

Reoxygenation induces reactive oxygen species production and ferroptosis in renal tubular epithelial cells by activating aryl hydrocarbon receptor

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Abstract. During the reperfusion phase of ischemia-reperfusion injury, reactive oxygen species (ROS) production aggravates the course of many diseases, including acute kidney injury. Among the various enzymes implicated in ROS production are the enzymes of the cytochromes P450 superfamily (CYPs). Since arylhydrocarbon receptor (AhR) controls the expression of certain CYPs, the involvement of this pathway was evaluated in reperfusion injury. Because AhR may interact with the nuclear factor erythroid 2-related factor 2 (Nrf2) and the hypoxia-inducible factor-1 α (HIF-1 α), whether such an interaction takes place and affects reperfusion injury was also assessed. Proximal renal proximal tubular epithelial cells were subjected to anoxia and subsequent reoxygenation. At the onset of reoxygenation, the AhR inhibitor CH223191, the HIF-1 α activator roxadustat, or the ferroptosis inhibitor α -tocopherol were used. The activity of AhR, Nrf2, HIF-1 α , and their transcriptional targets were assessed with western blotting. ROS production, lipid peroxidation and cell death were measured with colorimetric assays or cell imaging. Reoxygenation induced ROS production, lipid peroxidation and cell ferroptosis, whereas CH223191 prevented all. Roxadustat did not affect the above parameters. Reoxygenation activated AhR and increased CYP1A1, while CH223191 prevented both. Reoxygenation with or without CH223191 did not alter Nrf2 or HIF-1 α activity. Thus, AhR is activated during reoxygenation and induces ROS production, lipid peroxidation and ferroptotic cell death. These detrimental effects may be mediated by AhR-induced CYP overexpression, while the Nrf2 or the HIF-1 α pathways remain unaffected. Accordingly, the AhR

pathway may represent a promising therapeutic target for the prevention of reperfusion injury.

Introduction

Ischemia-reperfusion (I-R) injury plays an important role in the pathogenesis of several diseases, such as stroke, coronary heart disease and multiorgan failure (1-3). Due to the high metabolic demands of renal tubular cells, the kidney is exceptionally vulnerable to I-R, and I-R injury is the most common cause of acute kidney injury (4). I-R injury consists of two consecutive, but distinct, stages. During ischemia, cell energy collapse induces cell death, whereas during reperfusion, cell death results from increased reactive oxygen species (ROS) production (1-3). Regarding renal tubular epithelial cells, a study demonstrated that under anoxia, cells die through apoptosis, whereas reoxygenation induces lipid peroxidation-induced cell death, also known as ferroptosis (5,6). The latter has also been detected in isolated mouse renal tubules, confirming the different pathophysiology of ischemia- and reperfusion-induced cell injury (7).

Ferroptosis is a type of regulated iron-dependent cell necrosis mainly caused by increased redox imbalance and characterized by extensive lipid peroxidation and, eventually, severe cell membrane dysfunction (8). Unlike uncontrolled cell necrosis, which occurs acutely after a severe physical, chemical, or mechanical insult, ferroptosis and other types of regulated cell necrosis involve a genetically encoded machinery, occur in a delayed manner, and could be considered as a part of an adaptive response that unsuccessfully attempts to restore cellular homeostasis (8). Thus, contrary to uncontrolled necrosis, for ferroptosis, pharmacological intervention is possible (8). Apoptosis is another type of programmed cell death, which differs in many ways from the various types of regulated cell necrosis. A notable difference is that during apoptosis, cell membrane rupture does not take place, and the release of cytoplasmic content and the ensuing inflammation are avoided (8).

When metabolizing their substrates, the enzymes of the cytochrome P450 superfamily (CYP) produce ROS (9). By oxidizing their substrates, often drugs or xenobiotics, CYPs increase their polarity, aiding to their excretion.

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Substrate oxidation by CYPs is achieved by a six-step reaction during which heme-thiolate iron fluctuates between ferric (Fe^{+3}) and ferrous (Fe^{+2}) form, interacts with oxygen, and eventually oxidize the substrate. The general reaction is $\text{RH} + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{ROH} + \text{H}_2\text{O}$. However, two shunts exist in the six-step CYP catalytic pathway. The first takes place in step 3 of the reaction and gives rise to superoxide. The second occurs in step 4 of the reaction and releases hydrogen peroxide (9,10). In addition to the above mechanism of CYP-induced ROS production, a supplementary mechanism has also been identified. CYPs metabolize arachidonic acid to produce 20-hydroxyeicosatetraenoic acid (20-HETE). The latter increases NADPH oxidase activity resulting in further ROS production (11,12). The role of CYPs in producing ROS and eventually cell death has been confirmed in experimental models of heart and liver I-R injury (13-17).

Certain CYPs are under the transcriptional control of the aryl hydrocarbon receptor (AhR). Non-ligand bound AhR is retained in the cytoplasm as an inactive complex with various factors, which also protect AhR from proteasomal degradation. When activated by exogenous or endogenous ligands, AhR is released, translocates into the nucleus, forms a complex with HIF-1 β , and transcribes the CYPs CYP1A1, CYP1A2, and CYP1B1 (18,19). Interestingly, activated AhR becomes vulnerable to proteasomal degradation, leading to decreased AhR cellular levels (20-24).

Several studies have suggested that AhR-induced CYP overexpression results in ROS production (25-29). For instance, in human aortic endothelial cells, 2,3,7,8-tetrachlorodibenzo-p-dioxin activates AhR and induces CYP1A1 expression and ROS production. In this model, small interfering RNA targeting of AhR or CYP1A1 decreased ROS production significantly, confirming that CYPs could mediate AhR-induced ROS production (27). However, the role of this pathway during reperfusion remains unclear.

Although AhR induces oxidative stress by increasing CYPs, it also promotes the expression of nuclear factor erythroid 2-related factor 2 (Nrf2) (30-32). Nrf2 is kept in the cytoplasm by kelch-like ECH-associated protein 1 (KEAP1) and cullin-3. Cullin-3 ubiquitinates Nrf2 leading it to proteasomal degradation. Under increased ROS levels, the conformation of KEAP1 changes releasing Nrf2, which translocates into the nucleus and transcribes genes encoding antioxidant and anti-ferroptotic proteins, as well as proteins required for detoxification (33,34). At first glance, AhR-induced Nrf2 might attenuate the oxidative stress produced by AhR-induced CYPs (30-32). A study has demonstrated that the kynurenine-AhR pathway mediates brain damage after experimental stroke, a condition that involves both phases of I-R injury (35). Moreover, inhibition of AhR through downregulation of CYPs and ROS protects newborn rat lungs from hyperoxia (25). Thus, evidence indicates that in the context of oxidative stress, the balance between AhR-induced CYPs and AhR-induced Nrf2 tilts towards the former. Besides the detection of a possible detrimental effect of AhR activation in I-R injury or hyperoxia, the aforementioned studies did not assess Nrf2, nor the effect the reperfusion per se. Thus, the precise interaction between AhR and Nrf2 during the reperfusion stage of I-R-injury has not been evaluated.

