DMOG, a Prolyl Hydroxylase Inhibitor, Increases Hemoglobin Levels without Exacerbating Hypertension and Renal Injury in Salt-Sensitive Hypertensive Rats

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ABSTRACT

Prolyl hydroxylase (PHD) inhibitors are being developed as alternatives to recombinant human erythropoietin (rHuEPO) for the treatment of anemia in patients with chronic kidney disease (CKD). However, the effects of PHD inhibitors and rHuEPO on blood pressure and CKD in animal models susceptible to hypertension and nephropathy have not been studied. The present study compared the effects of dimethyloxaloylglycine (DMOG), a PHD inhibitor, and rHuEPO on the development of hypertension and renal injury in Dahl salt-sensitive rats fed an 8% salt diet for 3 weeks. DMOG and rHuEPO were equally effective at raising hemoglobin levels. Systolic blood pressure rose to a greater extent in rHuEPO-treated rats (267 ± 10 vs. 226 ± 4 mm Hg) than in rats given DMOG (189 ± 8 mm Hg). Urinary protein excretion increased to 568 ± 54 versus 353 ± 25 mg/day in rats treated with rHuEPO and vehicle; however, it only rose to 207 ± 21 mg/day in rats receiving DMOG. DMOG significantly attenuated the degree of glomerulosclerosis and renal interstitial fibrosis as compared with that in vehicle and rHuEPO-treated rats. This was associated with lower renal levels

of monocyte chemoattractant protein-1 and interleukin-1 β and increased vascular endothelial growth factor expression in cortex and medulla. These results indicate that DMOG and rHuEPO are equally effective in increasing hemoglobin levels in Dahl S rats; however, rHuEPO aggravates hypertension and renal injury, whereas DMOG has marked renoprotective effects. These results suggest that PHD inhibitors may have a therapeutic advantage for the treatment of anemia in CKD.

SIGNIFICANCE STATEMENT

Prolyl hydroxylase (PHD) inhibitors are in phase 3 clinical trials as alternatives to recombinant human erythropoietin (rHuEPO) for the treatment of anemia in chronic kidney disease (CKD). The present study reveals that dimethyloxaloylglycine (DMOG), a PHD inhibitor, and rHuEPO are equally effective in increasing hemoglobin levels in Dahl S rats; however, rHuEPO aggravated hypertension and renal injury, whereas DMOG attenuated the development of hypertension and prevented renal injury. PHD inhibitors may provide a safer therapeutic option for the treatment of anemia in CKD.

Introduction

Erythropoietin (EPO) acts on its receptor to activate the Janus-activating kinase 2 signaling cascade to stimulate the differentiation of erythroid progenitor cells into erythrocytes (Koury and Haase, 2015; Kuhrt and Wojchowski, 2015). EPO is produced in EPO-producing cells found in the renal cortical interstitium (Obara et al., 2008; Paliege et al., 2010). In chronic kidney disease (CKD), these cells transform into myofibroblasts and lose the ability to produce EPO (Asada et al., 2011; Souma et al., 2013). Consequently, patients with CKD develop anemia. The prevalence of anemia is 20% in patients with stage 3 CKD and 60% and 75% in patients with stage 4 or stage 5 CKD (McFarlane et al., 2008).

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Recombinant human EPO (rHuEPO) is widely used to compensate for the deficiency in EPO production to treat anemia and improves the quality of life in patients with CKD (Evans et al., 1990). On the other hand, some issues are associated with the use of rHuEPO for the treatment of anemia in patients with CKD. rHuEPO is given by chronic injections and is painful for non-dialysis patients. Chronic administration of exogenous rHuEPO promotes the development of anti-rHuEPO antibodies, which neutralizes endogenous EPO (Means, 2016). Treatment of rHuEPO has also been reported to promote the development of hypertension, perhaps by increasing blood viscosity (Letcher et al., 1981; Raine, 1988; Steffen et al., 1989). Furthermore, three pivotal clinical studies have raised concerns about the safety of high-dose rHuEPO for the treatment of anemia. In the CREATE and the TREAT studies, the risks of hypertension, dialysis, and stroke were higher when the target hemoglobin level was high (Drüeke et al., 2006; Pfeffer et al., 2009). In the CHOIR study, high doses of rHuEPO increased the risk of

