Morg1 heterozygous deficiency ameliorates hypoxia-induced acute renal injury

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Running Title: Systemic hypoxia and renal damage

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ABSTRACT

Acute kidney injury is a common complication of critically ill patients and may occur as a result of various factors and coexisting previous illnesses. Some pathophysiological responses seen in critical illness can be similar to the human physiological response to extreme environmental challenges, such as hypoxia from reduced oxygen availability at high altitudes (systemic hypoxia). Due to oxygen deficiency mammalian cells activate the transcriptional factor HIF (hypoxia-inducible factor), its degradation is regulated by PHD3 (prolyl hydroxylase 3) in interaction with the scaffold protein Morg1 (MAPK organizer 1). While homozygous Morg1−/− mice are embryonic lethal, the kidneys of heterozygous Morg1+/− mice reveal elevated HIF protein levels and increased serum erythropoietin compared to wildtype Morg1+/+ mice. In this study, we exposed wildtype and Morg1+/− mice to 10% oxygen in a hypoxic chamber for three days. This reduced oxygen concentration leads to a deterioration of renal function, an increase in renal inflammation and significantly more tubular damage and apoptosis in the kidneys of wildtype (Morg1+/+) mice. In sharp contrast, Morg1+/− kidneys were protected against systemic hypoxia. They show significantly less renal lesions, reduced or no inflammation, less tubular damage and apoptosis. Thus short-term systemic and subsequently renal hypoxia which may occur in many patients in the intensive care unit, induces in wildtype mice renal injury which is ameliorated by Morg1 deficiency. Our findings suggest that therapeutical manipulation of Morg1 may be an interesting novel target to prevent hypoxia-associated renal damage.

KEY WORDS: acute renal injury, critical illness, systemic hypoxia, Morg1, HIF-α
INTRODUCTION

Acute kidney injury (AKI) is a common complication of critical illness affecting one third to two thirds of patients in the intensive care unit and is associated with high mortality (approx. 20 and 60%) (6, 43). AKI is classically defined as an abrupt (hours to days) and sustained decrease in the kidney’s excretory function (6, 43). The causes of AKI are traditionally grouped into three categories: prerenal, renal (intrinsic kidney disease), and postrenal. Whereas intrinsic renal disease has been the most common cause in the past, AKI now often accompanies systemic or extrarenal illness and can be part of multiorgan failure syndrome, for example subsequent to sepsis, especially in elderly people (30). AKI often develops as a consequence of one or more consecutive insults such as sepsis, volume depletion, renal ischaemia, or shock (30). Acute changes in systemic and renal pO2 are frequently found in intensive care unit patients and it is widely accepted that renal hypoxia plays a crucial role in the pathogenesis of AKI. The high sensitivity to changes in oxygen delivery makes the kidney prone to hypoxic injury (12, 17, 38). A classic systemic adaptation to hypoxia is the increase in erythropoietin (EPO) with subsequently enhanced red blood cell production, that is orchestrated by hypoxia-inducible factors (HIFs) (18). HIFs are pleiotropic oxygen-sensitive, heterodimeric transcription factors that have key roles in the regulation of various biological processes (17). Whereas under normoxic conditions, hydroxylation of HIF-α leads to polyubiquitylation and rapid degradation in proteasomes; under hypoxic conditions, HIF-α remains stable, heterodimerizes with HIF-β, and transcriptionally activates a large number of genes, including the gene encoding EPO (52). Post-translational HIF-α hydroxylation is catalyzed by specific HIF-prolyl hydroxylases (PHDs) (38). All three identified PHDs (PHD1, PHD2, and PHD3) are expressed in the kidney, where they control HIF activity (17). Compared with PHD2, PHD1 and PHD3 are more abundant in the glomeruli, whereas all three
PHDs appear to be expressed at higher levels in the distal renal tubule compared with the proximal tubular epithelium (17). PHDs are regulated on three levels: transcription, protein abundance/stability, and enzymatic activity (52). At the transcriptional level, for example, it has been shown that PHD2 and PHD3, but not PHD1, are HIF target genes induced under hypoxic conditions (52). In addition to transcriptional regulation, newly identified PHD interaction partners are able to regulate various aspects of PHD function (52). A number of proteins with putative functions, including protein stability and molecular scaffold, have recently been identified as interacting with PHDs (52). We identified Morg1, a WD-repeat protein, as a molecular scaffold that directly binds PHD3 in vitro and in vivo (24). We showed that Morg1 decreases HIF-mediated reporter gene activity and that this effect is additive by co-expression of PHD3 (24). Suppression of Morg1 results in a marked increase of HIF activity (24) and we show that heterozygous Morg1 knockout mice (Morg1+/−), generated by homologous recombination, are protected from experimentally induced focal cerebral ischemia (50) as well as from acute renal ischemia-reperfusion injury (19). The decreased injury of ischemic kidneys from Morg1+/− mice compared to the wildtype, reflected by less tubular damage, a smaller serum creatinine increase, less inflammation and apoptosis, was associated with a stronger increase in HIF-1α and HIF-2α expression as well as with enhanced serum erythropoietin levels (19). There are different modes of HIF signal enhancement: (i) chemical inhibition of PHDs by hypoxia-mimetics (e.g. CoCl2), (ii) molecular biology techniques (e.g. on-Hippel-Lindau knockout), and (iii) inhibition of PHDs by the induction of cellular physiological hypoxia (e.g. arterial clamping) (22). The potential protective impact of HIF upregulation has been studied in various acute organ injuries, e.g. myocardial injury, lung injury, neuronal injuries, and acute kidney injury (22). Although the cellular and molecular consequences of chronic or intermittent
hypoxia on the kidney are less obvious, some insights into the effects of subacute or chronic hypoxic signalling on kidneys can be gained from studies of humans permanently living at high altitudes (17). Although they may be genetically adapted to diminished oxygen availability, they show an increased prevalence of microalbuminuria and proteinuria as well as glomerular hypertrophy (4, 27, 33). A new paradigm suggests that the physiological and pathophysiological responses to extreme environmental challenge (e.g. high-altitude hypoxia) may be similar to responses seen in critical illness (16). Therefore, studying responses to hypoxia as a result of breathing a low fractional inspired oxygen tension, such as at high-altitudes, may offer important insights into the pathophysiology of critical illness (16). In this study, we exposed wildtype (Morg1+/+) and heterozygous Morg1+/- mice to diminished oxygen availability (10% oxygen for 3 days) and tested the hypothesis that downregulation of Morg1, as a preconditional activation of HIFs, may protect the kidney from AKI induced by systemic hypoxia.