Another transcription factor that is inevitably affected by I-R is hypoxia-inducible factor-1 α (HIF-1 α). Under normoxia, HIF-prolyl-hydroxylases are active, leading to HIF-1 α proteasomal degradation. Under anoxia, hydroxylation does not take place, and HIF-1 α is released from the Von Hippel-Lindau E3 ubiquitin ligase, escaping proteasomal degradation. The accumulated HIF-1 α forms a heterodimer with HIF-1 β and transcribes many genes necessary for cellular adaptation to anoxia (36,37). However, activated AhR also forms a heterodimer with HIF-1 β (33, 34). In certain experimental models of AhR activation, the competition between AhR and HIF-1 α for HIF-1 β results in reduced HIF-1 α transcriptional activity, and *vice versa* (38-40). However, this interaction between AhR and HIF-1 α has not been evaluated in models of I-R injury.

Most studies have evaluated I-R-injury as a one-step process (1-3), yet I-R consists of two consecutive but different stages, the first of ischemia, and the second of reperfusion (1-3). The present study aimed to evaluate the role of AhR during the reoxygenation phase, and more precisely, its effect on ROS producing CYPs, Nrf2, HIF-1 α and cell survival. A cell culture model was established, in which primary murine renal proximal tubular epithelial cells (RPTECs) were subjected to anoxia, a condition that imitates ischemia, and then to reoxygenation and replenishment with fresh culture medium, a condition that mimics reperfusion. RPTECs are particularly vulnerable to I-R injury (4).

To evaluate the role of AhR in reoxygenation-induced cell death the specific AhR inhibitor CH223191 was applied at the onset of reoxygenation (41). The HIF-prolyl-hydroxylase inhibitor roxadustat was used to assess the possible role of interaction between AhR and HIF-1 α (42). Finally, the ferroptosis inhibitor α -tocopherol (43) was applied at the onset of reoxygenation to examine the effect of AhR on ferroptotic cell death specifically.

Materials and methods

Cell culture and treatment. Primary C57BL/6 murine RPTECs (cat. no. C57-6015) and Complete Epithelial Cell Medium kit (cat. no. M6621) were purchased from Cell Biologics. Primary cells were used instead of genetically altered cell lines in order to obtain reliable results. Second-passage RPTECs were cultured in 96-well plates (10^4 cells/well) or 6-well plates (3×10^5 cells/well) at 37°C . In order to reproduce ischemic conditions, cells were placed in a GasPak™ EZ Anaerobe Container System with Indicator (cat. no. 26001; BD Biosciences), which reduces the concentration of oxygen to $<1\%$. To replicate reperfusion, cells were then removed from the Anaerobe Container System and washed with PBS. Fresh culture medium was added, and cells were cultured in a humidified atmosphere containing $5\% \text{CO}_2$.

The selection of appropriate culture time points was based on a previous study demonstrating that primary mouse RPTECs died after 48 h of anoxia or 4 h of reoxygenation (5). The latter was also confirmed in the present study. Thus, in this study, cells were subjected to anoxia for 24 h and subsequently to reoxygenation for 2 h, as after these time points, cell death precludes any further reliable analysis.

At the onset of the reoxygenation period, 3 μM of the AhR inhibitor CH223191 (cat. no. C8124; Sigma-Aldrich; Merck KGaA) or 100 μM of the ferroptosis inhibitor α -tocopherol (cat. no. T3251; Sigma-Aldrich; Merck KGaA) or the HIF-1 α activator roxadustat at a concentration of 10 $\mu\text{g}/\text{ml}$ (cat. no. FG-4592; Selleck Chemicals) were added.

RPTECs cultured under normoxic conditions for a total of 26 h were used as control. Two hours before the end of the 26-h period, control cells were washed with PBS and fresh culture medium was added.

Cell imaging. Cell images were captured at the onset of reoxygenation, then at 2-h intervals for 24 h. An Axiovert 40C inverted microscope (Carl Zeiss AG) equipped with a digital camera (3MP USB2.0 Microscope Digital Camera; Amscope) and related software (Amscope; v. x64, 3.7.3036) were used.

Assessment of ROS production, lipid peroxidation and cell necrosis. To assess ROS production, RPTECs were cultured in 96-well plates. At the end of the reoxygenation period, 5 μM of the fluorogenic probe CellROX[®] Deep Red Reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was added, and cells were incubated at 37°C for 30 min. Then, cells were washed with PBS, and fluorescence signal intensity was measured with an EnSpire[®] Multimode plate reader (PerkinElmer, Inc.).

To assess lipid peroxidation, RPTECs were cultured in 6-well plates. At the end of the reoxygenation period, the end product of lipid peroxidation malondialdehyde (MDA) was measured fluorometrically in cell lysates using the Lipid Peroxidation (MDA) Assay Kit (cat. no. ab118970; Abcam). The required cell lysis buffer was provided along with the aforementioned kit. The lower detection limit of the kit is 0.1 nmol. Prior to MDA measurement, a Bradford assay was performed to adjust lysate volumes to an equal protein mass of 100 μg .

To assess cell necrosis and the effect of AhR, RPTECs were cultured in 96-well plates in the presence or absence of the ferroptosis inhibitor α -tocopherol or the AhR inhibitor CH223191. The above inhibitors were added into the cell cultures at the beginning of the reoxygenation period. At the end of the reoxygenation period, cell necrosis was evaluated using a lactate dehydrogenase (LDH) release assay with the Cytotox Non-Radioactive Cytotoxic Assay kit (Promega Corporation). Cell necrosis was calculated as (LDH in the supernatant/Total LDH) x 100.

Western blot analysis. RPTECs were cultured in 6-well plates. Once the reoxygenation period was over, cells were lysed with the T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Inc.) supplemented with phosphatase inhibitor (Roche Diagnostics) and protease inhibitor (Sigma-Aldrich; Merck KGaA). Protein was quantified using a Bradford assay (Sigma-Aldrich; Merck KGaA). For western blotting, 10 μg of protein from each sample were electrophoresed in SDS-PAGE (4-12% Bis-Tris) gels (Thermo Fisher Scientific, Inc.) and transferred to PVDF membranes (Thermo Fisher Scientific, Inc.). Tris-buffered saline with 0.1% Tween-20 and 5% non-fat dry milk was used as blocking buffer. Blots were incubated at 4°C for 16 h with the primary antibody, then at room temperature for 30 min with the secondary antibody. For the enhanced

chemiluminescent detection of the western blot bands, the LumiSensor Plus Chemiluminescent HRP Substrate kit (GenScript Corporation) was used. Whenever reprobing of the PVDF blots was necessary, the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Inc.) was used. The Image J software v. 1.51t (National Institutes of Health) was used for densitometric analysis.

