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Morg1 heterozygous deficiency ameliorates hypoxia-induced acute renal injury

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Loeffler I, Wolf G. Morg1 heterozygous deficiency ameliorates hypoxia-induced acute renal injury. Am J Physiol Renal Physiol 308: F511–F521, 2015. First published December 30, 2014; doi:10.1152/ajprenal.00236.2014.—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 hypoxia-inducible factor (HIF); its degradation is regulated by prolyl hydroxylase 3 (PHD3) in interaction with the scaffold protein MAPK organizer 1 (Morg1). While homozygous Morg1−/− mice are embryonically lethal, the kidneys of heterozygous Morg1+/− mice reveal elevated HIF protein levels and increased serum erythropoietin compared with wild-type Morg1+/+ mice. In this study, we exposed wild-type and Morg1+/− mice to 10% oxygen in a hypoxic chamber for 3 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 wild-type (Morg1+/+) mice. In sharp contrast, Morg1+/− kidneys were protected against systemic hypoxia. They show significantly less renal lesions, reduced or no inflammation, and 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 wild-type mice renal injury, which is ameliorated by Morg1 deficiency. Our findings suggest that therapeutic manipulation of Morg1 may be an interesting novel target to prevent hypoxia-associated renal damage.

acute renal injury; critical illness; systemic hypoxia; Morg1; HIF-α

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 (~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 a 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 ischemia, 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, which 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). Posttranslational 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 coexpression of PHD3 (24). Suppression of Morg1 results in a marked increase in 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 with wild-type, reflected by less tubular damage, a smaller serum creatinine increase, and less inflammation and apoptosis, was associated with a stronger increase in HIF-1α and HIF-2α expression as well as with enhanced serum EPO levels (19). There are different modes of HIF signal enhancement: 1) chemical inhibition of PHDs by hypoxia-mimetics (e.g., CoCl2), 2) molec-
ular biology techniques (e.g., on-Hippel-Lindau knockout), and 3) 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., myocardiogenic injury, lung injury, neuronal injuries, and AKI (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 signaling 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 wild-type (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 wild-type littermates. All mice were 12–15 wk old, and only male mice were used to control for potential hormonal effects. Wild-type and Morg1+/− mice were subjected to systemic normobaric hypoxia (12 mice/group) by substituting normal compressed air with nitrogen using a small anesthetic bracket system with flowmeters for two gases (Hugo Sachs Elektronik-Harvard Apparatus, March-Hugstetten, Germany) at a constant gas flow rate of 2 liters/min in an air-tight chamber (Hugo Sachs Elektronik-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% O2 for 3 days of continuous hypoxia. Control mice (normoxia) (n = 12) were placed in the same chamber with normal air (21% O2) with a flow rate of 2 liters/min for 3 days. At the end of the experiment, all mice were placed in metabolic cages (Tecniplast, 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% O2 for 3 days of continuous hypoxia. Control mice (normoxia) (n = 12) were placed in the same chamber with normal air (21% O2) with a flow rate of 2 liters/min for 3 days. At the end of the experiment, all mice were placed in metabolic cages (Tecniplast, Buguggiate, Italy) in room air (21% O2) overnight to collect the urine. At the end of the experiment, all mice were placed in metabolic cages (Tecniplast, Italy) in room air (21% O2) overnight to collect the urine.

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 Chemical, Ann Arbor, MI). NGAL concentrations in the urine were determined by using mouse an NGAL ELISA according to the manufacturer’s instructions (Bioporto Diagnostics, Gentofte, Denmark). Mouse serum creatinine and urea levels were measured with Fuji Dri-chem Slides CRE-PIII and BUN-PIII, respectively (Fujifilm Europe, Düsseldorf, Germany). Serum EPO levels were determined with a specific mouse EPO ELISA (Quantikine, R&D Systems, Wiesbaden, Germany) and serum IL-6 concentrations with a specific mouse IL-6 ELISA (Quantikine, R&D Systems).

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, Roth, Karlsruhe, Germany). Histological changes due to tubular damage were graded (0 = none, 1 = 11–25%, 2 = 26–45%, 3 = 46–75%, and 4 = >75%) 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), polyclonal rabbit anti-PHD3 (Santa Cruz Biotechnology, Santa Cruz, CA), polyclonal rabbit anti-CTGF, anti-TNF-α (Abcam, Cambridge, UK), polyclonal goat anti-HIF-1α, anti-HIF-2α (R&D Systems), and a monoclonal mouse anti-aquaporin-1A (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, anti-mouse, or anti-goat IgG antibody, respectively (KPL, Gaithersburg, MD). Apoptosis was detected by the terminal deoxynucleotidyl 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 a digital camera and AxioVision 4.8 software was used (Carl Zeiss). Five nonoverlapping fields of each individual kidney sample (12 mice/group) were scanned in the monochrome mode of the camera (magnification: ×200). 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 an 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 1 shows the sequences and annealing temperature of all primer pairs. Transcript levels were normalized to the mean value of samples from the normoxic wild-type animals.