METHODS

Animal model and study protocol.

All animal experiments were approved by the local Ethics Committee and were done in accordance with the German Animal Protection Law. We studied Morg1+/- mice of C57bl6 background, which were generated as described before (16), and their wildtype littermates. All mice were 12 - 15 weeks old and only male mice were used to control for potential hormonal effects. Wildtype and Morg1+/- mice were subjected to systemic normobaric hypoxia (12 mice per group) by substituting normal compressed air with nitrogen using a small anesthetic bracket system with flowmeters for two gases (Hugo Sachs Elektronic – Harvard Apparatus GmbH, March-Hugstetten, Germany) at a constant gas flow rate of 2 l/min in an air-tight
chamber gases (Hugo Sachs Elektronic – Harvard Apparatus GmbH, Germany). All animals were provided with food and water, allowed to adjust to the hypoxic environment by gradually decreasing the oxygen concentration from 21 to 10% during an adaptation time of 1 h, and kept at 10% O\textsubscript{2} for three days of continuous hypoxia. Control mice (normoxia) (n=12) were placed in the same chamber with normal air (21% O\textsubscript{2}) with a flow rate of 2 l/min for three days. At the end of the experiment all mice were placed in metabolic cages (Tecniplast, Buguggiate, Italy) in room air (21% O\textsubscript{2}) over night to collect the urine samples (in the text called as ‘reoxygenation’). For some immunohistochemical analysis additional time points of hypoxia (1h, 24h, 72h, and 10 days) were chosen, at which no reoxygenations were performed. We assessed hematocrit, hemoglobin, MCV (mean corpuscular volume), MCH (mean corpuscular haemoglobin), and MCHC (mean corpuscular haemoglobin concentration) with pocH-100iV DIFF (Sysmex, Norderstedt, Germany). Mouse tail vein blood glucose levels were measured with Free Style Lite (Abbott Diabetes Care, Wiesbaden, Germany). Mice were killed and the kidneys were removed. One kidney per mouse was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological and immunohistochemical studies. The remaining kidney was mechanically homogenized with the homogenizer SpeedMill P12 (Analytik Jena Bio Solutions, Jena, Germany) and total RNA or protein were isolated as described below.

Assessment of renal function.

To quantify albuminuria the urinary albumin-to-creatinine ratio (ACR) was determined. Urinary albumin excretion was measured using an ELISA specific for mouse albumin (Cell Trend, Luckenwalde, Germany) and urinary creatinine was measured with a standard enzymatic assay (Cayman chemicals, Ann Arbor, USA).
NGAL concentrations in the urine were determined by using mouse NGAL ELISA according to the manufacturer’s instructions (Biporto Diagnostics, Gentofte, Denmark). Mouse serum creatinine and urea levels were measured with Fuji Dri-chem Slides CRE-PIII respectively BUN-PIII (Fujifilm Europe, Düsseldorf, Germany). Serum erythropoietin (EPO) levels were determined with a specific mouse erythropoietin ELISA (Quantikine, R&D Systems, Wiesbaden, Germany) and serum interleukin 6 (IL6) concentrations with a specific mouse IL6 ELISA (Quantikine, R&D Systems, Wiesbaden, Germany).

Histology, immunohistochemistry, and analysis of apoptosis.

For histology, paraffin-embedded kidneys were sectioned at 4 µm and stained with periodic acid-Schiff (PAS) (PAS staining Kit from Roth, Karlsruhe, Germany). Histological changes due to tubular damage were graded as follows: 0 = none, 1 = <10%, 2 = 11–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >76% by an investigator who was unaware of the treatment groups. For immunohistochemistry, deparaffinized kidney sections, 4 µm thick, were subjected to heat-mediated antigen retrieval in citrate buffer (pH 6.0) (except for CTGF) and then incubated with 3% H₂O₂ for 10 min at room temperature to block endogenous peroxidase. As primary antibodies a polyclonal rabbit anti-Morg1 (Biotrend, Berlin, Germany), a polyclonal rabbit anti-PHD3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), polyclonal rabbit anti-CTGF, anti-TNFα (Abcam, Cambridge, UK), polyclonal goat anti-HIF-1α, anti-HIF-2α (R&D Systems, Wiesbaden, Germany), and a monoclonal mouse anti-AQP1 (Abcam) antibody were used. Diaminobenzidine (DAB) was used for detection as the chromogen (peroxidase substrate kit DAB; Vector Laboratories, Burlingame, CA) after incubation of the sections with peroxidase-labeled goat anti-rabbit resp. anti-mouse or anti-goat IgG antibody (KPL, Gaithersburg, MD). Apoptosis was detected...
by the terminal transferase-dUTP-nick-end labeling (TUNEL) method using an In Situ Cell Death Detection Kit from Roche Diagnostics (Penzberg, Germany).

For imaging and documentation, a computer-assisted microscope with digital camera and AxioVision 4.8 software was used (Carl Zeiss). Five nonoverlapping fields of each individual kidney sample (12 mice per group) were scanned in the monochrome mode of the camera (magnification: 200x). For each parameter, all images were taken under constant conditions as appropriate. For the quantitative assessment of staining intensities, the area was highlighted and the mean densitometric gray levels were measured. Finally, the average of gray labels obtained for each individual kidney sample was used as an equivalent for the respective staining intensity.

Reverse transcription and real-time PCR.

Total RNA was isolated from kidney homogenates using the RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), possible DNA contaminations were eliminated using the RNase-Free DNase Set (Qiagen), and 1 µg total RNA was reverse-transcribed using the Reverse Transcription System from Promega (Madison, WI). The expression levels of genes were determined as previously described (19). Table 3 shows the sequences and annealing temperature of all primer pairs. Transcript levels were normalized to the mean value of samples from the normoxic wildtype animals.

Western blot analysis.