ABBREVIATIONS: BUN, blood urea nitrogen; CKD, chronic kidney disease; DMOG, dimethyloxaloylglycine; EPO, erythropoietin; HIF, hypoxia-inducible factor; IL-1 β , interleukin-1 β ; KIM-1, kidney injury marker 1; NAG, *N*-acetyl- β -D-glucosaminidase; PHD, prolyl hydroxylase; rHuEPO, recombinant human EPO; TGF β 1, transforming growth factor β 1.

cardiovascular events independent of the target hemoglobin level (Singh et al., 2006; Szczech et al., 2008).

Potential alternatives to rHuEPO for the treatment of anemia are prolyl hydroxylase (PHD) inhibitors. PHD hydroxylates proline residues of hypoxia-inducible factor α (HIF α), which is a master regulator of the hypoxic response (Epstein et al., 2001). Hydroxylated HIF α is recognized by a ubiquitin ligase, von Hippel-Lindau protein, and degraded through the ubiquitin-proteasome pathway (Jaakkola et al., 2001). In hypoxic conditions, the PHD activity is reduced, and HIF α escapes from hydroxylation and subsequent degradation. Once HIF α is stabilized, HIF α binds to the hypoxia response element together with cAMP response element binding protein (CREB) binding protein/p300 and constitutively active HIF β to upregulate the expression of target genes (Haase, 2006). As a consequence, vascularity is increased through HIF1 α , and hemoglobin levels are elevated through the actions of HIF2 α (Elson et al., 2001; Percy et al., 2008). PHD inhibitors are under the investigation in ongoing phase 3 clinical trials to treat renal anemia in both nondialysis and dialysis patients (Cernaro et al., 2019). Recently, roxadustat was approved for the treatment of anemia in CKD in China (Dhillon, 2019). PHD inhibitors are orally active (Flamme et al., 2014; Kato et al., 2018) and upregulate the formation of endogenous EPO and other renoprotective factors such as vascular endothelial growth factor (VEGF). Therefore, it is important to compare the safety profile of PHD inhibitors and rHuEPO in models of renal disease.

Several studies have investigated the effects of rHuEPO on renal function in several CKD models in normotensive strains of rats and mice (Lee et al., 2005; Katavetin et al., 2007; Toba et al., 2009; Cañadillas et al., 2010; Rjiba-Touati et al., 2012). In these studies, rHuEPO reduced renal inflammation and fibrosis regardless of hematopoietic effect. On the other hand, hypertension and diabetes are the primary risk factors for the development of CKD, and these patients may be more susceptible to potential adverse effects of rHuEPO on blood pressure and hypertension-induced renal injury. To explore this possibility, we compared the effects of rHuEPO and dimethylx-aloylglycine (DMOG), an injectable PHD 1/2/3 pan inhibitor (Epstein et al., 2001), on the development of hypertension and renal injury in Dahl S rats, which are highly susceptible to the development of salt-sensitive hypertension and renal injury. This study demonstrated that DMOG markedly attenuated the development of hypertension and renal injury in this model, whereas rHuEPO had the opposite effect. These findings suggest that PHD inhibitors may provide a safer therapeutic option for the treatment of anemia in diabetes- and hypertension-induced CKD.

Materials and Methods

General. DMOG was synthesized by Medical Chemistry Laboratories in Taisho Pharmaceutical Co., Ltd. rHuEPO (PROCRIT) was purchased from Centocor Ortho Biotech Products, L.P. These experiments were performed using male Dahl S rats obtained from inbred colonies maintained in the University of Mississippi Medical Center. They were maintained on a 0.4% salt diet from weaning to the start of the experiments. All the experiments were approved by the Animal Care Committee of the University of Mississippi Medical Center.