Primary antibodies were specific for AhR (1:200; cat. no. sc-133088; Santa Cruz Biotechnology, Inc.), cytochrome P450 family 1 subfamily A member 1 (CYP1A1; 1:500; cat. no. sc-25304; Santa Cruz Biotechnology, Inc.), Nrf2 (1:1,000; cat. no. TA343586; OriGene Technologies, Inc.), superoxide dismutase 3 (SOD-3; 1:100; cat. no. sc-271170; Santa Cruz Biotechnology, Inc.), cystine-glutamate antiporter (xCT, also known as SLC7A11; 1:1,000; cat. no. ANT-111; Alomone Labs), HIF-1 α (1:500; cat. no. sc-10790; Santa Cruz Biotechnology, Inc.), LDH-A (1:1,000; cat. no. 2012; Cell Signaling Technology, Inc.), activated cleaved caspase-3 (CC3; 1:1,000; cat. no. ab13847; Abcam) and β -actin (1:2,500; cat. no. 4967; Cell Signaling Technology, Inc.). Anti-mouse IgG, HRP-linked antibody (1:1,000; cat. no. 7076; Cell Signaling Technology, Inc.) or anti-rabbit IgG, HRP-linked antibody (1:1,000; cat. no. 7074; Cell Signaling Technology, Inc.) were used as secondary antibodies.

Statistical analysis. One-sample Kolmogorov-Smirnov test verified that all, except one, variables were normally distributed. One-way analysis of variance and Bonferroni's correction test were used for comparison of means. Results are presented as the mean \pm SEM of six experiments. The analysis of the cell imaging results was carried out using the Kruskal-Wallis H test and Dunn's post hoc test, since this variable did not follow the normal distribution. Statistical analysis was performed with the SPSS version 20 (IBM Corp.). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Reoxygenation activates AhR and increases CYP1A1 expression, while CH223191 prevents both. Reoxygenation decreased AhR levels to 0.68 ± 0.05 of the control ($P = 0.001$). The AhR inhibitor CH223191 inhibited the reoxygenation-induced changes in AhR levels, increasing the AhR levels 0.99 ± 0.08 of the control (P not significant compared with the control; $P = 0.003$ compared with cells subjected to reoxygenation only). Of note, under normoxic conditions, CH223191 increased AhR level to 1.29 ± 0.04 of the control ($P = 0.03$). Since AhR is degraded after its activation, these results indicated that reoxygenation activated AhR, and that this effect was inhibited by the AhR specific inhibitor CH223191 (Fig. 1A and B).

The expression of the AhR transcriptional target CYP1A1 confirmed the aforementioned conclusion. Reoxygenation increased CYP1A1 expression to 1.44 ± 0.01 of the control ($P = 0.033$). CH223191 prevented the reoxygenation-induced change of CYP1A1 expression since, in this case, CYP1A1 level equaled 1.04 ± 0.07 of the control (P not significant compared with the control; $P = 0.014$ compared with cells subjected to reoxygenation only). Notably, under normoxic conditions, CH223191 decreased CYP1A1 expression levels to 0.66 ± 0.05 of the control ($P = 0.033$; Fig. 1A and C).

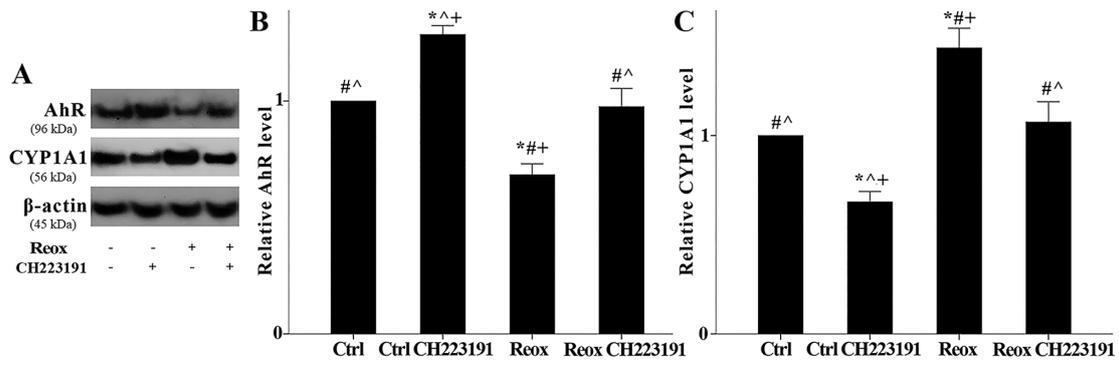


Figure 1. Reox activates AhR and induces CYP1A1 expression, while CH223191 inhibits both. RPTECs were cultured under ctrl conditions or subjected to Reox in the presence or absence of the AhR inhibitor CH223191. (A) Representative AhR and CYP1A1 western blot images. (B and C) Statistical analysis of the western blots. Reox decreases AhR levels, indicating its activation. CH223191 increases AhR levels both in ctrl cells and cells subjected to Reox. Reox enhanced CYP1A1 expression, whereas CH223191 reduced CYP1A1 under both conditions (C). Data are presented as the mean \pm SEM of six independent experiments. * P <0.05 vs. ctrl; $^{\#}P$ <0.05 vs. ctrl CH223191; $^{\wedge}P$ <0.05 vs. Reox; ^{+}P <0.05 vs. Reox CH223191. AhR, arylhydrocarbon receptor; CYP1A1, cytochrome P450 family 1 subfamily A member 1; RPTEC, renal proximal tubular epithelial cell; ctrl, control; Reox, reoxygenation.