Proteins were extracted from renal homogenates using ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Merck, Darmstadt, Germany). 50 micrograms of protein lysate from membrane fraction was loaded into each lane. SDS-PAGE and Western blotting were performed as described elsewhere (19). To assess the protein
expression of TNFα and the caspase 3 cleavage, the membrane was further
incubated overnight with a polyclonal rabbit antibody against mouse TNFα (Abcam)
at a dilution of 1:100 resp. a polyclonal rabbit antibody against mouse caspase 3
(Abcam) at a dilution of 1:500 in 5% BSA-TBST, washed several times with TBST,
and incubated for one hour with a horseradish peroxidase-conjugated goat anti-rabbit
secondary antibody (1:2000; KPL, Gaithersburg, MD). Equal loading was controlled
by reincubation with a monoclonal antibody against vinculin (Sigma-Aldrich, St. Louis,
MO). After intensive washing, the proteins were visualized with ECL detection
reagent (Roth). For quantification, the band densities were measured using the
ImageJ software provided by NIH on their website and normalized for the respective
densities of vinculin bands as loading controls.

Statistical analysis.
The values given in this article are presented as mean ± S.E.M. Results were
analyzed using SPSS statistics (IBM company, Armonk, NY). The Kruskal-Wallis test
was first used for multigroup comparison followed by the Mann-Whitney rank sum
test to compare two groups of mice. A $P$ value of $\leq 0.05$ was considered significant.

RESULTS

Influence of systemic hypoxia on clinical and laboratory parameters.
The effects of systemic hypoxia (10% O₂, 3 days) compared with normoxia (21% O₂,
three days) on clinical and laboratory parameters are summarized in Table 1. The
results reveal that, even at normoxic levels, in Morg1⁺/⁻ mice the total number of red
blood cells, hemoglobin as well as hematocrit are significantly higher than in wildtype
mice. In wildtype mice these parameters were significantly increased after hypoxic
treatment to a level similar to those of the normoxic Morg1⁺/⁻ mice. Interestingly, in
contrast to the heterozygous Morg1 mice, hypoxia leads to a significant rise of white
blood cells in the wildtype mice, suggesting that Morg1 deficiency may somewhat
protect against hypoxia-induced systemic inflammatory response.

The hypoxia-induced decline of kidney function is reduced in Morg1\(^+/-\) mice.

Data from various renal function analyses (Table 2) reveal that urine output
significantly decreases after exposure to hypoxia independent of genotype, but more
precise parameters of kidney function, such as ACR (albumin-to-creatinine ratio),
serum urea and serum creatinine, show significant increases in response to hypoxia
only in the wildtype and not (or significantly less) in the Morg1\(^+/-\) mice (Table 2).
These data may suggest that Morg1\(^+/-\) animals are protected against systemic
hypoxia-induced deterioration of kidney function.

Wildtype and Morg1\(^+/-\) mice differ in HIF activity after systemic hypoxia.

To study the PHD3/HIF axis in response to systemic hypoxia, we investigated the
renal expression of mRNA and protein and tested the activity of HIF-1\(\alpha\) and HIF-2\(\alpha\)
via expression of EPO. In the case of wildtype mice we observed the expected
results: systemic hypoxia induces mRNA (Fig. 1, A) and protein (Fig. 1, B-G)
expression of PHD3 and Morg1 as well as of HIF-1\(\alpha\) and HIF-2\(\alpha\). Figures 1 B and C
demonstrate a hypoxia-induced increase of Morg1, PHD3, HIF-1\(\alpha\) and HIF-2\(\alpha\)
protein expression even after 1 hour of hypoxia. Although on day 4 (three days 10%
\(\text{O}_2\) with afterwards reoxygenation over night) a significant increase of HIF-1\(\alpha\)/HIF-2\(\alpha\)
mRNA and protein were no longer detectable (Fig. 1, A, F and G), the systemic
serum concentration of the typical target gene of HIF, EPO, were significantly
elevated after systemic hypoxia (Fig. 1, H). In the Morg1\(^+/-\) mice exposed to hypoxia,
renal immunhistochemical data show also an induction of PHD3 and Morg1 over the
time, which was significantly less compared with wildtype animals (Fig. 1, B, D and E). Similar to wildtype mice, in Morg1\(^{+/−}\) mice the renal HIF expression was first increased during exposure to 10% O\(_2\) and then no more significantly altered at the time point after hypoxia and reoxygenation, but in the kidneys of the Morg1\(^{+/−}\) mice the basal level of both HIF-1\(α\) and HIF-2\(α\) were already significantly higher than in the wildtype animals (Fig. 1, C, F and G). Figure 1 H clearly reveals that under normoxic conditions, the serum EPO was significantly higher in Morg1\(^{+/−}\) mice than in the wildtype. Interestingly, this elevated level of EPO decreased in the Morg1\(^{+/−}\) mice after three days of hypoxia (Fig. 1, H). Immunohistochemical analysis of Morg1, PHD3, HIF-1\(α\) and HIF-2\(α\) protein in the mouse kidneys after 10 days of hypoxia showed no differences in the expression compared to the 72h time point.

Hypoxia-induced tubular damage and pre-fibrotic changes are reduced in Morg1\(^{+/−}\) mice.

To investigate the changes in tubular structure after hypoxia, we initially used PAS staining to assess overall morphology (Fig. 2, A). It was clearly visible that, in contrast to the heterozygous Morg1\(^{+/−}\) mice, tubuli of the wildtype mice exposed to 10% O2 for three days, show obvious loss of the brush borders as well as numerous exfoliated degenerated cells (Fig. 2, A and B). It has been previously shown that hypoxia promotes fibrogenesis in renal cells and induces extracellular matrix (ECM) synthesis (40-42). Therefore, the potential influence of hypoxia on ECM production in our model was tested by looking at renal protein expression of collagen type I (Col-I) using immunohistochemistry (Fig. 2, C and D). Whereas in wildtype kidneys Col-I is markedly elevated after hypoxia, no significant differences between normoxia and hypoxia were seen in the Morg1\(^{+/−}\) mice (Fig. 2, C and D). In addition, we tested the effects of low oxygen on different markers for kidney damage, in particular neutrophil
gelatinase-associated lipocalin (NGAL), the kidney injury molecule-1 (KIM1), the profibrotic factor connective tissue growth factor (CTGF) and aquaporin 1 (AQP1), a proximal tubular marker upregulated in a variety of renal diseases (Fig. 2, E-J). In contrast to the Morg1⁺⁻ mice, the mRNA expression of all tested markers was significantly increased after hypoxia in wildtype mice, notably the KIM1 and NGAL expression (Fig. 2, E). Measurement of urinary NGAL concentrations confirms the finding that kidneys of wildtype mice are damaged by hypoxia, whereas the NGAL concentrations in the urine of Morg1⁺⁻ mice were only marginally increased (Fig. 2, F). Immunohistochemical staining of CTGF, a profibrotic marker, showed an elevation of this protein after exposure of Morg1⁺⁻ mice to 10% O2. However, in heterozygous mice significantly less renal staining for CTGF was found compared to wildtype animals after hypoxia (Fig. 2, G and H). The expression of the water channel AQP1, as studied by immunohistochemistry, dramatically increased after three days of hypoxia in both genotypes without significant difference (Fig. 2, I and J).