Effect of DMOG on EPO Expression. These experiments were performed on male 9-week-old Dahl S rats. A control blood sample was collected from the jugular vein, and the rats received intraperitoneal

injection of saline or DMOG at a dose of 600 mg/kg. Blood samples were collected from jugular vein 4, 8, and 24 hours later. The samples were centrifuged at 2130g for 10 minutes, and serum EPO levels were measured using an ELISA kit (R&D Systems, Minneapolis, MN). Additional groups of rats were given DMOG 600 mg/kg, and the kidneys were removed before (0 hours) and 1, 2, and 4 hours after administration. mRNA was extracted from the renal cortex using TRIZOL (Life Technologies, Grand Island, NY). The samples were reverse transcribed using a poly t and random hexamer primers. EPO mRNA levels were determined using quantitative reverse transcription-polymerase chain reaction. EPO was amplified using the following forward and reverse primers: 5-GCTCCAATCTTTGTG GCATCT-3 and 5-TGGCTTCGTGACCCCTCTGT-3. β -Actin forward and reverse primers were 5-CCTCTATGCCAACACAGTGC-3 and 5-GTACTCCTGCTTGCTGATCC-3.

Time Course of the Effects of DMOG and rHuEPO. Male 9-week-old Dahl S rats were randomly assigned to four groups and treated with vehicle, DMOG at 60 mg/kg, DMOG at 600 mg/kg, or rHuEPO at 100 U/kg. DMOG was dissolved in 0.9% NaCl solution, and rHuEPO was diluted with a 0.5% BSA in a 0.9% NaCl solution. DMOG was given intraperitoneally, whereas rHuEPO was given subcutaneously, three times per week starting from day 1.

Baseline urinary protein excretion, blood pressure, hemoglobin, and hematocrit were measured during a control period while the rats were fed a normal-salt diet containing 0.4% NaCl. Then, they were switched to a high-salt diet (8.0% NaCl), DMOG or EPO was administered for 3 weeks, and samples were collected weekly. Urine samples were collected using metabolic cages, and urine protein concentrations were determined by Bradford method (Bio-Rad Laboratories, Hercules, CA). Blood pressure was measured using a tail-cuff device (Hatteras Instruments, Cary, NC). Hemoglobin levels were determined in 10 μ l of blood using HemoCue Hb 201+ device (HemoCue, Brea, CA), and hematocrit was determined in 80- μ l blood samples collected in a hematocrit tube from tail vein.

Histology. After 3 weeks of treatment with vehicle, DMOG, or rHuEPO, the rats were sacrificed, and the left kidneys were fixed in 10% buffered formalin. Paraffin sections (3 μ m) were stained with Masson's trichrome and analyzed for the degree of glomerulosclerosis and glomerular and renal interstitial fibrosis. Glomerulosclerosis was scored on a scale from 0 to 4, where 0 represents a normal glomerulus, 1 represents 1%–25% loss of capillary area, 2 represents 26%–50% loss, 3 represents 51%–75% loss, and 4 represents >75% loss. The images were captured using a Nikon Eclipse 55i microscope equipped with a Nikon DS-Fi1 color camera (Nikon Instruments Inc., Melville, NY). The degree of renal interstitial fibrosis was calculated as the percentage of area stained blue using the NIS Elements 3.0 software (Nikon Instruments Inc.). Protein casts were determined as the percentage of area stained red in the sections (Muroya et al., 2015).

Measurement of Renal Injury Biomarkers. Blood urea nitrogen (BUN) levels were determined using a BUN detection kit (Arbor Assays, Ann Arbor, MI). Serum creatinine levels were determined using a LabAssay Creatinine kit (Wako Pure Chemical Industries, Osaka, Japan). Urinary *N*-acetyl- β -D-glucosaminidase (NAG) and kidney injury marker 1 (KIM-1) were determined using a NAG assay kit (SIGMA, St. Louis, MO) and a rat KIM-1 ELISA kit (R&D Systems).