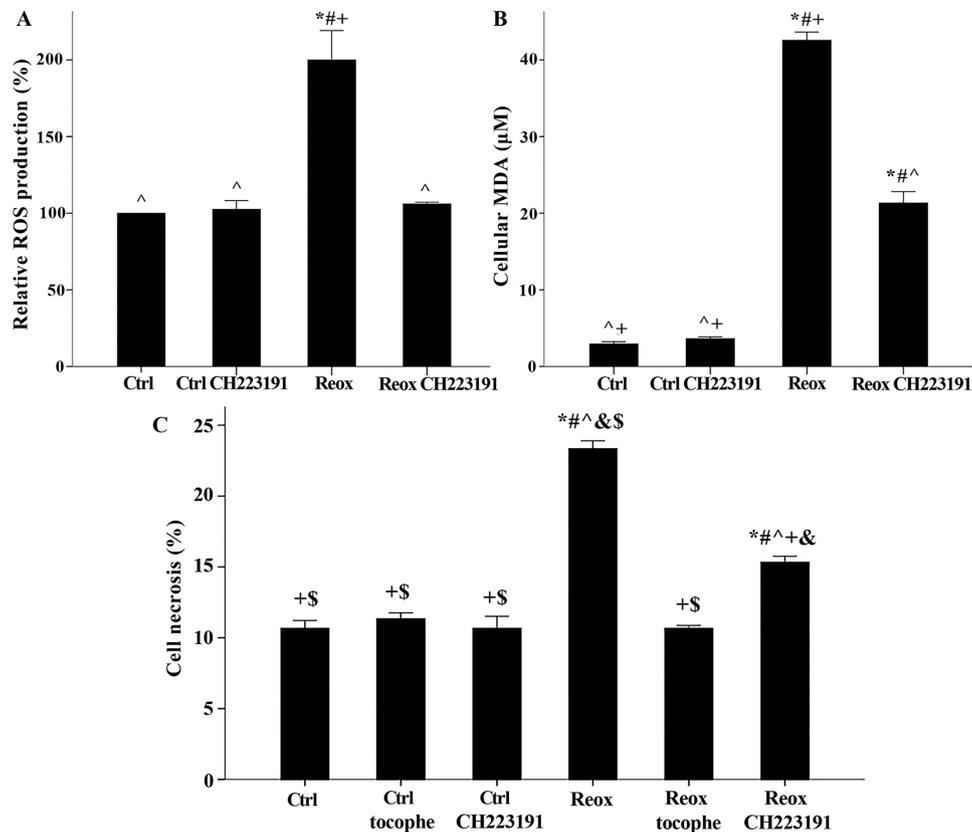


Figure 2. Inhibition of AhR prevents Reox-induced ROS production and attenuates lipid peroxidation and ferroptosis. RPTECs were cultured under ctrl conditions or subjected to Reox, with or without the AhR inhibitor CH223191 or the ferroptosis inhibitor tocophe. (A and B) Reox induced ROS production and lipid peroxidation, assessed by MDA levels. CH223191 prevented Reox-induced ROS production and attenuated lipid peroxidation. Reox caused cell necrosis. Necrosis was abrogated by α -tocopherol, demonstrating ferroptosis. * P <0.05 vs. ctrl; $^{\#}P$ <0.05 vs. ctrl CH223191; $^{\wedge}P$ <0.05 vs. Reox; ^{+}P <0.05 vs. Reox CH223191. (C) CH223191 ameliorated Reox-induced cell necrosis. Data are presented as the mean \pm SEM of six independent experiments. * P <0.05 vs. ctrl; $^{\#}P$ <0.05 vs. ctrl tocophe; $^{\wedge}P$ <0.05 vs. ctrl CH223191; $^{\wedge}P$ <0.05 vs. Reox; $^{\&}P$ <0.05 vs. Reox tocophe; $^{\$}P$ <0.05 vs. Reox CH223191. AhR, arylhydrocarbon receptor; RPTEC, renal proximal tubular epithelial cell; ROS, reactive oxygen species; MDA, malondialdehyde; ctrl, control; Reox, reoxygenation; tocophe, α -tocopherol.

Inhibition of AhR prevents reoxygenation-induced ROS production and ameliorates lipid peroxidation. Reoxygenation increased ROS production. Considering the ROS production in control cells as 100%, in cells subjected to reoxygenation, ROS levels doubled, compared with the control ($199.9 \pm 9.6\%$; P <0.001). The AhR inhibitor CH223191 prevented reoxygen-

ation-induced ROS production, with ROS level only reaching $106.0 \pm 0.6\%$ of the control (P not significant compared with the control; P <0.001 compared with cells subjected to reoxygenation only). Under normoxic conditions, CH223191 did not affect ROS production ($102.5 \pm 2.9\%$ of the control; P not significant; Fig. 2A).

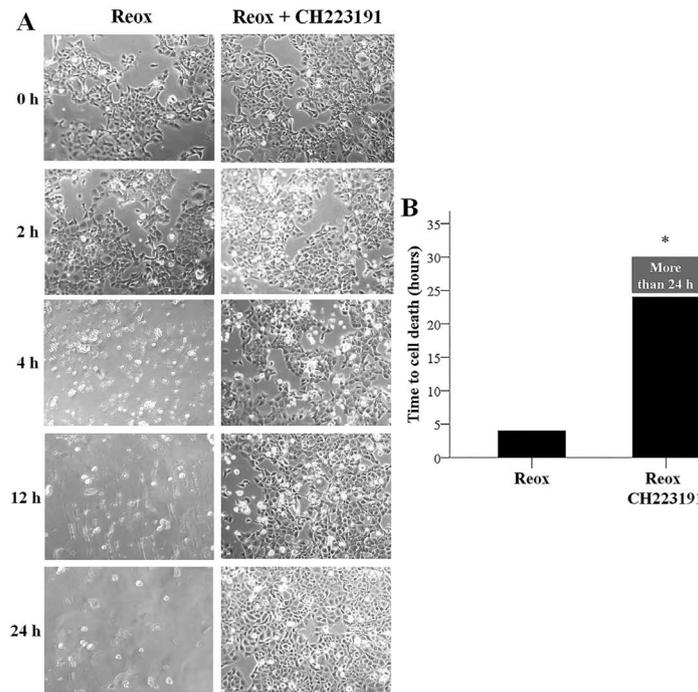


Figure 3. Inhibition of AhR protects from Reox-induced cell death. RPTECs were subjected to Reox with or without the AhR inhibitor CH223191. (A) Representative images of one of the experiments. Magnification, x100. (B) Statistical analysis of all experiments. RPTECs died within 4 h of Reox, while CH223191 rescued the cells. Data are presented as the mean \pm SEM of six independent experiments. * $P < 0.05$ vs. Reox. AhR, arylhydrocarbon receptor; RPTEC, renal proximal tubular epithelial cell; Reox, reoxygenation.

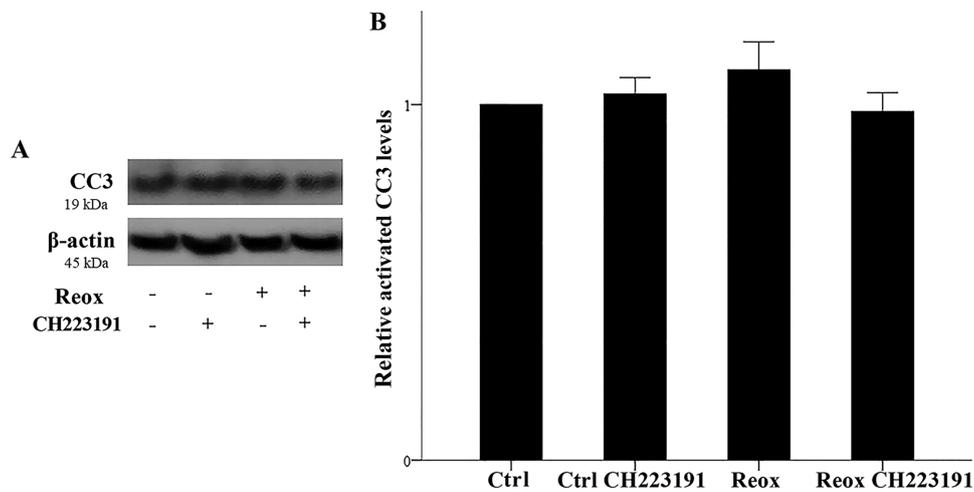


Figure 4. Apoptosis does not play a role in Reox-induced cell death and is not affected by inhibition of AhR. RPTECs were cultured under ctrl conditions or subjected to Reox in the presence or not of the AhR inhibitor CH223191. (A) Representative CC3 western blot images. (B) Statistical analysis of the western blots. Neither Reox nor CH223191 affects cell apoptosis. Data are presented as the mean \pm SEM of six independent experiments. AhR, arylhydrocarbon receptor; RPTEC, renal proximal tubular epithelial cell; ctrl, control; Reox, reoxygenation; CC3, cleaved caspase-3.