Kidneys from hypoxic Morg1⁺⁻ mice express less inflammatory cytokines compared with wildtype.

As hypoxia can trigger inflammatory responses, we tested for this effect in Morg1⁺⁻ mice (Fig. 3). Using real-time PCR, we quantified the mRNA expression of various pro-inflammatory cytokines (Fig. 3, A) and chemokines (Fig. 3, B). As expected, kidneys of the wildtype mice exposed to hypoxia expressed significantly more transcripts for proinflammatory cytokines, such as TNF-α (tumor necrosis factor-alpha) and IL6 (interleukin 6), as well as the acute phase protein SAA (serum amyloid A) (Fig. 3, A). An increase in chemokine expression was only seen for MCP1 (monocyte chemotactic protein-1) and MIP2 (macrophage inflammatory protein-2),
but not for HMGB1 (High-mobility group protein B1) (Fig. 3, B). No differences in the renal expression of all tested inflammatory markers were found between normoxia and hypoxia in Morg1 heterozygous mice (Fig. 3, A and B). To monitor the protein level of TNF-α, additional immunohistochemistry (Fig. 3, C and D) and western blot analysis (Fig. 3, E and F) were performed. In agreement with the mRNA data, kidneys of the wildtype animals show a strong hypoxia-induced increase of TNF-α protein expression whereas no response was found in Morg1+/− kidneys. Similar results were seen for serum IL6 concentrations, as measured with ELISA (Fig. 3, G).

In contrast to wildtype animals no significant increase in apoptosis was detectable in the kidneys of Morg1+/− mice after hypoxia.

To test whether the hypoxia-induced decline of kidney function in wildtype is associated with renal cell death, we initially investigated the mRNA expression of pro-apoptotic proteins (Bax and Bad) and the anti-apoptotic protein Bcl-Xl (Fig. 4, A). There were no significant changes of Bax, Bad and BCL-XI in wildtype mice exposed to hypoxia compared with normoxia (Fig. 4, A). However, Bax and Bad, but not BCL-XI mRNA expression significantly decreased in the Morg1+/− mice exposed to 10% O₂ compared with animals with normoxia (Fig. 4, A). We further assessed apoptotic cells using TUNEL staining. Figures 4 (B and C) strikingly show how reduced oxygen availability for a duration of three days significantly increases the number of TUNEL-positive cells in wildtype Morg1+/+ kidneys, whereas hypoxia had no significant effects on tubular TUNEL-positive cells in Morg1+/− mice. To confirm the findings of TUNEL analysis, another assay for apoptosis was performed. Immunoblot analysis for cleaved caspase 3 shows in kidney homogenates from hypoxic wildtype mice a significant increase in active caspase 3 compared to homogenates from
normoxic animals (Fig. 4, D and E). In contrast, this increased apoptotic activity was not detectable in Morg1+/− mice after 3 days hypoxia (Fig. 4, D and E).

DISCUSSION

Acute kidney injury (AKI) frequently occurs in the critically ill and is associated with high mortality and morbidity (15, 53). AKI in the intensive care unit is rarely an isolated event and often progresses into a multiorgan dysfunction syndrome, ultimately leading to death (53). In AKI, circumstantial evidence suggests that hypoxic injury to renal cells plays a significant role (12). In humans, it is difficult to determine whether AKI is a cause or rather the consequence of excess morbidity (15). There are various animal models to study the pathophysiology of AKI, which may reduce the complexity and experimental limitations encountered in human studies (6, 53). The use of “single insult” models of AKI, e.g. the most commonly studied renal ischemia reperfusion, however, clearly fails to reflect the multifactorial causes of AKI that is thought to occur in the clinical setting (53). Recently, a novel theory has emerged which suggests that the human response to hypoxia induced by high altitude reflects some elements of the complex pathophysiology of critical illness (16). The present study was carried out to investigate the effects of short-term exposure to 10% oxygen (systemic hypoxia) versus 21% O2 (normal air) on the kidneys of mice as well as the role of Morg1 heterozygous deficiency in these settings. Ten percent oxygen availability corresponds to the conditions of an altitude more than 5,000 m high, which is defined as a very high altitude (14). It is well known that inspiratory hypoxia increases the number of red blood cells, the hemoglobin concentration and hematocrit (4, 21, 33, 49). It has been suggested that the hypoxia-induced rise in hematocrit helps to maintain tissue oxygen delivery (33).
We could confirm in wildtype mice a hypoxia-induced increase in hematocrit, but in 
the heterozygous Morg1 animals the hypoxia-induced effects were not significant 
compared with 21% O$_2$. Moreover, their normoxic basal levels of this parameter were 
higher than in the Morg1$^{+/−}$ mice compared with the wildtype animals. The elevated 
number of red blood cells in untreated Morg1$^{+/−}$ mice likely results from enhanced 
erythropoiesis, which is due to the increase in HIF activity when Morg1 is diminished 
(24). HIFs regulate red blood cell production by induction of cell-type specific gene 
expression changes that result in increased erythropoietin (EPO) production in the 
kidney and liver (18). We detected in the kidneys of normoxic Morg1$^{+/−}$ mice 
significantly more HIF-1$α$/2$α$ proteins compared with the wildtype animals under 
normoxic conditions. This was associated with high systemic basal EPO levels in the 
serum of the heterozygous mice, even under normoxia. After induction of short-term 
hypoxia (three days) the wildtype animals show increasing serum EPO 
concentrations as well as enhanced Morg1 and PHD3 mRNA and protein 
expressions. It has been previously shown that PHD3 is also a HIF target gene, 
which in turn downregulates the HIF-α protein (52). The HIF-PHD regulatory loop and 
the fact that hypoxia-induced HIF-α proteins are rapidly degraded in the presence of 
oxygen (48), leads to the logical result that an increase in HIF-α expression following 
hypoxia was no longer detectable at the studied time-point. Morg1 and PHD3 
expression were also inducible by hypoxia in the heterozygous mice, but to a lesser 
extent than in the wildtype. Since PHD3 had relatively more influence on HIF-2$α$ than 
HIF-1$α$ hydroxylation (3), this finding may explain the trend of more HIF-2$α$ proteins 
in the kidneys of the Morg1$^{+/−}$ mice after induction of hypoxia. Although several 
studies have suggested that both HIF-1$α$ and HIF-2$α$ contribute to the EPO-mediated 
response, recent molecular genetic studies in mice have reported a dominant role for
HIF-2α in induction of EPO production (17, 18, 39, 47). Interestingly, in this study we found that after three days of systemic hypoxia the serum EPO concentrations of the Morg1+/− mice fall to levels similar to those of normoxic wildtype mice. Studies of the effects of respiratory hypoxia on plasma EPO in humans who move from sea level to high altitude have shown the same phenomenon: EPO levels show a rapid increase, peaking on the second day of hypoxic exposure followed by a decrease during successive days (21, 49). Plasma EPO of high-altitude residents does not differ from those living at sea-level, because of high hematocrit levels that negatively influence renal EPO synthesis (52). The plasma EPO half-life is not constant and can be modified by varying metabolic clearances or by increased receptor binding (10, 49). Whether the decreased EPO concentration detected in the plasma of hypoxic Morg1+/− mice results from a diminished EPO production due to adaptation or is due to increased EPO activity remains open to further studies. However, the major finding of this study is that mice with diminished Morg1 expression are protected from renal injury induced by hypoxia compared with wildtype mice.

