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Original Paper

Endothelin-1 Overexpression Improves Renal Function in eNOS Knockout Mice

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Key Words
Chronic kidney disease • Endothelial nitric oxide synthase • Endothelin • Mice • Nitric oxide

Abstract
Background/Aims: To investigate the renal phenotype under conditions of an activated renal ET-1 system in the status of nitric oxide deficiency, we compared kidney function and morphology in wild-type, ET-1 transgenic (ET+/+), endothelial nitric oxide synthase knockout (eNOS-/-) and ET+/+eNOS-/- mice. ***Methods:*** We assessed blood pressure, parameters of renal morphology, plasma cystatin C, urinary protein excretion, expression of genes associated with glomerular filtration barrier and tissue remodeling, and plasma metabolites using metabolomics. ***Results:*** eNOS-/- and ET+/+eNOS-/- mice developed hypertension. Osteopontin, albumin and protein excretion were increased in eNOS-/- and restored in ET+/+eNOS-/- animals. All genetically modified mice developed renal interstitial fibrosis and glomerulosclerosis. Genes involved in tissue remodeling (serpine1, TIMP1, Col1a1, CCL2) were up-regulated in eNOS-/-, but not in ET+/+eNOS-/- mice. Plasma levels of free carnitine and acylcarnitines, amino acids, diacyl phosphatidylcholines, lysophosphatidylcholines and hexoses were descreased in eNOS-/- and were in the normal range in ET+/+eNOS-/- mice. ***Conclusion:*** eNOS-/- mice developed renal dysfunction, which was partially rescued by ET-1 overexpression in eNOS-/- mice. The metabolomics results suggest that ET-1 overexpression on top of eNOS knockout is associated with a functional recovery of mitochondria (rescue effect in β-oxidation of fatty acids) and an increase in antioxidative properties (normalization of monounsaturated fatty acids levels).

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Introduction

Endothelin-1 (ET-1) and nitric oxide (NO) closely interact in a manner of a local network, i.e. ET-1 stimulates NO production, NO in turn inhibits ET-1 expression proportionally in different cell-types [1]. An *in vivo* example of interaction between ET-1 and NO production was demonstrated in transgenic mice overexpressing ET-1 (ET+/+), which have a normal blood pressure. The vasoconstrictory effects of ET-1 were antagonized by an increased production of NO, the endothelial counterpart of ET-1 in animals overexpressing ET-1 [2].

An excessive renal production of ET-1 is often observed in patients with chronic kidney disease (CKD) of diabetic and non-diabetic origin [3]. In contrast, in CKD the overall NO availability is decreased, in part due to endothelial dysfunction [4]. To date, the only known agents to block the activity of ET-1, which have been approved for clinical use, are ET receptor antagonists [5], although they have not so far been validated for a therapeutic use in CKD due to adverse effects such as water and salt retention [3, 6].

We have previously generated crossbred animals of ET transgenic mice (ET+/+) and endothelial nitric oxide synthase knockout (eNOS-/-) mice to investigate the role of ET-1 overproduction in endothelial function and blood pressure in the conditions of NO deficiency [7]. In another study performed by our group,we demonstrated that both eNOS-/- and ET+/+eNOS-/- mice developed high blood pressure in contrast to wild-type (WT) and ET+/+ mice, which remained normotensive [8]. Interestingly, left ventricular catheterization showed that only eNOS-/- mice, but not ET+/+eNOS-/- developed diastolic dysfunction [8]. The aim of the present work was to investigate renal phenotypic consequences and mechanisms of ET/eNOS interaction using crossbred ET+/+eNOS-/- mice.

Materials and Methods

Mouse model

Human ET-1 transgenic mice (line 856) [9] and eNOS-/- mice [10] were generated as described previously. Only homozygous mice were used for breeding. We ensured the steadiness of the genotype over the generations. Crossbred mice were created by breeding female eNOS-/- mice with male ET+/+. Study groups were composed as follows: WT, ET+/+, eNOS-/-, and ET+/+eNOS-/- mice. The number of animals per group was 14. Only male mice were used in the study. All mice were sacrificed at the age of 9 months. The protocol was approved by the Committee on the Ethics of Animal Experiments of the city of Berlin, Germany.

Blood pressure

Systolic blood pressure (SBP) was measured by the tail cuff method [7] at two time points (3 and 9 months).

Histological studies

The parameters of renal morphology were measured as previously described [9, 11]. Briefly, interstitial fibrosis was evaluated in Sirius Red stained kidney sections. A ratio of Sirius Red-stained area to the total area of the picture was analyzed using the program ImageJ (shareware by National Institutes of Health, Bethesda, MD, USA). The extent of glomerulosclerosis was quantified as percentage of periodic acid-Schiff positive area within the glomerulus by a semi-quantitative score system using a score of 1-4 by two investigators who were blinded to the study groups. Scores 1, 2, 3, and 4 represented mesangial matrix expansion involving 0-25%, 25-50%, 50-75%, and 75-100% of the glomerular tuft area, respectively.

Plasma and urinary analyses

Urinary concentrations of albumin, H-FABP, and osteopontin were determined in urine samples collected in a six hours time period in metabolic cages. ELISA assays were conducted to determine the levels of albumin (CellTrend GmbH kit. no. 50200, Luckenwalde, Germany), H-FABP (Hycult Biotech, kit no. HK403, Uden The Netherlands), osteopontin (R&D Systems, kit no. MOST00, Wiesbaden, Germany) according to the

protocols provided by the suppliers of the assays. Optical density of the samples was measured at 450 nm using an automatic ELISA reader (Tecan Infinite M200, Tecan Group Ltd. Männedorf, Switzerland). Urinary creatinine, albumin, and protein were quantitatively determined in the samples using a Cobas Integra 400 Plus analyzer (Roche Diagnostics Deutschland GmbH, Mannheim, Germany) according to the manual of the manufacturer. Plasma cystatin C was measured using mouse/rat cystatin C Quantikine ELISA kit from R&D Systems Inc. (Minneapolis, MN, USA) according to manufacturer's guidelines with readout performed with a Tecan Infinite M200 ELISA Reader (Crailsheim, Germany).

Western blotting

Western blotting was performed as previously described [7]. Briefly, whole kidney protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Amersham™ Hybond™ ECL, GE Healthcare, Milwaukee, USA) in transfer buffer (184 mM Glycin, 24 mM Tris, 20% methanol) at 110 V for 2.5 hours. Membranes were blocked in 5% non-fat milk TBS-T for 1 hour at room temperature. Primary antibodies against ET-1 type A receptor (ETAR,sc-33536), ET-1 type B receptor (ETBR,sc-33538, both from Santa Cruz Biotechnology, Santa Cruz, CA, USA), eNOS (ALX-210-509), inducible NOS (iNOS,ALX-210-515, both from Alexis Biochemicals, San Diego, CA, USA), actin (A5060,Sigma-Aldrich, Steinheim, Germany) were used.