Measurement of Renal Inflammatory Markers and VEGF Levels. The right kidneys were separated into cortex and medulla, and they were homogenized in a Tris buffer containing 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, and a proteinase inhibitor cocktail. The homogenate was centrifuged at 11,000g for 5 minutes at 4°C, and the monocyte chemoattractant protein-1 (MCP-1), interleukin-1 β (IL-1 β), transforming growth factor β 1 (TGF β 1), and VEGF levels in the supernatant were measured using ELISA kits (R&D Systems). Samples for measurement of TGF β 1 were first activated by acidification for 5 minutes and then measured using an ELISA kit from R&D systems.

Statistics. Data are presented as mean values $\pm$ 1 S.E. The statistical significances of differences were determined using the Student's *t* test for comparisons between two groups, a one-way ANOVA and Holm-Sidak test for multiple comparisons, or a two-way ANOVA for repeated measures followed by a Holm-Sidak test for the time course studies (Sigma Plot 11; Systat Software, San Jose, CA).

Results

Effect of DMOG on EPO Expression. We first addressed the effect of DMOG on renal EPO mRNA expression and serum EPO concentration after administration of 600 mg/kg of DMOG. EPO mRNA levels in the renal cortex started to increase 1 hour after the administration of DMOG. EPO mRNA levels at 4 hours were 70-fold higher than those seen at baseline (Fig. 1A). Serum EPO concentration in the vehicle-treated group was below detection 24 hours after administration. The EPO concentration in the 600 mg/kg of the DMOG-treated group increased to 890 pg/ml, 4 hours after administration. The levels returned to the baseline 24 hours after the administration of DMOG (Fig. 1B).

Comparison of the Hematopoietic Effects of Chronic DMOG and rHuEPO Administration. The hematopoietic effects of DMOG (60 or 600 mg/kg, i.p.) or rHuEPO (100 U/kg, s.c.) given three times per week were studied in Dahl S rats fed an 8% NaCl diet for 3 weeks. There was no difference in food intake or body weight among the groups (data not shown), indicating that these drugs were well tolerated. Hemoglobin and hematocrit levels in the groups treated with vehicle and 60 mg/kg of DMOG remained unchanged throughout the study. The hemoglobin and hematocrit levels in the group treated with 600 mg/kg of DMOG increased from 16.3 ± 0.2 g/dl and $56.1\% \pm 2.0\%$ to 18.3 ± 0.4 g/dl and $63.1\% \pm 1.4\%$ after 3 weeks of treatment. These values were comparable to the rise in hemoglobin and hematocrit levels seen in the rHuEPO-treated group (18.8 ± 0.4 g/dl and $63.3\% \pm 1.8\%$, respectively) (Fig. 2).

Effects of DMOG rHuEPO on the Development of Hypertension and Renal Injury. A comparison of the effects of DMOG or rHuEPO treatment on blood pressure and urinary protein excretion in Dahl S rats fed a high-salt diet is presented in Fig. 3. Baseline systolic blood pressure averaged 161 ± 3 mm Hg in the vehicle-treated group and rose to 226 ± 4 mm Hg after 3 weeks on a high-salt diet (Fig. 3A). Blood pressure and proteinuria were not different in the

vehicle-treated group and the group treated with 60 mg/kg of DMOG (Fig. 3). Blood pressure and proteinuria increased to a greater extent in rats treated with rHuEPO. Chronic treatment with 600 mg/kg of DMOG attenuated the development of hypertension and proteinuria, and systolic blood pressure only increased to 189 ± 8 mm Hg over the 3-week course of the study.

Effects of DMOG and rHuEPO on Renal Injury. At baseline, glomerular injury and fibrosis were rare (Fig. 4, A and B). The degree of glomerulosclerosis and fibrosis was markedly enhanced in the vehicle-treated group fed a high-salt diet for 3 weeks (Fig. 4C). They also exhibited some degree of renal interstitial fibrosis (Fig. 4D). The degree of glomerulosclerosis and renal interstitial fibrosis was markedly enhanced in rats receiving rHuEPO (Fig. 4, G and H). In contrast, the degree of glomerulosclerosis and glomerular fibrosis, as well as renal interstitial fibrosis, was markedly reduced in rats treated with 600 mg/kg of DMOG (Fig. 4, E and F).