The clinical diagnosis of AKI is difficult, because AKI is typically asymptomatic (6). The laboratory hallmarks of AKI are oliguria and the presence of an increased serum creatinine and/or plasma urea (6, 43). We show here that, in contrast to Morg1+/− mice, serum creatinine and urea are slightly increased in hypoxic wildtype mice. Moreover, the urinary albumin concentration, a characteristic of impaired glomerular ultrafiltration and/or tubular handling of albumin, was significantly higher in wildtype than in heterozygous Morg1 mice after exposure to hypoxia. During critical illness, oliguria is often used as a clinical marker of AKI (45). Although, we found the same magnitude of decrease in urine output independent from genotype, it has also been reported that oliguria is neither specific nor sensitive enough to monitor AKI (6). Furthermore, we tested the mRNA and protein expression of AQP1, a water channel,
which is known to be induced by hypoxia (1). It has been shown that reduced AQP1 leads to the inability to concentrate urine in response to volume depletion (35), which conversely implies that an overexpression reduces the urine output. Our finding that AQP1 protein in both genotypes is significantly increased by hypoxia without any difference between wildtype and Morg1+/− mice could be a possible explanation for the similar urine output.

To confirm the ameliorated hypoxia-induced kidney injury in Morg1+/− mice compared to wildtype, we investigated the promoter for kidney fibrosis KIM1 and the biomarker of AKI NGAL, which appears to increase significantly earlier than the changes in serum creatinine (5, 6, 8, 26, 34, 36). In contrast serum creatinine, which increases only when more than 50% of the glomerular filtration rate is reduced (6), NGAL, a small secreted polypeptide that is detectable in the urine, is easily detected in the urine immediately after induction of acute renal failure in mice and rats as well as even after mild renal ischemia in mice (1). The results from NGAL expression and urine analysis clearly revealed that kidneys from Morg1+/− mice were less damaged than those from the wildtype mice. Reasons for the deteriorated renal functions by hypoxia are changes in extracellular matrix metabolism and increased level of profibrogenic factors (28, 40, 41). Furthermore, the finding that KIM1 is increased in kidneys of hypoxic wildtype animals confirm this assumption. For KIM1, a highly induction of expression after acute injury and in fibrotic kidneys has been shown (26).

To circumstantiate fibrotic actions after induction of hypoxia, we investigated the expression of collagen I and CTGF, which are known to be induced in renal cells by hypoxia (28, 40). Whereas in wildtype mice a significant increase was detectable, in the kidneys of the Morg1+/− mice we found no significant effects of hypoxia on the expression of Col-I as well as CTGF.
Experimental studies of AKI, including our recently published ischemic AKI work, show that inflammation plays an important role in the initiation and extension phases of AKI (2, 13). From the observation of the slightly elevated number of circulating white blood cells and the indications of kidney injury in wildtype animals exposed to hypoxia we expected an inflammatory response. The investigation of the renal expression of several proinflammatory cytokines (e.g. TNF$\alpha$ and IL-6), chemokines (e.g. MCP1 and MIP2), and the acute phase protein SAA, that have been previously shown to be increased after induction of AKI and to mediate ischemic AKI (2, 51, 53), confirmed the hypothesis that only in wildtype mice hypoxia induces an inflammatory response. Among profibrotic changes, which are inducible by KIM1, it has been also shown that KIM1 expression correlates with MCP1 expression (26).

Apoptosis has been investigated in a wide range of AKI models including renal ischaemia-reperfusion injury and it has been shown that hypoxia can induce apoptosis, dependent on transcriptional activation of apoptotic factors (11, 19, 32). Recently, we reported that tubular apoptosis is decreased in ischemic and reperfused Morg1$^{-/-}$ kidneys compared with ischemic kidneys of wildtype mice (19). The results of the present study revealed that systemic hypoxia leads to a marked increase of TUNEL-positive cells and caspase 3 activity in wildtype mice, whereas no significant induction of apoptosis was observed when Morg1 is diminished. Ratanen et al. showed that PHD3 activates apoptosis in an oxygen- and hydroxylase activity-dependent manner (46). Therefore, the diminished Morg1 and PHD3 expression in heterozygous Morg1 mice and the subsequently decreased activity of PHD3 may result in the attenuated apoptosis independently of the HIF axis.

