onto the tissue sections. They were then covered with glass cover-slips and examined with a fluorescence microscope equipped with UV epi-illumination.

**Statistical Analyses** - Results were expressed as mean ± SD following statistical analyses. Student’s $t$ test was used to compare the data between two groups. A $p$ value of $<0.05$ was considered to be statistically significant.

**RESULTS**

The findings described below in this section include the induction of MIOX expression and activity in a hyperlipidemic state via phosphorylation of its potential PKC-, PKA-, and PDK1-phosphorylating sites, and how high fat diet (HFD) administration or exposure of fatty acids to renal tubular cells *in vitro* affects its translational and transcriptional activities. The latter seemingly to be largely influenced by unique DNA E-Box overlapping sites for the binding of transcription factor Sterol Regulatory Element-Binding Protein (SREBP) localized in its promoter. Intriguingly, fatty acid induced MIOX upregulation also led to a notable oxidant stress in renal tubular cells.

**Effect of high fat diet (HFD) and albumin-bound fatty acids on MIOX expression and activity** - Administration of HFD to CD1 mice over a period of 2 - 6 weeks induced a tremendous increase in the expression of MIOX, as assessed by immunofluorescence microscopy (Figures 1B versus 1A). The increased expression, highlighted by green fluorescence, was exclusively confined to the tubular compartment of kidney cortex, while no expression was seen in the glomeruli (arrowheads). Also, besides the increased expression in superficial cortical tubules it also extended into the deeper cortex. The expression was time-dependent since it steadily increased over a period of 6 weeks, as assessed by immunoblot analyses, while β-actin expression was unchanged (Figure 1C). Likewise, MIOX activity was increased notably up to 4 weeks, although a mild further increase was also seen at 6 weeks (Figure 1D). This increase in the activity was reduced to normal levels with treatment of protein $\lambda$-phosphatase ($\lambda$-PPase), suggesting that the increase in the activity was phosphorylation-dependent. These *in vivo* observations were confirmed by *in vitro* experiments where LLCPK-1 cells were exposed to various concentrations of palmitate conjugated bovine serum albumin (BSA). A dose-dependent increase in MIOX expression and activity was observed at a concentration range of 10 - 300 µM of palmitate:BSA, while no change was observed with BSA treatment alone (Figures 1E & 1F). Similar to *in vivo* results the $\lambda$-PPase treatment reduced MIOX activity to basal levels in LLCPK-1 cells. During these *in vitro* experiments a certain degree of apoptosis was observed in these cells, and thus in light of these observations apoptogenic Bax expression was assessed to rule out any cytotoxic effect. Concomitant with MIOX upregulation a dose-dependent increased Bax protein expression was observed following
Effect of high fat diet (HFD) and albumin-bound Fatty Acids on the phosphorylation of MIOX and its activity - With respect to phosphorylation of MIOX the next question addressed was as to which amino acid residues are affected by HFD or palmitate:BSA treatment since one of our previous publications suggests that MIOX is differentially phosphorylated in a diabetic state or under high glucose ambience. This was accomplished by using antibodies directed against various phosphorylated amino acid residues. Immuno-precipitation was performed on kidney homogenates or cellular lysates with substrate-specific phospho antibodies to PKA (Ser/Thr) and PKC (Ser) and to phosphothreonine and phosphotyrosine residues. This was followed by Western blot analyses with anti-MIOX antibody using equal concentrations of proteins in immunoprecipitated samples. A time-dependent (2-6 weeks) increased phosphorylation was observed following HFD administration in kidney samples where immunoprecipitation was carried out with phosphoantibodies directed against serine and threonine residues (Figures 2A). No increase was observed in samples immunoprecipitated with anti-phosphotyrosine antibody. Similarly, a dose-dependent increase in phosphorylation at serine and threonine residues was observed in cells treated with various concentrations of palmitate:BSA (Figure 2B). No increase in phosphorylation of tyrosine residues was observed, suggesting that these residues in MIOX are unaffected by phosphorylation following HFD administration or palmitate:BSA treatment. The latter treatment also led to an increase in MIOX activity in LLCPK-1 cells. Concomitant treatment with palmitate:BSA and activators of PKA (forskolin), PDK/PI3K (insulin) and PKC (TPA) further increased MIOX activity (Figure 2C). While concomitant treatment with respective inhibitors, i.e., H89 (PKA), wortmannin (PI3K) and calphostin (PKC) reduced the activity below the levels induced by palmitate:BSA alone, confirming that fatty acid induced activity is phosphorylation dependent (Figure 2C). Since fatty acids seem to modulate expression of MIOX we proceeded to investigate the status of proteins that regulate fatty acid and cholesterol biosynthesis.

Effect of hyperlipidemia, palmitate:BSA and insulin on MIOX and SREBP1 expression - Treatment of HK-2 cells with palmitate:BSA (100 μM) for 24 hrs induced an increased MIOX expression with a concomitant decrease in membrane-bound precursor form of pre-SREBP1 (pSREBP1, 120 kDa) in the cytoplasmic fraction. Simultaneously, with its recruitment and cleavage the mature form (mSREBP1, 68 kDa) translocated into the nucleus, as assessed by immunoblot analyses (Figure 3A). No change in the expression of β-actin or laminB1 was observed. The dissociation of mSREBP from Golgi sacules following cleavage with increase and decrease in the nuclear and cytoplasmic expression, respectively, was quantified and included as bar graphs (Figures 3B & 3C). SREBP1 is believed to serve as a link in changes in insulin levels and expression of genes that modulate systemic energy metabolism, e.g., fatty acid synthase and leptin (30). Thus, it is activated during nutritional abundance and adiposity and is associated with excessive secretion of insulin in early phases of obesity, a vital component of metabolic syndrome with increased insulin and glucose levels and certain degree of insulin resistance (30-32). Since MIOX is transcriptionally up-regulated by high glucose ambience we evaluated if insulin causes concomitant increase in the expression of MIOX and mSREBP1. A dose-dependent increase in the expression of MIOX was observed following insulin (10 - 1,000 nM) treatment (Figures 3D & 3F). At the same time a dose-dependent increase in the mSREBP1 was observed (Figures 3E & 3G), suggesting a temporal relationship between the upregulation of MIOX and translocation of mSREBP1. The in vivo results in mice or rats were similar to those seen in cultured cells exposed to palmitate:BSA. Like MIOX the HFD administration over a period of six weeks resulted in a marked time-dependent upregulation of mSREBP1 (Figure 3H). No change in the laminB1 expression was observed. These observations were further substantiated by immuno-histochemical (IHC) studies performed on kidney sections of mice fed with control and HFD diets, using anti-SREBP1 antibody (Life Span Biosciences).
contrast to other SantaCruz SREBP-1 antibodies this antibody was found to be suitable for IHC. In control animals, mild staining of both the cytoplasm as well as of nucleus of the kidney tubules, indicative of basal expression of SREBP, was observed (Figure 3I). Minimal staining of the glomerular cells was observed, which authenticated specificity of the antibody. Following HFD administration, a remarkable increase in the expression in renal proximal tubules was observed (Figure 3J). Intriguingly, increased expression was seen in both cytoplasmic and nuclear (Figures 3J versus 3I, arrowheads) compartments of the tubular cells, while there was very mild increase in glomerular cells. This suggested a specific SREBP1 response following HFD that is selectively confined to tubular cells. In aggregate, these observations suggested responsiveness of MIOX and SREBP expression to insulin as well as fatty acids and this led us to examine the promoter characteristics of MIOX.

**Effect of palmitate:BSA on MIOX promoter activity and binding of SREBP to the sterol responsive elements (SRE) of its promoter** - To explore mechanisms that up-regulate expression of MIOX by palmitate-BSA, human MIOX gene upstream of open reading frame (ORF) was cloned by genomic PCR amplification, and it was used for promoter activity analyses. First, three constructs were generated spanning MIOX gene -1500 bp upstream of ORF. The construct spanning entire region had maximum MIOX basal promoter activity, as schematically shown (Figure 4A). This construct was enriched with E-Box sites while at the same time was inclusive of multiple Sterol Response Elements (SRE) sites. The SRE and E-Box motifs (CANNTG) congregated at -1047 to -1499 in MIOX promoter region. A total of seven SRE motifs were localized to -1499 (GTCCCA), -1328 (AGGGGC), -1271 (GGTGGTGGA), -1196 (TTAAAT), -1137 (CTTGTT), -1090 (AAGCCCA) and -1047 (CACCCCA) bp regions. In addition, a mutant construct was generated by modifying the SRE sites by site directed mutagenesis. All these four constructs were transfected into HK-2 cells and also co-transfected with the luciferase constructs to measure the luciferase activity. The basal activity was designated as one, and following palmitate:BSA treatment a variable increase in luciferase activity was observed in all the constructs (Figure 4B). The highest increase in activity (~2-folds) was observed in transfected construct inclusive of E-Box and SRE sites. A marginal increase in activity in HK-2 cells transfected with mutant construct was observed, which indicated that responsiveness of MIOX promoter to fatty acids is mediated via the SRE sites. This suggested that SREBP, a basic helix-loop-helix-leucine zipper transcription factor that is known to regulate cholesterol biosynthesis and fatty acid metabolism (33-35), can potentially bind to promoter region of MIOX and modulates its transcription following palmitate:BSA treatment.

