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α/β hydrolase 1 is upregulated in D5 dopamine receptor knockout mice and reduces O_2^- production of NADPH oxidase

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ABSTRACT

Renal dopamine receptors have been shown to play a critical role in ROS-dependent hypertension. D5 dopamine receptor deficient (D5−/−) mice are hypertensive and have increased systemic oxidative stress which is manifested in the kidney and the brain. To further investigate the underlying mechanisms of hypertension in D5−/− mice, we used RNA arrays to compare mRNA levels of kidneys from wildtype and D5−/− mice. Our data show, that the mRNA level of α/β hydrolase 1 (ABHD1) is significantly upregulated in D5−/− mice. Additionally, overexpression of ABHD1 in a new established renal proximal tubule cell line reduced the amount of O_2^- produced by the NADPH oxidase. Therefore the upregulation of ABHD1 in D5−/− mice could be an answer to the increased oxidative stress. While oxidative stress is an important factor for the development of hypertension, ABHD1 could play a protective role in the pathogenesis of hypertension.

Hypertension is a common cause of end-stage renal failure and nearly 80% of chronic kidney disease patients develop hypertension. Numerous studies show that renal dopamine receptors are involved in hypertension [1]. The D5 dopamine receptor (D5 DAR) is of special interest because it is constitutively active and has a high affinity for dopamine. Moreover, the D5 DAR locus is linked to human essential hypertension. D5 dopamine receptor deficient (D5−/−) mice have been shown to develop hypertension and increased systemic oxidative stress [2]. Hypertension in D5−/− mice partly attributes to upregulated phospholipase D and NADPH oxidase expression and activity [3]. Additionally, stimulation of the D5 DAR results in an upregulation of antioxidant enzymes.

Oxidative stress is a known prohypertensive factor, caused by reactive oxygen species (ROS) [4]. Under physiological conditions ROS are produced at low concentrations in a controlled manner acting as regulating factors in cell proliferation, differentiation or apoptosis. Increased ROS production results in oxidative stress which leads to damage of lipids, proteins and DNA [5]. Several studies have shown that ROS production is elevated in genetic and essential hypertension, antioxidant capability is decreased in essential hypertension and antioxidant treatment lowers blood pressure. Animal models confirmed these results: it was shown that oxidative stress can cause hypertension, oxidative stress causes renal damage, and that reversal of oxidative stress with antioxidants decreases blood pressure [6,7].

A primary source of ROS in vascular tissues and kidney is NADPH oxidase, a multi-subunit enzyme that catalyzes the production of O_2^- [8]. Discrepancies in NADPH oxidase expression and activity are closely associated with hypertension [9].

To identify D5 DAR dependent genes that might be involved in the pathogenesis of hypertension in D5−/− mice we compared mRNA levels of kidneys from wildtype (D5+/+) and D5−/− mice.

Subjects and methods

Reagents. Culture media and antibiotics were obtained from PAA (Cölbe, Germany) and Invitrogen (Mannheim, Germany). Reagents were from Sigma–Aldrich (Deisenhofen, Germany).

Mice. Proximal tubules for establishment of the M-PT cell line where obtained from the immorto H-2Kb-tsA58 mice [10]. Generation and characterization of D5−/− mice were previously described [2]. We exclusively used >20th-generation littermate mice. Mice were genotyped and treated in accordance with NIH guidelines for ethical treatment and handling of animals in research and in accordance to German animal protection laws.

Antibodies. Monoclonal antibodies directed against β-tubulin, vinculin and the Flag-tag were purchased from Sigma–Aldrich, monoclonal antibody against ZO-1 was purchased from Invitrogen.

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Fluorochrome-conjugated secondary antibodies as well as phalloidin coupled to Alexa 594 were purchased from Molecular Probes (Göttingen, Germany). Horseradish peroxidase-conjugated secondary antibody was purchased from Dianova (Hamburg, Germany).