Results in total from this study show for the first time that heterozygous Morg1 deficiency protects against kidney injury, induced by systemic hypoxia, which is an important factor in critical illness. In agreement with our results, the potential
protective impact of HIF upregulation has been extensively studied in AKI and the
use of pharmacologic HIF activation in the prevention of AKI is supported by
preclinical studies (7, 17, 22, 23). In addition, accumulating evidence suggests that
EPO has organ protective effects, which may be useful in the prevention of AKI (37,
44). On the basis of our results, we propose that Morg1 inhibition may have a
significant novel therapeutic potential to limit kidney injury in critically ill patients.

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No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTION
Author contributions: I.L. and G.W. conception and design of research; I.L. performed
experiments; I.L. and G.W. analyzed data, interpreted results of experiments,
prepared figures, drafted manuscript, and approved final version of manuscript.

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e28385, 2011.


LEGENDS TO FIGURES

Fig. 1. A-H: The PHD3/HIF axis in response to different oxygen levels depends on the Morg1 genotype. A: Real-time PCR analysis of Morg1, PHD3, HIF-1α, and HIF-2α expression after 3 days of hypoxia and reoxygenation. At this time point, only an increase of Morg1 and PHD3 mRNA in wildtype by hypoxia is detectable. Values were normalized to 18SrRNA expression, and the controls (wildtype normoxia) were assigned as an arbitrary value of 1. B and C: Representative stainings for Morg1, PHD3, HIF-1α and HIF-2α at different time points of hypoxia (magnification: x100;...
bars: 200µm). Black-rimmed image: higher magnification (x400; bar: 50µm) of marked area in the images of time point 72 h + reoxygenation. A hypoxia-induced increase of the expression of all proteins is detectable at 1h, 24h and 72h. After 10 days of hypoxia no further changes compared to the 72h time point were detectable. The images from the 72h plus reoxygenation confirm the findings of real-time PCR analysis, but additionally an increase of Morg1 and PHD3 expression in Morg1<sup>+/−</sup> mice after hypoxia was found, which was semiquantitative analyzed for Morg1 in D), for PHD3 in E), for Hif-1α in F), and for Hif-2α in G) (for details see METHODS). H:

Serum erythropoietin (EPO) level are elevated in wildtype mice after induction of hypoxia for 3 days and reoxygenation. Data represent mean values ± SEM; n = 12 mice per group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 hypoxia versus normoxia; #P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001 Morg1<sup>+/−</sup> versus wildtype (Morg1<sup>+/+</sup>).

Fig. 2. A-J. Heterozygous deficiency of Morg1 reduces tubular damage and prefibrotic changes induced by systemic hypoxia. Representative examples of Periodic acid-Schiff (PAS) staining (A), relative PAS staining intensity (B), representative examples of immunohistochemical staining for collagen type I staining (C), semiquantitative analysis (see METHODS) of collagen I (Col-I) staining (D). E:
mRNA expressions in whole kidneys for neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule-1 (KIM1), connective tissue growth factor (CTGF) and aquaporin 1 (AQP1) using real-time PCR. Measurement of urinary NGAL excretion (F), representative examples of immunohistochemical staining for CTGF (G), semiquantitative analysis for CTGF staining (H), representative examples of immunohistochemical staining for AQP1 (I), semiquantitative analysis for AQP1 staining (J). Magnification: x400 (B), x200 (A, E, F); Bars: 50µm (B), 100µm (A, E, F). Black-rimmed image in (A): higher magnification (x400; bar: 50µm) of marked area in
the image of hypoxic wildtype. Data represent mean values ± SEM; n = 12 mice per
670 group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 hypoxia versus normoxia; #P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001 Morg1+/− versus wildtype. A – J represent the data after 3 days of
673 hypoxia with following reoxygenation overnight.

674 Fig. 3. A-G. In contrast to wildtype mice no hypoxia-induced expression of various
cytokines is detectable in Morg1+/− mice. Analysis of mRNA expression, using real-
time PCR normalized to the expression of 18SrRNA, of various pro-inflammatory
cytokines tumor necrosis factor alpha (TNFα), interleukin 6 (IL6), and of the acute
phase proteins serum amyloid A (SAA) (A), chemokines monocyte chemotactic
protein-1 (MCP1) and macrophage inflammatory protein-2 (MIP2), as well as of high-
mobility-group-pProtein B1 (HMGB1) and Hyaluronan synthase 3 HAS3, the protein
encoded by this gene is involved in the synthesis of the unbranched
glycosaminoglycan hyaluronic acid, which is a major constituent of the extracellular
matrix (B). Representative examples of protein for TNFα, immunhistochemical
staining (C), quantitative analysis of TNFα staining (c) (D) (magnification: 100x; Bars:
200 µm). Representative western blot for TNFα protein expression using whole
kidney lysates (E), quantitative analysis of TNFα protein expression in western blots
normalized to vinculin as loading control (n=3 independent animals) (F). G:
Measurement of serum IL6 concentrations via ELISA shows significant increase in
wildtype mice after hypoxia, but not in Morg1+/− mice. Data are mean values ± SEM;
n=3 mice for western blots and n = 12 mice per group for all other experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 hypoxia versus normoxia; #P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001 Morg1+/− versus wildtype. A – G represent the data after 3 days of hypoxia with
following reoxygenation overnight.
Fig. 4. A–E. Systemic hypoxia induces apoptosis in wildtype mice, but not in Morg1+/- mice. A: Real-time PCR analysis of the pro-apoptotic proteins Bcl-2-associated X protein (Bax), Bcl-2-associated death promoter (Bac), and the antiapoptotic protein B-cell lymphoma-extra large protein (Bcl-Xl) mRNA expression show no significant changes, merely a slight reduction of Bax and Bad in hypoxic Morg1+/- mice. Values are normalized to 18S rRNA expression. B: Representative terminal transferase-dUTP-nick-end labelling (TUNEL) stainings for apoptosis, and (C) quantitative analysis of the number of TUNEL positive nuclei per area. Kidney sections of hypoxic wildtype mice show a strong elevation of the number of tubular cells stained TUNEL positive, whereas in heterozygous Morg1 mice no significant differences between normoxia and hypoxia were detectable. Mean values ± SEM; n = 12 mice per group. Magnification in B upper panel: 200x; Bars: 100 µm. Black-rimmed images: higher magnification (x400; bar: 50µm) of marked areas in the images from the upper panel. Negative and positive (DNase treatment) TUNEL stainings are shown. Representative western blot for caspase 3 cleavage in whole kidney lysates (D). Quantitative analysis of activated caspase 3 in western blots normalized to vinculin as loading control (n=3 independent animals) (E). Kidney lysates of hypoxic wildtype mice show active caspase 3, which is another readout for apoptotic activity. *P ≤ 0.05, **P ≤ 0.01 hypoxia versus normoxia; #P ≤ 0.05, ##P ≤ 0.01 Morg1+/- versus wildtype. A – E represent the data after 3 days of hypoxia with following reoxygenation overnight.
1A

