Kidney injury by cyclosporine A is aggravated in heme oxygenase-1 deficient mice and involves regulation of microRNAs

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Cyclosporine A (CsA), a widely used immunosuppressive drug, exerts nephrotoxic activities, as demonstrated by increased tubulointerstitial fibrosis, inflammation and podocyte damage. Recently, a number of microRNAs expressed in the kidney have been reported to be elevated during renal damage. Our aim was to investigate the effect of CsA on selected microRNAs in the mouse kidney after CsA treatment. Moreover, as heme oxygenase-1 (HO-1, encoded by the *Hmox1* gene) was shown to play a protective role during kidney disorders, we assessed whether HO-1 deficiency *in vivo* influences the CsA-regulated microRNAs' expression. We have observed that the pro-fibrotic miR-21 and pro-apoptotic miR-34a expression was upregulated in kidneys of HO-1 deficient mice and it was further enhanced by CsA. Concomitantly, the level of anti-fibrotic microRNAs, belonging to miR-29 and miR-200 families, was down-regulated after CsA treatment. Generally, *Hmox1* knock-out (*Hmox1*^{-/-}) animals were more susceptible to CsA treatment, as the mortality rate was 4 out of 9 *Hmox1*^{-/-} mice, and increased fibrosis (*Tgfb2*, *Pai1*), inflammation (*Il6*) and apoptosis (*Cdkn1a-p21*) were noticed in the HO-1 deficient kidneys. In summary, our data demonstrate that CsA induces significant changes in the expression of renal microRNAs and emphasize HO-1 deficiency as an important factor contributing to the CsA-mediated renal toxicity.

INTRODUCTION

Cyclosporine A (CsA), a calcineurin inhibitor, is one of the most effective and widely used immunosuppressants in solid organ transplantation and autoimmune diseases; however, it exerts strong nephrotoxic effects. Inflammation, apoptosis, and fibrosis associated with oxidative stress play a pivotal role in producing structural and functional kidney impairment in CsA-induced renal injury (Bennett *et al.*, 1996; Camilleri *et al.*, 2016; Naesens *et al.*, 2009). Recently, modulation of microRNAs' expression has been suggested as a possible new mechanism of action of nephrotoxic agents.

Previously, we have observed increased expression of miR-21 and miR-34a in the mouse kidney after ochratoxin A (OTA) stimulation, a factor with known nephrotoxic activity (Loboda *et al.*, 2017a; Loboda *et al.*, 2017b). The role of microRNAs in CsA nephrotoxicity has been also evaluated in other recent studies showing, among others, up-regulation of pro-fibrotic miR-21 both, *in vitro* (Chen *et al.*, 2015) and *in vivo* (Gooch *et al.*, 2017).

Heme oxygenase-1 (HO-1, encoded by the *Hmox1* gene) not only catalyzes conversion of heme to biliverdin, carbon monoxide and iron ions, but it also exerts anti-inflammatory, anti-apoptotic and anti-oxidant properties (Loboda *et al.*, 2016a). The level of HO-1 expression in human population can vary because of the known *HMOX1* promoter polymorphism – the number of guanosine thymidine dinucleotide $(GT)_n$ repeats in the promoter region is inversely associated with *HMOX1* mRNA expression and HO enzyme activity (Loboda *et al.*, 2008; Taha *et al.*, 2010). Importantly, HO-1 was shown to play a pivotal role in the kidney protection against harmful insults in several models of kidney disorders, including unilateral ureteral obstruction (UUO) (Correa-Costa *et al.*, 2010), diabetic induced-glomerular injury (Elmarakby *et al.*, 2012) and transplant rejection (Li *et al.*, 2011). We have already demonstrated that HO-1 protects against OTA-induced nephrotoxicity, as lack of HO-1 increased inflammation, apoptosis and fibrosis, whereas its induction with cobalt protoporphyrin (CoPPIX) halted OTA toxicity (Loboda *et al.*, 2017a).

The importance of HO-1 in CsA-induced kidney failure was already suggested (Shin *et al.*, 2010); however, the involvement of microRNAs in such a process has not been evaluated until now. However, we have previously found that HO-1 is a regulator of microRNAs expression and biogenesis in satellite cells (Kozakowska *et al.*, 2012), it controls miR-206-dependent myogenic program in rhabdomyosarcoma (Ciesla *et al.*, 2016) and modulates miR-378 effect in human non-small cell lung carcinoma (NSCLC) development (Skrzypek *et al.*, 2013). More importantly, there is a cross-talk between HO-1 and microRNAs in the kidney cells (Stachurska *et al.*, 2013).