Relative quantification of mRNA by RT-PCR

Total RNA was extracted from whole kidney samples using RNeasy fibrous tissue kit (Qiagen, Hilden, Germany) and checked for integrity on a Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). For reverse transcription, 1 µg of total RNA was first digested with RNase-free DNase I (Gibco, Invitrogen Corp. Carlsbad, CA, USA) for 15 minutes at room temperature and then reverse-transcribed with the use of ImProm II Reverse Transcription System (Promega Corp. Fitchburg, WI, USA) in a total reaction volume of 40 µL according to the standard protocol of the kit supplier. Real-time polymerase chain reaction was performed by ABI Prism Sequence Detection System (Applied Biosystems ABI Prism 7700 Sequence Detector). cDNA samples were amplified with a PCR mix containing Taq polymerase (qPCR MasterMix Plus, Eurogentec, Liege, Belgium) and primer sets (Table 1) with 6-FAM and TAMRA labeled probes (Sigma Genosys) in 384-well microtiter plates. cDNA samples (20 ng) were run in triplicates in reaction volumes of 20 µl (384-well microtiter plates) under standard thermocycler conditions. TaqMan probe sets were generated by using Primer3Plus (*http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus*). Relative gene expression was calculated using the ddCt term (Applied Biosystems, User Bulletin No. 2) related to endogenous controls ribosomal protein L32 and β-actin. Real Time PCR primers were deduced from reference gene sequences of the following NCBI GenBank accession numbers (Table 1).

Targeted metabolomic measurements

Metabolites were simultaneously quantified in plasma samples using the Absolute*IDQ*™ p150 kit (BIOCRATES Life Science AG, Innsbruck, Austria). These measurements are based on FIA-MS/MS (FIA: flow injection analysis) [12, 13]. The assay includes free carnitine, 40 acylcarnitines (Cx:y), 14 amino acids (13 proteinogenic + ornithine), hexoses (sum of hexoses – about 90-95 % glucose), 92 glycerophospholipids (15 lysophosphatidylcholines (lysoPC) and 77 phosphatidylcholines (PC)), and 15 sphingolipids (SMx:y). The abbreviations Cx:y are used to describe the total number of carbons and double bonds of all chains, respectively [13]. Mass spectrometric (MS) analyses were done on a API 4000 system (AB Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL auto sampler (CTC Analytics, Zwingen, Switzerland) controlled by the Analyst software version 1.4.1 (Applied Biosystems, Foster City, CA, USA). Data evaluation for quantification of metabolite concentrations as well as quality assessment was performed with the Met*IQ*™ software, which is an integral part of the Absolute*IDQ*™ kit (both from Biocrates Life Sciences AG, Innsbruck, Austria).

The Absolute*IDQ*™ p150 kit has been proven to be in conformance with FDA-Guidline "Guidance for Industry - Bioanalytical Method Validation" (May 2001) [14], which implies proof of reproducibility within a given error range. The metabolite panel covered by targeted metabolomics approach has been succesfully used to evaluate mechanistic background of phenotypes observed in human [15] and mouse [16]. Metabolomics has been instrumental in analysing kidney disfunction [17–19].

Table 1. Real Time PCR primers

Gene	Acc. No.	5'-Primer	3'Primer	Probe
Rpl32	NM_172086.2	ACTGGAGGAAACCCAGAGGC	CATCAGGATCTGGCCCTTGA	TCGACAACAGGGTGCGGAGAAGG
Actb (ß-Actin)	NM_007393.3	TTGCTGACAGGATGCAGAAG	TGCTCTGGCTCCTAGCACCATGA	GTACTTGCGCTCAGGAGGAG
Col1a1	NM_007742.3	AAGAAGCACGTCTGGTTTGGA	AGCATGACCGATGGATTCCCG	AGCCCTCGCTTCCGTACTC
CCL2 (MCP-1)	NM_011333.3	AAGAGGATCACCAGCAGCAG	TCCCAAAGAAGCTGTAGTTTTTGTCACCA	TTCTTGGGGTCAGCACAGA
IL-18	NM_008360.1	TGACCCTCTCTGTGAAGGATAGTA	TCAGGTGGATCCATTTCCTC	CCCTCTCCTGTAAGAACAAGATCATTTCCT
Serpine1	NM_008871.2	GGCCGACTTCACAAGTCTTTC	CGATCCTGACCTTTTGCAGT	ACCAAGAGCAGCTCTCTGTAGCACAGG
TIMP1	NM_001044384.1	GTCCCAGAACCGCAGTGAAG	CTGATGTGCAAATTTCCGTT	TTTCTCCATCACGGGCCGCCTAA
ACE2	AB053182.1	GCCTAAAATCAGCTCTTGGAGCTA	ACAGATGATCGGAACAGGAACA	TGCATATGAATGGACCAACAACGAA
Ctgf	NM_010217	CTCTGCATGGTCAGGCCCTG	TCAAACTTGACAGGCTTGGC	CATTAAGAAAGGGCAAAAAGTGCATCCG
Podxl (podocalyxin-like protein 1)	NM_013723	CTCTACGGCTGCTGTCACC	CCACTGTCTGCAGCTCCTCT	AGGAAGGACCAGCAACGGCTCA
Nphs1 (nephrin)	NM_019459	TCTGGGGACCCCTCTATGAT	TAGATTCCCCTTGGGTCCTC	CCTTCGCTGGCCTGAGGTCAAA
Itga3 (integrin alpha 3 subunit)	NM_013565	AGGCTGACCGACGACTACTG	GTAGTAGCGGGTCGGCTTAAA	CGGCCCACCCGGTGTGACT
Col4a1	NM_009931.2	GCTCGCCACCATAGAGAGAA	CCCTGCCTTCAAGGTGGAT	CGAGATGTTCAAGAAGCCCACGC
CD34	BC006607.1	CAGCTTATGGAAAAGCACCA	GTGGCTCCCGATATCTTGTT	TGAGAAAGCTGGGGATCCAAAGC
Pecam1 (CD31)	NM_008816.2	GTACAGTGAGATCCGGAAGG	GTGCACTGCCTTGACTGTCT	CGACCCTAAGAACGGAAGGCTCCC
Lamb2 (laminin beta 2)	NM_008483.3	GGAGCTTCGAGAACTTATCCAG	CCGAGTCGCTACCATTTCAA	CAGGAGGGAGCCGATCCTGACA
Lamc1 (laminin gamma 1)	NM_010683.2	GATGATGCCGACCAGGAC	GCATTGAGCTCAGCCTCCT	ATGGCAGGGATGGCTTCGCAG

Statistical analysis

All data are expressed as means ± SEM. Statistical analyses were performed using GraphPad Prism 5 software (La Jolla, CA, USA). For comparison of two parametrically distributed groups of data unpaired t-test with equal or unequal variance (Welch's correction) was applied. For nonparametrically distributed groups of data the Mann-Whitney U-test was used. Differences were considered statistically significant when $P < 0.05$.

Results

Systolic blood pressure

SBP was significantly elevated in eNOS-/- (137.6 ± 3.6 mmHg; $P < 0.001$ versus WT) and in ET+/+eNOS-/- (129.1 ± 5.0 mmHg; $P < 0.05$ versus WT) compared to WT (111.9 ± 4.2 mmHg) and ET+/+ groups (109.7 ± 7.5 mmHg) in 9 month old mice. The aforementioned tendencies were revealed starting as early as 3 months of age (Table 2). SBP in eNOS-/- group was not significantly different from that in ET+/+eNOS-/- mice.

Renal function

Plasma cystatin C was elevated in both groups with eNOS genetic ablation ($P < 0.05$ versus WT), but not in ET+/+ mice (Fig. 1a). In contrast, albuminuria and proteinuria were both significantly ($P < 0.01$ versus WT) increased only in eNOS-/- compared to WT (15.0- and 3.1-fold increase versus WT respectively, Fig. 1b-c), while the other genetically modified groups did not show significant differences in albuminuria and proteinuria levels versus control. Interestingly, in crossbred mice both albuminuria and proteinuria levels were observed to be diminished compared to eNOS-/- ($P < 0.01$ versus eNOS-/-).