To investigate the existence of SRE sites and binding of SREBP1 with the MIOX promoter electrophoretic mobility shift assays (EMSA) were performed, using nuclear extracts of HK-2 cells in a non-denaturing minigel system. The reaction mixtures included nuclear extracts, unlabeled cold or biotin-labeled double stranded oligonucleotide probes (-1048 to -1017 bp) or anti-SREBP-1 antibody. A shifted band was seen in the biotin labeled oligonucleotides probe (Figure 4C, lower arrow, middle lane). The unlabeled cold oligonucleotides probe effectively competed with the labeled probe in nuclear protein reaction mixture since the shifted band disappeared (Figure 4C, left lane). A non-specific low molecular band and free labeled oligos were seen in this lane. With the inclusion of anti-SREBP1 antibody in reaction mixture a super-shifted band to higher molecular weight was seen, which suggested the formation of SRE:oligonucleotides:nuclear protein complex and existence of an SREBP1 binding SRE site in the promoter region of the MIOX gene (Figure 4C, upper arrow, right lane). To investigate if binding of SREBP1 with MIOX promoter was dose-dependent ChIP assays were carried out on HK-2 cells treated with BSA or palmitate-BSA (10-100 μM) for 24 hrs. ChIP PCR products were amplified from input-positive control (Input), a negative control normal rabbit IgG (Neg), and an antibody specific for SREBP1 (ChIP). ChIP assays was followed by PCR analyses of SRE region (-1499 to -1229) with the expected PCR product size of 271 bp. A dose-dependent increase in binding of SREBP1 to SRE sites was observed following palmitate:BSA treatment (Figure 4D). No band was seen in negative control samples where normal rabbit IgG was substituted for SREBP1 antibody.
Finally, in order to ensure the functionality of SREBP1 in transfected cells that this transcription factor can modulate MIOX expression a 1,500 bp SREBP1 cDNA was cloned into the pcDNA3.1/V5-His-TOPO. This vector was transfected into cells by utilizing FuGENE 6 and stable transfectents were generated. Expression of transfected SREBP1, His protein and MIOX was confirmed by Western blot analysis using antibodies directed against -His tag, -SREBP1 and -MIOX. His tagged antibody could confirm the presence of His fusion protein in cells transfected with pcDNA3.1/V5-His TOPO SREBP1, while no band was seen in cells transfected with empty vector (Figure 4E). The pcDNA transfected cells also yielded an increased expression of both SREBP1 and MIOX, suggesting SREBP1 is functional in transfected cells and can modulate the MIOX expression. This validated the findings of promoter analyses studies, which indicated that fatty acids by upregulating SREBP1 enables it to bind to MIOX promoter and modulates MIOX transcription (Figure 4E).

Effect of HFD diet on SREBP1, tubular cytoplasmic & nuclear alterations, senescence, apoptosis and urinary protein excretion in mice and rats - Since studies detailing the effect of HFD on kidney tubules in the literature are limited we therefore examined cytoplasmic and nuclear changes in the context of SREBP-1 and MIOX pathobiology. We examined both rat and mouse kidneys to assess the morphologic and biochemical differences and similarities between two species, utilizing various IHC markers. Overall, major changes were seen in the metabolically active tubular compartment of the kidney in both the species. In mice, notable morphological changes were observed in the tubular compartment in kidney sections stained with PAS. There was a partial loss of the pink staining luminal brush border (Figures 5B versus 5A, arrows) with extrusion and falling of nuclei from tubular cells (arrowheads). Bcl2 expression was decreased while conversely that of apoptogenic Bax was remarkably increased in tubular cells (Figures 5C-5F). Damage to tubular nuclear DNA was assessed by IHC staining with anti-ph2AX antibody which localizes phosphorylated form of histone variant H2AX at Ser139 residue. Many nuclei with prominent ph2AX staining were observed in kidney tubules of mice fed with HFD (Figures 5H versus 5G, arrowheads). Tubular damage was reflected by pathophysiologic changes as well. There was an increased urinary excretion of low molecular proteins ranging in 30 - 45 kDa (Figures 5Q, arrows). Proteinuria was time-dependent up to 6 weeks, and a mild increase in albumin excretion was also observed, suggesting that glomerular damage is less at this time interval. Likewise, changes were seen in kidney tubules of rats fed with HFD. A tremendous increase in the expression of MIOX in kidney tubules was observed, and it had identical spatio-temporal distribution in the tubular compartment as seen in mice following 2-6 weeks of HFD administration (Figures 1A & 1B). SREBP1 expression was increased in both the cytosolic and nuclear compartment, as reflected by increased antibody staining in cytoplasm and nuclei (Figures 5J versus 5I). This suggested that HFD-induced induction of MIOX is mediated via SREBP1 transcription factor in rats as well. Associated with it was increased expression of apoptogenic Bax, and it had both cytoplasmic and perinuclear distribution (Figures 5L versus 5K). Increased expression of Bax following HFD administration was time dependent up to 6 weeks, as assessed by Immunoblot analyses (Figure 5R). HFD-induced nuclear damage in rats was reflected by increased degree of apoptosis as highlighted by TUNEL staining (Figures 5N versus 5M). In view of the HFD-induced cellular and nuclear changes the replicative capacity of tubular cells was investigated by using senescence associated β-galactosidase (SA- β-Gal) activity marker. Strikingly, a marked increase in SA-β-Gal staining was observed (Figures 5P versus 5O, arrowheads), suggesting that HFD administration has affected the overall homeostasis of renal tubules concomitant with increased expression of MIOX and SREBP1.

Effect of Rapamycin on HFD-induced MIOX expression and associated changes in the renal tubules - Since mammalian target of rapamycin (mTOR) is activated in states of obesity studies were carried out in vivo to assess the effect of rapamycin on HFD-induced expression of MIOX, SREBP1 and changes in biomarkers associated with cytoplasmic or nuclear damage and related functional parameters. Six weeks of HFD
administration resulted in a significant increase in body weight, blood glucose and serum insulin levels (Figures 6A, 6C & 6D). In overweight animals obesity was mainly due to increase in abdominal fat, and these changes were normalized to basal levels with concomitant administration of rapamycin. Along with increase in abdominal fat there was ~40% increase in serum cholesterol levels in mice fed with HFD, i.e., 134±15.56 vs 188±8.94 mg/dl, which was reversed with rapamycin treatment. Also, pathologic changes, i.e., loss of proximal tubular brush border and extrusion of nuclei (arrow heads) were restored to normal by administration of rapamycin (Figures 6E-6G).

Interestingly, HFD-induced expression of MIOX (mRNA and protein), Bax and of pH2AX were restored to basal expression following rapamycin treatment as well (Figures 6B, 6H-6P), suggesting a relationship between obesity, MIOX expression, apoptogenic Bax, nuclear DNA damage, reflected by pH2AX staining, and blood glucose, cholesterol and insulin levels, parameters that are conceivably deranged in metabolic syndrome (MetS).

**Effect of Rapamycin on Palmitate:BSA-induced MIOX, SREBP1 and p53 expression and apoptosis in renal tubular cells** - To establish mechanistic causal relationship between fatty acid injury leading to upregulated expression of MIOX via transcription factor SREBP1 in vitro experiments were performed using HK-2 cells. A basal expression of MIOX and SREBP1 was observed in HK-2 cells treated with BSA (Figures 7A & 7D). With the treatment of palm:BSA (100 μM) there was a marked simultaneous increase in expression of MIOX and SREBP1 (Figures 7B & 7E), and it was dramatically reduced with rapamycin treatment (Figures 7C & 7F). At high magnification the expression of SREBP1 was equally localized to both the cytoplasm and nucleus under basal conditions, and it was notably increased in the nuclear compartment following palm:BSA treatment (Figures 7G & 7H, arrowhead). Nuclear expression was remarkably reduced following rapamycin, suggesting that mTOR pathway is involved in upregulation of MIOX and SREBP1 (Figure 7I). Along with increase in expression of MIOX and SREBP1 there was a concomitant increase of apoptosis following palm:BSA treatment, which was notably reduced by rapamycin, as assessed by TUNEL method.

TO-PRO-3 dye was used as nuclear stain, it yields a red color whereas cells undergoing apoptosis acquire yellow color (Figures 7J - 7L). Apoptosis observed was most likely triggered by p53 since there was increased expression p53, which could be inhibited by rapamycin treatment, as indicated by the immunoblots (Figure 7M). Interestingly, this increase and decrease of p53 was congruent with expression of MIOX following various treatments. To elucidate a direct causal relationship between MIOX and SREBP1, siRNA experiments were performed. With SREBP1-siRNA treatment MIOX expression was dramatically reduced in palm:BSA-treated cells, while scramble oligo had no effect (Figure 7N). Specificity of SREBP1-siRNA was confirmed by measuring mRNA expression. SREBP1-siRNA caused a remarkable reduction in expression of SREBP1 in palm:BSA-treated cells, while scramble oligo had minimal effect (Figure 7O). Next, we proceeded to address the question that whether SREBP-1 induced MIOX expression leads to changes in cellular redox.