As HO-1 may regulate the expression of microRNAs and it may exert protective effect against CsA-induced kidney damage, we aimed to investigate a possible involvement of microRNAs in CsA toxicity. We used *Hmox1* knock-out mice in order to elucidate the role of HO-1 in the observed effects. For our study, we decided to use a relatively low dose of CsA (15 mg/kg/bw), based on the literature data and the fact that

Hmox1 knock-out animals are generally more sensitive to various stressors. According to the work by Yadav *et al.*, daily intraperitoneal injection of CsA (15 mg/kg/day for 30 days) caused renal dysfunction in *Tmbim6* knock-out mice (Yadav *et al.*, 2015). In another study, various concentrations of CsA (7.5–30 mg/kg/day injected i.p for 4 weeks) were used and already the lowest dose was able to exert a profibrotic effect (Lim *et al.*, 2012). In conclusion, we have demonstrated that microRNAs affecting such processes as fibrosis and apoptosis might be regulated by CsA and, of importance, the effect of immunosuppressant is accelerated in HO-1 deficient mice.

MATERIALS AND METHODS

Ethics statement. All animal experiments were performed in accordance with the national and European regulations upon approval by the First Local Ethical Committee on Animal Testing at the Jagiellonian University in Krakow. Animals were maintained under a constant 12 h dark/light cycle at the environmental temperature of $22 \pm 2°C$, and were provided with normal laboratory pellet diet and water *ad libitum*.

Animals. Experiments were performed on C57BL/6 × FVB mice with normal level of *Hmox1* (*Hmox1$^{+/+}$*) and on littermate mice lacking both *Hmox1* alleles (*Hmox1$^{-/-}$*), of both sexes. The mice were generated from the *Hmox1$^{+/-}$* breeding pairs kindly gifted by Dr. Anupam Agarwal (Birmingham, AL, USA). Animals were genotyped three weeks after birth by PCR using tail DNA, as described previously (Poss & Tonegawa, 1997).

Experimental procedure. 6-month-old mice fed a low salt diet (Na$^+$ 0.02%) were injected every other day for 19 days intraperitoneally (i.p.) with 15 mg/kg bw CsA dissolved in ethanol and diluted in olive oil, producing a working solution (10 mg/ml). As a control, olive oil with ethanol (10%) was used. On the last day of experiment, the mice were sacrificed and blood and kidneys were taken for the downstream analysis.

Analysis of renal and liver markers. Serum level of creatinine, blood urea nitrogen (BUN), alanine *aminotransferase* (ALAT) and aspartate *aminotransferase* (ASPAT) were determined in blood by using a standard commercial kit (Spotchem Arkray) according to the manufacturer's instructions with the Spotchem EZ SP-4430 analyzer.

Reverse Transcription and quantitative real-time PCR. Total RNA was isolated by using a modified Chomczynski and Sacchi protocol (Chomczynski and Sacchi, 1987). Kidney fragments were homogenized in 1 ml of QIAzol Total RNA Isolation Reagent by using a TissueLyser (Qiagen). Then, samples were subjected to chloroform extraction and isopropanol precipitation. RNA pellets were dissolved in nuclease-free water and subsequently RNA concentration and purity were determined spectrophotometrically by using NanoDrop 1000 (Thermo Fisher Scientific). RevertAid Reverse Transcriptase (Thermo Fisher Scientific) or NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen) were used for reverse transcription, as described earlier (Stachurska *et al.*, 2013). Quantitative real-time PCR was done with the use of SYBR Green PCR Master Mix (Sigma-Aldrich), specific reverse and forward primers, and 30 ng of cDNA, utilizing StepOnePlus™ Real-Time PCR System (Applied Biosystems). *Eef2* (encoding eukaryotic translation elongation factor 2, EF-2) and U6 snRNA were used to normalize the expression of examined mRNAs and microRNAs, respectively.

Preparation of the tissue lysates. Fragments of kidney isolated from each animal were homogenized by using an automatic Tissue Lyser (Qiagen) in ice-cold PBS containing 1% Triton X-100 and protease inhibitors (1 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Then, the samples were incubated for 30 min on ice and centrifuged ($12\,000 \times g$, 10 min, 4°C). Clear supernatants were collected and total protein concentration was determined by the bicinchoninic acid protein assay kit (Sigma-Aldrich), according to the vendor's protocol. Kidney lysates were subjected to luminex analysis, ELISA and/or Western blotting analysis.

Luminex analysis. Kidney lysates, as well as mouse serum, were used for determination of the cytokine content using multiplex Luminex technology. The concentration of interleukin (IL)-6 and plasminogen activator inhibitor-1 (PAI-1) was measured according to the manufacturer's instructions (Millipore, Billerica, MA, USA). Data were analyzed by using a Flexmap 3D instrument and Luminex xPONENT® 4.0 Software and Luminex Analyst Program (Luminex Corporation, Austin, Texas, USA).