Relative mRNA expression normalized to 18SrRNA

- Morg1 wildtype
- Morg1 heterozygous

- Normoxia
- Hypoxia

- PHD3
- HIF-1α
- HIF-2α
1C

HIF-1α

WT

HZ

HIF-2α

WT

HZ

normoxia  1 h  24 h  72 h  72 h + reox  10 d

hypoxia
1D

**relative Morg1 staining intensity**

- **normoxia**
  - Morg1 wildtype
  - Morg1 heterozygous

- **hypoxia**
  - Morg1 wildtype
  - Morg1 heterozygous

The bar graph shows a significant increase in staining intensity in hypoxia compared to normoxia for Morg1 wildtype, indicated by the asterisks (** **). For Morg1 heterozygous, there is a trend towards increased staining intensity in hypoxia, indicated by the double dagger (##).
relative PHD3 staining intensity

<table>
<thead>
<tr>
<th>Condition</th>
<th>Morg1 Wildtype</th>
<th>Morg1 Heterozygous</th>
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<td>Normoxia</td>
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</tr>
<tr>
<td>Hypoxia</td>
<td><strong>2</strong></td>
<td><strong>2</strong></td>
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</table>

**P-values:**
- Hypoxia vs. Normoxia in Morg1 Wildtype: **P < 0.01**
- Hypoxia vs. Normoxia in Morg1 Heterozygous: **P < 0.01**
relative Hif-1α staining intensity

<table>
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<td>Morg1 heterozygous</td>
<td><img src="image3.png" alt="Graph" /></td>
<td><img src="image4.png" alt="Graph" /></td>
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**Relative Hif-2α Staining Intensity**

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</tr>
<tr>
<td>Hypoxia</td>
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</table>

*Significance:
- # indicates a significant difference between normoxia and hypoxia in Morg1 wildtype.
- ## indicates a significant difference between normoxia and hypoxia in Morg1 heterozygous.*
serum EPO (pg/ml)

1H

Morg1 wildtype

Morg1 heterozygous
2A

PAS

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<td>Morg1 wildtype</td>
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2B

<table>
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<th>Hypoxia</th>
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<tr>
<td>Morg1 Heterozygous</td>
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Relative PAS staining intensity.
2C
Collagen I

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<table>
<thead>
<tr>
<th>Normoxia</th>
<th>Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morg1 heterozygous</td>
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</tbody>
</table>
2D

**relative Col-I staining intensity**

- **normoxia**  
  - Morg1 wildtype
  - Morg1 heterozygous

- **hypoxia**  
  - ***
  - ###
relative mRNA expression normalized to 18S rRNA

- **NGAL**
- **KIM1**
- **CTGF**
- **AQP1**

**Morg1 wildtype**

**Morg1 heterozygous**
2F

urinary NGAL (mg/g)

normoxia hypoxia normoxia hypoxia

Morg1 wildtype

Morg1 heterozygous

***

*
2G
CTGF

normoxia hypoxia
Morg1 wildtype

normoxia hypoxia
Morg1 heterozygous
relative CTGF staining intensity

2H

<table>
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<tr>
<td>Morg1 Heterozygous</td>
<td><img src="image" alt="Bar Graph" /></td>
<td>###</td>
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</table>

The graph shows the relative CTGF staining intensity under normoxia and hypoxia conditions for Morg1 wildtype and Morg1 heterozygous mice. The staining intensity is significantly higher in hypoxia compared to normoxia for both genotypes.
2I

AQP1

normoxia  hypoxia
Morg1 wildtype

normoxia  hypoxia
Morg1 heterozygous
relative AQP1 staining intensity

normoxia hypoxia normoxia hypoxia

Morg1 wildtype Morg1 heterozygous

2J
3A

The figure shows relative mRNA expression normalized to 18S rRNA under normoxia and hypoxia conditions for Morg1 wildtype and Morg1 heterozygous mice. The expression levels for TNFalpha, IL6, and SAA are compared. Significant differences are indicated by asterisks (*) for TNFalpha and IL6, and by hash marks (#) for SAA. The expression levels are significantly higher in hypoxia compared to normoxia for SAA in both genotypes.
3B

**Graph:**

- **X-axis:** Normoxia, Hypoxia (Morg1 Wildtype, Morg1 Heterozygous)
- **Y-axis:** Relative mRNA expression normalized to 18S rRNA
- **Legend:**
  - HMGB1
  - MCP1
  - MIP2
  - HAS3

**Note:**
- Hypoxia conditions show significant upregulation compared to Normoxia.
- Significance indicated by asterisks: * (p < 0.05), # (p < 0.01).
3C

TNFα

normoxia  hypoxia  normoxia  hypoxia
Morg1 wildtype  Morg1 heterozygous
Relative TNFα staining intensity under normoxia and hypoxia conditions for Morg1 wildtype and heterozygous mice.
3E

- 17 kDa: TNFα
- 130 kDa: Vinculin

Conditions:
- Normoxia
- Hypoxia
- Morg1 wildtype
- Morg1 heterozygous
Relative TNFα protein expression under normoxia and hypoxia conditions for Morg1 wildtype and heterozygous mice.
serum IL6 (pg/ml)

3G

Morg1 wildtype

Morg1 heterozygous

normoxia
hypoxia

0 6 12 18 24 30

serum IL6 (pg/ml)
4B

200x
TUNEL
400x

normoxia  hypoxia  normoxia  hypoxia
Morg1 wildtype  Morg1 heterozygous

negative control  positive control
**Figure 4C**

Relative number of TUNEL positive nuclei in normoxia and hypoxia conditions for Morg1 wildtype and heterozygous mice.