**Effect of palmitate:BSA or HFD-induced perturbations in cellular redox in renal tubular cells** - Cells were treated with different concentrations of Palmitate:BSA for 24 hrs, and cellular ROS generation was assessed by 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H2DCF-DA) staining followed by flow cytometric analysis. A dose-dependent increase in mean fluorescence intensity (MFI) related to DCFDA staining was observed within the concentration range of 30 - 300 μM palmitate:BSA (Figure 8A). Likewise a dose-dependent increase in mitochondrial ROS was observed following treatment with palmitate:BSA, as assessed by MitoSox staining and flow cytometry (Figure 8B). These in vitro observations were confirmed by in vivo experiments. To assess the effect of HFD on cellular redox, kidney sections from mice fed with control and HFD were processed for CM-H2DCF-DA and dihydroethidium (DHE) staining. A notable increase in both CM-H2DCFDA and DHE staining was observed in kidney sections of mice fed with HFD, suggesting increased intracellular levels of ROS (Figures 8D & 8F vs 8C & 8E). DCF staining was mainly confined to the cytoplasm of the tubules (Figure 8D). Glomerular cells did not yield any distinct notable increase in DCF staining.
DHE staining was also confined to tubular compartment, and it was seen exclusively localized to nuclei (Figure 8F). No notable DHE staining was seen in kidney sections of mice fed normal diet (Figure 8E).

Effect of SREBP1 & MIOX siRNA on palmitate:BSA-induced perturbations in cellular redox of renal tubular cells - Keeping in view that HFD administration to mice or palmitate:BSA treatment to HK-2 cells leads to increased MIOX expression, we addressed the question whether increased generation of ROS is specific to MIOX and is mediated via SREBP1 transcription factor. HK2 cells treated with palmitate:BSA were subjected to siRNA gene disruption, and then they were stained with CM-H2DCFDA. Following which, ROS levels were monitored by flow cytometry and fluorescent microscopy. A significant increase in DCF staining and MFI following palmitate:BSA treatment was observed (Figure 9B vs 9A). However, following SREBP1 siRNA or MIOX siRNA treatment the palmitate:BSA-induced increase in MFI was notably reduced (Figures 9C-9E). The reduction in MFI was comparable following the MIOX siRNA or SREBP1 siRNA treatment. Fluorescent microscopy of HK-2 cells subjected to various treatments confirmed the findings of flow cytometric analyses. By fluorescent microscopy a notable increase in intensity of staining was observed following palmitate:BSA treatment (Figure 9G vs 9F). Both the SREBP1 siRNA and MIOX siRNA treatments remarkably reduced intensity of fluorescence, suggesting that palmitate:BSA-induced ROS perturbations are specific to MIOX and are mediated via SREBP1 (Figures 9H & 9I).

DISCUSSION

The results of this investigation highlight that in states of obesity there is an upregulation of a kidney specific metabolic enzyme, i.e., myo-inositol oxygenase (MIOX) with activation of glucuronate-xylulose (G-X) pathway in which myo-inositol via series of reactions gets converted into xylulose and ribulose along with the generation of reactive oxygen species (ROS). The latter would likely adversely affect the pathobiology of the tubulo-interstitial compartment of the kidney. In such a setting, the tubulo-interstitial injury besides the glomerular injury would be reflected in compromised renal functions. In this regard, currently obesity has emerged as a significant risk factor, independent of diabetes and hypertension, in the progression of chronic kidney disease (CKD) on a long term basis (36). So far most of the mechanistic studies concerning development of CKD in states of obesity have focused on the glomerular compartment, especially on podocyte, whereas the proximal tubular cell compartment has not received much attention, although it is equally affected by various adverse metabolic assaults (6,36,37).

Previously, we reported the status of MIOX in the context of type 1 and 2 models of diabetes while focusing on the biology of the proximal tubular compartment (20,22,24). Since this enzyme is conserved across various specie lines, we utilized different proximal tubular cell lines and mice and rats to investigate its pathobiology in states of obesity and exposure of cells to albumin-bound fatty acids. The latter strategy would mimic the in vivo renal disease states, such as, nephrosis, where the tubular cells are subjected to assault by the filtered albumin-bound fatty acids. Like in type 1 or 2 diabetes the mice receiving high fat diet (HFD) had a marked time-dependent (1-6 weeks) increased expression of MIOX and activity in the proximal tubules while the glomeruli were unaffected (figure 1). One may regard the time-dependent increase in expression dose-dependent as well since cholesterol levels gradually increased with time reaching significantly high at six weeks. In fact, in vitro exposure of palmitate:BSA induced an increased expression and activity of MIOX in LLC-PK1 cells in a dose-dependent manner. The question of toxicity of palmitate:BSA leading to cellular disintegration was ruled out since Bax expression was concomitantly increased up to a concentration range of 300 µM. MIOX activity was reduced to basal levels with the treatment of lambda protein phosphatase (λ-PPase), suggesting that phosphorylation may be essential for the activity of this enzyme. Phosphorylation can induce a conformational change in the protein in order to associate or dissociate with other proteins or increase or decrease substrate affinity and thereby reduce or accentuate the activity of a given enzyme (38,39). Kinase induced phosphorylation or phosphatase-mediated dephosphorylation
processes target serine, threonine or tyrosine residues. All these potential sites are included in the MIOX protein and are amenable to phosphorylation in different biologic processes thereby increasing its expression and activity (22). Like in the diabetic state or the cells under high glucose ambience there was an increased expression and activity at the serine or threonine residues following HFD administration or treatment of LLC-PK1 cells with palmitite:BSA (figure 2). The role of phosphorylation in regulation of MIOX activity by fatty acids was further substantiated by the fact that various activators or inhibitors of PKC, PKA and PDK1 were able to boost or inhibit palmitate:BSA-induced MIOX activity. Interestingly, since insulin also could remarkably induce MIOX activity this would suggest its significance and a link in various pathobiologic processes like diabetes and obesity, the latter relating to perturbation in the fatty acid metabolism.

A number of genes that are involved in the synthesis of fatty acids, cholesterol, triglycerides and phospholipids, are regulated by transcription factors known as sterol regulatory element-binding proteins (SREBPs) (32-35). They belong to the basic helix-loop-helix-leucine zipper (bHLH-Zip) family of transcription factors, and they are bound to endoplasmic reticulum in an inactive precursor form, i.e., pSREBP. Upon activation by various stimuli, e.g., hyperinsulinemia, SREBP is escorted by SREBP cleavage-activating protein (SCAP) into the Golgi apparatus where it undergoes successive cleavage by Site-1 protease (S1P) and Site-2 protease (S2P). The mature form (mSREBP) translocates into the nucleus and binds to sterol response elements (SRE) to initiate transcription of various genes (32-35). Given the above considerations we investigated if HFD-induced upregulation of MIOX was mediated by insulin via utilizing SREBP transcription factors. In line with this notion we observed that palmitite:BSA increased the expression of mSREBP1 concomitant with the upregulation of MIOX and decreased expression of pSREBP1 (figure 3). Similarly, HFD diet increased the expression of mSREBP1 in proximal tubular compartment (figure 3). However, no significant increase in SREBP1 expression or glomerulosclerosis, was observed following HFD administration. Conceivably, these differences may be related to the duration of HFD administration, i.e., 6 weeks versus 12 weeks or use of different strains of mice, i.e., CD1 versus C57BL/6J (40). Insulin is also known to increase the expression of SREBP1 in adipocytes (30), and likewise a dose-dependent increase in HK-2 cells with a concomitant upregulation of MIOX was also observed (figure 3).

Collectively, the above observations suggest that the biology of SREBP1 and MIOX is interlinked in states of high insulin or fatty acid ambience. This led us to investigate the transcriptional regulation of MIOX. The MIOX promoter included multiple Sterol Response Elements (SREs) and E-Box motifs localized to -1047 to -1099 bp (figure 4). This suggested that MIOX is a potential target candidate of SREBP-1 that has dual DNA-binding specificity (30,41). Although SREBPs have many potential targets, but two well-characterized genes to which adipocyte determination- and differentiation-dependent factor 1 (ADD1)/SREBP1, with dual DNA-binding specificity, can bind include fatty acid synthase (FAS) and leptin, both involved in fatty acid metabolism (30,42). It is known that many transcription factors bind to the E-box, including TFE3 and USF1, the latter is involved in the synthesis of fatty acids (43,44). The USF1 and SREBP1 cooperate with one another to regulate the transcription of FAS. Thus, conceivably, USF1 in cooperation with SREBP1 could regulate the transcription of MIOX following HFD administration. Among several MIOX promoter constructs, a maximal activity was confined to the construct containing the entire promoter segment inclusive of SREs and E-box motifs. A marked increase in the activity was noted following palmitate-BSA treatment, and it was reduced to basal levels in cells transfected with a mutant construct (figure 4). EMSA and Chip Assay further provided the evidence that MIOX transcription is modulated by SREBP1 in states of high fatty acids ambience.