Western blotting. Western blotting was performed as described previously (Mucha *et al.*, 2018). Membranes were probed with primary antibodies against HO-1 (ADI-SPA-894, Enzo Life Sciences) and α-tubulin (CP06, Calbiochem), overnight at 4°C. Antibodies were diluted in a blocking buffer (0.1% Tween20 in TBS with 5% nonfat dry milk). HRP activity was detected using Immobilon Western Chemiluminescent HRP Substrate (WBKLS0500 EMD Millipore).

ELISA for HO-1. HO-1 protein quantification in the kidney lysates was performed by using a Mouse Heme Oxygenase 1 ELISA Kit (ab204524, Abcam) according to the manufacturer's instructions.

Masson's trichrome staining. Paraffin-embedded kidney sections (6 μm) were sliced, deparaffinized and rehydrated. Kidney fibrosis was assessed with the Masson's trichrome stain (Sigma-Aldrich). Results were observed under light microscopy. Evaluation of fibrosis was performed by a person that was blind to the mice genotype and treatment, using the following assessment – 0 – no signs of collagen deposition; 1 – any sign of collagen deposition; 2 – clearly visible collagen deposition; 3 – collagen deposition on half of field of view; 4 – fibrosis took up more than half of field of view.

Statistical analysis. Data are presented as mean ± standard error (SEM). Statistical assessment was done by analysis of variance (ANOVA), followed by a Bonferroni post-hoc test for multiple comparisons, or with Student's t-test for two group comparisons. Differences were accepted as statistically significant at $p < 0.05$.

RESULTS

CsA exerts nephrotoxic effects in HO-1 deficient mice

At first, we confirmed our animal model (HO-1 deficient mice) by assessing the expression of HO-1 and we investigated the effect of CsA treatment on HO-1 expression in the kidneys of wild type animals. We were not able to detect HO-1 in *Hmox1* knock-out animals (not shown) and we did not observe any effect of CsA treatment on HO-1 expression in WT mice (Fig. 1A–C).