Table 2. Systolic Blood Pressure. Systolic blood pressure in 3 and 9 months old mice, mm Hg. Values are given as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs WT

Age	WT	ET+/+	eNOS-/-	ET+/+eNOS-/-
3 Months	91.0 +/- 4.4	91.0 +/- 2.7	108.9 +/- 5.2*	107.8 +/- 3.8**
9 Months	111.9 +/- 4.2	109.7 +/- 7.5	137.6 +/- 3.6***	129.1 +/- 5.0*

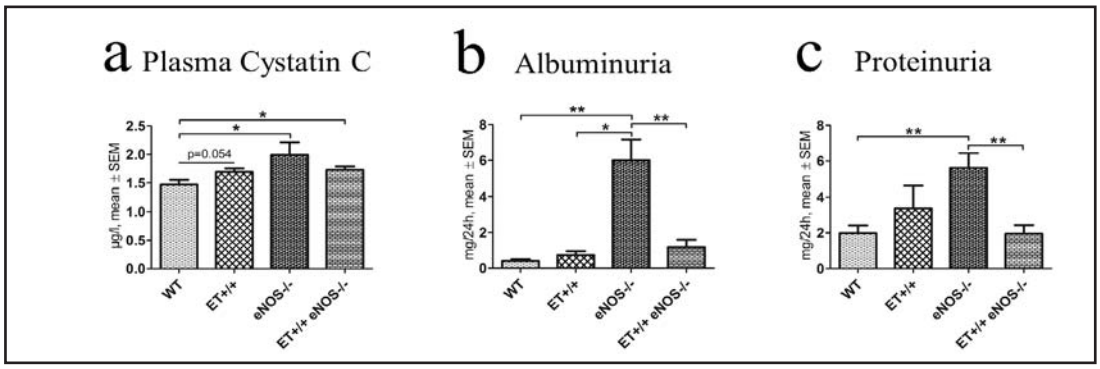
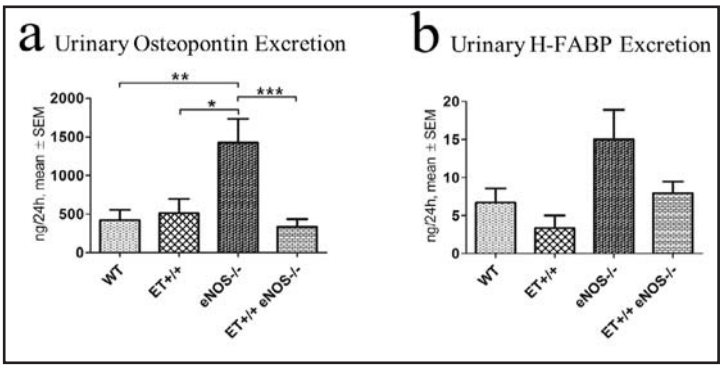


Fig. 1. Plasma cystatin C levels (a), urinary albumin (b) and protein (c) daily excretion. Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.01$.

Fig. 2. Values are given as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



Urine biomarkers

Urinary excretion of osteopontin was 3.4-fold increased in eNOS-/- mice versus WT ($P < 0.01$) and restored in crossbred mice versus eNOS-/- ($P < 0.001$, Fig. 2a). Urinary excretion of heart muscle-type fatty acid binding protein (H-FABP, measured in ng/24h) was 6.69 +/- 1.85 in WT; 3.36 +/- 1.64 in ET+/+; 14.99 +/- 3.91 in eNOS-/-; and 7.91 +/- 1.56 in ET+/+eNOS-/-. There were no significant differences in H-FABP urinary excretion between the genotypes (Fig. 2b).

Kidney morphology

The absolute and relative kidney weight was significantly higher in ET+/+ and ET+/+eNOS-/- versus WT, whereas in the eNOS-/- group it was lower compared to all other groups (Table 3). ET+/+, eNOS-/- and ET+/+eNOS-/- mice all developed glomerulosclerosis, as assessed by increased deposits of periodic acid Schiff (PAS) positive material within the glomeruli and interstitial fibrosis, as evidenced by an increased percentage of fibrotic area compared to WT (Table 3). Perivascular fibrosis, media-to-lumen ratio (Table 3), glomeruli size and number (data not shown) revealed no significant differences between the study groups.

Renal profile of ET- and NOS- associated proteins

The eNOS protein expression was increased in the ET+/+ group ($P < 0.01$ versus WT). ETAR, ETBR, and iNOS showed no significant differences between the study groups as shown in Table 4.

Table 3. Renal Morphology. The parameters of renal morphology in 9 months old mice. Values are given as mean ± SEM. * $P < 0.05$; *** $P < 0.001$ vs WT; §§§ $P < 0.001$ vs eNOS-/-

Parameter	Units	WT	ET+/+	eNOS-/-	ET+/+eNOS-/-
Total Weight of Left and Right Kidneys	g	0.47 +/- 0.02	0.55 +/- 0.02*,§§§	0.40 +/- 0.02*	0.52 +/- 0.01§§§ P = 0.050 vs WT
Kidney Weight / Body Weight Ratio	mg/g	12.20 +/- 0.46	14.10 +/- 0.46*,§§§	9.32 +/- 0.39***	11.86 +/- 0.26§§§
Interstitial Fibrosis	Percentage of Fibrotic Area per Total Section Area	4.84 +/- 0.40	6.73 +/- 0.53*	6.04 +/- 0.32*	5.91 +/- 0.22*
Glomerulosclerosis Index	Score	2.38 +/- 0.45	3.27 +/- 0.77*	3.32 +/- 0.84*	3.19 +/- 0.59*
Media-to-lumen Ratio	Ratio	0.59 +/- 0.03	0.61 +/- 0.09	0.63 +/- 0.06	0.59 +/- 0.05
Perivascular Fibrosis	Score	1.49 +/- 0.16	1.14 +/- 0.08	1.76 +/- 0.09	1.74 +/- 0.13

Table 4. Renal Profile of Endothelin-1 and Nitric Oxide Synthase- Associated Proteins. Relative renal protein expression of endothelin receptor type A (ET-A), endothelin receptor type B (ET-B), endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) in 9 months old mice. Values are given as mean ± SEM. ** $P < 0.01$ vs WT

Protein	WT	ET+/+	eNOS-/-	ET+/+eNOS-/-
ET-A	1.00 +/- 0.10	0.92 +/- 0.09	1.02 +/- 0.14	1.13 +/- 0.02
ET-B	1.00 +/- 0.08	1.11 +/- 0.07	1.10 +/- 0.26	1.26 +/- 0.18
eNOS	1.00 +/- 0.07	1.40 +/- 0.09**	0.0 +/- 0.0	0.0 +/- 0.0
iNOS	1.00 +/- 0.21	0.71 +/- 0.11	1.02 +/- 0.14	1.46 +/- 0.28

Glomerular filtration barrier- associated gene expression

Podocyte-associated genes (podocalyxin-like protein 1, nephrin and integrin alpha 3 subunit) showed only minor regulation patterns (Fig. 3a-c). In contrast, glomerular basement membrane (GBM) – associated genes were differently regulated. In ET+/+ and eNOS-/- mice a down-regulation of collagen IV gene expression was observed, whereas the gene encoding one of the laminin subunits (Lamc1) was up-regulated. Lamb2, a gene responsible for the synthesis of another laminin subunit, showed a trend (P = 0.056 versus WT) towards up-regulation in eNOS-/- mice. Crossbred animals showed only a minor regulation of GBM associated genes (Fig. 3d-f). Endothelial cells-associated genes CD34 and CD31 were significantly down-regulated in ET+/+eNOS-/- mice versus WT and eNOS-/- (Fig. 3g-h). Tissue remodeling associated genes (Serpine1, TIMP1 and CCL2) were significantly up-regulated in eNOS-/- animals compared to WT. Col1a1 gene showed a trend towards up-regulation (P = 0.077) in eNOS-/- mice compared to WT. The Ctgf gene was not regulated (Fig. 4a-e). mRNA expression levels of Serpine1, TIMP1, CCL2 and Col1a1 were improved in the ET+/+eNOS-/- mice. Interleukin 18 gene expression was up-regulated in ET+/+ mice versus all other groups (Fig. 4f). The angiotensin I-converting enzyme 2 (ACE2) gene was down-regulated in eNOS-/- animals compared to all other groups (Fig. 4g).