Plasmids. cDNA of murine ABHD1 was amplified by PCR using a M-PT cDNA bank and the following primers: sense – 5′ GCGACGCGTATGGAATACCCCTACACAACCAAGATGCTG 3′, antisense – 5′ GCATGCATGCGGCCGCTCAGCTCTTTCCATCTTCAGGAGTGAG 3′. The fragment was subcloned into the pcDNA3.1 (+) vector (Invitrogen), in the pIRES-EGFP vector and in a modified pLSXN vector (Clontech, Saint-Germain-en-Laye, France) using standard techniques.

Tissue preparation. To obtain kidneys or proximal tubules, mice were sacrificed and perfused with heparin-containing PBS. Kidneys were recovered, the capsules were stripped away and a small piece of each kidney was used for RNA preparation. PT segments were isolated as previously described [11].

Microarray. Total RNA isolation, the NIA 15K Mouse cDNA microarray-analysis as well as the hybridization were performed as previously described. Twenty-four micrograms of either tissue or reference-RNA was transcribed into cDNA in the presence of Cy3- and Cy5-labeled dUTP, using Superscript II reverse transcriptase (Invitrogen). All other steps were performed as described recently [12–14].

Cell culture. For establishment of an immortalized proximal tubule cell line, isolated tubule segments from H-2Kb-tsA58 mice were seeded on collagen-I coated dishes and grown in Ham's F12/DMEM medium containing L-glutamine supplemented with 2% fetal calf serum (Biochrom, Berlin, Germany), 20 mM HEPES, 5 μg/L EGF (Invitrogen), 5 mg/L insulin–transferrin–sodium–selenit supplement, 50 nM dexametasone, 1 nM triiodotyronine, and 100 U/ml γ-interferon (Roche, Mannheim, Germany). After 5–10 days of primary culture single cell clones were replated and incubated at 33 °C in medium containing 10 U/ml γ-interferon. Following characterization one clone was chosen for further cultivation. To induce differentiation, cells were maintained at 37 °C without supplementation with γ-interferon. HEK293T cells were cultured in DMEM with supplements [15]. Cells were passaged to fresh media every second day or 24–48 h before the experiments.

Generation of ABHD1 overexpressing cell line. Retroviral transduction of M-PT cells was performed as described before [15]. Transgene expression was monitored by reverse transcription PCR (RT-PCR) and Western blot analysis. For further cultivation of stable transduced M-PT Flag-ABHD1 cells, the medium was supplemented with 75 μg/ml neomycin.

Ultrastructural studies. M-PT cells were seeded on Transwell membrane chambers (Greiner bio-one, Frickenhausen, Germany) and cultivated until differentiation. The preparation procedure for scanning electron microscopy has been described elsewhere [16]. Imaging was performed with an S-450 scanning electron microscope (Hitachi Ltd., Tokyo, Japan) with secondary electrons at 20 kV acceleration voltage at room temperature. Micrographs were recorded from a high-resolution cathode-ray tube using negative film (Agfapan, APX 100; Agfa-Geraert AG, Leverkusen, Germany).

PCR. Total RNA isolation of M-PT cells was performed with Molzym RNA kit (Omni Life Science, Raynham, MA, USA) according to the manufacturer's instruction. cDNA first-strand synthesis was performed using the MMLV reverse transcriptase (Promega). One microliter of cDNA first-strand reaction mixture was then subjected to a 25-μl PCR reaction with 10 U GoTaq DNA polymerase (Promega, primer details see Table 1, Supplementary data). PCR products were analyzed by agarose gel electrophoresis and DNA sequencing.

Total RNA isolation of kidneys and proximal tubules for quantitative Real Time PCR (qPCR) was performed using the RNAeasy Kit (Qiagen, Hilden, Germany). One microgram of RNA was transcribed using the Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's instruction. qPCR was performed using an ABI 7900HT-Sequence Detection System, with SYBR Green Master Mix (Eurogentec, Cologne, Germany). Details of primers and conditions are given in Table 2, Supplementary data.