Importantly, we have demonstrated that *Hmox1* knock-out mice were more susceptible to CsA toxicity. Accordingly, CsA treatment (15 mg/kg bw delivered
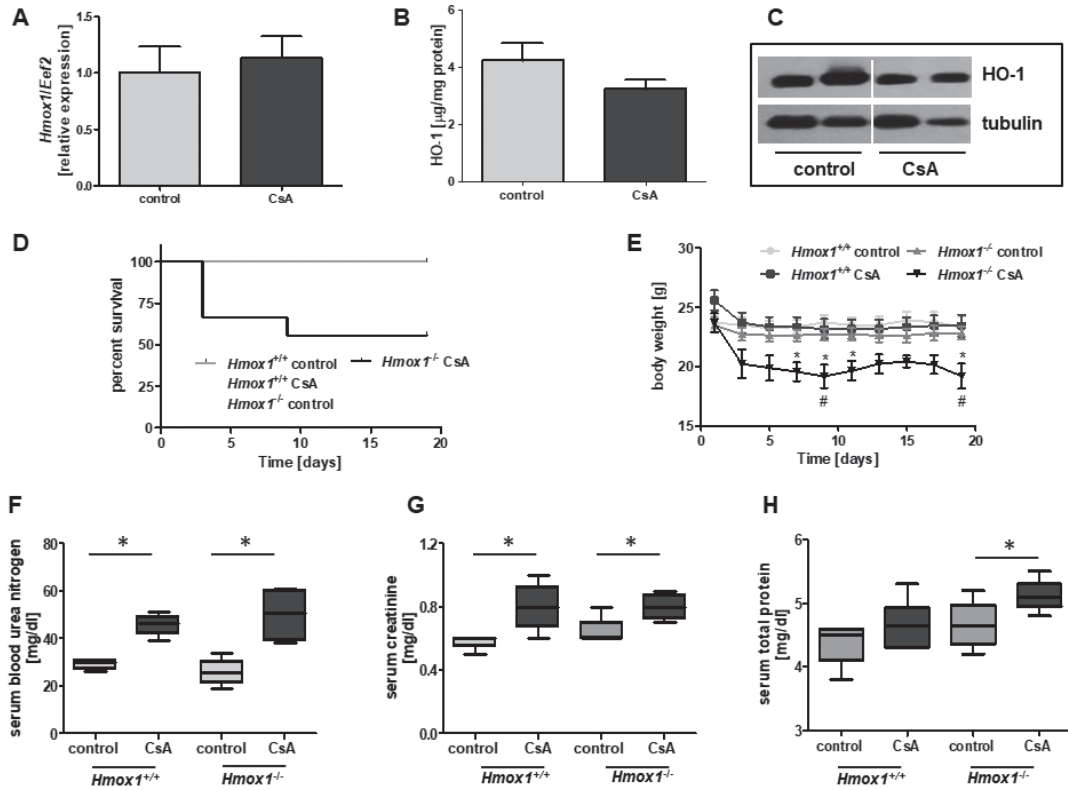
Figure 1. HO-1 expression is not regulated by CsA in wild type animals but *Hmox1* knock-out (*Hmox1⁻/⁻*) mice are more susceptible to CsA toxicity.
Hmox1 mRNA expression assessed by real-time PCR (**A**), protein level assessed by ELISA (**B**) and western blot (**C**) in the mouse kidneys is not regulated by CsA in wild-type animals. After CsA treatment, 4 out of 9 *Hmox1⁻/⁻* mice had died, whereas in wild-type animals no mortality was observed (**D**). The biggest decrease in body weight was found in the *Hmox1* knock-out animals after CsA treatment (**E**). The markers of renal function, blood urea nitrogen (**F**) and creatinine level (**G**) were upregulated in mice of both genotypes, whereas total protein level (**H**) was increased only in the *Hmox1⁻/⁻* mice treated with CsA. Mean of 5–9 animals/group, *$p<0.05$ *vs.* control.
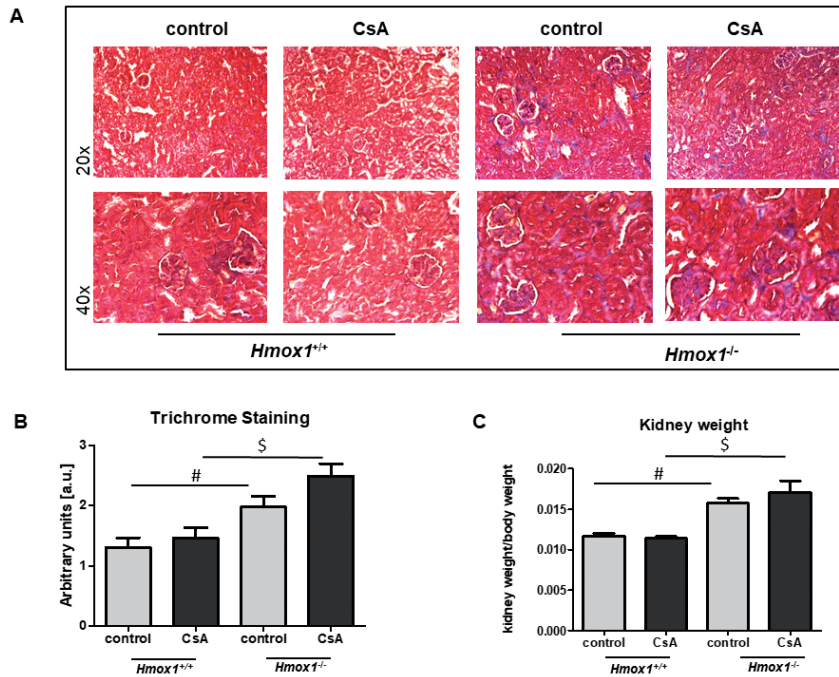