A comparison of the glomerulosclerosis score and degree of renal fibrosis is presented in Fig. 4, I–K. The glomerular sclerosis index and degree of glomerular fibrosis were similar in rats treated with vehicle versus rHuEPO. However, rHuEPO markedly enhanced the development of renal interstitial fibrosis. The degree of glomerulosclerosis and fibrosis and renal interstitial fibrosis was markedly attenuated in a dose-dependent manner in rats treated with DMOG.

A comparison of plasma BUN and serum creatinine levels among the groups is presented in Table 1. Plasma BUN and creatinine levels increased similarly in all the groups following 3 weeks on a high-salt diet. Urinary NAG and KIM-1 excretion increased following the development of hypertension in the vehicle-treated rats. rHuEPO increased urinary KIM-1 excretion to a greater extent than that seen in the vehicle-treated group. The increases in NAG and KIM-1 excretion were markedly attenuated in the rats treated with DMOG (Fig. 5).

The degree of fibrosis in the outer medulla of the kidney was markedly elevated in vehicle-treated rats fed a high-salt diet for 3 weeks. rHuEPO aggravated renal medullary fibrosis (Fig. 6G), whereas it was completely prevented in rats treated with DMOG (Fig. 6E). The formation of protein casts was increased dramatically following the development of hypertension in the vehicle-treated group (Fig. 6, B and D). DMOG, as well as rHuEPO, attenuated the formation of protein casts

Fig. 1. Time-course study of the effects of DMOG (600 mg/kg) on EPO mRNA expression and serum EPO concentration. (A) DMOG increased EPO mRNA expression level in renal cortex. (B) Serum EPO concentration increased 4 hours after the DMOG administration and returned to the baseline level thereafter. Open circles represent serum EPO concentration in vehicle-treated animals, and closed circles represent levels in DMOG-treated animals. Data are represented as mean values $\pm$ 1 S.E.M. from $n = 3$ animals per group.

Fig. 2. Effect of DMOG and rHuEPO on (A) hemoglobin and (B) hematocrit in high-salt-fed Dahl S rats before and after chronic administration of vehicle (open circle), 60 mg/kg of DMOG (filled circle), 600 mg/kg of DMOG (open triangle), and 100 U/kg of rHuEPO (filled triangle). The high-salt diet and the drug administration were started together on day 1 after the control period. Hemoglobin and hematocrit were measured at 1 week before the start of the study through week 3. Data are presented as the mean values $\pm$ 1 S.E.M. The number of animals studied in each group is shown in parentheses. *, significant difference from the corresponding value in vehicle-treated rats.

(Fig. 6, F, H, and J). Quantitative analysis revealed that DMOG prevented the renal medullary fibrosis and protein cast formation in a dose-dependent manner (Fig. 6, I and J).

Effects of DMOG and rHuEPO on Renal Inflammatory Markers and Growth Factors. High-salt treatment increased renal cortical and medullary IL-1 β and MCP-1 compared with baseline levels (Fig. 7A). rHuEPO had no significant effect on the increase in renal IL-1 β and MCP-1 levels. In contrast, DMOG treatment prevented the increase of IL-1 β and MCP-1 levels in a dose-dependent manner.

Exposure to a high-salt treatment increased TGF β 1 levels in the medulla in the vehicle-treated group, and rHuEPO further increased the TGF β 1 levels. DMOG treatment had no significant effect on the rise in TGF β 1 expression.

The levels of VEGF in both cortex and medulla fell following 3 weeks on a high-salt diet in the vehicle-treated group. rHuEPO had no effect on the fall in VEGF expression (Fig. 8). In contrast, DMOG dose dependently increased renal VEGF levels by 7-fold in cortex and 60-fold in medulla compared with values seen in the vehicle-treated group.