Next, we addressed the question as to which of the genes co-localize with MIOX that would be reflective of proximal tubular injury and are induced following HFD administration. Expression studies were carried out both in rats and mice since different species/strains display differential susceptibility to HFD-induced tubular injury (40). In either species there was loss of the proximal...
tubular brush border with increased low-molecular weight proteinuria (figure 5). Such low molecular weight proteinuria besides albuminuria has also been reported in states of obesity in children (45). A mild increase in albumin excretion was also observed which may be due to the shorter duration of HFD administration, i.e., 6 weeks vs 12 weeks (40). HFD administration also induced cellular and nuclear damage, which was readily discernible by TUNEL assay and by delineating the changes in the expression of various genes and markers, i.e., Bcl2/Bax and -pH2AX and SA-β-Gal (figure 5). Overall, HFD markedly perturbed cellular homeostasis with increased expression of SREBP1 and concomitant up-regulation of MIOX. Upstream modulators of SREBP1 are believed to be a group of kinases, including PI3Kinase, Akt/protein kinase and more importantly another protein kinase known as mTORC1. The latter regulates cell growth by coordinating protein anabolism, nucleotide synthesis, gluconeogenesis and lipogenesis (46,47). The mTORC1 is also modulated by 5' AMP-activated kinase (AMPK), an enzyme that is a key regulator of carbohydrate as well as lipid metabolism (3,48). Among these enzymes mTROC1 seems to rest at the cross-roads of various kinase-mediated signaling pathways and is activated by fatty acids and in states of obesity; and its inhibition by Rapamycin leads to amelioration of palmitate-induced endoplasmic reticulum stress and obesity-mediated autophagy insufficiency (48-50). Most of these studies relate to glomerular biology focusing on podocyte injury culminating into glomerulosclerosis, and there are few studies concerning obesity or metabolic syndrome related to tubulo-interstitial injury (45,51). The current investigation addresses an interlinked pathobiology of MIOX, mTORC1 and SREBP1 in the context of tubular injury and obesity/metabolic syndrome. It is likely that the scenario that one is dealing with here is reminiscent of metabolic syndrome since the mice had HFD-induced obesity (body weight), and high blood sugar, cholesterol and insulin levels. Interestingly, these physiological parameters were normalized along with restoration of villous architecture of proximal tubules and reduction in expression of MIOX and markers of cellular/nuclear damage (figure 6). To interlink the biological relationship between MIOX, SREBP1 and mTORC1 in vitro experiments utilizing HK-2 cell, rapamycin and siRNA techniques were carried out. Treatment of cells with rapamycin concomitantly reduced the palmitate:BSA-induced expression of MIOX and SREBP-1 (figure 7). Nuclear translocation of SREBP1 was also remarkably reduced, suggesting that inhibition of mTROC1 led to a down-regulation of MIOX that is most likely mediated via SREBP1 transcription factor. The fact that palmitate:BSA-induced MIOX and SREBP1 expression was reduced by SREBP1-siRNA would strongly suggest an existence of mTORC1-SREBP1 signaling pathway that modulates the pathobiology of MIOX. In addition, the extent of apoptosis was remarkably reduced by rapamycin, and this process, that influences the regulation of MIOX, was conceivably mediated through p53 since latter’s expression was reduced (figure 7). The p53 is a redox active transcription factor that modulates cellular responses in the setting of various stresses that lead to genomic instability and apoptosis (52). With respect to cellular redox ROS may serve as upstream or downstream modulators of p53 signaling, but a long held view is that ROS are downstream mediators of p53-dependent apoptosis (53).

In view of the above discussion and the fact that over-expression of MIOX leads to ROS generation (29) we examined the status of ROS in HFD- or palmitate-induced upregulation of MIOX in proximal tubules. ROS, as assessed by staining with DCFDA and DHE staining, indicative of hydrogen peroxide and superoxide anion generation, were notably increased in the proximal tubular compartment of kidneys of mice fed with HFD for 6 weeks (figure 8). Likewise in vitro experiments a dose-dependent increase in the Mean Fluorescence Intensity of DCFDA and MITOSOX following palmitate:BSA treatment was observed, which suggested that most likely the ROS generated are derived from both mitochondrial and cytosolic fractions of HK-2 cells, as assessed by flow cytometric analysis (figure 8). Although, the generation of ROS followed the upregulation of MIOX, it is worth mentioning here that MIOX promoter includes both oxidant and anti-oxidant response elements (22). Therefore, it is conceivable that MIOX activation may lead to setting up of a positive regulatory loop with sustained generation of ROS thus accentuating the oxidant stress in proximal tubules. Besides in the kidney, HFD-diet induced generation of ROS is
also seen in other organ systems, including skeletal muscle, liver, generalized vasculature and importantly in adipose tissues (2,6,14,54-57). However, peculiar to the kidney is that HFD-diet induced ROS generation would be sustained because of unique oxidant response elements in the MIOX promoter. Here, another relevant question that needs to be addressed is whether ROS generation directly relates to increased expression of SREBP1 and MIOX and whether this process is specific to the MIOX pathobiology? With this in mind siRNA experiments were performed. Both SREBP-siRNA and MIOX-siRNA reduced the DCFDA related Mean Fluorescence Intensity, as measured by flow cytometric analyses, as well as gauged by fluorescence microscopy (figure 9). The degree of suppression of ROS generation was comparable following the treatment with either of the siRNAs, suggesting an intertwined pathobiology of SREBP1 and MIOX.

In summary, this study elucidates mechanisms involved in HFD- or fatty acids-induced transcriptional, translational and post-translational regulation of MIOX expression as well as its activity. Importantly, this investigation also highlights MIOX as a novel target of the transcription factor SREBP1, and a new mTORC1/SREBP1/MIOX pathway (figure 10) in the generation of ROS culminating into tubulo-interstitial injury in states of obesity.

Acknowledgements: Authors apologize for not citing or mis-quoting any investigator’s work. Supported by NIH grant DK60636, and Manpei Suzuki Diabetes Foundation.

Conflict of Interest: None

Author Contributions: TT, RKD and DJ performed the experiments and wrote the manuscript. TD, JKR and YSK guided the investigation and edited the manuscript.

REFERENCES

1. Bayliss, G., Weinrauch, L. A., and D'Elia, J. A. (2012) Pathophysiology of obesity-related renal dysfunction contributes to diabetic nephropathy. *Curr Diab Rep* **12**, 440-446
2. Tanner, R. M., Brown, T. M., and Muntner, P. (2012) Epidemiology of obesity, the metabolic syndrome, and chronic kidney disease. *Curr Hypertens Rep* **14**, 152-159
3. Singhal, S. S., Figarola, J., Singhal, J., Reddy, M. A., Liu, X., Berz, D., Natarajan, R., and Awasthi, S. (2013) RLIP76 protein knockdown attenuates obesity due to a high-fat diet. *J Biol Chem* **288**, 23394-23406
4. Gregg, E. W., Li, Y., Wang, J., Burrows, N. R., Ali, M. K., Rolka, D., Williams, D. E., and Geiss, L. (2014) Changes in diabetes-related complications in the United States, 1990-2010. *N Engl J Med* **370**, 1514-1523
5. Kanwar, Y. S., Sun, L., Xie, P., Liu, F. Y., and Chen, S. (2011) A glimpse of various pathogenetic mechanisms of diabetic nephropathy. *Annu Rev Pathol* **6**, 395-423
6. Redon, J., and Lurbe, E. (2015) The kidney in obesity. *Curr Hypertens Rep* **17**, 555
7. Sahin-Efe, A., Katsikeris, F., and Mantzoros, C. S. (2012) Advances in adipokines. *Metabolism* **61**, 1659-1665
8. Fasshauer, M., and Bluher, M. (2015) Adipokines in health and disease. *Trends Pharmacol Sci* **36**, 461-470
9. Tartaglia, L. A., Dembski, M., Weng, X., Deng, N., Culpepper, J., Devos, R., Richards, G. J., Campfield, L. A., Clark, F. T., Deeds, J., Muir, C., Sanker, S., Moriarty, A., Moore, K. J., Smutko, J. S., Mays, G. G., Wool, E. A., Monroe, C. A., and Tepper, R. I. (1995) Identification and expression cloning of a leptin receptor, OB-R. *Cell* **83**, 1263-1271
10. Coleman, D. L. (2010) A historical perspective on leptin. *Nat Med* **16**, 1097-1099
11. Lusis, A. J., Attie, A. D., and Reue, K. (2008) Metabolic syndrome: from epidemiology to systems biology. *Nat Rev Genet* **9**, 819-830
12. Giacco, F., and Brownlee, M. (2010) Oxidative stress and diabetic complications. *Circ Res* **107**, 1058-1070
13. Vallon, V. (2011) The proximal tubule in the pathophysiology of the diabetic kidney. *Am J
14. Zhang, H. M., Dang, H., Kamat, A., Yeh, C. K., and Zhang, B. X. (2012) Geldanamycin derivative ameliorates high fat diet-induced renal failure in diabetes. *PLoS One* 7, e32746