Plasma metabolites

Free carnitine and acyl carnitines (Fig. 5), diacyl phosphatidylcholines (PCaa), lysophosphatidylcholines (LPCs) (Fig. 6), most amino acids (Fig. 7), and hexoses (Fig. 8a) exhibited a similar pattern, in which the metabolites in eNOS-/- or transgenic ET+/+ were lower in concentration, whereas these metabolites of crossbred (ET+/+eNOS-/-) animals were comparable in concentration to those found in WT animals. We did not observe changes in the activity of mitochondrial carnitine palmitoyl transferase I in any of the transgenic models, as depicted by a stable CPT-I ratio (calculated from octadecanoylcarnitine + hexadecanoylcarnitine/carnitine free (C18+C16)/C0). The oxidative degradation of even-

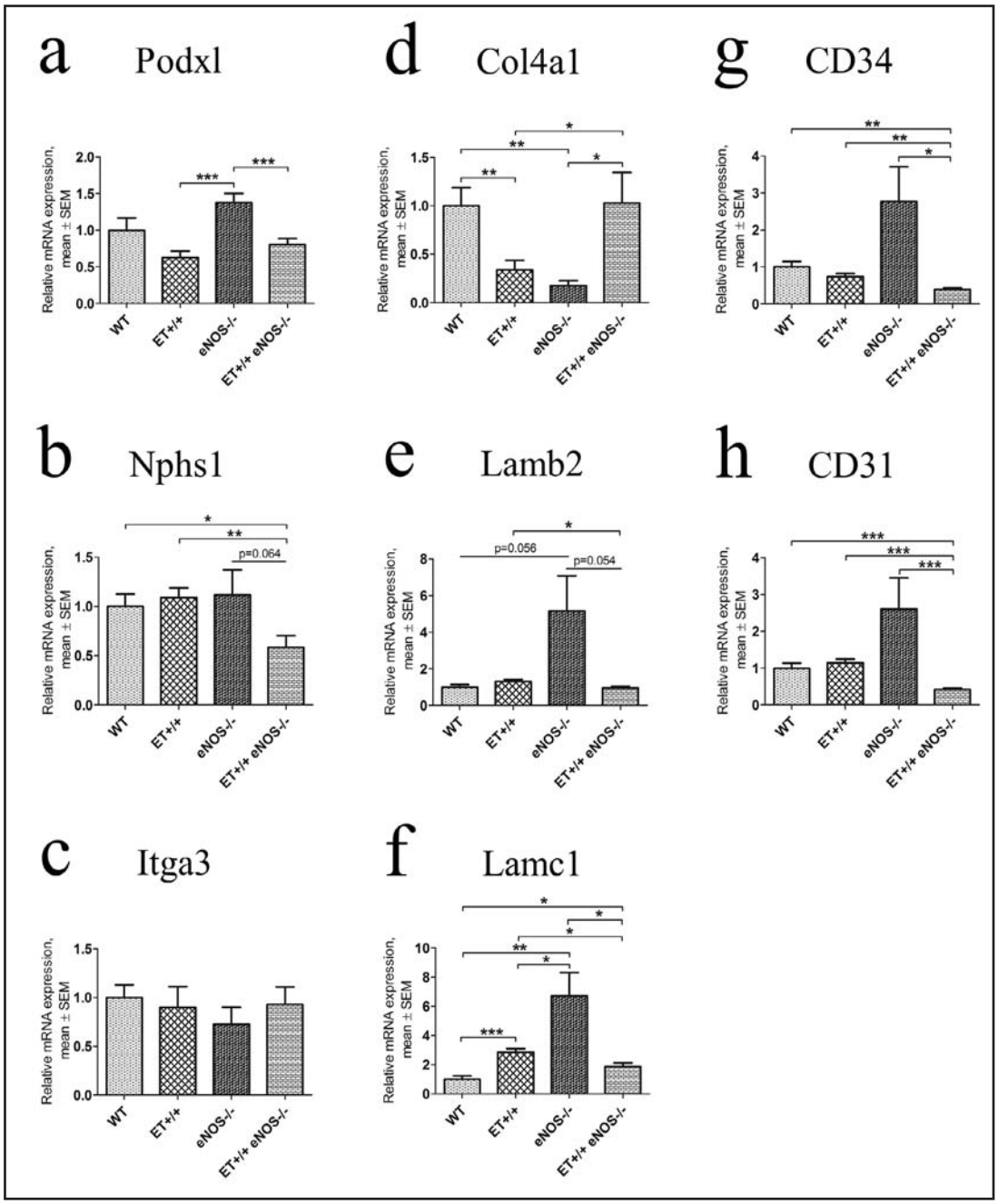


Fig. 3. Relative expression of podocyte- (podocalyxin-like protein 1 (a), nephrin (b), integrin alpha 3 subunit (c)), glomerular basement membrane- (collagen IV alpha 1 (d), laminin beta 2 (e), laminin gamma 1 (f)) and endothelial cells- associated genes (cluster of differentiation 34 (g) and cluster of differentiation 31 (h)) in whole kidney. Values are given as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

numbered fatty acids in mitochondria (characterized by carnitine free/acetylcarnitine (C2/C0) ratio) compared to the overall activity of ß-oxidation (measured by acetylcarnitine + propionylcarnitine/carnitine free (C2+C3)/C0) ratio) revealed the aforementioned pattern (Fig. 5b-d). Herein, we present selected molecules (ranked by statistical significance), representative of each metabolite class.

Desaturation of fatty acid chains in the PC(aa) or acyl carnitines abrogated the differences observed between WT, eNOS-/- and ET+/+ (Fig. 5e-h; Fig. 6a-c). LPCs, regardless of their saturation level, revealed differences between WT, eNOS-/- and ET+/+ (Fig. 6d-f).