**Graph Details:**
- x-axis: Normoxia (Morg1 wildtype) and Hypoxia (Morg1 wildtype) for both conditions.
- y-axis: Relative number of TUNEL positive nuclei (0 to 100).
- Significant differences indicated by asterisks (*) and double asterisks (**) compared to normoxia conditions.
4D

- **Pro-Caspase 3**
  - 32 kDa
  - 17 kDa
  - 10 kDa

- **active Caspase 3**

- **Vinculin**
  - 130 kDa

**Morg1**
- Wildtype
- Heterozygous

**Conditions**
- Normoxia
- Hypoxia
relative cleaved caspase 3 intensity

4E

Morg1 wildtype

Morg1 heterozygous

normoxia hypoxia normoxia hypoxia

*
**Table 1**: Clinical/laboratory data in wildtype mice and in Morg1 heterozygous mice after normoxia or hypoxia.

<table>
<thead>
<tr>
<th></th>
<th>Morg1 wildtype normoxia</th>
<th>Morg1 wildtype hypoxia</th>
<th>Morg1 heterozygous normoxia</th>
<th>Morg1 heterozygous hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>body weight (g)</strong></td>
<td>21.5±0.4</td>
<td>19.9±0.2*</td>
<td>21.3±0.3</td>
<td>20.1±0.4*</td>
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<tr>
<td><strong>blood glucose (mmol/l)</strong></td>
<td>6.9±0.5</td>
<td>5.9±0.5</td>
<td>6.7±0.6</td>
<td>5.8±0.5</td>
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<tr>
<td><strong>white blood cells (10^9/l)</strong></td>
<td>2.3±0.3</td>
<td>3.5±0.4</td>
<td>1.9±0.2</td>
<td>2.4±0.3</td>
</tr>
<tr>
<td><strong>red blood cells (10^{12}/l)</strong></td>
<td>8.9±0.3</td>
<td>10.0±0.2*</td>
<td>9.8±0.1#</td>
<td>10.0±0.3</td>
</tr>
<tr>
<td><strong>platelets (10^9/l)</strong></td>
<td>940±65</td>
<td>839±64</td>
<td>1007±49</td>
<td>838±63</td>
</tr>
<tr>
<td><strong>hemoglobin (mmol/l)</strong></td>
<td>13.6±0.3</td>
<td>14.9±0.2*</td>
<td>14.4±0.3</td>
<td>15.0±0.4</td>
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<tr>
<td><strong>hematocrit (l/l)</strong></td>
<td>0.44±0.01</td>
<td>0.51±0.07*</td>
<td>0.48±0.01#</td>
<td>0.51±0.01</td>
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<tr>
<td><strong>MCV (µm³)</strong></td>
<td>48.6±0.3</td>
<td>50.7±0.2*</td>
<td>49.4±0.2#</td>
<td>50.3±0.2*</td>
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<tr>
<td><strong>MCH (fmol)</strong></td>
<td>0.92±0.01</td>
<td>0.93±0.01</td>
<td>0.92±0.004</td>
<td>0.92±0.01</td>
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<tr>
<td><strong>MCHC (mmol/l)</strong></td>
<td>18.9±0.3</td>
<td>18.4±0.1</td>
<td>18.6±0.1</td>
<td>18.3±0.1</td>
</tr>
</tbody>
</table>

* * hypoxia versus normoxia of the same genotype

# Morg1 heterozygous versus Morg1 wildtype under same conditions
MCV, mean corpuscular volume.
MCH, mean corpuscular hemoglobin.
MCHC, mean corpuscular hemoglobin concentration.
Values are means ± SEM (n = 12).
**Table 2:** Parameters of kidney function in wildtype mice and in Morg1 heterozygous mice after normoxia or hypoxia.

<table>
<thead>
<tr>
<th></th>
<th>Morg1 wildtype normoxia</th>
<th>Morg1 wildtype hypoxia</th>
<th>Morg1 heterozygous normoxia</th>
<th>Morg1 heterozygous hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>urine output per day (µl)</td>
<td>1358±93</td>
<td>400±63* ( P^{1E-07} )</td>
<td>1121±116</td>
<td>358±114* ( P^{0.0004} )</td>
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<tr>
<td>ACR (mg/g)</td>
<td>21.4±1.5</td>
<td>79.3±8.5* ( P^{5E-06} )</td>
<td>24.8±2.2</td>
<td>45.4±3.3* ( P^{0.0003} ) # ( P^{0.006} )</td>
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<tr>
<td>serum urea (mg/dl)</td>
<td>19.3±1.2</td>
<td>24.9±1.6* ( P^{0.02} )</td>
<td>20.9±0.9</td>
<td>21.0±0.7</td>
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<tr>
<td>serum creatinine (µmol/l)</td>
<td>19.6±0.4</td>
<td>21.4±0.6* ( P^{0.03} )</td>
<td>19.7±0.4</td>
<td>19.8±0.6</td>
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</table>

* hypoxia versus normoxia of the same genotype

\( P^* \) Morg1 heterozygous versus Morg1 wildtype under same conditions

ACR, albumin-to-creatinine ratio.

Values are means ± SEM (n = 12).
Table 3. Sequences of sense and antisense primers for real-time PCR and Annealing Temperatures

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<th>Gene</th>
<th>Primer Sequences</th>
<th>$T_{\text{Ann}}$, °C</th>
<th>Ref.</th>
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<td>TNFα</td>
<td>5'-AATTTCGACGACAGACCTTGTA-3' (fwd)</td>
<td>60</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>5'-CTTCCAGAGAATCCGGAATG-3' (rev)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

18SrRNA, 18S ribosomal RNA; AQP1, aquaporin 1; Bad, Bcl-2-associated death promoter; Bax, Bcl-2-associated X protein; Bcl-XI, B-cell lymphoma-extra large protein; CTGF, connective tissue growth factor; HAS3, hyaluronan synthase 3; HIF-
1α, hypoxia-inducible factor 1alpha; HIF-2 α, hypoxia-inducible factor 2alpha; HMGB1, high-mobility-group protein B1; IL6, interleukin 1; KIM1, kidney injury molecule-1; MCP1, monocyte chemotactic protein 1; MIP2, macrophage inflammatory protein 2; NGAL, neutrophil gelatinase-associated lipocalin; PHD3, prolyl hydroxylase 3; SAA, serum amyloid A; TNFα, tumor necrosis factor alpha.