Western blot analysis. Proteins were extracted from renal homogenates using a ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Merck, Darmstadt, Germany). Fifty 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 TNF-α and caspase 3 cleavage, the membrane was further incubated overnight with, respectively, a polyclonal rabbit antibody against mouse TNF-α (Abcam) at a dilution of 1:100 and a polyclonal rabbit antibody against mouse caspase 3 (Abcam) at a dilution of 1:500 in 5% BSA-TBST, washed...
The effects of systemic hypoxia (10% O₂, 3 days) on clinical and laboratory parameters are summarized in Table 2. 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 wild-type mice. In wild-type mice, these parameters were significantly increased after hypoxic treatment to a level similar to those of normoxic Morg1⁺/+ mice. Interestingly, in contrast to heterozygous Morg1 mice, hypoxia leads to a significant rise in white blood cells in wild-type mice, suggesting that Morg1 deficiency may somewhat protect against the hypoxia-induced systemic inflammatory response.

**Table 1. Sequences of sense and antisense primers for real-time PCR and annealing temperatures**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences</th>
<th>T&lt;sub&gt;an&lt;/sub&gt;, °C</th>
<th>Ref.</th>
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<tr>
<td>18S rRNA</td>
<td>5'-CACGCGCCTACGAGCTGAC3' (fwd)</td>
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<td>25</td>
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<td>AQP1</td>
<td>5'-GATCTGGATCCAGAGCTGCTG3' (fwd)</td>
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<td>31</td>
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<td>Bad</td>
<td>5'-GATCCGCCTAGTGGTGGCTG3' (fwd)</td>
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<td>20</td>
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<tr>
<td>Bax</td>
<td>5'-GCCTGAGCTGCCGATGGC3' (rev)</td>
<td>59</td>
<td>59</td>
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<tr>
<td>Bcl-xl</td>
<td>5'-GCCGAGCTAAGTCGATGGG3' (rev)</td>
<td>58.4</td>
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<tr>
<td>CTGF</td>
<td>5'-GCCGTGGCAAGGAGCTGAC3' (fwd)</td>
<td>58</td>
<td>29</td>
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<td>HAS3</td>
<td>5'-GGACAGGTTCCTCTGAGGAC3' (fwd)</td>
<td>62</td>
<td>19</td>
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<tr>
<td>HIF-1α</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>HIF-2α</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<tr>
<td>IL-6</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>19</td>
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<td>KIM1</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>MCP1</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>MIP2</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>19</td>
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<td>Morg1</td>
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<td>NGAL</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>36</td>
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<td>PHD3</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>9</td>
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<tr>
<td>SAA</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
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<td>51</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-GGGCGATGAGCTGAC3' (rev)</td>
<td>58</td>
<td>9</td>
</tr>
</tbody>
</table>

T<sub>an</sub>, annealing temperature; 18S rRNA, 18S ribosomal RNA; AQP1, aquaporin-1; Bad, Bcl-2-associated death promoter; Bax, Bcl-2-associated X protein; Bcl-Xl, B cell lymphoma-extra large protein; CTGF, connective tissue growth factor; HAS3, hyaluronan synthase 3; HIF-1α, hypoxia-inducible factor 1α; HIF-2α, hypoxia-inducible factor 2α; HMG1B1, high-mobility group protein B1; KIM1, kidney injury molecule-1; MCP1, monocyte chemotactic protein 1; MIP2, macrophage inflammatory protein 2; NGAL, neutrophil gelatinase-associated lipocalin; PHD3, poly-2 hydroxylase 3; SAA, serum amyloid A.

** RESULTS **

**Influence of systemic hypoxia on clinical and laboratory parameters.** The effects of systemic hypoxia (10% O₂, 3 days) compared with normoxia (21% O₂, 3 days) on clinical and laboratory parameters are summarized in Table 2. 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 wild-type mice. In wild-type mice, these parameters were significantly increased after hypoxic treatment to a level similar to those of normoxic Morg1⁺/+ mice. Interestingly, in contrast to heterozygous Morg1 mice, hypoxia leads to a significant rise in white blood cells in wild-type mice, suggesting that Morg1 deficiency may somewhat protect against the hypoxia-induced systemic inflammatory response.