Lipid peroxidation, assessed by MDA levels, followed the same trend as ROS. In control cells, cellular MDA concentration was $3.0 \pm 0.2 \mu\text{M}$, while under reoxygenation, MDA concentration increased to $42.6 \pm 0.5 \mu\text{M}$ ($P < 0.001$). CH223191 decreased MDA in RPTECs subjected to reoxygenation to $21.3 \pm 0.7 \mu\text{M}$ ($P < 0.001$, compared with cells subjected to reoxygenation only). Notably, inhibition of AhR under control conditions did not alter MDA significantly ($3.6 \pm 0.3 \mu\text{M}$; Fig. 2B).

Inhibition of AhR attenuates reoxygenation-induced ferroptosis, while apoptosis was not observed. Cell imaging

indicated that RPTECs were extremely vulnerable to reoxygenation since in all performed experiments they died after 4 h. The specific AhR inhibitor CH223191 rescued RPTECs from reoxygenation induced cell death since, in this case, in all experiments, cells remained alive at the 24-h timepoint ($P < 0.001$; Fig. 3A and B).

In the control cells, LDH release assay indicated a percentage of necrotic cells equal to $10.7 \pm 0.6\%$. In cells subjected to reoxygenation, the percentage of necrotic cells increased significantly to $23.3 \pm 0.6\%$, compared with control cells ($P < 0.001$). The inhibitor of ferroptosis α -tocopherol

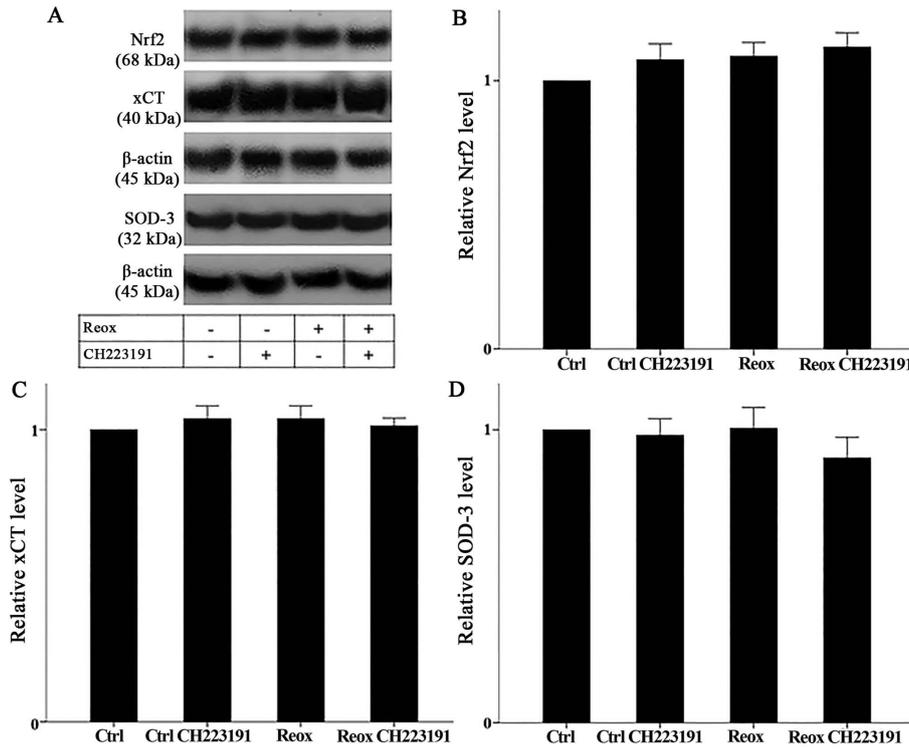


Figure 5. AhR activation status does not affect Nrf2 activation or transcriptional activity. RPTECs were cultured under ctrl conditions or subjected to Reox with or without the AhR inhibitor CH223191. (A) Representative western blots of Nrf2 levels (corresponding to its activation status) and the expression of the Nrf2 transcriptional targets xCT (SLC7A11) and SOD-3. (B-D) Statistical analysis of the western blots. Neither Reox nor CH223191 affects Nrf2 activity, or the expression of xCT and SOD-3. Data are presented as the mean \pm SEM of six independent experiments. AhR, arylhydrocarbon receptor; Nrf2, nuclear factor erythroid 2-related factor 2; xCT, cystine-glutamate antiporter; SOD-3, superoxide dismutase; RPTEC, renal proximal tubular epithelial cell; ctrl, control; Reox, reoxygenation.

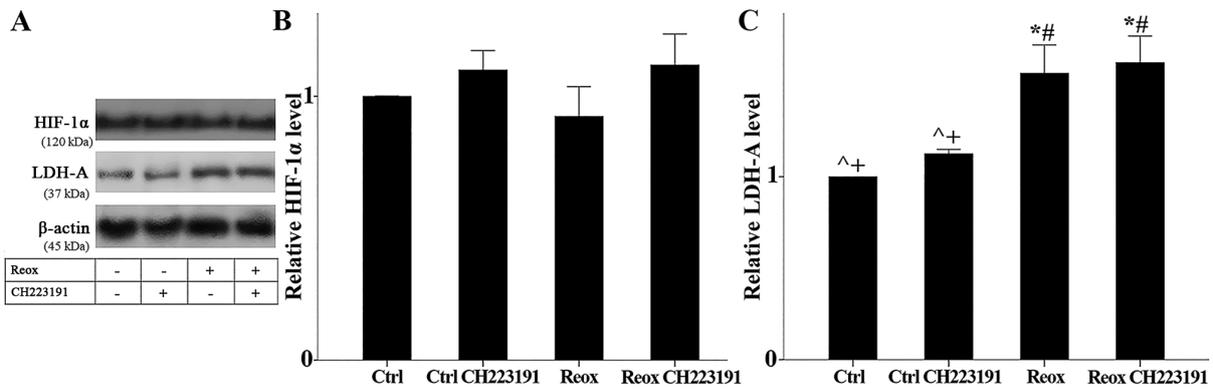


Figure 6. AhR activation status does not affect HIF-1α levels or its transcriptional activity. RPTECs were cultured under ctrl conditions or subjected to Reox with or without the AhR inhibitor CH223191. (A) Representative western blot images of HIF-1α levels (corresponding to its activation status) and its transcriptional target LDH-A. (B and C) Statistical analysis of the western blots. Neither Reox nor CH223191 affected HIF-1α levels. LDH-A levels increased under Reox, yet CH223191 did not affect LDH-A expression in both ctrl cells and cells subjected to Reox. Data are presented as the mean \pm SEM. * P <0.05 vs. ctrl; # P <0.05 vs. ctrl CH223191; [^] P <0.05 vs. Reox; ⁺ P <0.05 vs. Reox CH223191. AhR, arylhydrocarbon receptor; RPTEC, renal proximal tubular epithelial cell; HIF-1α, hypoxia-inducible factor 1α; LDH-A, lactate dehydrogenase-A; ctrl, control; Reox, reoxygenation.

prevented reoxygenation-induced cell necrosis completely since, in this case, the percentage of cell necrosis was equal to that detected in control cells ($10.7 \pm 0.2\%$; P not significant compared to the control cells; $P < 0.001$ compared to cells subjected to reoxygenation only). Thus, reoxygenation-induced cell necrosis may be mediated through the ferroptotic pathway. Moreover, the AhR inhibitor CH223191 ameliorated reoxygenation-induced cell necrosis considerably. Compared with the cells subjected to reoxygenation, in cells subjected to

reoxygenation and treated with the AhR inhibitor, cell necrosis percentage decreased from 23.3 ± 0.6 to $15.3 \pm 0.4\%$ ($P < 0.001$). Of note, under control conditions, CH223191 was not toxic for RPTECs, since the percentage of cell necrosis was $10.7 \pm 0.8\%$ (P not significant; Fig. 2C).