Lysate preparation. Cells were either scraped directly into sample buffer and boiled or into lysis buffer (LB, 20 mM Tris–HCl (pH 7.5), 25 mM NaCl, 50 mM NaF, 15 mM $Na_4P_2O_7$, 1% Triton X-100, 1 mM EDTA, *Complete* Protease Inhibitor Mix (Roche)) and lysed by 10 passes through a 26-gauge needle. LB-lysates were centrifuged at 14,000*g* and supernatants were stored at −20 °C.

Western blotting. Western blotting was performed using standard techniques [17]. Finally, the membranes were washed and developed using the Lumilight chemiluminescence detection kit (Roche) and an X-ray film developer.

Immunofluorescence. M-PT cells were seeded on collagen-I coated coverslides until differentiation and then fixed in 4% paraformaldehyde in PBS. Proteins were detected by subsequent incubation of the cells with primary and fluorochrome-coupled secondary antibodies. After washing, specimen were mounted in Vectastain mounting medium (Vector Laboratories, Burlingame, CA, USA) and analyzed on a fluorescence microscope. Substitution of primary antibodies by non-immune serum served as negative control.

NADPH oxidase activity. Measurement of superoxide anion production was performed as described recently [18]. Bioluminescence was measured with Lumat LB9501 (Berthold GmbH, Bad Wildbad, Germany). Cells were subsequently lysed and total protein concentration was quantified by BCA test. To calculate the amount of superoxide produced, ROS production was normalized using the total protein concentration. Total counts were analyzed by integrating the area under the signal curve. These values were compared to a standard curve that was generated by using xanthine/xanthine oxidase.

Statistical analysis. Data are expressed as means ± SD; Student's *t* test was used for a two-group comparison, $p < 0.05$ was considered statistically significant.

Results

Expression analysis D5−/− versus wildtype mice

To identify differentially regulated genes in D5−/− mice, we performed microarrays with the NIA 15K mouse chip which contains at least 15,000 genes [13]. Data analysis of three wildtype and three D5 dopamine receptor deficient mice kidneys revealed that up to 3.5% of all genes are putatively regulated in D5−/− mice compared with D5+/+ mice ($p < 0.01$). We selected genes that were at least 1.5-fold up- or downregulated with a p-value < 0.05. According to these criteria we identified 27 genes. We grouped the genes in five functional categories: metabolism ($n = 10$), cell cycle ($n = 8$), signaling ($n = 4$), adhesion ($n = 2$) and unknown function ($n = 3$). The strongest regulated gene was the gene for the murine α/β hydrolase 1 (ABHD1) which was more than 5-fold upregulated in D5−/− mice compared to D5+/+ mice (Table 3, Supplementary data). To confirm the microarray data, we performed qPCR experiments. We found that ABHD1 mRNA expression in kidneys of D5−/− mice was strongly upregulated compared to kidneys of D5+/+ mice (Fig. 1A). As the major site of dopamine production in the nephron is the proximal tubule, we performed qPCR with RNA from proximal tubules of D5−/− and D5+/+ mice, too. ABHD1 expression was significantly upregulated in proximal tubules of D5−/− mice (Fig. 1B). We tried to generate a specific ABHD1 antibody to confirm the data at the protein level, however all strategies to produce an anti-ABHD1 antibody failed.

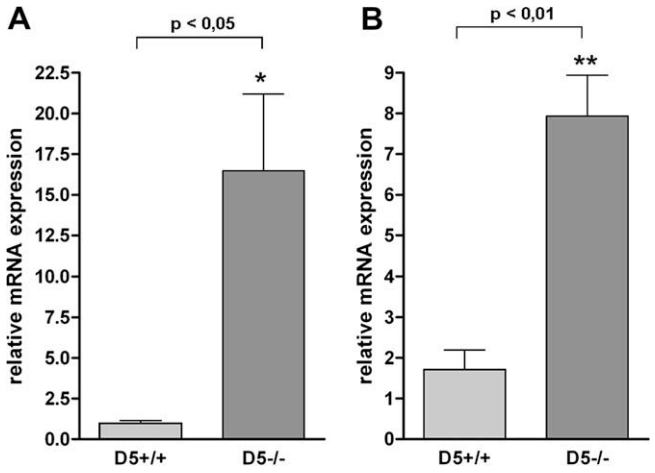

Fig. 1. (A) Relative mRNA expression levels of ABHD1 in kidneys of wildtype and D5−/− mice. (B) Relative mRNA expression levels of ABHD1 in proximal tubules of wildtype and D5−/− mice. Results (means ± SD) are from three pairs of kidneys, each evaluated at least in duplicate. $^{*}p < 0.05$; $^{**}p < 0.01$.