**Hypoxia-induced decline in kidney function is reduced in Morg1⁺/+ mice.** Data from various renal function analyses (Table 3) reveal that urine output significantly decreases after exposure to hypoxia independent of genotype, but more precise parameters of kidney function, such as ACR, serum urea, and serum creatinine, show significant increases in response to hypoxia only in wild-type and not (or significantly less) in Morg1⁺/+ mice (Table 3). These data may suggest that Morg1⁺/+ animals are protected against systemic hypoxia-induced deterioration of kidney function.

**Wild-type 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-1α and HIF-2α via expression of EPO. In the case of wild-type mice, we observed the expected results: systemic hypoxia induces mRNA (Fig. 1A) and protein (Fig. 1B–G) expression of PHD3 and Morg1 as well as of HIF-1α and HIF-2α. Figure 1, B and C, demonstrates a hypoxia-induced increase in Morg1, PHD3, HIF-1α, and HIF-2α protein expression even after 1 h of hypoxia. Although on day 4 (3 days of 10% O2 with reoxygenation afterward overnight) a significant increase in HIF-1α/ HIF-2α mRNA and protein was no longer detectable (Fig. 1, A, F, and G), the systemic serum concentration of the typical hypoxia marker upregulated in a variety of renal diseases (Fig. 2, F514 SYSTEMIC HYPOXIA AND RENAL DAMAGE

Table 2. Clinical/laboratory data in wild-type and Morg1 heterozygous mice after normoxia or hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Morg1 Wild-Type Normoxia</th>
<th>Morg1 Wild-Type Hypoxia</th>
<th>Morg1 Heterozygous Normoxia</th>
<th>Morg1 Heterozygous Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>21.5 ± 0.4</td>
<td>19.9 ± 0.2 * P = 0.003</td>
<td>21.3 ± 0.3</td>
<td>20.1 ± 0.4 * P = 0.004</td>
</tr>
<tr>
<td>Blood glucose, mmol/l</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>
</tr>
<tr>
<td>White blood cells, 10^9/l</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>Red blood cells, 10^12/l</td>
<td>8.9 ± 0.3</td>
<td>10.0 ± 0.2 * P = 0.009</td>
<td>9.8 ± 0.1 * P = 0.02</td>
<td>10.0 ± 0.3</td>
</tr>
<tr>
<td>Platelets, 10^12/l</td>
<td>940 ± 65</td>
<td>839 ± 64</td>
<td>1,007 ± 49</td>
<td>838 ± 63</td>
</tr>
<tr>
<td>Hemoglobin, mmol/l</td>
<td>13.6 ± 0.3</td>
<td>14.9 ± 0.2 * P = 0.006</td>
<td>14.4 ± 0.3</td>
<td>15.0 ± 0.4</td>
</tr>
<tr>
<td>Hematocrit, l/l</td>
<td>0.44 ± 0.01</td>
<td>0.51 ± 0.07 * P = 0.006</td>
<td>0.48 ± 0.01 * P = 0.03</td>
<td>0.51 ± 0.01</td>
</tr>
<tr>
<td>MCV, μm^3</td>
<td>48.6 ± 0.3</td>
<td>50.7 ± 0.2 * P = 0.06</td>
<td>49.4 ± 0.2 * P = 0.04</td>
<td>50.3 ± 0.2 * P = 0.02</td>
</tr>
<tr>
<td>MCH, fmol</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>MCHC, mmol/l</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>

Values are means ± SE (n = 12). Morg1, MAPK organizer 1; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration *P hypoxia vs. normoxia of the same genotype. #P Morg1 heterozygous vs. Morg1 wild-type under same conditions.

HIF-1α, demonstrates a hypoxia-induced increase in Morg1, PHD3, HIF-1α, and HIF-2α protein expression even after 1 h of hypoxia. Although on day 4 (3 days of 10% O2 with reoxygenation afterward overnight) a significant increase in HIF-1α/ HIF-2α mRNA and protein was no longer detectable (Fig. 1, A, F, and G), the systemic serum concentration of the typical target gene of HIF, EPO, was significantly elevated after systemic hypoxia (Fig. 1H). In the Morg1+/− mice exposed to hypoxia, renal immunohistochemical data show also an induction of PHD3 and Morg1 over time, which was significantly less compared with wild-type animals (Fig. 1, B, D and E). Similar to wild-type mice, in Morg1+/− mice renal HIF expression was first increased during exposure to 10% O2 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-1α and HIF-2α were already significantly higher than in the wild-type animals (Fig. 1, C, F, and G). Figure 1H clearly reveals that under normoxic conditions, serum EPO was significantly higher in Morg1−/− than in wild-type. Interestingly, this elevated level of EPO decreased in the Morg1−/− mice after 3 days of hypoxia (Fig. 1H). Immunohistochemical analysis of Morg1, PHD3, HIF-1α, and HIF-2α protein in the mouse kidneys after 10 days of hypoxia showed no differences in expression compared with the 72-h time point.