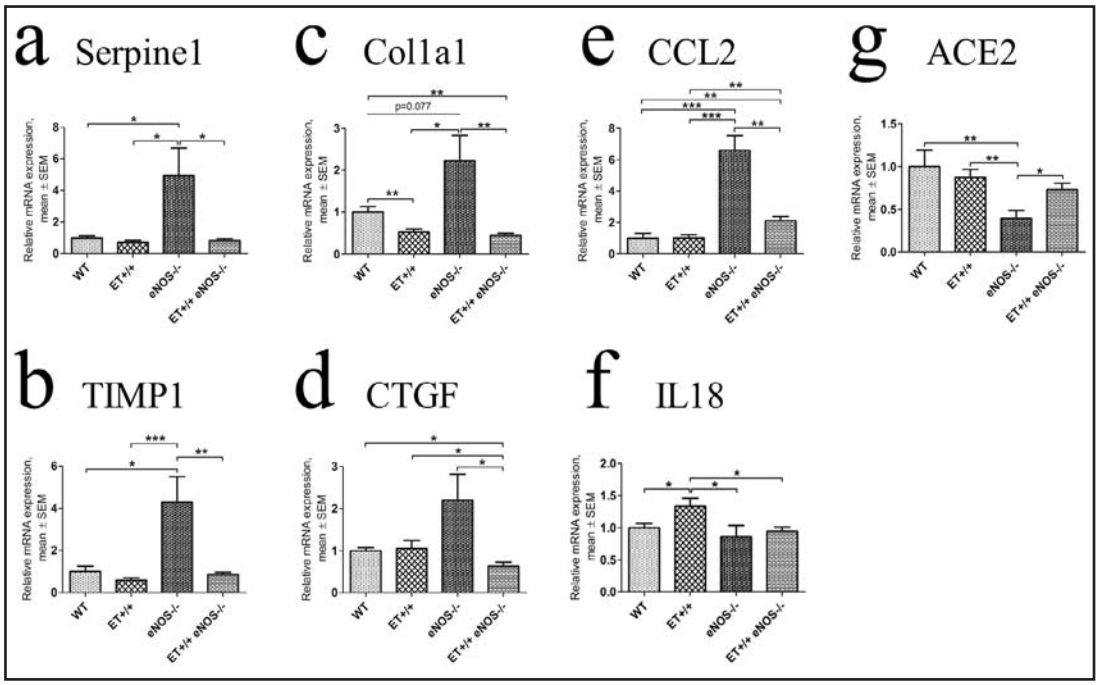


Fig. 4. Relative expression of genes involved in tissue remodeling processes in whole kidney. Serpine1 / plasminogen activator inhibitor-1 (a), tissue inhibitor of metalloproteinase 1 (TIMP-1) (b), collagen type I alpha 1 (c), connective tissue growth factor (d), chemokine (C-C motif) ligand 2 / monocyte chemoattractant protein-1 (e), interleukin 18 (f), angiotensin I-converting enzyme 2 (ACE2) (g). Values are given as mean ± SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

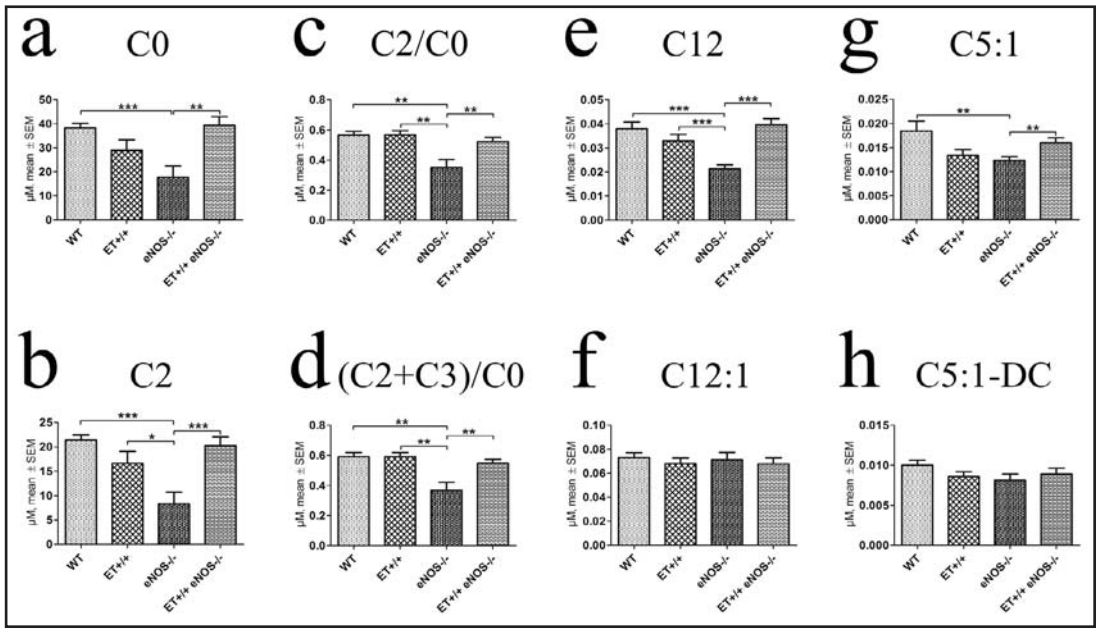


Fig. 5. Plasma levels of free carnitine (a), acetylcarnitine (b), carnitine free/acetylcarnitine ratio (c), acetylcarnitine + propionylcarnitine/carnitine free ratio (d), dodecenoyl-L-carnitines C12 and C12:1 (e and f), tiglyl-L-carnitine (g), glutaconyl-L-carnitine (h). Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Total alkyl-alkyl phosphatidylcholines PCaa showed a rescue effect but total acyl-alkyl-phosphatidylcholines (PCae) did not (Fig. 6g-i).