Generation and characterization of a new murine immortalized proximal tubule cell line

The D5 DAR is expressed in proximal tubules and dopamine synthesis takes place in proximal tubule cells. Additionally, the reabsorption of proximal tubule cells plays a major role in natriuresis. For examining the functional role of ABHD1 upregulation we generated a new murine conditionally immortalized proximal tubule cell line (M-PT) expressing the D5 dopamine receptor.

The new M-PT cell line harbors SV40 strain tsA58 early region coding sequences under the control of the mouse H-2K^{b} class 1 promoter [10]. Therefore, M-PT cells proliferate under permissive conditions whereas under non-permissive conditions (37 °C w/o Interferon) proliferation was reduced. Fig. 2 A shows the typical low density growth of our M-PT cells. M-PT cells harbor a small cell body until they get in contact with each other (left panel). At high density, M-PT cells show a cobblestone, epithelium like morphology and tent to grow over each other (Fig. 2A, right panel). On EM, differentiated M-PT cells show a well developed brush border at the apical side with a typical microvilli structure indicating cellular polarity (Fig. 2B). In immunofluorescence studies, M-PT cells show an actin cytoskeleton with a distinct accumulation of actin stress fibers in the periphery and a huge nucleus compared to the overall cell body size. Tubulin fibers accumulate around the nucleus with a crosslinking throughout the cell body and, the ZO-1 stained cell–cell contacts display the tight cobblestone formation of M-PT cells. The vinculin staining shows that M-PT cells build up several focal adhesions which vary in number and size with a focus on the periphery, especially in cell protrusions (Supplementary data Fig. S1A–D).

RT-PCR was used to screen for the mRNA expression of kidney and proximal tubule specific markers (Fig. 2C). We detect the