Hypoxia-induced tubular damage and prefibrotic 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. 2A). It was clearly visible that, in contrast to the heterozygous Morg1+/− mice, tubuli of the wild-type mice exposed to 10% O2 for 3 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 wild-type 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), 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, mRNA expression of all tested markers was significantly increased after hypoxia in wild-type mice, notably KIM1 and NGAL expression (Fig. 2E). Measurement of urinary NGAL concentrations confirms the finding that kidneys of wild-type mice are damaged by hypoxia, whereas the NGAL concentrations in the urine of Morg1+/− mice were only marginally increased (Fig. 2F). 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 with wild-type animals after hypoxia (Fig. 2, G and H). The expression of the water channel AQP1, as studied by immunohistochemistry, dramatically increased after 3 days of hypoxia in both genotypes without significant difference (Fig. 2, I and J).

Table 3. Parameters of kidney function in wild-type and Morg1 heterozygous mice after normoxia or hypoxia

<table>
<thead>
<tr>
<th></th>
<th>Morg1 Wild-Type Normoxia</th>
<th>Morg1 Wild-Type Hypoxia</th>
<th>Morg1 Heterozygous Normoxia</th>
<th>Morg1 Heterozygous Hypoxia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine output per day, μl</td>
<td>1.358 ± 0.93</td>
<td>400 ± 63 * P &lt; 0.007</td>
<td>1,121 ± 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 = 0.006</td>
<td>24.8 ± 2.2</td>
<td>45.4 ± 3.3 * P = 0.0003 * P = 0.0006</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>
</tr>
<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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Values are means ± SE (n = 12). ACR, albumin-to-creatinine ratio. *P hypoxia vs. normoxia of the same genotype. #P Morg1 heterozygous vs. Morg1 wild-type under same conditions.
Fig. 1. A–H: the prolyl hydroxylase 3 (PHD3)/hypoxia-inducible factor (HIF) axis in response to different oxygen levels depends on the MAPK organizer 1 (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 in Morg1 and PHD3 mRNA in wild-type by hypoxia is detectable. Values were normalized to 18S rRNA expression, and the controls (wild-type normoxia) were assigned an arbitrary value of 1.

**B and C:** representative stainings for Morg1, PHD3, HIF-1α/H9251, and HIF-2α/H9251 at different time points of hypoxia (magnification: ×100; bars = 200 µm). Black-rimmed image: higher magnification (×400; bar = 50 µm) of marked area in the images of time point 72 h reoxygenation. Hypoxia-induced increase in the expression of all proteins is detectable at 1, 24, and 72 h. After 10 days of hypoxia, no further changes were detectable. The images from 72 h reoxygenation confirm the findings of real-time PCR analysis, but additionally an increase in Morg1 and PHD3 expression in Morg1 Heterozygous mice after hypoxia was found, which was semiquantitatively analyzed for Morg1 (D), PHD3 (E), Hif-1α (F), and Hif-2α (G). For details, see methods. 

**H:** serum erythropoietin (EPO) levels are elevated in wild-type mice after induction of hypoxia for 3 days and reoxygenation. Values are means ± SE; n = 12 mice/group. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 hypoxia vs. normoxia. #P ≤ 0.05, ##P ≤ 0.01, ###P ≤ 0.001 Morg1 Heterozygous vs. wild-type (Morg1 Wildtype).
Kidneys from hypoxic Morg1−/− mice express fewer inflammatory cytokines compared with wild-type. 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 proinflammatory cytokines (Fig. 3A) and chemokines (Fig. 3B). As expected, kidneys of wild-type mice exposed to hypoxia expressed significantly more transcripts for proinflammatory cytokines, such as TNF-α and IL-6, as well as the acute-phase protein serum amyloid (SAA) (Fig. 3A). An increase in chemokine expression was only seen for monocyte chemotactic protein-1 (MCP1) and macrophage inflammatory protein-2 (MIP2), but not for high-mobility group protein B1 (HMGB1) (Fig. 3B).