Apoptosis was assessed using the levels of CC3. Neither reoxygenation nor CH223191 had any effect on CC3 levels. Considering the level of CC3 in the control cells as 1, CC3 level equaled 1.0 ± 0.05 in cells treated with the inhibitor,

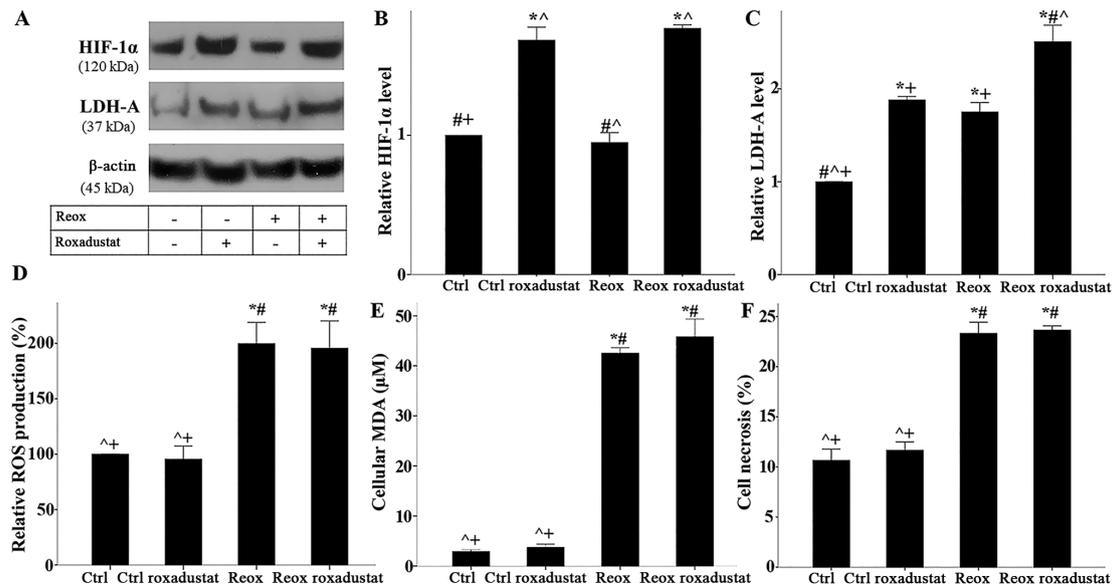


Figure 7. Roxadustat increases HIF-1α level and transcriptional activity but does not affect ROS production, lipid peroxidation, or cell necrosis. RPTECs were cultured under ctrl conditions or subjected to Reox with or without the HIF-1α activator roxadustat. (A) Representative western blot images of HIF-1α levels (corresponding to its activation status) and its transcriptional target LDH-A. (B and C) Statistical analysis of the western blots. Roxadustat enhanced HIF-1α levels in both ctrl cells and cells subjected to Reox. Roxadustat also upregulated LDH-A expression in both ctrl cells and cells subjected to Reox. Compared to ctrl cells, LDH-A levels increased in cells subjected to Reox. (D) ROS production, (E) lipid peroxidation and (F) cell necrosis were also assessed. Roxadustat did not affect Reox-induced ROS production, lipid peroxidation, and cell necrosis. Data are presented as the mean ± SEM. *P<0.05 vs. ctrl; ^P<0.05 vs. ctrl roxadustat; #P<0.05 vs. Reox; #P<0.05 vs. Reox roxadustat. RPTEC, renal proximal tubular epithelial cell; HIF-1α, hypoxia-inducible factor 1α; LDH-A, lactate dehydrogenase-A; ROS, reactive oxygen species; MDA, malondialdehyde; ctrl, control; Reox, reoxygenation.

1.1±0.08 in RPTECs subjected to reoxygenation, and 1.0±0.03 in cells subjected to reoxygenation and treated with CH223191 (P not significant; Fig. 4).

AhR activation status does not affect Nrf2 activation or transcriptional activity. Neither reoxygenation nor the AhR inhibitor CH223191 affected Nrf2 levels (which correspond to its activation). In RPTECs subjected to reoxygenation, Nrf2 levels equaled 1.01±0.07 of the control (P not significant). In RPTECs subjected to reoxygenation and treated with CH223191, Nrf2 levels equaled 0.97±0.03 of the control (P not significant, compared with control cells). Under normoxia, RPTECs treated with CH223191, Nrf2 levels equaled 0.98±0.06 of the control (P not significant; Fig. 5A and B). Thus, AhR does not affect Nrf2 activation status.

The stable expression of the Nrf2 transcriptional targets xCT and SOD-3 confirmed the aforementioned conclusion. Under reoxygenation, xCT and SOD-3 expression remained stable and equaled 1.04±0.04 and 1.01±0.07 of the control, respectively (P not significant in both cases). In RPTECs subjected to reoxygenation and treated with CH223191, xCT and SOD-3 expression also remained stable. In this case, xCT and SOD-3 levels equaled 1.01±0.03 and 0.91±0.07 of the control, respectively (P not significant in both cases). Moreover, under normoxic conditions, CH223191 did not alter the expression of the above proteins. xCT and SOD-3 expression levels equaled 1.04±0.04 and 0.98±0.06 of the control, respectively (P not significant in both cases; Fig. 5A, C and D).

AhR activation status does not affect HIF-1α levels or its transcriptional targets. Neither reoxygenation nor CH223191 affected HIF-1α levels (which correspond to its activation). In

cells treated with the AhR inhibitor CH223191, cells subjected to reoxygenation, and cells subjected to reoxygenation and treated with CH223191, HIF-1α levels were 1.10±0.07, 0.92±0.11, and 1.12±0.12 of the control, respectively (P not significant in any case; Fig. 6A and B). Thus, AhR does not alter HIF-1α activity.

The AhR inhibitor CH223191 did not affect the expression of the HIF-1α transcriptional target LDH-A, confirming the aforementioned conclusion. Compared to the control cells, in cells treated with CH223191, LDH-A expression remained stable (1.12±0.02 of the control; P not significant). Reoxygenation increased LDH-A expression to 1.56±0.16 of the control (P=0.008). However, CH223191 did not result in any further change to LDH-A expression, (1.62±0.15 of the control; P not significant compared with reoxygenation only; Fig. 6A and C).

Roxadustat increases HIF-1α level and transcriptional activity but does not affect ROS production, lipid peroxidation, or cell survival. Under normoxia, roxadustat increased HIF-1α to 1.68±0.09 of the control (P<0.001). Reoxygenation did not alter HIF-1α, compared with the control cells (0.95±0.07 of the control; P not significant). In RPTECs subjected to reoxygenation and treated with roxadustat, HIF-1α increased to 1.77±0.03 of the control (P<0.001, compared with the control and cells subjected to reoxygenation alone; Fig. 7A and B). Thus, the HIF-1α activator roxadustat increased HIF-1α level both in control cells and in cells subjected to reoxygenation.