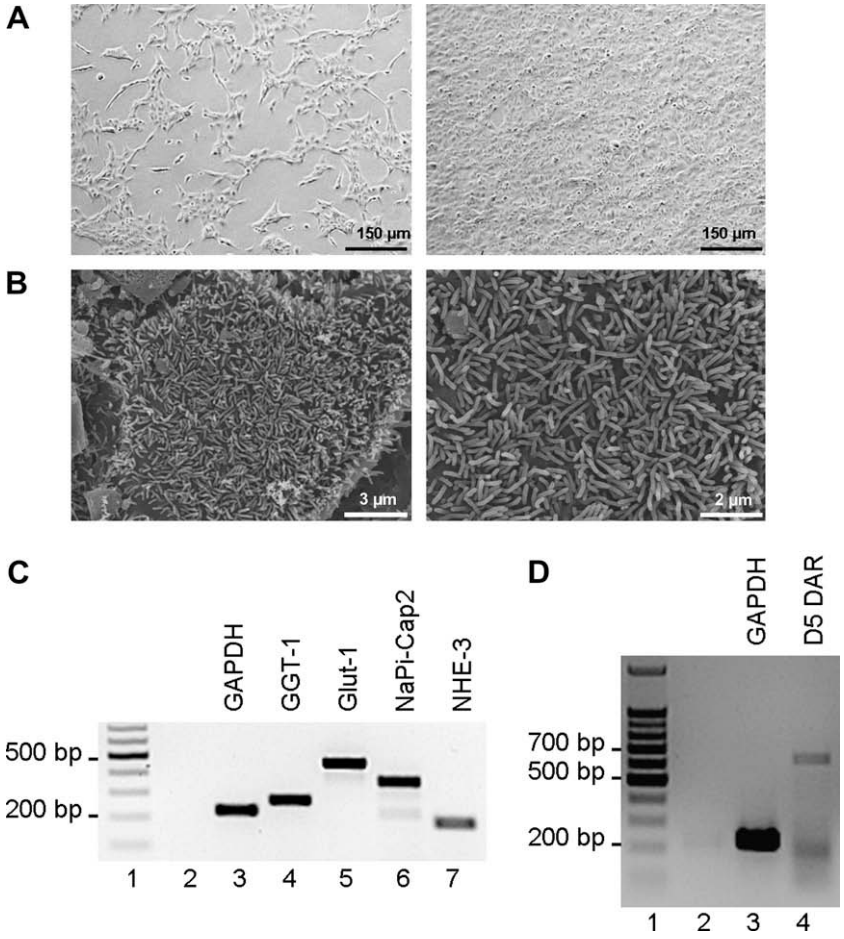

Fig. 2. (A) Low and high density cultured M-PT cells. (B) Scanning EM images of differentiated M-PT cells, showing the brush border side (left) with microvillis at higher magnification (right). (C) RT-PCR analysis demonstrating the expression of kidney and proximal tubule marker proteins in M-PT cells; lane 1: marker, lane 2: GAPDH negative control, lane 3: GAPDH, lane 4: GGT-1, lane 5: Glut-1, lane 6: NaPi-Cap2, lane 7: NHE-3. (D) RT-PCR analysis demonstrating the expression of the D5 DAR in M-PT cells; lane 1: DNA marker, lane 2: GAPDH negative control, lane 3: GAPDH, lane 4: D5 dopamine receptor.

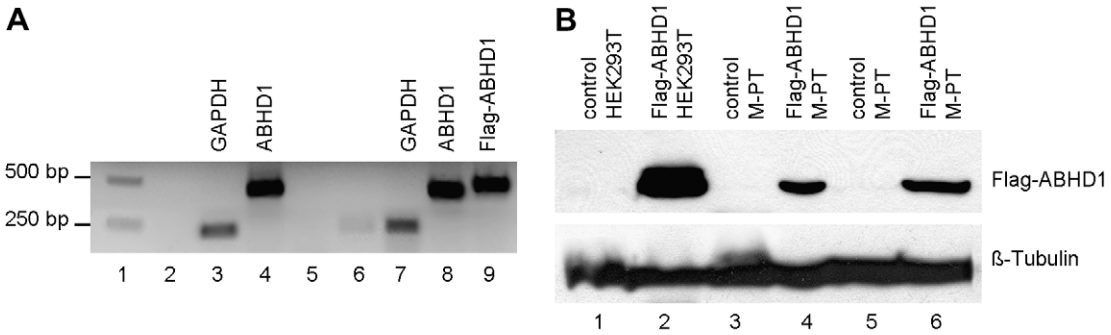

Fig. 3. (A) RT-PCR analysis of endogenous ABHD1 and Flag-ABHD1 in stable transduced M-PT cells; lane 1: DNA marker, lane 2: GAPDH negative control wildtype M-PT cells, lane 3: GAPDH of wildtype M-PT cells, lane 4: endogenous ABHD1 of wildtype M-PT cells, lane 5 showing that no Flag-ABHD1 is expressed in wildtype M-PT cells, lane 6: GAPDH negative control of transduced M-PT cells, lane 7: GAPDH of transduced M-PT cells, lane 8: endogenous ABHD1 of transduced M-PT cells, lane 9: Flag-ABHD1 in transduced M-PT cells. (B) Flag-ABHD1 protein expression in stable transduced M-PT cells over a period of 15 passages; lane 1: untransfected HEK293T cells, lane 2: Flag-ABHD1 expression in transiently transfected HEK293T cells, lane 3 and 5 showing that no Flag-ABHD1 is expressed in wildtype M-PT cells, lane 4: Flag-ABHD1 expression in transduced M-PT cells at passage 21, lane 6: Flag-ABHD1 expression in transduced M-PT cells at passage 36.