Discussion

rHuEPO is widely used to treat anemia and improves the quality of life in patients with CKD (Evans et al., 1990). Several studies have explored the effects of rHuEPO on renal function in a variety of experimental models of CKD (Lee et al., 2005; Katavetin et al., 2007; Toba et al., 2009; Cañadillas

et al., 2010; Rjiba-Touati et al., 2012). In general, these studies indicate that rHuEPO reduced renal inflammation and fibrosis regardless of its hematopoietic effect. However, subsequent clinical trials indicate that the use of rHuEPO increased the risks of hypertension, stroke, cardiovascular events, and dialysis in patients with CKD (Drüeke et al., 2006; Singh et al., 2006; Szczec et al., 2008; Pfeffer et al., 2009). Because hypertension and diabetes are the primary risk factors for the development of CKD; it is possible that these patients may be more susceptible to potential adverse effects of rHuEPO on blood pressure and hypertension-induced renal injury. To explore this possibility, we compared the effects of rHuEPO and DMOG, an injectable PHD inhibitor, on the development of hypertension and renal injury in Dahl S rats, which is an experimental model that is highly susceptible to the development of salt-sensitive hypertension and renal injury.

Treatment of Dahl S rats fed a high-salt diet with rHuEPO not only increased hemoglobin levels but also augmented the degree of hypertension. The treatment of the rats with rHuEPO also aggravated proteinuria and fibrosis in the renal cortex and medulla. These effects were associated with increased expression of TGF β 1. These results are consistent with the clinical findings that stimulation of hematopoiesis by rHuEPO treatment in patients with CKD is associated with increased risk of hypertension and renal dysfunction (Raine, 1988; Drüeke et al., 2006). Interestingly, in other experimental models of renal disease, such as cyclosporine-induced nephropathy, unilateral ureter obstruction, and diabetic

Fig. 3. Effect of DMOG and rHuEPO on (A) systolic blood pressure and (B) urinary protein excretion in Dahl S rats fed a high-salt diet before and after chronic administration of vehicle (open circle), 60 mg/kg of DMOG (filled circle), 600 mg/kg of DMOG (open triangle), and 100 U/kg of rHuEPO (filled triangle). The high-salt diet and the drug administration were started together on day 1 after the control period. The drugs were given three times per week. Systolic blood pressure and urinary protein excretion were measured at 1 week before the start of the study through week 3. Data are presented as the mean values $\pm$ 1 S.E.M. The number of animals studied per group is shown in parentheses. *, significant difference from the corresponding value in vehicle-treated rats.

Fig. 4. Effect of DMOG and rHuEPO on glomerular injury and renal interstitial fibrosis in the renal cortex. Representative renal micrographs of the rats at baseline (A and B) and after treatment with vehicle (C and D), 600 mg/kg of DMOG (E and F), or rHuEPO (G and H) are shown. (A, C, E, and G) are micrographs of glomerulus, and (B, D, F, and H) are micrographs of the interstitium. Original magnification, 200 $\times$. Glomerulosclerosis (I), glomerular fibrosis (J), and interstitial fibrosis (K) are quantified and shown in the bar graphs. Thirty glomeruli were scored for the degree of glomerulosclerosis, and 10 fields were quantified for fibrosis in each animal. The number of animals studied per group is presented on the graphs. #, significant difference from the corresponding baseline value; *, significant difference from the corresponding value in the vehicle-treated group; †, significant difference from the corresponding value in the EPO-treated group; BL, baseline; Ve, vehicle.

nephropathy, and in normotensive strains of rats and mice that are not salt sensitive, blood pressure did not increase following administration of rHuEPO. Moreover, rHuEPO suppressed TGF β 1 expression and reduced renal inflammation and fibrosis in several of these studies (Lee et al., 2005; Park et al., 2007; Toba et al., 2009). Because the blockage of TGF β 1 attenuates hypertension and renal fibrosis (Dahly et al., 2002; Murphy et al., 2012), these results suggest that in models in which rHuEPO does not increase blood pressure,

rHuEPO can attenuate renal interstitial fibrosis by reducing TGF β 1 expression. In contrast, our results indicate that when rHuEPO increased both hemoglobin levels and blood pressure, the rise in blood pressure may increase renal fibrosis.