15. Thomas, M. E., Harris, K. P., Walls, J., Furness, P. N., and Brunskill, N. J. (2002) Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinuria. *Am J Physiol Renal Physiol* 283, F640-647

16. Arici, M., Brown, J., Williams, M., Harris, K. P., Walls, J., and Brunskill, N. J. (2002) Fatty acids carried on albumin modulate proximal tubular cell fibronectin production: a role for protein kinase C. *Nephrol Dial Transplant* 17, 1751-1757

17. Weinberg, J. M. (2006) Lipotoxicity. *Kidney Int* 70, 1560-1566

18. Birn, H., and Christensen, E. I. (2006) Renal albumin absorption in physiology and pathology. *Kidney Int* 69, 440-449

19. Ruggiero, C., Elks, C. M., Kruger, C., Cleland, E., Addison, K., Noland, R. C., and Stadler, K. (2014) Albumin-bound fatty acids but not albumin itself alter redox balance in tubular epithelial cells and induce a peroxide-mediated redox-sensitive apoptosis. *Am J Physiol Renal Physiol* 306, F896-906

20. Yang, Q., Dixit, B., Wada, J., Tian, Y., Wallner, E. I., Srivastava, S. K., and Kanwar, Y. S. (2000) Identification of a renal-specific oxido-reductase in newborn diabetic mice. *Proc Natl Acad Sci U S A* 97, 9896-9901

21. Arner, R. J., Prabhu, K. S., Thompson, J. T., Hildenbrandt, G. R., Liken, A. D., and Reddy, C. C. (2001) myo-Inositol oxygenase: molecular cloning and expression of a unique enzyme that oxidizes myo-inositol and D-chiro-inositol. *Biochem J* 360, 313-320

22. Nayak, B., Kondeti, V. K., Xie, P., Lin, S., Viswakarma, N., Raparia, K., and Kanwar, Y. S. (2011) Transcriptional and post-translational modulation of myo-inositol oxygenase by high glucose and related pathobiological stresses. *J Biol Chem* 286, 27594-27611

23. Prabhu, K. S., Arner, R. J., Vunta, H., and Reddy, C. C. (2005) Up-regulation of human myo-inositol oxygenase by hyperosmotic stress in renal proximal tubular epithelial cells. *J Biol Chem* 280, 19895-19901

24. Nayak, B., Xie, P., Akagi, S., Yang, Q., Sun, L., Wada, J., Thakur, A., Danesh, F. R., Chugh, S. S., and Kanwar, Y. S. (2005) Modulation of renal-specific oxidoreductase/myo-inositol oxygenase by high-glucose ambience. *Proc Natl Acad Sci U S A* 102, 17952-17957

25. Xie, P., Kondeti, V. K., Lin, S., Haruna, Y., Raparia, K., and Kanwar, Y. S. (2011) Role of extracellular matrix renal tubulo-interstitial nephritis antigen (TINag) in cell survival utilizing integrin (alpha)vbeta3/local adhesion kinase (FAK)/phosphatidylinositol 3-kinase (PI3K)/protein kinase B-serine/threonine kinase (AKT) signaling pathway. *J Biol Chem* 286, 34131-34146

26. Debacq-Chainiaux, F., Erusalimsky, J. D., Campisi, J., and Toussaint, O. (2009) Protocols to detect senescence-associated beta-galactosidase (SA-beta gal) activity, a biomarker of senescent cells in culture and in vivo. *Nat Protoc* 4, 1798-1806

27. Cousin, S. P., Hugl, S. R., Wrede, C. E., Kajio, H., Myers, M. G., Jr., and Rhodes, C. J. (2001) Free fatty acid-induced inhibition of glucose and insulin-like growth factor I-induced deoxyribonucleic acid synthesis in the pancreatic beta-cell line INS-1. *Endocrinology* 142, 229-240

28. Charalampous, F. C., and Lytras, C. (1957) Biochemical studies on inositol. IV. Conversion of inositol to glucuronic acid by rat kidney extracts. *J Biol Chem* 228, 1-13

29. Xie, P., Sun, L., Oates, P. J., Srivastava, S. K., and Kanwar, Y. S. (2010) Pathobiology of renal-specific oxidoreductase/myo-inositol oxygenase in diabetic nephropathy: its implications in tubulointerstitial fibrosis. *Am J Physiol Renal Physiol* 298, F1393-1404

30. Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B., and Spiegelman, B. M. (1998) Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. *J Clin Invest* 101, 1-9

31. Roberts, C. K., Hevener, A. L., and Barnard, R. J. (2013) Metabolic syndrome and insulin
resistance: underlying causes and modification by exercise training. Compr Physiol 3, 1-58

Shimomura, I., Hammer, R. E., Richardson, J. A., Ikemoto, S., Bashmakov, Y., Goldstein, J. L., and Brown, M. S. (1998) Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. Genes Dev 12, 3182-3194

Yokoyama, C., Wang, X., Briggs, M. R., Admon, A., Wu, J., Hua, X., Goldstein, J. L., and Brown, M. S. (1993) SREBP-1, a basic-helix-loop-helix-leucine zipper protein that controls transcription of the low density lipoprotein receptor gene. Cell 75, 187-197

Kim, J. B., and Spiegelman, B. M. (1996) ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. Genes Dev 10, 1096-1107

Horton, J. D., Goldstein, J. L., and Brown, M. S. (2002) SREBP: activators of the complete program of cholesterol and fatty acid synthesis in the liver. J Clin Invest 109, 1125-1131

Ritz, E., Koleganova, N., and Piecha, G. (2011) Is there an obesity-metabolic syndrome related glomerulopathy? Curr Opin Nephrol Hypertens 20, 44-49

Wickman, C., and Kramer, H. (2013) Obesity and kidney disease: potential mechanisms. Semin Nephrol 33, 14-22

32. Narayanan, A., and Jacobson, M. P. (2009) Computational studies of protein regulation by post-translational phosphorylation. Curr Opin Struct Biol 19, 156-163

33. Xue, Y., Liu, Z., Cao, J., Ma, Q., Gao, X., Wang, Q., Jin, C., Zhou, Y., Wen, L., and Ren, J. (2011) GPS 2.1: enhanced prediction of kinase-specific phosphorylation sites with an algorithm of motif length selection. Protein Eng Des Sel 24, 255-260

34. Jiang, T., Wang, Z., Proctor, G., Moskowitz, S., Liebman, S. E., Rogers, T., Lucia, M. S., Li, J., and Levi, M. (2005) Diet-induced obesity in C57BL/6J mice causes increased renal lipid accumulation and glomerulosclerosis via a sterol regulatory element-binding protein-1c-dependent pathway. J Biol Chem 280, 32317-32325

35. Kim, J. B., Spotts, G. D., Halvorsen, Y. D., Shih, H. M., Ellenberger, T., Towle, H. C., and Spiegelman, B. M. (1995) Dual DNA binding specificity of ADD1/SREBP1 controlled by a single amino acid in the basic helix-loop-helix domain. Mol Cell Biol 15, 2582-2588

36. Rome, S., Lecomte, V., Meugnier, E., Rieusset, J., Debard, C., Euthine, V., Vidal, H., and Lefai, E. (2008) Microarray analyses of SREBP-1a and SREBP-1c target genes identify new regulatory pathways in muscle. Physiol Genomics 34, 327-337

37. Nakagawa, Y., Shimano, H., Yoshikawa, T., Ide, T., Tamura, M., Furusawa, M., Yamamoto, T., Inoue, N., Matsuzaka, T., Takahashi, A., Hasty, A. H., Suzuki, H., Sone, H., Toyoshima, H., Yahagi, N., and Yamada, N. (2006) TFE3 transcriptionally activates hepatic IRS-2, participates in insulin signaling and ameliorates diabetes. Nat Med 12, 107-113

38. Griffin, M. J., and Sul, H. S. (2004) Insulin regulation of fatty acid synthase gene transcription: roles of USF and SREBP-1c. JUBMB Life 56, 595-600

39. Csernus, K., Lanyi, E., Erhardt, E., and Molnar, D. (2005) Effect of childhood obesity and obesity-related cardiovascular risk factors on glomerular and tubular protein excretion. Eur J Pediatr 164, 44-49

40. Porstmann, T., Santos, C. R., Griffiths, B., Cully, M., Wu, M., Levers, S., Griffiths, J. R., Chung, Y. L., and Schulze, A. (2008) SREBP activity is regulated by mTORC1 and contributes to Akt-dependent cell growth. Cell Metab 8, 224-236