Similarly, roxadustat increased the expression of the HIF-1α transcriptional target LDH-A, confirming that this compound activates HIF-1α at the used concentration of 10 μg/ml. Under normoxic conditions, roxadustat enhanced LDH-A expression

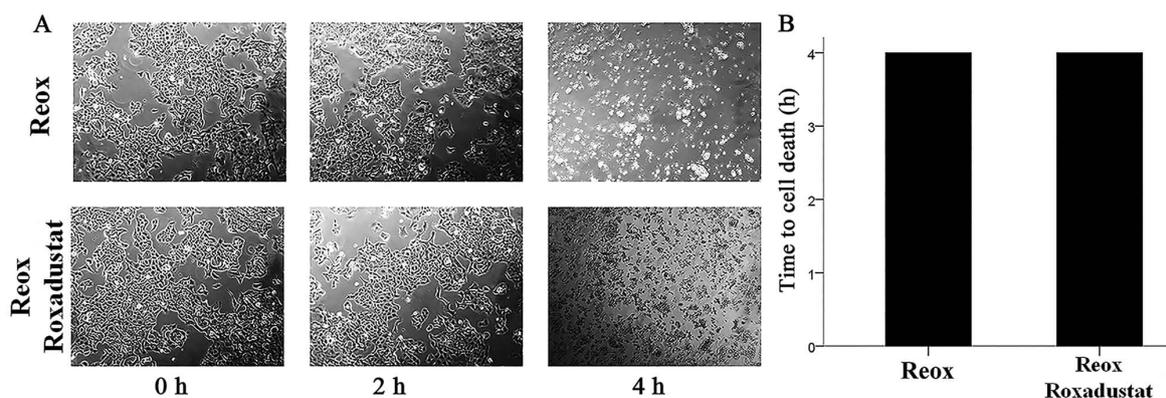


Figure 8. Activation of HIF-1 α protects from Reox-induced cell death. RPTECs were subjected to Reox with or without the HIF-1 α activator roxadustat. (A) Representative images of one of the experiments. Magnification, $\times 100$. (B) Statistical analysis of all experiments. Roxadustat did not alter RPTEC resistance to Reox-induced cell death, since both roxadustat-treated and untreated cells died within 4 h of observation. Data are presented as the mean \pm SEM of six independent experiments. RPTEC, renal proximal tubular epithelial cell; HIF-1 α , hypoxia-inducible factor 1 α ; Reox, reoxygenation.

to 1.88 ± 0.04 of the control ($P < 0.001$, compared with control cells). Reoxygenation increased LDH-A to 1.75 ± 0.10 of the control ($P < 0.001$, compared with control cells), although roxadustat treatment of RPTECs subjected to reoxygenation further increased LDH-A expression to 2.51 ± 0.18 of the control ($P < 0.001$, compared to control cells and cells subjected to reoxygenation alone; Fig. 7A and C).

Roxadustat did not affect ROS production or lipid peroxidation. Treatment of control cells with roxadustat did not alter relative ROS signal intensity. Considering the ROS production in the control cells as 100%, ROS levels reached $95.6 \pm 5.9\%$ of the control in cells under normoxia and treated with roxadustat (P not significant). Reoxygenation increased ROS signal intensity to $199.9 \pm 9.6\%$ ($P < 0.001$, compared with control cells), a value that remained stable when roxadustat was administered ($195.7 \pm 12.3\%$, P not significant, compared with cells subjected to reoxygenation alone; Fig. 7D). In RPTECs treated with roxadustat, cellular MDA did not change significantly, compared with the control group ($3.0 \pm 0.2 \mu\text{M}$ and 3.6 ± 0.6 , respectively; P not significant). Reoxygenation increased MDA concentration to $42.6 \pm 0.5 \mu\text{M}$ ($P < 0.001$, compared to the control cells). When roxadustat was administered at the onset of reoxygenation, MDA did not alter considerably ($45.8 \pm 1.8 \mu\text{M}$, P not significant), compared to cells subjected to reoxygenation only; Fig. 7E).

As regards the hard end-point of cell survival, LDH release assay revealed that in control cells, roxadustat did not alter the percentage of cell necrosis compared with the control (10.7 ± 0.6 vs. $11.7 \pm 0.4\%$, respectively; P not significant). Reoxygenation increased cell necrosis to $23.3 \pm 0.6\%$ ($P < 0.001$, compared to the control cells), while roxadustat did not affect necrosis ($23.7 \pm 0.2\%$; P not significant compared with cells subjected to reoxygenation only; Fig. 7F). Cell imaging confirmed the above LDH release assay results since it showed that roxadustat treatment did not offer any survival benefit in RPTECs subjected to reoxygenation. In all performed experiments, both treated and untreated cells died within 4 h of reoxygenation (P not significant; Fig. 8).

Discussion

I-R injury contributes to the pathogenesis of many diseases, including the acute kidney injury (1-4). As a consequence,

clarifying the pathogenesis of I-R injury is decisive in developing novel therapeutic strategies. This study aimed to evaluate the role of AhR during the reoxygenation phase of I-R injury.

Some data support a detrimental role of AhR during the anoxic phase of I-R injury. Inhibition of AhR/CYP1A1 protects murine hippocampal cells from anoxia-induced apoptosis (44). However, no data are available concerning the role of AhR during the subsequent reoxygenation. To evaluate the role of AhR in RPTECs subjected to reoxygenation, a cell culture system designed to study anoxia and reoxygenation separately was established.

In the present study, reoxygenation decreased AhR levels. The decrease in AhR level indicates its activation since activated AhR becomes vulnerable to proteasomal degradation, leading to decreased AhR cellular levels (20-24). Accordingly, reoxygenation increased the expression of CYP1A1, an AhR transcriptional target (18,19). The specific AhR inhibitor CH223191 increased AhR level and decreased CYP1A1 expression. Thus, AhR was activated during reoxygenation, and CH223191 successfully inhibited AhR activation. The exact endogenous AhR ligands, which results in its activation during reoxygenation, remain to be defined. Initially, AhR was considered a critical factor for the CYP-mediated metabolism of xenobiotics, such as dioxins, which are also AhR activators. However, endogenous AhR activators were identified subsequently (45,46). Examples of exogenous AhR activators include natural plant flavonoids, polyphenolics, and indoles, as well as dioxins-like and other synthetic compounds, collectively characterized as xenobiotics. Examples of endogenous AhR ligands include tryptophan derivatives, steroids, eicosanoids, and heme metabolites (45).

Observation of cell cultures under reoxygenation suggested that mouse RPTECs were particularly vulnerable to reoxygenation injury since they died within 4 hours. However, when the AhR inhibitor CH223191 was added at the onset of reoxygenation, it prevented cell death, with the cells remaining alive after 24 h of culture.