Figure 2. Increased kidney fibrosis is observed in *Hmox1* knock-out animals
Increased collagen deposition (assessed by Masson's trichrome staining) was observed in kidneys of *Hmox1* knock-out animals as indicated by the photos (**A**) and quantification of the staining (**B**). The difference in kidney weight was also prominent in the *Hmox1* knock-out animals (**C**). Mean of 5–9 animals/group, #$p<0.05$ *vs.* *Hmox1⁺/⁺* control animals, \$$p<0.05$ *vs.* *Hmox1⁺/⁺* CsA-treated mice.
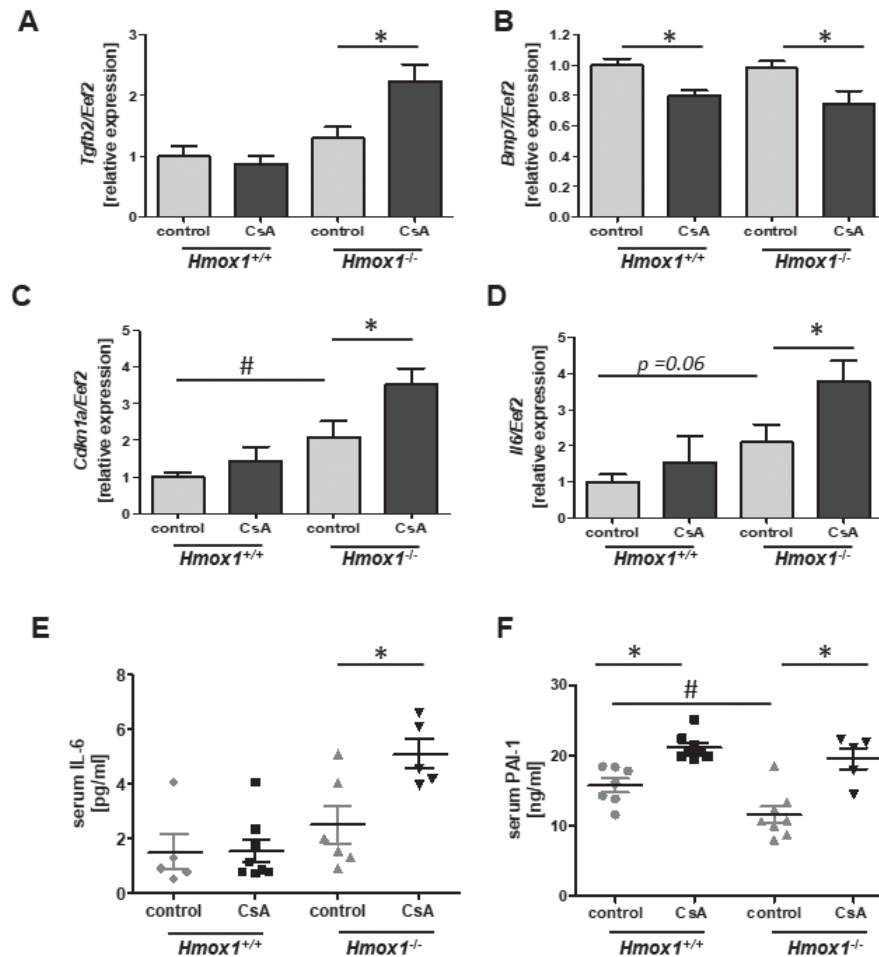
Figure 3. CsA treatment influences the expression of fibrotic, apoptotic and inflammatory markers in the kidneys of *Hmox1*^{+/+} and *Hmox1*^{-/-} mice.
Pro-fibrotic *Tgfb2* (**A**), anti-fibrotic *Bmp7* (**B**), pro-apoptotic *Cdkn1a* (**C**) and pro-inflammatory *Il6* (D) transcript levels (normalized to *Eef2*) were assessed using qRT-PCR. Protein level of IL-6 (E) and PAI-1 (F) was checked by luminex in the mouse serum. Data are presented as a fold change *over* control *Hmox1*^{+/+} mice. Mean of 5–9 animals/group, *$p<0.05$ vs.* control in each genotype, #$p<0.05$ vs. *Hmox1*^{+/+} control animals.

intraperitoneally every other day for 19 days) resulted in death of 4 out of 9 *Hmox1*^{-/-} mice (~44% mortality) (Fig. 1D). Moreover, CsA caused the highest decrease in the total body weight in HO-1 deficient mice (Fig. 1E). The levels of kidney function markers, namely BUN (Fig. 1F) and creatinine (Fig. 1G) were up-regulated in the mouse serum of both, the *Hmox1*^{+/+} and *Hmox1*^{-/-} mice, whereas the total protein level (Fig. 1H) was increased in a statistically significant way only in the *Hmox1* knock-out animals treated with CsA. In contrast, markers of the liver injury (ALAT and ASPAT) were not changed (data not shown).

Markers of fibrosis, inflammation and apoptosis are elevated after CsA treatment mostly in mice lacking HO-1

CsA treatment may lead to fibrosis, inflammation and apoptosis (1). In our experimental model, fibrosis (collagen deposition) assessed by Trichrome staining (Fig. 2A, B) was mostly affected by lack of HO-1. Also, the kidney weight was increased in *Hmox1* knock-out animals (Fig. 2C), but was not influenced by CsA treatment. However, a more detailed molecular analysis indicated elevated expression of pro-fibrotic transforming growth factor-β2 (*Tgfb2*) in the *Hmox1*^{-/-} animals (Fig. 3A), whereas no statistically significant difference in the expression of *Tgfb1* after CsA treatment was observed (not shown). On the other hand, the level of anti-fibrotic *Bmp7* was decreased irrespectively of the mice genotype (Fig. 3B). Expression of *Cdkn1a* (*p21*), a p53-inducible gene (Georgakilas *et al.*, 2017) was significantly increased by CsA in HO-1 deficient mice (Fig. 3C). Similarly, *Il6* mRNA expression (Fig. 3D) and IL-6 protein synthesis (Fig. 3E) were increased mostly in the *Hmox1*^{-/-} mice. Interestingly, in the mouse serum, a higher level of plasminogen activator inhibitor-1 (PAI-1) induced by CsA was detected (Fig. 3F), independently of HO-1 presence.