mRNA expression of the gamma-glutamyltranspeptidase 1 (GGT-1), the facilitated glucose transporter 1 (Glut-1), natrium-phosphate cotransporter IIa C-terminal-associated protein 2 (NaPi-Cap2) and sodium–proton exchanger type 3 (NHE-3, Fig. 2C). mRNA expression of these markers was stable throughout 30 days of differentiation (data not shown). Additionally, the expression of sodium–proton exchanger 1, sodium–calcium exchanger 1 (NCX-1), sodium phosphate cotransporter 2 (NaPi2), organic cationtransporter 1–3 (OCT1–3) and the angiotensin II receptor type 1 (AT1R) could be detected (data not shown). Finally, we showed the expression of the D5 DAR (Fig. 2D).

Generation of an ABHD1 overexpressing M-PT cell line

The M-PT cell line was used for retroviral transduction to generate an ABHD1 stable overexpressing cell line (Fig. 3). Therefore we used a plasmid coding for full length ABHD1 including an aminoterminal Flag-tag. The overexpression of Flag-tagged ABHD1 as well as endogenous ABHD1 was proven with RT-PCR (Fig. 3A) and western blotting (Fig. 3B). Fig. 3 also shows that the expression of Flag-ABHD1 is stable throughout 15 passages. Stable ABHD1 overexpressing M-PT cells appear normal in size and proliferation/differentiation characteristics including the expression of marker proteins (data not shown).

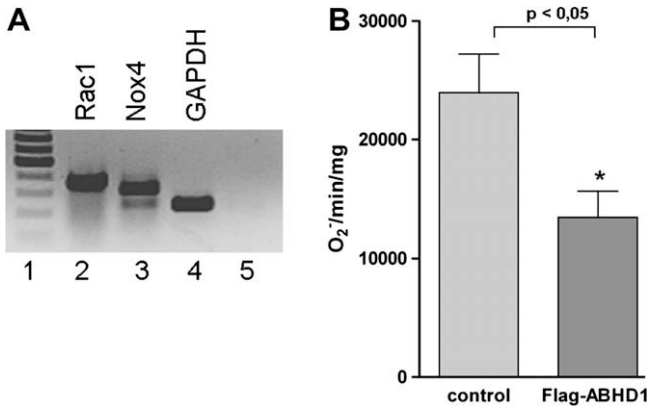

Fig. 4. (A) RT-PCR analysis demonstrating the expression of NADPH oxidase subunits in wildtype M-PT cells; lane 1: DNA marker, lane 2–5: Nox-4, Rac1, GAPDH, and GAPDH negative control. (B) Effect of ABHD1 overexpression on O_2^- production by NADPH oxidase. NADPH oxidase assays were performed either with empty vector transduced M-PT cells (□) or Flag-ABHD1 stable transduced M-PT cells (■). Results (means ± SD) are from 12 samples per cell line, $^*p < 0.05$.

NADPH oxidase assays

To reveal if ABHD1 has an effect on O_2^- production, we measured NADPH oxidase activity in Flag-ABHD1 overexpressing M-PT cells. However, before NADPH oxidase assays were performed, we checked if M-PT cells express the NADPH oxidase subunit Nox-4 and the small G-protein Rac-1. Indeed, the kidney specific subunit Nox-4 as well as Rac-1, which is necessary for assembly of the NADPH oxidase complex, are expressed (Fig. 4A) indicating that M-PT cells harbor a functional NADPH oxidase complex. Hence, we compare the NADPH oxidase driven O_2^- production in empty vector transduced M-PT cells with O_2^- production in Flag-ABHD1 overexpressing M-PT cells. Fig. 4B shows that O_2^- production in Flag-ABHD1 overexpressing M-PT cells was significantly reduced to one third compared to the control. These data show, that overexpression of ABHD1 has an inhibiting effect on NADPH oxidase driven O_2^- production.