DMOG is a PHD 1/2/3 pan inhibitor that stimulates erythropoiesis by stabilizing HIFs (Epstein et al., 2001; Barrett et al., 2011). We compared the effects of rHuEPO and that of DMOG on blood pressure and renal function. In our experiments, 600 mg/kg of DMOG increased endogenous EPO concentration in serum and increased hemoglobin levels as potently as rHuEPO. However, in sharp contrast to rHuEPO, DMOG attenuated the development of hypertension and proteinuria in the Dahl S rats. Furthermore, DMOG prevented the increase of renal inflammatory markers in the kidney along with renal fibrosis and medullary protein cast formation. These results are consistent with the previous findings that the activation of HIF1 α attenuated the rise of blood pressure in high-salt-treated Dahl S rats (Zhu et al., 2012, 2014). However, unlike the results of the previous study, we could not confirm an increase in heme oxygenase-1 expression in the kidney of Dahl S rats (data not shown). We instead found that renal VEGF levels were very low in high-salt-treated Dahl S rats and that it increased in the rats

TABLE 1
BUN and serum creatinine
Mean values $\pm$ 1 S.E.M. are presented.

	n	BUN (mg/dl)	sCre (mg/dl)
Baseline	8–10	10.8 $\pm$ 0.4	0.56 $\pm$ 0.02
Vehicle	8	25.6 $\pm$ 1.9*	1.07 $\pm$ 0.12*
DMOG			
60 mg/kg	6	31.6 $\pm$ 3.2	0.96 $\pm$ 0.10
600 mg/kg	8	36.2 $\pm$ 1.8	0.89 $\pm$ 0.03
EPO			
100 U/kg	7	33.6 $\pm$ 4.1	0.97 $\pm$ 0.05

*Indicates $P < 0.05$ from the pooled baseline value. sCre, serum creatinine concentration.

Fig. 5. Effect of DMOG and rHuEPO on (A) urinary NAG and (B) KIM-1 excretion in Dahl S rats fed a high-salt diet. The number of animals studied in each group is shown on the graphs. #, significant difference from the corresponding baseline value; *, significant difference from the corresponding value in the vehicle-treated group; †, significant difference from the corresponding value in the EPO-treated group.

treated with DMOG. The renoprotective effects of VEGF have been reported elsewhere (Kang et al., 2001; Suga et al., 2001; Ma et al., 2011; Sivaskandarajah et al., 2012). Suppression of VEGF signaling using a humanized monoclonal antibody, genetic knockout of VEGF, or pharmacological blockade (Eremina et al., 2008; Lankhorst et al., 2017) has been shown

to increase blood pressure. Interestingly, Eremina et al. (2008) found that glomerular injury preceded the development of hypertension in VEGF knockout mice. Their observations are consistent with the present results that renal fibrosis and injury were attenuated in the group treated with 60 mg/kg of DMOG, whereas the hemoglobin levels and blood pressure

Fig. 6. Effect of DMOG and rHuEPO on renal injury in the medulla. Representative renal micrographs of the rats at baseline (A and B) and after treatment with vehicle (C and D), 600 mg/kg of DMOG (E and F), or 100 U/kg of rHuEPO (G and H) are shown. (A, C, E, and G) are micrographs of medullas, and (B, D, F, and H) are micrographs of protein casts. Original magnification, 100 $\times$. Medullary fibrosis (I) and protein cast area (J) are quantified and shown in the bar graphs. Ten fields were quantified for fibrosis, and five fields were quantified for protein cast area in each animal. The number of animals studied per group are shown on the graphs. #, significant difference from the corresponding baseline value; *, significant difference from the corresponding value in the vehicle-treated group; †, significant difference from the corresponding value in the EPO-treated group; BL, baseline; Ve, vehicle.