CsA regulates expression of microRNAs in an HO-1 dependent manner

Based on the importance of some microRNAs in the kidney pathophysiology and on our previous studies with nephrotoxic agent, OTA (Loboda *et al.*, 2017b; Loboda *et al.*, 2016b; Metzinger-Le Meuth *et al.*, 2018), we have assessed if CsA modulates expression of selected microRNAs in the mouse kidney and if the HO-1 level has any impact on such regulation. As indicated in Fig. 4A, B, the level of anti-fibrotic microRNAs, namely miR-29b and miR-200c, was slightly decreased
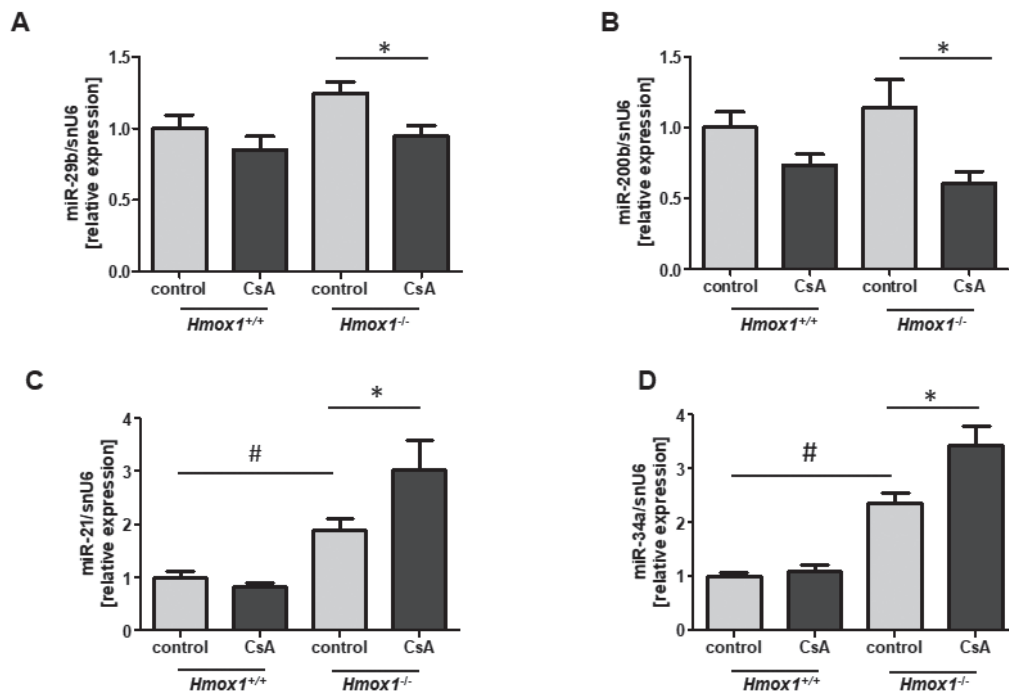
Figure 4. CsA treatment influences expression of microRNAs in the mouse kidneys in an HO-1 dependent way.
Expression of anti-fibrotic miR-29b (**A**) and miR-200b (**B**) is decreased in *Hmox1* knock-out animals treated with CsA. Expression of major pro-fibrotic microRNAs, miR-21 (**C**) and miR-34a (**D**) is increased by HO-1 deficiency and is further up-regulated by CsA. Transcript levels were normalized to snU6 and is shown as a fold of change over control *Hmox1*$^{+/+}$ mice. Mean of 5-9 animals/group, *$p<0.05$ *vs.* control in each genotype, #$p<0.05$ *vs.* *Hmox1*$^{+/+}$ control animals.

by CsA in WT animals, but the effect was greater (and statistically significant) in mice lacking HO-1. Moreover, the expression of pro-fibrotic miR-21 and pro-apoptotic miR-34a was increased in the kidneys of *Hmox1*$^{-/-}$ mice in comparison to WT counterparts. Notably, CsA treatment further increased their level only in the *Hmox1* knock-out animals (Fig. 4C, D).

DISCUSSION

Cyclosporine A (CsA)-induced glomerulosclerosis and tubulointerstitial fibrosis is a well-described side effect of CsA treatment that limits its long-term use (Bennett *et al.*, 1996). The mechanisms of CsA toxicity have been investigated for the past several years and new possible factors implicated in its nephrotoxic effect have been demonstrated (Camilleri *et al.*, 2016; Naesens *et al.*, 2009). The aim of our study was to evaluate the involvement of HO-1 in the effects triggered by CsA in mice. Moreover, we have studied contribution of small non-coding RNA molecules – microRNAs in CsA-induced nephrotoxicity.