41. Betz, C., and Hall, M. N. (2013) Where is mTOR and what is it doing there? J Cell Biol 203, 563-574

42. Satriano, J., and Sharma, K. (2013) Autophagy and metabolic changes in obesity-related chronic kidney disease. Nephrol Dial Transplant 28 Suppl 4, iv29-36

43. Yasuda, M., Tanaka, Y., Kume, S., Morita, Y., Chin-Kanasaki, M., Araki, H., Isshiki, K., Araki, S., Koya, D., Haneda, M., Kashiwagi, A., Maegawa, H., and Uzu, T. (2014) Fatty acids are novel nutrient factors to regulate mTORC1 lysosomal localization and apoptosis in podocytes. Biochim Biophys Acta 1842, 1097-1108
50. Yamahara, K., Kume, S., Koya, D., Tanaka, Y., Morita, Y., Chin-Kanasaki, M., Araki, H., Ishiki, K., Araki, S., Haneda, M., Matsusaka, T., Kashiwagi, A., Maegawa, H., and Uzu, T. (2013) Obesity-mediated autophagy insufficiency exacerbates proteinuria-induced tubulointerstitial lesions. J Am Soc Nephrol 24, 1769-1781

51. Thomas, M. E., and Schreiner, G. F. (1993) Contribution of proteinuria to progressive renal injury: consequences of tubular uptake of fatty acid bearing albumin. Am J Nephrol 13, 385-398

52. Liu, B., Chen, Y., and St Clair, D. K. (2008) ROS and p53: a versatile partnership. Free Radic Biol Med 44, 1529-1535

53. Johnson, T. M., Yu, Z. X., Ferrans, V. J., Lowenstein, R. A., and Finkel, T. (1996) Reactive oxygen species are downstream mediators of p53-dependent apoptosis. Proc Natl Acad Sci U S A 93, 11848-11852

54. Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., and Shimomura, I. (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest 114, 1752-1761

55. Carmiel-Haggai, M., Cederbaum, A. I., and Nieto, N. (2005) A high-fat diet leads to the progression of non-alcoholic fatty liver disease in obese rats. FASEB J 19, 136-138

56. Jain, S. S., Paglialunga, S., Vigna, C., Ludzki, A., Herbst, E. A., Lally, J. S., Schrauwen, P., Hoeks, J., Tupling, A. R., Bonen, A., and Holloway, G. P. (2014) High-fat diet-induced mitochondrial biogenesis is regulated by mitochondrial-derived reactive oxygen species activation of CaMKII. Diabetes 63, 1907-1913

57. Youn, J. Y., Siu, K. L., Lob, H. E., Itani, H., Harrison, D. G., and Cai, H. (2014) Role of vascular oxidative stress in obesity and metabolic syndrome. Diabetes 63, 2344-2355

FIGURE LEGENDS

Figure 1. Modulation of MIOX expression and activity and of Bax by High fat diet (HFD) and albumin-bound fatty acids - Following HFD administration an increased expression of MIOX was seen in kidney cortical tubules (green fluorescence, panels 1A and 1B), while no expression was seen in glomeruli (arrowheads). MIOX expression extended into deeper cortex in mice fed HFD diet (panel 1B). By Immunoblot analyses, the expression seemed to be time-dependent and it increased over a period of 6 weeks while the β-actin expression was unchanged (panel 1C). A concomitant time-dependent increase in MIOX activity was also observed, which was normalized following protein λ-phosphatase (λ-PPase) treatment, suggesting that the activity was phosphorylation-dependent (panel 1D). In vitro a dose-dependent increase in the expression and activity of MIOX was also observed in cells treated with palmitate:BSA (panels 1E & 1F). Similarly, a dose-dependent increase in the BAX’s expression was observed with the palmitate:BSA treatment, suggesting preserved cellular integrity at least within the concentration range used (panels 1G – 1I). * p < 0.01 versus control, N = 4.

Figure 2. Modulation of phosphorylation of MIOX and its activity by high fat diet (HFD) and albumin-bound fatty Acids - Kidney homogenates or cellular lysates were immunoprecipitated with substrate-specific phospho antibodies to PKA (Ser/Thr) and PKC (Ser) and to phosphothreonine and phosphotyrosine residues followed by Western blot analyses with anti-MIOX antibody. A time-dependent (2-6 weeks) increased phosphorylation was observed with phosphoantibodies directed against serine and threonine residues (panel 2A). Similarly, a dose-dependent increase in the phosphorylation at serine and threonine residues was observed in cells treated with various concentrations of palmitate:BSA (panel 2B). No increase was observed in samples immunoprecipitated with anti-phosphotyrosine antibody. Concomitant treatment with palmitate:BSA and activators of PKA (forskolin), PDK/PI3K (insulin) and PKC (TPA) further increased the MIOX activity (panel 2C). While concomitant treatment with respective inhibitors, i.e., H89 (PKA), wortmannin (PI3K) and calphostin (PKC) reduced the activity.
below the levels induced by palmitate:BSA alone, confirming that fatty acid induced activity is phosphorylation dependent (panel 2C). * p < 0.01 versus control, or inhibitor versus activator N = 4.

Figure 3. Modulation of MIOX and SREBP1 expression by hyperlipidemia, palmitate:BSA and insulin - Treatment of HK-2 cells with palmitate:BSA (100 μM) increased MIOX expression with a concomitant decrease in membrane-bound precursor form of pre-SREBP1 (pSREBP1, 120 kDa) in cytoplasmic fraction and a simultaneous recruitment and cleavage mature form (mSREBP1, 68 kDa) translocated into the nucleus (panels 3A-3C). No change in the expression of β-actin or laminB1 was observed. In view of the fact that SREBP1 is activated and is associated with excessive secretion of insulin, expression of MIOX and mSREBP1 was determined. A dose-dependent increase in expression of MIOX and mSREBP1 was observed following insulin (10 - 1,000 nM) treatment, suggesting a temporal relationship between the upregulation of MIOX and translocation/expression of mSREBP1 (panels 3D - 3G). The in vivo results were similar to those seen in cultured cells exposed to palmitate:BSA. HFD administration over a period of six weeks to mice resulted in a time-dependent upregulation of mSREBP1 (panel 3H). Immuno-histochemical (IHC) studies confirmed the upregulation of SREBP in renal proximal tubules, both in cytoplasmic and nuclear fractions (panels 3I & 3J, arrowheads). * p < 0.01 versus control, N = 4.

Figure 4. Modulation of MIOX promoter activity by palmitate:BSA and characterization of binding of SREBP to sterol responsive elements (SRE) - Three constructs were generated spanning MIOX gene -1500 bp upstream of ORF, and one spanning the entire region had maximum MIOX basal promoter activity (panel 4A). This construct was populated with E-Box sites and multiple Sterol Response Elements (SRE) sites. A mutant construct was generated by modifying the SRE sites by site directed mutagenesis. The constructs were transfected into HK-2 cells and co-transfected with the luciferase constructs to measure the luciferase activity. Basal promoter activity was designated as one, and following the palmitate:BSA treatment a variable increase in luciferase activity was observed in all constructs (panel 4B). The highest palmitate-induced increase in activity (~2-folds) was observed in the transfected construct inclusive of E-Box and SRE sites. A marginal increase in activity in HK-2 cells transfected with mutant construct was observed, which indicated that the responsiveness of MIOX promoter to fatty acids is mediated via SRE sites. Electrophoretic mobility shift assays (EMSA) revealed a shifted band where a biotin labeled oligonucleotides probe was included in the incubation mixture (panel 4C, lower arrow, middle lane). Unlabeled cold oligonucleotides probe effectively competed since the shifted band disappeared (panel 4C, left lane). With inclusion of anti-SREBP1 antibody in the reaction mixture a super-shifted band to a higher molecular weight was seen, (panel 4C, upper arrow, right lane). To investigate if binding of SREBP1 with the MIOX promoter was dose-dependent ChIP assays followed by PCR were performed following palmitate-BSA treatment with an expected PCR product size of 271 bp. A dose-dependent increase in the binding of SREBP1 to SRE sites was observed following palmitate:BSA-treatment (panel 4D). No band was seen in negative control samples where normal rabbit IgG was substituted for SREBP1 antibody. Functionality of SREBP1 in transfected cells was confirmed by Western blot analysis using antibodies directed against -His tag, -SREBP1 and -MIOX. His tagged antibody could confirm the presence of His fusion protein in cells transfected with pcDNA3.1/V5-His TOPO SREBP1, while no band was seen in cells transfected with empty vector (panel 4E). The pcDNA transfected cells also yielded an increased expression of both SREBP1 and MIOX, suggesting SREBP1 is functional in transfected cells and can modulate MIOX expression (panel 4E). These experiments validated that fatty acids by upregulating SREBP1 enables it to bind to MIOX promoter and modulates MIOX transcription. * p < 0.01 versus control, N = 4.