In accordance with previous experiments with the same cell type and similar experimental conditions (5), reoxygenation-induced cell death was not apoptotic since the levels of the activated CC3, in which all the apoptotic pathways

converge (47), was not altered. Of note, CH223191 did not enhance CC3, indicating that at the used concentration, this inhibitor does not induce apoptosis in RPTECs.

The primary factor that induces cell injury during the reperfusion phase of I-R is the burst of ROS production, which follows the restoration of oxygen supply to the ischemic tissues (1-3). CYPs play a significant role in ROS production during I-R injury. In experimental models of heart and liver I-R injury, inhibition of CYPs ameliorates ROS production and organ dysfunction (13-17). AhR transcribes certain CYP genes (18,19), and consequently, AhR activation during reoxygenation may contribute to cell injury. Indeed, in experimental models of heart and lung I-R injury, inhibition of AhR was beneficial (25,35). However, in the aforementioned studies, AhR and CYPs were investigated separately, and not as a part of a pathway. Importantly, the aforementioned studies did not discriminate between events taking place under the anoxic phase and from those occurring during the reoxygenation phase of I-R injury. The effect of AhR on ROS production was evaluated in RPTECs subjected to reoxygenation. Reoxygenation induced ROS production, which was prevented by the AhR inhibitor CH223191. Thus, AhR may play a pivotal role in the burst of ROS production that follows reoxygenation.

With regards reoxygenation-induced cell death, previous studies using primary RPTECs and similar experimental conditions have demonstrated that reoxygenation leads to lipid peroxidation and ferroptotic cell death (5,6). Elegant research using isolated murine renal tubules detected the same (7). Interestingly, besides ROS production, CYPs can directly induce lipid peroxidation (48), which causes ferroptosis (43). In our model, reoxygenation increased lipid peroxidation considerably, as identified by cellular MDA. Nevertheless, when AhR was inhibited, reoxygenation-induced lipid peroxidation decreased significantly. In accordance with microscopic observation of cell cultures, the LDH release assay confirmed the reoxygenation-induced cell necrosis biochemically. Administration of the ferroptosis inhibitor α -tocopherol prevented reoxygenation-induced cell necrosis completely, indicating the ferroptotic nature of cell death. The inhibition of AhR attenuated reoxygenation-induced cell death considerably, suggesting great therapeutic potential. Of note, in control RPTECs, CH223191 did not induce cell necrosis, proving non-toxic for RPTECs at the used concentration. Thus, during reoxygenation, AhR is activated and increases certain CYPs expression, which in turn, by increasing ROS, induces ferroptotic cell death. The endogenous AhR activators that participate in reoxygenation-induced cell injury remain to be identified. Interestingly, indoleamine 2,3-dioxygenase metabolizes tryptophan to kynurenine, an endogenous AhR activator. Inhibition of this enzyme was protective for murine kidneys subjected to I-R injury. However, whether the beneficial effect of indoleamine 2,3-dioxygenase inhibition was mediated by the suppression of AhR-CYPs pathway was not evaluated (49). The exact impact of indoleamine 2,3-dioxygenase on this pathway deserves further investigation.

Several studies have suggested that AhR induces the expression of Nrf2 (30-32), a transcription factor implicated in the expression of many antioxidant and anti-ferroptotic proteins (33,34). From a teleological point of view, this achieves a balance between AhR-induced CYP-mediated

oxidative stress and AhR-induced Nrf2-mediated antioxidant defense (31,32). However, in our model, and despite the reoxygenation-induced AhR activation, the cellular Nrf2 level remained stable. Since activated Nrf2 is released from the ubiquitin ligase cullin-3 and avoids proteasomal degradation, its cellular level corresponds to its activity (33,34). The nuclear Nrf2 levels were not assessed in order to better determine its activation status; instead, the expression of the Nrf2 transcriptional targets SOD-3 and xCT were measured (33). SOD-3 catalyzes the dismutation of superoxide radical into either molecular oxygen or hydrogen peroxide (50), while xCT enters the necessary for glutathione synthesis cystine into the cell and prevents ferroptosis (43). In the present study, the expression of both Nrf2 transcriptional targets SOD-3 and xCT remained stable, also indicating that reoxygenation left Nrf2 activation status unaffected. This was in agreement with a previous study showing that reoxygenation failed to enhance Nrf2 activity in mouse RPTECs (51). Interestingly, in RPTECs from the hibernator Syrian hamster, reoxygenation activates Nrf2 contributing to the resistance of these cells to reoxygenation-induced ferroptosis (51). Moreover, in the present study, the specific AhR inhibitor CH223191 was used. Cell treatment with the AhR inhibitor did not alter Nrf2, SOD-3, and xCT level, offering additional support to the idea that in the context of reoxygenation, AhR does not affect the Nrf2 pathway.

Another transcription factor implicated in I-R is HIF-1 α . HIF-1 α is upregulated during the ischemic phase, forms a complex with HIF-1 β , and transcribes many genes required for adaptation to anoxic conditions. For instance, LDH upregulation promotes the anaerobic catabolism of glucose (36,37). However, activated AhR also associates with HIF-1 β (33,34). In certain experimental models, this competition between AhR and HIF-1 α for HIF-1 β results in reduced HIF-1 α transcriptional activity in the case of AhR activation, or to decreased AhR transcriptional activity in the case of HIF-1 α activation (38-40). However, the interaction between AhR and HIF-1 has not been evaluated during the reperfusion phase of I-R injury. In the present experimental model, inhibition of AhR during reoxygenation did not affect either HIF-1 α levels (36,37), or the expression of its transcriptional target LDH-A. Interestingly, in RPTECs under reoxygenation, HIF-1 α level was similar to the control cells, whereas the expression of LDH-A was increased. The latter may result from rapid HIF-1 α degradation when oxygen is available. By contrast, the increased LDH-A levels may be the result of a slower turnover of this enzyme, which is upregulated during the anoxic phase.

To delineate the role of HIF-1 α further, RPTECs were subjected to reoxygenation with the HIF-1 α activator roxadustat. As expected, roxadustat increased HIF-1 α levels and LDH-A expression in both control cells and cells subjected to reoxygenation. However, roxadustat did not affect ROS production, lipid peroxidation or cell death. Thus, during reoxygenation, HIF-1 α does not play a pivotal role in cell survival.

Certainly, the *in vitro* nature of our study is a limitation. However, this approach allowed us to apply strict experimental conditions and to evaluate the effect of AhR on cell survival solely during the reoxygenation phase. As already noted, I-R injury consists of two consecutive but pathophysiologically distinct phases, anoxia and reoxygenation (1-3). A better understanding of the molecular mechanisms that govern the

two stages of I-R injury may result in an efficient combination of therapeutic approaches targeting both stages.

In conclusion, in RPTECs, AhR is activated during reoxygenation and induces ROS production, lipid peroxidation and ferroptotic cell death. These detrimental effects may be mediated by AhR-induced CYP overexpression, while AhR does not affect the Nrf2 or the HIF-1 α pathways. Thus, the AhR pathway may represent a promising therapeutic target for the prevention of reperfusion injury.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TE designed the study. GP and TE performed the experiments. TE, GP, GF, VL and IS analyzed the results. TE wrote the manuscript with help from GP. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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