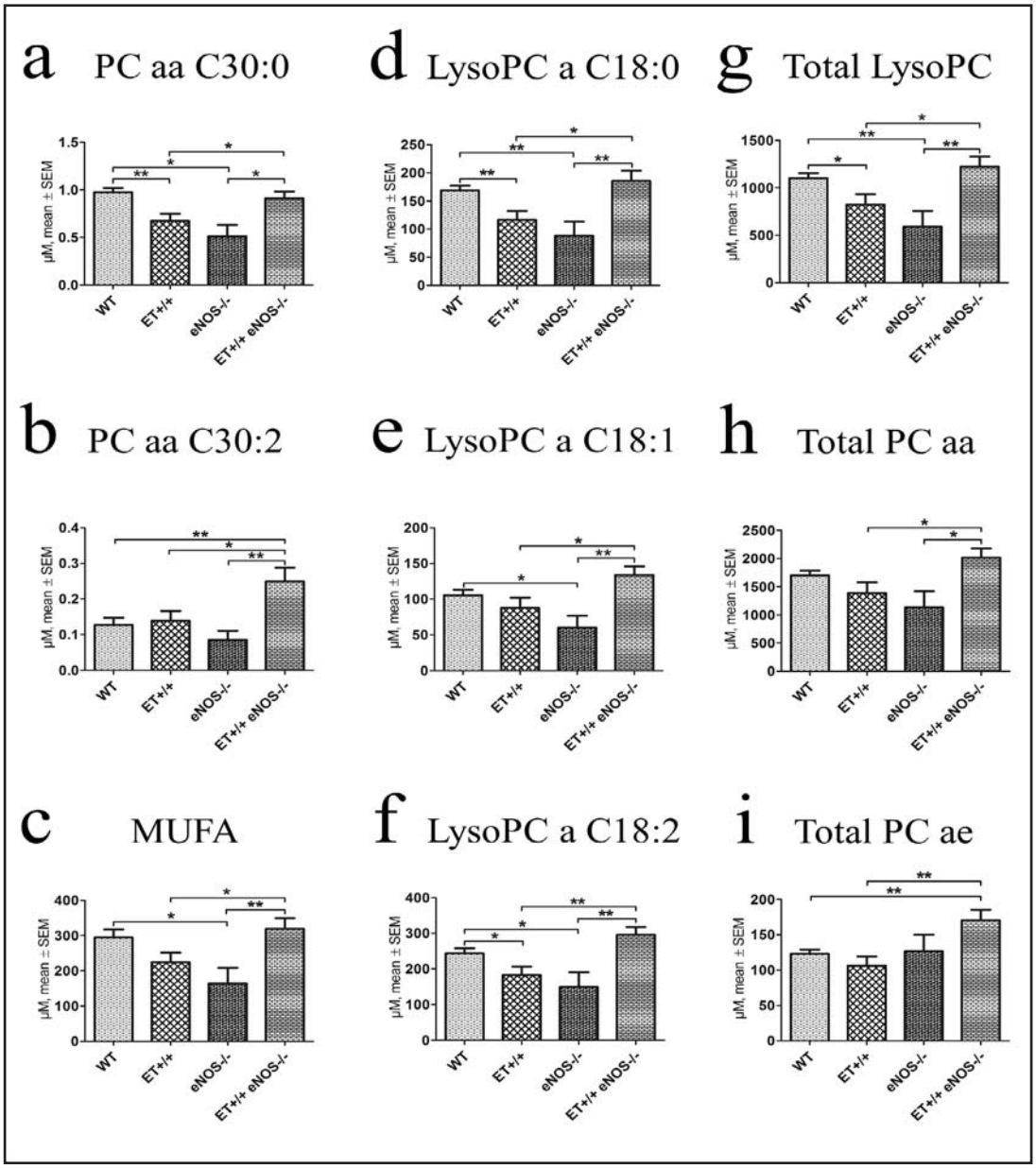


Fig. 6. Plasma levels of phosphatidylcholine diacyls C30:0 (a) and C30:2 (b), mono-unsaturated glycerophosphocholines (c), lysophosphatidylcholine acyls C18:0 (d), C18:1 (e) and C18:2 (f), total lysophosphatidylcholines (g), total diacyl-containing phosphatidylcholines (h) and total acyl-alkyl- containing phosphatidylcholines (i). Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.01$.

Discussion

We compared kidney function and morphology in wild-type mice, ET-1 transgenic (ET+/+) mice, endothelial nitric oxide synthase knockout (eNOS-/-) mice, and ET+/+eNOS-/- crossbred mice to investigate the renal phenotype under conditions of an activated renal ET-1 system in a status of NO deficiency. Our study demonstrates that genetic deficiency of eNOS leads to the development of proteinuria, which is improved by overexpression of ET-1 (Fig. 1). Analysis of genes involved in the glomerular filtration barrier (Fig. 3) indicated that the restoration of the markedly increased protein/albumin excretion in eNOS knockout mice might be due to a normalization of gene expression involved in glomerular basement

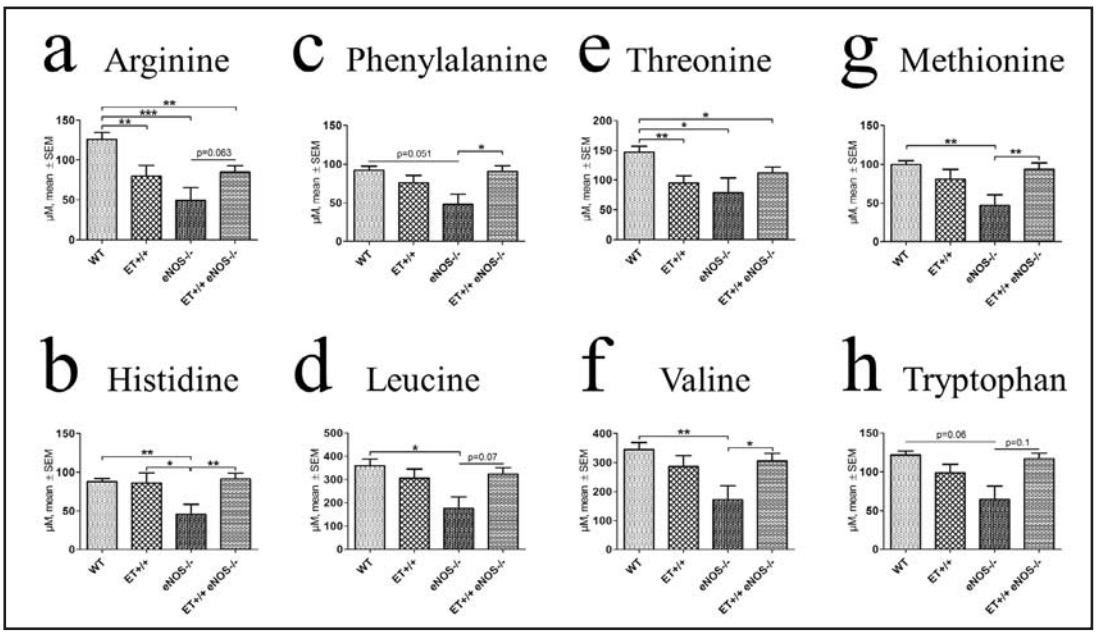


Fig. 7. Plasma levels of arginine (a), histidine (b), phenylalanine (c), leucine (d), threonine (e), valine (f), methionine (g) and tryptophan (h). Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

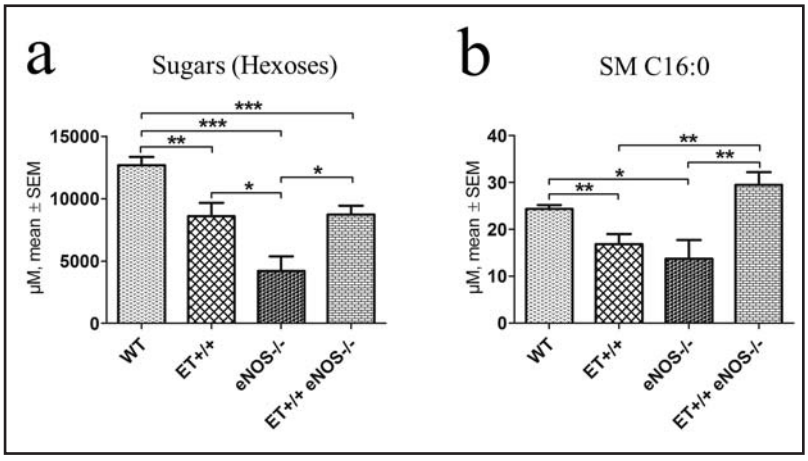


Fig. 8. Plasma levels of hexoses (a) and sphingomyeline C16:0 (b). Values are given as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

integrity. Increased urinary osteopontin excretion, (Fig. 2a) as well as renal expression of genes involved in kidney fibrosis (serpine1, TIMP-1, chemokine ligand 2), and decreased antifibrotic ACE2 [20] gene expression in eNOS-/- mice (see Fig. 4) were likewise improved by ET-1 overexpression. In order to identify potential mechanisms leading to the restoration of the renal phenotype of eNOS-/- mice, we performed metabolomic analyses. These analyses indicated that ET-1 overexpression, in addition to an eNOS knockout is associated with functional recovery of mitochondria (rescue effect in β-oxidation of fatty acids) and an increase in antioxidative properties (normalization of monounsaturated fatty acids levels).