We and others have reported that an elevated level of HO-1 exerts protective effects under various stressful conditions (reviewed in: Loboda *et al.*, 2016a; Loboda *et al.*, 2008) and previous studies have shown induction of HO-1 after CsA treatment, both *in vitro* in the rat tubular epithelial NRK-52E cells (Shin *et al.*, 2010) and *in vivo* in the rat kidney (Capasso *et al.*, 2008). In our hands, no differences in the HO-1 level upon CsA treatment were detected, possibly because of the different experimental scheme with a relatively low dose of CsA used. Nonetheless, as shown by us, the lack of HO-1 causes accumulation of collagen and increased kidney weight (kidney hypertrophy). Moreover, HO-1 deficiency promoted expression of miR-21 and miR-

34a, the markers of fibrosis and apoptosis, indicating the importance of this protective enzyme in the kidney homeostasis. Such regulation was also observed by us previously in other experimental settings in the mouse kidney (Loboda *et al.*, 2017a) and in fibroblasts isolated from the *Hmox1* knock-out mice (Stepniewski *et al.*, 2018). Through its anti-apoptotic, anti-inflammatory and anti-oxidant properties, HO-1 may also protect against toxic insults as the extent of fibrosis was accelerated in the HO-1 knock-out animals treated with OTA (Loboda *et al.*, 2017a; Stachurska *et al.*, 2013).

Interestingly, we have identified several microRNAs that are regulated in the kidneys exposed to OTA, but this effect was HO-1-independent (Loboda *et al.*, 2017a). In contrast, as shown in the study presented here, the differences in the expression of pro-fibrotic miR-21, anti-fibrotic miR-29b and miR-200b, as well as pro-apoptotic miR-34a, were aggravated by the lack of HO-1 in the CsA-induced nephrotoxicity. Notably, miR-21 was previously found to mediate CsA nephrotoxicity via the PTEN/AKT signaling pathway in the human proximal tubular epithelial cells (HPTECs) (Chen *et al.*, 2015). Recent study by Gooch *et al.* revealed 76 differentially expressed microRNAs in kidneys of mice that received CsA (20 mg/kg) or vehicle treatment daily for six weeks (Gooch *et al.*, 2017). Except changes in particular microRNAs which were previously linked to renal fibrosis, like let-7d, miR-21, miR-29, miR-30, miR-130, miR-192, and miR-200a, microRNAs that have not been reported to be related to nephrotoxicity or immunosuppression (such as miR-186 and miR-709) were also found to be dysregulated (Gooch *et al.*, 2017). Interestingly, we did not observe induction of miR-21 in the wild type animals. However, even upon more frequent and longer administration of CsA applied by Gooch *et al.*, induction

of miR-21 in healthy wild type mice was rather moderate (less than 2 fold induction was measured by quantitative real-time PCR analysis and 2.19 fold induction by RNA sequencing). miR-21 was suggested to be a universal biomarker of kidney pathology and was found to be increased in response to various stimuli, not only in this organ but also in the serum, plasma, and urine (reviewed in: Loboda et al., 2016b). Thus, it would be of interest to further extend our analysis beyond the kidney to verify its utility as a biomarker in our experimental scheme.

In our study, we also observed an increased expression of miR-34a, regulated by the p53 signaling pathway, in the CsA-treated HO-1 knock-out mice. DNA damage and cell cycle arrest accompanied with elevated p53 level are found to be early events in the CsA nephrotoxicity, both in vitro (Lally et al., 1999) and in vivo (Shihab et al., 1999). We did not assess expression of p53 or p21 regulator in this study, but we have shown previously that treatment the with another nephrotoxic agent, OTA, leads to a significant and concomitant upregulation of miR-34a, p53, p21 and p53 upregulated modulator of apoptosis (PUMA) expression (Loboda et al., 2017a). Increased apoptosis, including p21 induction, was already observed in the renal proximal tubular cells in vitro after CsA stimulation (Jennings et al., 2007).

In addition, Yuan et al. pointed out to miR-494 as responsible for CsA-induced nephrotoxicity and epithelial to mesenchymal transition (EMT) progression (Yuan et al., 2015). Finally, Sun and coworkers (Sun et al., 2017) identified miR-181c as a factor protecting renal tissues from CsA-induced nephrotoxicity.

Our results indicate that selected microRNAs, which were known to be altered in the diseased kidneys, are regulated by CsA in an HO-1-dependent way. It would be also interesting to study if CsA may influence the total pool of microRNAs, as well as the expression of microRNAs processing enzymes. In microRNAs biogenesis, DiGeorge syndrome critical region gene 8, (DGCR8), a heme-dependent protein, plays a critical role in the first step of primary microRNAs (pri-miRNAs) processing (Faller et al., 2007). In our previous study, DGCR8 was shown to be decreased by HO-1 overexpression (Kozakowska et al., 2012), whereas its level was upregulated in porcine renal epithelial cells stimulated by OTA, which concomitantly inhibited HO-1 expression (Stachurska et al., 2013). Therefore, it might be suggested that the DGCR8 level, as well as its properties, depend on the HO-1 activity.