Fig. 7. Effect of DMOG and rHuEPO on (A) IL-1 β , (B) MCP-1, and (C) TGF β 1 levels in the renal cortex and medulla in Dahl S rats fed a high-salt diet. The number of animals studied per group is shown on the graphs. #, significant difference from the corresponding baseline value; *, significant difference from the corresponding value in the vehicle-treated group; †, significant difference from the corresponding value in the EPO-treated group.

were similar to the vehicle-treated group. Overall, these findings suggest that the reduction of renal VEGF expression could be one of the mechanisms contributing to the development of hypertension and renal injury in Dahl S rats. Based on this mechanism, DMOG could oppose the development of nephropathy by induction of renal VEGF expression and the attenuation of hypertension.

HIF1 α induces CD73 (ecto-5-prime-nucleotidase) (Synnestevedt et al., 2002) and increases adenosine signaling. In the kidney, adenosine is a vasoconstrictor involved in tubuloglomerular feedback responses (Schnermann, 2015; Romero and Carretero, 2019). Upregulation of CD73 has been reported to protect the kidney from ischemia-reperfusion injury and diabetic nephropathy (Tak et al., 2014; Sung et al., 2017). Therefore, PHD inhibitors may protect the kidney from salt-sensitive hypertension and diabetes-induced renal injury by enhancing tubuloglomerular feedback responsiveness and reducing transmission of systemic pressure to the glomerulus.

Fig. 8. Effect of DMOG and rHuEPO on VEGF expression in the renal cortex and medulla. The number of animals studied in each group is shown on the graphs. #, significant difference from the corresponding baseline value; *, significant difference from the corresponding value in the vehicle-treated group; †, significant difference from the corresponding value in the EPO-treated group.

Currently, PHD inhibitors are under the investigation of phase 3 clinical trials to treat renal anemia in nondialysis and dialysis patients (Cernaro et al., 2019), and roxadustat was first approved for the treatment of anemia in China (Dhillon, 2019). The potential adverse events of three PHD inhibitors—molidustat, daprodustat, and vadadustat—have been investigated in recent trials. Although the administration of molidustat was as effective as rHuEPO in increasing hemoglobin in patients with CKD, the incidence of hypertension was lower in the molidustat-treated group (Macdougall et al., 2019). Daprodustat was also effective in increasing hemoglobin levels by more than 2 g/dl, but it did not affect blood pressure (Brigandi et al., 2016). Additionally, vadadustat increased hemoglobin levels by 1.4 g/dl without altering blood pressure rise in a 6-week study (Martin et al., 2017); however, the number of patients with hypertension was higher in the vadadustat-treated group in a 20-week study (Pergola et al., 2016). Although the statistical power in these clinical trials was insufficient to detect the change in blood pressure, it is noteworthy that the results from these clinical trials suggest that treatment of anemia with PHD inhibitors did not raise blood pressure. In the present study, we found that the blood pressure was lower in the DMOG-treated group than in the vehicle-treated group. This result is consistent with the results of the previous clinical trials and contrasts with the prohypertensive effects seen in rats treated with rHuEPO.

DMOG has been reported to increase VEGF expression in the eye (Safran et al., 2006). Thus, there is a risk of retinopathy. However, in the present study, we did not observe any adverse effects in the Dahl S rats chronically treated with 60 or 600 mg/kg per day of DMOG.

In summary, rHuEPO and DMOG are equally effective in increasing hemoglobin levels in Dahl S rats fed a high-salt diet. However, rHuEPO aggravated the degree of hypertension, proteinuria, and renal injury. This was associated with induction of the expression of TGF β 1. In contrast, DMOG attenuated the development of hypertension and renal injury

through induction of renal VEGF expression. These results suggest that PHD inhibitors may provide an alternative and safer therapeutic option for the treatment of anemia in patients with diabetes- and hypertension-induced CKD.

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Authorship Contributions

Participated in research design: Kato, Takahashi, Miyata, Roman.
Conducted experiments: Kato.

Performed data analysis: Kato, Roman.

Wrote or contributed to the writing of the manuscript: Kato, Roman.

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