Importantly, the phenotype of the ET-1 transgenic mice did not exhibit a measurable decrease of glomerular filtration rate (GFR) at the age of 9 months, which is in line with earlier reports indicating that kidney function in these animals starts to decrease in later stages (>12 months) [9]. Protein excretion was only seen in eNOS-/- mice [21–23], but not in ET+/+ as reported earlier [9, 24, 25]. ET-1 overexpression on its own does not cause proteinuria, but in cases of underlying proteinuric renal diseases, such as pediatric nephrotic syndrome or diabetic renal disease, an activated ET-system enhances protein excretion [26].

Blood pressure was normal in ET-1+/+ mice, most likely due to an activated NO system

[27]. This hypothesis is in line with findings in ET+/+eNOS-/- crossbred mice, which have higher blood pressure than eNOS-/- mice [7]. However, as seen in our study, in later generations of ET+/+eNOS-/- mice, the blood pressure was similar in eNOS-/- mice and ET+/+eNOS-/- mice [8, 24, 25]. The reasons why the blood pressure phenotype changes slightly over the years is still unknown.

One of the reasons for a leakage of plasma proteins into the urine might be a damaged glomerular filtration barrier (GFB). GFB is composed of three layers: podocytes, glomerular basement membrane (GBM) and endothelial cells. Albumin- and proteinuria can be caused by disturbances of any GFB element [28]. In order to better understand the morphology of GFB damage, we measured expression of genes specific for each of its layers. The GBM is lined by a layer of interdigitating podocyte foot processes from the urinary-space side. Our data do not indicate that podocyte- and endothelials cell specific processes contribute to the significant reduction of the markedly increased protein/albumin excretion in eNOS-/- mice seen in ET+/+eNOS-/- mice (Fig. 3).

The GBM itself is composed of extracellular matrix proteins assembled through an interweaving of type IV collagen with laminins, nidogen, and sulfated proteoglycans [29]. Any changes in the balance of extracellular matrix proteins within GBM may lead to an increased permeability. The eNOS-/- mice were characterized by pronounced changes in expression of GBM associated genes, namely by a down-regulation of Col4α1. Lamb2 and Lamc1 are the genes responsible for synthesis of laminin-521 (α5β2γ1) trimer, one of the main components of GBM [30]. In eNOS-/- mice, the Lamc1 gene was significantly up-regulated ($P < 0.01$ versus WT), while Lamb2 showed a trend towards up-regulation ($P = 0.056$ versus WT). Similar, although less pronounced changes were revealed in ET+/+ mice, whereas in crossbreds GBM associated gene expression was similar to WT (Fig. 3d-f). Mutations in genes encoding Lamb2, Col4α3, Col4α4, and Col4α5 cause glomerular disease in humans as well as in mice [31] which is in line with our findings. Laminins together with other osteopontin binding proteins, including integrins are known to act as osteopontin receptors [32]. Although their interaction in the context of renal dysfunction is not clear yet, it is known that osteopontin is associated with tissue remodeling processes, inflammation and fibrosis [33]. An increase of osteopontin observed in eNOS-/- (Fig. 2) was consistent with an elevation of SBP (Table 1). In addition, we further analyzed renal expression of genes involved in tissue remodeling. In agreement with our assumption, increased urinary osteopontin excretion together with an up-regulation of osteopontin binding proteins Lamb2 and Lamc1 was accompanied by an up-regulation of serpine1, TIMP-1, chemokine (C-C motif) ligand 2 mRNA expression, especially pronounced in eNOS knockouts (Fig. 4). Similar to our findings, Liu *et al*. previously reported that decreased NO levels in the kidney aggravated renal dysfunction, damage, hypertrophy, inflammation, and fibrosis as evidenced by decreased creatinine clearance and increased serum creatinine, blood urea nitrogen and urinary protein levels, accompanied by increases in the expression of the profibrotic genes collagen I, transforming growth factor-β, and tissue inhibitors of metalloproteinases [34]. Therefore, we suggest that the proteinuric phenotype in eNOS knockout mice is caused, at least partially, by an impaired GBM composition, which is improved in crossbred animals (Fig. 3d-f).

Our current data of an improvement of the functional consequences of a systemic eNOS knockout on GFR and protein excretion fit very well with an earlier study showing that eNOS-/-, but not ET+/+eNOS-/- mice, developed cardiac dysfunction manifested in increased end diastolic pressure and time of left ventricular relaxation [8]. Cardioprotective properties of ET-1 overexpression on top of eNOS knockout were explained by reduction of cardiac oxidative stress in these animals [8]. However, these findings are in conflict with several studies employing both ETAR selective and nonselective ET antagonists [35–37]. For instance, Peng et al. reported that in patients with CKD plasma ET-1 levels positively correlated with left ventricular hypertrophy [35]. The opposite effects of ET-1 overproduction in two different settings might be explained by a big heterogeneity within the patient cohort (concomitant chronic diseases, age, race etc. are the factors affecting organ function independently of ET-1 production) in the clinical trial, while our preclinical study was conducted in mice lines

of a known genotype and an identical age. Nakamura et al. stated that the ETAR selective antagonist T-0115 ameliorated glomerulosclerosis, the rise in SBP, and prevented left ventricular hypertrophy in rats with N^G-nitro-l-arginine methyl ester (L-NAME) induced inhibition of NO synthesis [36]. The differences in the results might be explained by the fact that in our study NO deficiency in ET+/+eNOS-/- mice was a life-long feature, whereas in the study by Nakamura et al. NO production was blocked only for a given time frame. In addition, L-NAME-induced NO deficiency may not have resulted in a complete inactivation of eNOS. Moreover, L-NAME may also have blocked the other isoforms of NOS, which was not the case in our model. In the present study, we observed a reduction of urinary protein excretion by ET-1 overexpression on top of eNOS-/- genotype. This is somewhat in contradiction with earlier trials [3, 6, 26], which reported that both ETAR selective and nonselective ET receptor antagonists ameliorate proteinuria. However, in these studies ETAR selective antagonists were shown to have much more pronounced renoprotective effect than nonselective ones. Nevertheless, the selective ETAR antagonist atrasentan showed no effects on protein urinary excretion, SBP, or kidney morphology parameters in uremic rats with 5/6 nephrectomy [38]. The aforementioned effects of ET antagonists were investigated in CKD models such as 5/6 nephrectomy or diabetic nephropathy, however the pathophysiological conditions induced in these models are different in comparison to the model used in our study, in which ET-1 overexpression was not combined with any other insult to the kidney.

The kidney is very active in carnitine metabolism and releases the metabolites into the plasma and urine [39]. The metabolic pattern observed in the ET+/+eNOS-/- animals suggests a functional recovery of mitochondria. This effect is prominent for internal mitochondrial enzymes but not for the carnitine shuttle (CPT-I, EC 2.3.1.21) [40]. Furthermore, the same rescue effect is seen in oxidation of even-numbered fatty acids and overall activity of β-oxidation of fatty acids. Treatment with ET-1 induces the redistribution of eNOS from the plasma membrane to the mitochondria [41] and therefore shall be reflected in overall mitochondrial function. Substitution of rats with L-carnitine has beneficial effects on oxidative damage in the renal cortex of hypertensive rats [42]. It results in an up-regulation of both antioxidant enzymes and eNOS [43]. Desaturation of fatty acid chains in the PC(aa) or carnitines abrogates the differences observed between WT, eNOS -/- and ET +/+, which is in agreement with the observation that saturated fatty acids (e.g. palmitic acids) activate eNOS [44]. In other studies on kidney dysfunction changes in specific lipid concentrations and the underlying mechanisms were reported. In erythrocyte membrane lipid composition monounstaturated fatty acids (MUFA) are increased in non-diabetic patients with continuous ambulatory peritoneal dialysis [45]. In addition, MUFAs are increased in the plasma of hemodialysis patients [46]. MUFAs regulate the reactive oxygen species (ROS) production via the NFκB pathway [47], whereas ROS also induce the Nrf2 antioxidative pathway [48]. Under hyperhomocysteinemia (caused by a methionine-high diet) ROS were shown to reduce NO activity [49]. Lysophosphatidylcholines (LPCs) are more sensitive to desaturation changes as they are composed of only one hydrocarbon chain instead of two present in the PCaas and PCaes. Peroxisomal metabolism of phosphatidylcholines is not affected in any of the models (neither single nor double mutants) included in our study as the levels of PC(ae)s were not affected [50].