HO-1 is a well-known anti-inflammatory factor (Loboda et al., 2016a, 2008). In the study presented here, expression of IL-6, a marker of inflammation, was induced by CsA at the mRNA level in the kidneys and at the protein level in the serum only in the Hmox1-/-animals, suggesting that the lack of HO-1 triggers an inflammatory reaction after CsA treatment. Similarly, Cdkn1a (p21) expression was elevated only in the HO-1 deficient kidneys.

PAI-1, not expressed in the kidneys under normal conditions, is rapidly induced in a wide variety of acute and chronic renal diseases. Accordingly, PAI-1 was shown to be implicated in glomerulosclerosis and tubulointerstitial fibrosis (reviewed in: (Eddy, 2009). Moreover, CsA-induced up-regulation of PAI-1 was observed in vitro in the rat mesangial cells (Waiser et al., 2002) or in vivo in the rat kidney (Pereira et al., 2006). Here, we report that in Hmox1 knock-out animals, the rate of induction of Pai1 expression after CsA is more pronounced than in wild-type mice (69% vs 34% induction, $p = 0.0098$). Concomitantly, increased collagen deposition

and expression of Tgfb2 demonstrates pro-fibrotic changes in the kidneys of HO-1 deficient mice subjected to CsA treatment.

Lack or only mild changes in the expression of examined genes in control mice after CsA treatment may suggest that our scheme of the experiment, including the chosen dose of CsA, was too gentle to cause potent fibrotic, inflammatory or apoptotic effects; however, it was sufficient to trigger such a response in mice lacking HO-1. We have also observed mortality in Hmox1 knockout animals after CsA injections, which might indicate (but not prove) an increased sensitivity to nephrotoxic insults. Hence, HO-1 seems to play a protective role against CsA-mediated toxicity in wild-type animals.

Altogether, our data indicate a higher renal damage in animals lacking HO-1. Based on these results, it might be proposed that in HO-1 deficient mice the disruption of kidney architecture might be related to disturbances in extracellular matrix synthesis and increased apoptosis and inflammation, which collectively can be considered as major mechanisms of CsA toxicity. As highlighted in this study, specific microRNAs are also implicated in this process.

It has to be emphasized that our results indicate an increased sensitivity of Hmox1 knock-out animals to CsA treatment, however, in the human population, instead of total HO-1 deficiency, a rather decreased level of HMOX1 mRNA expression and HO enzyme activity might be detected in people with longer $(GT)_n$ repeats in the HMOX1 promoter (Loboda et al., 2008; Taha et al., 2010). Thus, HO-1 heterozygous mice potentially could be used to recapitulate, at least partially, the situation observed in the human population. Nevertheless, one may speculate that CsA toxicity and its influence on microRNAs' expression might be patient-specific. However, such data are not available so far.

Our results may also help to understand some molecular aspects of CsA-related toxicity after heart transplantations. Triple therapy based on calcineurin inhibitors (CsA or tacrolimus), antiproliferative agents (azathioprine or mycophenolate mofetil) and steroids, can be used as the gold standard immunosuppressive therapy after cardiac transplantation (Guethoff et al., 2013; O'Neill et al., 2004). CsA monotherapy has been also tested and its effectiveness was compared with the double therapy (CsA with azathioprine) in the long-term heart transplant recipients, surviving more than 5 years (Sansone et al., 2010). No differences in the survival rate and major events, including heart failure, malignancy, infection and cardiac allograft vasculopathy (CAV), were reported, suggesting the usefulness of CsA treatment (Sansone et al., 2010). Nonetheless, data from retrospective analysis indicate that the renal failure is a frequent cause of death after heart transplantation, and 10 years after such operation around 6% of patients require dialysis and about 4% need renal transplantation (reviewed in: (Söderlund & Rådegran, 2015)). Those adverse effects could be the result of CsA toxicity, therefore, minimizing the use of this immunosuppressant is suggested to decrease chronic kidney diseases after heart transplantations. Recently, involvement of specific microRNAs in the toxic effects of CsA on cardiomyocytes has been also reported. Zhu and coworkers (Zhu et al., 2016) have identified miR-377 as the factor responsible for cardiomyocyte apoptosis after CsA treatment. More importantly, miR-21 level that was increased in the renal tissues after CsA treatment, as shown in our and other studies (Chen et al., 2015), was suggested to be a predictive marker of the development of acute kidney injury after cardiac surgery (Gaede et al., 2016).

In summary, we have shown that *Hmox1* knock-out mice are more susceptible to CsA toxic effects through the increased fibrosis, inflammation and apoptosis, including microRNA regulation. Therefore, the level of *Hmox1* expression (which can vary because of the known HO-1 promoter polymorphism) might be an important factor regulating patient's response to the CsA treatment, however, this requires further studies.

Conflict of interest statement

The authors declare that there is no conflict of interests.

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