Discussion

The aim of this study was to identify differentially regulated genes in D5 dopamine receptor knock out mice. We identified 27 genes that are differentially regulated in D5−/− mice compared to wildtype mice. The strongest regulated gene was ABHD1 which encodes the α/β hydrolase 1, a protein which function is so far unknown [19].

The α/β hydrolase 1 was first discovered in lung emphysema and contains an α/β hydrolase fold, a protein fold that is common to several hydrolytic enzymes [19,20]. ABHD1 is ubiquitously expressed in murine and human tissues [20]. Several α/β hydrolase protein family relations to oxidative stress have been reported. ABHD2, one of the two paralogues of ABHD1 in mice, plays a role in stress response and its expression increases during differentiation from monocytes to macrophages [21]. This could due to the beginning activity of phagocytic NADPH oxidase during differentiation of macrophages. Furthermore, the closest ABHD1 related protein concerning the protein structure is *Pseudomonas fluorescens* chloroperoxidase [19], a protein with antioxidative capability whose expression is induced by ROS [22]. Additionally, several studies reported that the mammalian soluble epoxide hydrolase (sEH), a key α/β hydrolase fold family enzyme is implicated in oxidative stress and blood pressure regulation [23]. Therefore, we speculated that ABHD1 is directly involved in ROS regulation, too. To further investigate this issue, we first generated a new murine immortalized proximal tubule cell line derived form the transgenic immorto H-2Kb-tsA58 mouse [10]. We chose proximal tubule cells with regard to the established attendance of proximal

tubule cells in dopamine production and blood pressure regulation [24–26]. A main advantage of our cell line represents the induction of differentiation. The combination of temperature and addition of γ-interferon assures the development of a proximal tubule phenotype without cancerous growth. M-PT cells exhibit common features of proximal tubule cells like cuboid shape, the formation of a completely developed brush border and the expression of proximal tubule marker proteins. Thus, we used this cell line and generate a stable ABHD1 overexpressing cell line. Previous studies showed that NADPH oxidase expression and activity in D5−/− mice is increased leading to oxidative stress which in part contributes to the development of hypertension. For this reasons, we performed NADPH oxidase assays to determine the effect of ABHD1 overexpression on NADPH oxidase-based ROS production. The NADPH oxidase represents a main source of ROS in the kidney [8] and abnormalities of NADPH oxidase expression and activity are often associated with hypertension [9,27]. The NADPH oxidase assays demonstrate that the overexpression of ABHD1 significantly reduces the amount of O_2^- produced by the NADPH oxidase. Whether ABHD1 directly metabolizes O_2^- or catalyzes its metabolization needs to be clarified but our data implicate that ABHD1, like its closely related protein chloroperoxidase, has a protective role during oxidative stress.

We suggest that upregulation of ABHD1 in D5−/− mice may contribute to the defense mechanism against oxidative stress. Recent studies suggest that D5 dopamine signaling events themselves lead to an antioxidant response. The D5 DAR inhibits oxidative stress in vascular smooth muscle cells through activation of PKA as well as suppression of phospholipase D (PLD) and PKC [28]. Additionally, Yang et al. demonstrate that stimulation of the D5 DAR inhibits PLD2 activity which product activates NADPH oxidase, and leads to an antioxidant response that is mediated by inhibition of NADPH oxidase activity and expression [3]. These data suggest that the increased blood pressure in D5−/− mice is partly due to an imbalance of ROS metabolism. Thus, the upregulation of ABHD1 could be a response to the increased oxidative stress in kidneys of D5−/− mice. Weather this upregulation indeed is an effect of the increased oxidative stress in D5−/− mice or perhaps directly depends on a missing regulation by the D5 DAR needs to be further investigated.

In summary, we have found 27 differentially expressed genes including highest ABHD1 which was significantly upregulated in kidneys as well as proximal tubules of D5−/− mice. By using our new M-PT cell line we demonstrate that overexpression of ABHD1 significantly reduced NADPH oxidase-based O_2^- production, suggesting that ABHD1 has a protective role during damage caused by ROS.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.12.008.

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