Figure 5. Modulation of SREBP1 expression, cytoplasmic & nuclear alterations, senescence, apoptosis and urinary protein excretion in mice and rats by HFD diet - Studies were performed to assess the effect of HFD on morphologic and biochemical changes in both the species in metabolically active tubular compartment. In mice, a loss of the pink staining luminal brush border (panel 5B versus
with extrusion of nuclei of tubular cells was observed (arrowheads). The Bcl2 expression was decreased while that of apoptotic Bax was increased (panels 5C-5F). Anti-pH2AX associated nuclear staining (reflective of nuclear DNA damage) was seen in many tubular nuclei of mice fed with HFD (panel 5H versus 5G, arrowheads). Tubular damage was also reflected in increased time-dependent urinary excretion of low molecular proteins ranging in 30 - 45 kDa (panel 5Q, arrows). Some of the changes seen in kidney tubules of rats fed with HFD were reversed by rapamycin treatment (panels 6E-6G). Also, the expression of MIOX (mRNA and protein), Bax and pH2AX were restored to basal levels following rapamycin treatment (panels 6B, 6H-6P).

* p < 0.01 versus control, N = 4.

A significant increase in body weight, blood glucose and serum insulin levels was observed following HFD administration and it was reduced following rapamycin treatment (panels 6A, 6C & 6D). HFD-induced pathologic changes, i.e., loss of proximal tubular brush border and extrusion of the nuclei (arrow heads) were reversed by rapamycin treatment (panels 6E-6G). Also, the expression of MIOX (mRNA and protein), Bax and pH2AX were restored to basal levels following rapamycin treatment (panels 6B, 6H-6P).

A notable increase in the expression of MIOX and SREBP1 was observed following treatment with palmitate:BSA compared to BSA treated cells alone (panels 7B & 7E versus 7A & 7D). The increase was predominantly confined to the nuclear fraction (panels 7G & 7H, arrowhead). The increased expression of MIOX and SREBP1 was reduced with rapamycin treatment (panels 7C, 7F & 7I), suggesting that the mTOR pathway is involved in the upregulation of MIOX and SREBP. Associated with the increased expression of MIOX and SREBP1 a concomitant increase of apoptosis (panel 7K, yellow colored nuclei) was seen, and it was reduced by rapamycin treatment, as assessed by the TUNEL assay (TO-PRO-3 dye) (panels 7L, red colored nuclei). The apoptosis observed was most likely mediated by p53 and its increased expression was also reduced by rapamycin (panel 7M).

A causal relationship between MIOX and SREBP1 was established by siRNA experiments where SREBP1-siRNA treatment reduced MIOX as well as SREBP1 mRNA expression (panels 7N & 7O). * p < 0.01 versus control, N = 4.

A significant increase in mean fluorescence intensity (MFI) related to 5- (and-6)-chloromethyl-2',7'-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H2DCF-DA) and MitoSox staining was observed in HK-2 cells following palmitate:BSA treatment (panels 8A & 8B). Likewise, a notable increase in both CM-H2DCFDA and dihydroethidium (DHE) staining was observed in kidney sections of mice fed with HFD, (panels 8D & 8F vs 8C & 8E). DCF staining was mainly confined to the cytoplasm of tubules while glomeruli were unaffected. * p < 0.01 versus control, N = 4.

A significant increase in mean fluorescence intensity (MFI), as assessed by flow cytometric analyses, following palmitate:BSA treatment was observed (panels 9B vs 9A, 9E). SREBP1 siRNA treatment reduced the MFI (panels 9C & 9E). Similarly, a reduction in MFI was observed following MIOX siRNA treatment (panels 9D & 9E). Likewise fluorescent microscopy of HK-2 cells revealed a notable increase in intensity of staining following palmitate:BSA treatment (panel 9G vs 9F). Both the SREBP1 siRNA and MIOX siRNA treatments remarkably reduced intensity of fluorescence,
suggesting that palmitate:BSA-induced ROS perturbations are specific to MIOX and are mediated via SREBP1 (panels 9H & 9I). * p < 0.01 versus control, N = 4.