Similarly to concentrations of carnitines and lipids, the amino acid levels are normalized in ET+/+eNOS-/- animals. This is of interest as these metabolites are biomarkers of complex diseases like diabetes type 2 and CKD. Arginine is a known precursor of NO [51] and induces oxidative stress through a modulation of NOS activity. Short-term exposure causes an increase, and long term exposure a decrease of NOS activity in human endothelial cells [52]. Metabolism of valine, isoleucine and leucine is reflected as well by isovarelylcarnitine (C5), tiglylcarnitine (C5:1) and glutaconylcarnitine (C5:1-DC) [53, 54]. The C5:1 is listed within a panel of biomarkers patented for the diagnosis of kidney disease [55]. Branched chain amino acids (BCAA, leucine, isoleucine and valine) constitute 40% of all amino acids from daily nutrition intake [56]. Changes in BCAA concentrations are risk biomarkers of diabetes type 2 [57] and accumulated BCAA are linked to congenital heart diseases and heart failure [58].

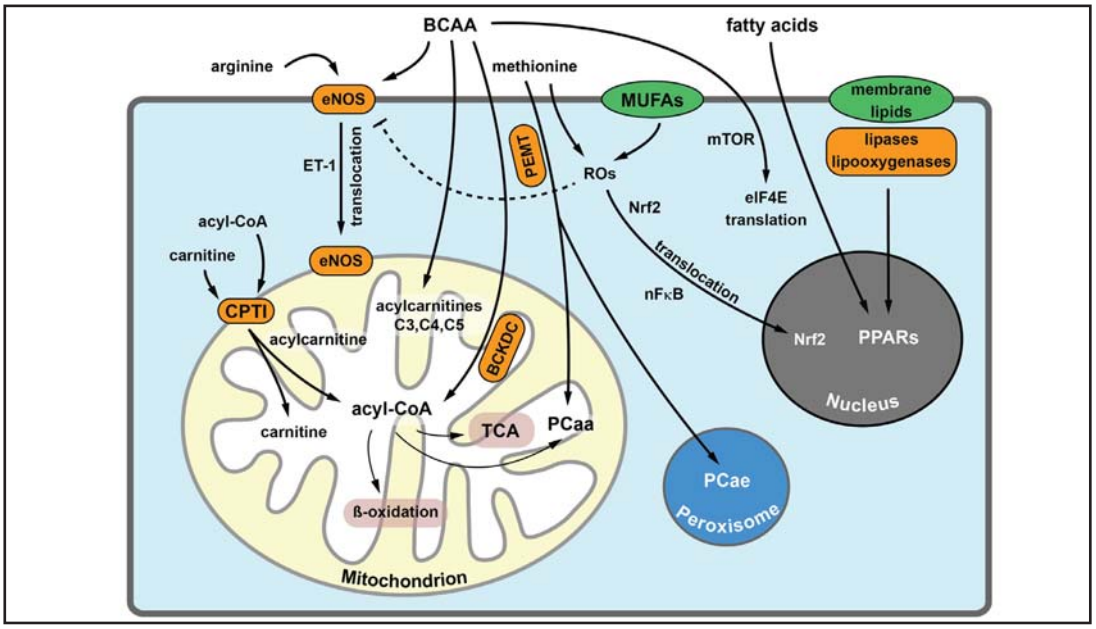


Fig. 9. Hypothesis: ET-1 overexpression on top of eNOS knockout may cause functional recovery of mitochondria in ET+/+eNOS-/- crossbred mice. ET-1 overexpression on top of eNOS knockout is associated with functional recovery of mitochondria (rescue effect in β-oxidation of fatty acids) and increase in antioxidative properties (normalization of monounsaturated fatty acids levels) possibly due to NFκB, Nrf2, and PPAR-alpha upregulation via ET-1 pathway. Pathways are discussed in the text with references therein. Key enzymes are given in orange rounded rectangles. Abbreviations: BCKDC - branched-chain alpha-keto acid dehydrogenase complex, PEMT - phosphatidyl ethanolamine methyltransferase, eNOS- endothelial nitric oxide synthase, CPTI – carnitine palmitoyltransferase I, PPARs- peroxisome proliferator activated receptor, PCae- phosphatidylcholine acylalkyl-chains, PCaa - phosphatidylcholine acylacyl-chains, TCA- tricarboxylic acid cycle, ET-1 – endothelin 1, Nrf2 - nuclear factor (erythroid-derived 2)-like 2, elF4E - eukaryotic translation initiation factor 4E, NFkB - nuclear factor kappa-light-chain-enhancer of activated B cells, mTOR – mammalian target of rapamycin, BCAA – branched chain amino acids, MUFAs – monounsaturated fatty acids, ROs - reactive oxygen species.

Valine but not other BCAAs were reported to reflect CKD [59]. Later studies indicated that patients with CKD showed a decrease in BCAA which might as well be caused by malnutrition [60]. Metabolic exchange of BCAA between different tissues was observed to modulate BCAA metabolism in CKD [61]. BCAA can enter energy generation in tricarboxylic acid cycle (TCA) through the action of branched-chain alpha-keto acid dehydrogenase complex [62]. Branched chain amino acids in dietary supply prolong lifespan through activation of mTOR and eNOS [63]. The BCAA action via mTOR might be independent from insulin through activation of translation via elF4E [64]. eNOS was shown to support both mitochondrial biogenesis and metabolic activity [65]. The restoration effects seen in ET+/+eNOS-/- animals in our study might be due to NFκB, Nrf2, and PPARalpha upregulation via the ET-1 pathway [43, 65]. Our interpretation of the processes is summarized in Fig. 9 and is based on published and our own records.

Our data may have considerable clinical impact. NO deficiency is a condition associated with CKD and heart failure. We have demonstrated that the functional consequences of a lack of eNOS in the kidney, such as impaired GFR and development of proteinuria, are improved by overexpression of ET-1. We suggest that this might be due to a functional recovery of mitochondria (rescue effect in β-oxidation of fatty acids) and increase in antioxidative properties (normalization of monounsaturated fatty acids levels). The current study is in agreement with a previous report showing that eNOS knockout mice develop diastolic

dysfunction which could be restored by ET-1 overexpression in the heart. This study furthermore suggested that cardiac ET-1 overexpression in eNOS deficiency interferes with the regulation of cardiac proteins playing a role in oxidative stress, myocyte contractility, and energy metabolism [8]. Thus, besides the fact that blockade of both the ETA and ETB receptors may lead to water and salt retention [3, 5, 6], our current data further challenges the idea to block the endothelin system in patients with endothelial dysfunction, as it is present in CKD due to diabetic nephropathy.

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Disclosure Statement

The authors have no financial interests relevant to the research

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