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 wild-type animals show a strong hypoxia-induced increase in TNF-α protein expression whereas no response was found in Morg1−/− kidneys. Similar results were seen for serum IL-6 concentrations, as measured with ELISA (Fig. 3G).

In contrast to wild-type animals, no significant increase in apoptosis was detectable in the kidneys of Morg1−/− mice after hypoxia. To test whether the hypoxia-induced decline in kidney function in wild-type is associated with renal cell death, we initially investigated the mRNA expression of proapoptotic proteins (Bax and Bad) and the antiapoptotic protein Bcl-XL (Fig. 4A). There were no significant changes in Bax, Bad, and BCL-XI in wild-type mice exposed to hypoxia compared with normoxia (Fig. 4A). However, Bax and Bad, but not BCL-XI
mRNA expression significantly decreased in Morg1+/− mice exposed to 10% O2 compared with animals with normoxia (Fig. 4A). We further assessed apoptotic cells using TUNEL staining. Figure 4 (B and C) strikingly shows how reduced oxygen availability for a duration of 3 days significantly increases the number of TUNEL-positive cells in wild-type 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 wild-type mice a significant increase in active caspase 3 compared with homogenates from normoxic animals (Fig. 4, D and E). In contrast, this increased apoptotic activity was not detectable in Morg1+/− mice after 3 days of hypoxia (Fig. 4, D and E).

DISCUSSION

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 with which 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 of which is renal ischemia-reperfusion, however, clearly
fails to reflect the multifactorial causes of AKI that are 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% O2 (systemic hypoxia) vs. 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 \( \text{H}11022 \) 5,000 m, which is defined as a very high altitude (14). It is well known that inspiratory hypoxia increases the number of red blood cells, hemoglobin concentration, and hematocrit (4, 21, 33, 49).
suggested that the hypoxia-induced rise in hematocrit helps
to maintain tissue oxygen delivery (33).

We could confirm in wild-type mice a hypoxia-induced increase in hematocrit, but in the heterozygous Morg1 animals the hypoxia-induced effects were not significant compared with 21% O₂. Moreover, normoxic basal levels of this parameter were higher in the Morg1⁺⁻⁻ mice compared with the wild-type 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 EPO production in the kidney and liver (18). We detected in the kidneys of normoxic Morg1⁺⁻⁻ mice significantly more HIF-1α/2α proteins compared with the wild-type 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 (3 days), wild-type mice 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 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) lead to the logical result that an increase in HIF-α expression after hypoxia was no longer detectable at the studied time point. Morg1 and PHD3 expression were also inducible by hypoxia in heterozygous mice, but to a lesser extent than in wild-type. Since PHD3 had relatively more influence on HIF-2α than HIF-1α hydroxylation (3), this finding may explain the trend of more HIF-2α proteins in the kidneys of the Morg1⁺⁻⁻ mice after induction of hypoxia. Although several studies have suggested that both HIF-1α and HIF-2α 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 3 days of systemic hypoxia serum EPO concentrations in Morg1⁺⁻⁻ mice fall to levels similar to those of normoxic wild-type mice. Studies of the effects of respiratory hypoxia on plasma EPO in humans who move from sea level to a 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 wild-type 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 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 wild-type mice. Moreover, urinary albumin concentration, a characteristic of impaired glomerular ultrafiltration and/or tubular handling of albumin, was significantly higher in wild-type 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 independently of genotype, it has also been reported that oliguria is neither specific nor sensitive enough to monitor AKI (6). Furthermore, we tested 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 urine output. Our finding that AQP1 protein in both genotypes is significantly increased by hypoxia without any difference between wild-type 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 with wild-type, 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 >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 wild-type 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 wild-type animals confirms this assumption. For KIM1, a high 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 wild-type 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 wild-type animals exposed to hypoxia, we expected an inflammatory response. The investigation of the renal expression of several proinflammatory cytokines (e.g., TNF-α and IL-6), chemokines (e.g., MCP1 and MIP2), and the acute phase protein SAA, which 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 wild-type mice does hypoxia induce 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 ischemia-reperfusion injury, and it has been shown that hypoxia can induce apoptosis, dependent on transcriptional activation of apoptotic factors (11, 19, 32). Recently, we have reported that tubular apoptosis is decreased in ischemic and reperfused Morg1+/− kidneys compared with ischemic kidneys of wild-type mice (19). The results of the present study revealed that systemic hypoxia leads to a marked increase in TUNEL-positive cells and caspase 3 activity in wild-type mice, whereas no significant induction of apoptosis was observed when Morg1 is diminished. Ratanen et al. (46) 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 subsequent decreased activity of PHD3 may result in 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 pharmacological 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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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

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