**Figure 10. Mechanism(s) of MIOX induction leading to tubulo-interstitial injury in states of obesity or metabolic syndrome.**  
**Panel A.** In states of obesity with the development of insulin resistance there is an activation of transcription factor, SREBP1, which by modulating fatty acid synthase (FAS) would increase the production of fatty acids in the liver. **Panel B.** Conceivably, the fatty acids reabsorbed by the proximal tubular cells activate mTORC1 protein kinase (refs. 48-50), which in turn would increase the expression of preSREBP1. The preSREBP1 is escorted by Scap from ER to Golgi via vesicular transport with simultaneous dissociation of Insig. In the Golgi sacules the preSREBP1 is successively cleaved by Site-1 and -2 proteases to generate a mature form, i.e., mSREBP1. The mSREBP1 translocates into the nucleus, binds to SREs and initiates transcription of MIOX. **Panel C.** Summary of signaling events leading up to MIOX activation, oxidant stress and thereby tubulo-interstitial injury are depicted. In addition, various steps where Rapamycin conceivably can interrupt mTORC1 signaling are also depicted. ER - endoplasmic reticulum, Scap - SREBP cleavage activating protein, Insig - insulin-induced gene, SRE - sterol response elements.
Figure 1. *Modulation of MIOX expression and activity and of Bax by High fat diet (HFD) and albumin-bound fatty acids* - Following HFD administration an increased expression of MIOX was seen in kidney cortical tubules (green fluorescence, panels 1A and 1B), while no expression was seen in glomeruli (arrowheads). MIOX expression extended into deeper cortex in mice fed HFD diet (panel 1B). By Immunoblot analyses, the expression seemed to be time-dependent and it increased over a period of 6 weeks while the β-actin expression was unchanged (panel 1C). A concomitant time-dependent increase in MIOX activity was also observed, which was normalized following protein λ-phosphatase (λ-PPase) treatment, suggesting that the activity was phosphorylation-dependent (panel 1D). *In vitro* a dose-dependent increase in the expression and activity of MIOX was also observed in cells treated with palmitate:BSA (panels 1E & 1F). Similarly, a dose-dependent increase in the BAX’s expression was observed with the palmitate:BSA treatment, suggesting preserved cellular integrity at least within the concentration range used (panels 1G – 1I). * p < 0.01 versus control, N = 4.
Figure 2. Modulation of phosphorylation of MIOX and its activity by high fat diet (HFD) and albumin-bound fatty Acids - Kidney homogenates or cellular lysates were immunoprecipitated with substrate-specific phospho antibodies to PKA (Ser/Thr) and PKC (Ser) and to phosphothreonine and phosphotyrosine residues followed by Western blot analyses with anti-MIOX antibody. A time-dependent (2-6 weeks) increased phosphorylation was observed with phosphoantibodies directed against serine and threonine residues (panel 2A). Similarly, a dose-dependent increase in the phosphorylation at serine and threonine residues was observed in cells treated with various concentrations of palmitate:BSA (panel 2B). No increase was observed in samples immunoprecipitated with anti-phosphotyrosine antibody. Concomitant treatment with palmitate:BSA and activators of PKA (forskolin), PDK/Pi3K (insulin) and PKC (TPA) further increased the MIOX activity (panel 2C). While concomitant treatment with respective inhibitors, i.e., H89 (PKA), wortmannin (PI3K) and calphostin (PKC) reduced the activity below the levels induced by palmitate:BSA alone, confirming that fatty acid induced activity is phosphorylation dependent (panel 2C). * p < 0.01 versus control, or inhibitor versus activator, N = 4.
Figure 3. Modulation of MIOX and SREBP1 expression by hyperlipidemia, palmitate:BSA and insulin - Treatment of HK-2 cells with palmitate:BSA (100 μM) increased MIOX expression with a concomitant decrease in membrane-bound precursor form of pre-SREBP1 (pSREBP1, 120 kDa) in cytoplasmic fraction and a simultaneous recruitment and cleavage mature form (mSREBP1, 68 kDa) translocated into the nucleus (panels 3A-3C). No change in the expression of β-actin or laminB1 was observed. In view of the fact that SREBP1 is activated and is associated with excessive secretion of insulin, expression of MIOX and mSREBP1 was determined. A dose-dependent increase in expression of MIOX and mSREBP1 was observed following insulin (10 - 1,000 nM) treatment, suggesting a temporal relationship between the upregulation of MIOX and translocation/expression of mSREBP1 (panels 3D - 3G). The in vivo results were similar to those seen in cultured cells exposed to palmitate:BSA. HFD administration over a period of six weeks to mice resulted in a time-dependent upregulation of mSREBP1 (panel 3H). Immuno-histochemical (IHC) studies confirmed the upregulation of SREBP in renal proximal tubules, both in cytoplasmic and nuclear fractions (panels 3I & 3J, arrowheads). *p < 0.01 versus control, N = 4.
Figure 4. Modulation of MIOX promoter activity by palmitate:BSA and characterization of binding of SREBP to sterol responsive elements (SRE) - Three constructs were generated spanning MIOX gene -1500 bp upstream of ORF, and one spanning the entire region had maximum MIOX basal promoter activity (panel 4A). This construct was populated with E-Box sites and multiple Sterol Response Elements (SRE) sites. A mutant construct was generated by modifying the SRE sites by site directed mutagenesis. The constructs were transfected into HK-2 cells and co-transfected with the luciferase constructs to measure the luciferase activity. Basal promoter activity was designated as one, and following the pamitate:BSA treatment a variable increase in luciferase activity was observed in all constructs (panel 4B). The highest palmitate-induced increase in activity (~2-folds) was observed in the transfected construct inclusive of E-Box and SRE sites. A marginal increase in activity in HK-2 cells transfected with mutant construct was observed, which indicated that the responsiveness of MIOX promoter to fatty acids is mediated via SRE sites. Electrophoretic mobility shift assays (EMSA) revealed a shifted band where a biotin labeled oligonucleotides probe was included in the incubation mixture (panel 4C, lower arrow, middle lane). Unlabeled cold oligonucleotides probe effectively competed since the shifted band disappeared (panel 4C, left lane). With inclusion of anti-SREBP1 antibody in the reaction mixture a super-shifted band to a higher molecular weight was seen, (panel 4C, upper arrow, right lane). To investigate if binding of SREBP1 with the MIOX promoter was dose-dependent ChIP assays followed by PCR were performed following palmitate-BSA treatment with an expected PCR product size of 271 bp. A dose-dependent increase in the binding of SREBP1 to SRE sites was observed following palmitate:BSA-treatment (panel 4D). No band was seen in negative control samples where normal rabbit IgG was substituted for SREBP1 antibody. Functionality of SREBP1 in transfected cells was confirmed by Western blot analysis using antibodies directed against -His tag, -SREBP1 and -MIOX. His tagged antibody could confirm the presence of His fusion protein in cells transfected with pcDNA3.1/V5-His TOPO SREBP1, while no band was seen in cells transfected with empty vector (panel 4E). The pcDNA transfected cells also yielded an increased expression of both SREBP1 and MIOX, suggesting SREBP1 is functional in transfected cells and can modulate MIOX expression (panel 4E). These experiments validated that fatty acids by upregulating SREBP1 enables it to bind to MIOX promoter and modulates MIOX transcription. * $p < 0.01$ versus control, $N = 4$. 
Figure 5. Modulation of SREBP1 expression, cytoplasmic & nuclear alterations, senescence, apoptosis and urinary protein excretion in mice and rats by HFD diet - Studies were performed to assess the effect of HFD on morphologic and biochemical changes in both the species in metabolically active tubular compartment. In mice, a loss of the pink staining luminal brush border (panel 5B versus 5A, arrows) with extrusion of nuclei of tubular cells was observed (arrowheads). The Bcl2 expression was decreased while that of apoptogenic Bax was increased (panels 5C-5F). Anti-pH2AX associated nuclear staining (reflective of nuclear DNA damage) was seen in many tubular nuclei of mice fed with HFD (panel 5H versus 5G, arrowheads). Tubular damage was also reflected in increased time-dependent urinary excretion of low molecular proteins ranging in 30 - 45 kDa (panel 5Q, arrows). Some of the changes seen in kidney tubules of rats fed with HFD are depicted here. Identical to the MIOX expression an increased expression of SREBP1 (panel 5J versus 5I) and BAX (panel 5L versus 5K) was observed. The increased expression of Bax was time dependent (panel 5R). This was associated with increased apoptosis, as highlighted by TUNEL staining (panel 5N versus 5M). In addition, a marked increase in SA-β-Gal staining was observed (panel 5P versus 5O, arrowheads), suggesting an increased replicative capacity of tubular cells following HFD-induced cellular damage. Overall, these changes suggest that HFD adversely affect the cellular homeostasis in both the species.
Figure 6. Rapamycin reverses HFD-induced MIOX expression and associated changes in the renal tubules - A significant increase in body weight, blood glucose and serum insulin levels was observed following HFD administration and it was reduced following rapamycin treatment (panels 6A, 6C & 6D). HFD-induced pathologic changes, i.e., loss of proximal tubular brush border and extrusion of the nuclei (arrow heads) were reversed by rapamycin treatment (panels 6E-6G). Also, the expression of MIOX (mRNA and protein), Bax and pH2AX were restored to basal levels following rapamycin treatment (panels 6B, 6H-6P). * p < 0.01 versus control, N = 4.
Figure 7. Rapamycin reverses Palmitate:BSA-induced MIOX, SREBP1 and p53 expression and apoptosis in renal tubular cells - A notable increase in the expression of MIOX and SREBP1 was observed following treatment with palmitate:BSA compared to BSA treated cells alone (panels 7B & 7E versus 7A & 7D). The increase was predominantly confined to the nuclear fraction (panels 7G & 7H, arrowhead). The increased expression of MIOX and SREBP1 was reduced with rapamycin treatment (panels 7C, 7F & 7I), suggesting that the mTOR pathway is involved in the upregulation of MIOX and SREBP. Associated with the increased expression of MIOX and SREBP1 a concomitant increase of apoptosis (panel 7K, yellow colored nuclei) was seen, and it was reduced by rapamycin treatment, as assessed by the TUNEL assay (TO-PRO-3 dye) (panels 7L, red colored nuclei). The apoptosis observed was most likely mediated by p53 and its increased expression was also reduced by rapamycin (panel 7M). A causal relationship between MIOX and SREBP1 was established by siRNA experiments where SREBP1-siRNA treatment reduced MIOX as well as SREBP1 mRNA expression (panels 7N & 7O). * p < 0.01 versus control, N = 4.
Figure 8. *Palmitate:BSA and HFD induced perturbations in cellular redox in renal tubular cells* - A dose-dependent increase in mean fluorescence intensity (MFI) related to 5-(and-6)-chloromethyl-2′,7′-dichlorodihydro-fluorescein diacetate, acetyl ester (CM-H2DCF-DA) and MitoSox staining was observed in HK-2 cells following palmitate:BSA treatment (panels 8A & 8B). Likewise, a notable increase in both CM-H2DCFDA and dihydroethidium (DHE) staining was observed in kidney sections of mice fed with HFD, (panels 8D & 8F vs 8C & 8E). DCF staining was mainly confined to the cytoplasm of tubules while glomeruli were unaffected. *p < 0.01 versus control, N = 4.
Figure 9. SREBP1 & MIOX siRNA reduced palmitate:BSA-induced perturbations in cellular redox in renal tubular cells -
A significant increase in mean fluorescence intensity (MFI), as assessed by flow cytometric analyses, following palmitate:BSA treatment was observed (Panels 9B vs 9A, 9E). SREBP1 siRNA treatment reduced the MFI (Panels 9C & 9E). Similarly, a reduction in MFI was observed following MIOX siRNA treatment (Panels 9D & 9E). Likewise fluorescent microscopy of HK-2 cells revealed a notable increase in intensity of staining following palmitate:BSA treatment (panel 9G vs 9F). Both the SREBP1 siRNA and MIOX siRNA treatments remarkably reduced intensity of fluorescence, suggesting that palmitate:BSA-induced ROS perturbations are specific to MIOX and are mediated via SREBP1 (panels 9H & 9I). * p < 0.01 versus control, N = 4.
Figure 10. **Mechanism(s) of MIOX induction leading to tubulo-interstitial injury in states of obesity or metabolic syndrome.** Panel A. In states of obesity with the development of insulin resistance there is an activation of transcription factor, SREBP1, which by modulating fatty acid synthase (FAS) would increase the production of fatty acids in the liver. Panel B. Conceivably, the fatty acids reabsorbed by the proximal tubular cells activate mTORC1 protein kinase (refs. 48-50), which in turn would increase the expression of preSREBP1. The preSREBP1 is escorted by Scap from ER to Golgi via vesicular transport with simultaneous dissociation of Insig. In the Golgi saccules the preSREBP1 is successively cleaved by Site-1 and -2 proteases to generate a mature form, i.e., mSREBP1. The mSREBP1 translocates into the nucleus, binds to SREs and initiates transcription of MIOX. Panel C. Summary of signaling events leading up to MIOX activation, oxidant stress and thereby tubulo-interstitial injury are depicted. In addition, various steps where Rapamycin conceivably can interrupt mTORC1 signaling are also depicted. ER - endoplasmic reticulum, Scap - SREBP cleavage activating protein, Insig - insulin-induced gene, SRE - sterol response elements.
Transcriptional and Translational Modulation of Myo-Inositol Oxygenase (MIOX) by Fatty acids: Implications in Renal Tubular Injury Induced in Obesity and Diabetes
Tatsuya Tominaga, Rajesh K. Dutta, Darukeshwara Joladarashi, Toshio Doi, Janardan K. Reddy and Yashpal S. Kanwar

*J. Biol. Chem.* published online November 17, 2015

Access the most updated version of this article at doi: 10.1074/jbc.M115.698191

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts