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Title:

DMOG, a prolyl hydroxylase inhibitor, increases hemoglobin levels without exacerbating hypertension and renal injury in salt-sensitive hypertensive rats.

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Renoprotective effects of DMOG

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Abbreviations: BUN, blood urea nitrogen; DMOG, dimethyloxaloylglycine; EPO, erythropoietin; HIF, hypoxia-inducible factor; KIM-1, kidney injury marker 1; NAG, N-acetyl-β-D-glucosaminidase; PHD, prolyl hydroxylase

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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 versus 226 ± 4 mmHg) than in rats given DMOG (189 ± 8 mmHg). 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 to that in vehicle and rHuEPO-treated rats. This was associated with lower renal levels of MCP-1, IL-1β, and increased VEGF 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 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, CKD patients develop anemia. The prevalence of anemia is 20% in patients with stage 3 CKD, and 60% and 75% in patients with stage 4 or 5 CKD (McFarlane et al., 2008).

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 CKD patients (Evans et al., 1990). On the other hand, some issues are associated with the use of rHuEPO for the treatment of anemia in CKD patients. 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, 3 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 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 alpha (HIFα), which is a master regulator of the hypoxic response (Epstein et al., 2001). Hydroxylated HIFα is recognized by a ubiquitin ligase, von Hippel-Lindau protein (VHL), and degraded through the ubiquitin-proteasome pathway (Jaakkola et al., 2001). In hypoxic conditions, the PHD activity is reduced, and HIFα escapes from hydroxylation and subsequent degradation. Once HIFα is stabilized, HIFα binds to the hypoxia response element together with CBP/p300 and constitutively active HIFβ to upregulate the expression of target genes (Haase, 2006). As a consequence, vascularity is increased through HIF1α, and hemoglobin levels are elevated through the actions of HIF2α (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 non-dialysis 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 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 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 that 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 diabetic and hypertension-induced CKD.

Materials and Methods

General

Dimethyloxaloylglycine (DMOG) was synthesized by Medical Chemistry Laboratories in Taisho Pharmaceutical Co., Ltd. Recombinant human EPO (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 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 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 2130 g for 10 min, 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 (0h) 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 qRT-PCR. EPO was amplified using the following forward and reverse primers: 5 -GCTCCAATCTTTGTGGCATCT- 3 and 5 -TGGCTTCGTGACCCTCTGT- 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 4 groups and treated with vehicle,

DMOG at 60 mg/kg, DMOG at 600 mg/kg or rHuEPO at 100 units/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 while rHuEPO was given subcutaneously, 3

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), and DMOG or EPO

were administered for 3 weeks, while 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 µL of blood using HemoCue Hb 201+ device (HemoCue, Brea, CA) and

hematocrit was determined in 80 µL 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 µm) were stained

with Masson's trichrome and analyzed for the degree of glomerulosclerosis, 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

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, Minneapolis, MN).

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 X100 and a proteinase

inhibitor cocktail. The homogenate was centrifuged at 11000 g for 5 minutes at 4°C, and the

MCP-1, IL-1β, TGFβ1 and VEGF levels in the supernatant were measured using ELISA

kits (R&D Systems, Minneapolis, MN). Samples for measurement of TGFβ1 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 ± 1 standard error. 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 that 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 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, intraperitoneally) or rHuEPO (100 U/kg, subcutaneously) given 3 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 vehicle and the 60 mg/kg of DMOG-treated groups remained unchanged throughout the study. The hemoglobin and hematocrit levels in the 600 mg/kg of DMOG-treated group increased from 16.3 ± 0.2 g/dL and 56.1 ± 2.0% to 18.3 ± 0.4 g/dL and 63.1 ± 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 ± 1.8%, respectively) (Fig. 2A, B).

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 are presented in Figure 3. Baseline systolic blood pressure averaged 161 ± 3 mmHg in the vehicle treated-group and rose to 226 ± 4 mmHg after 3 weeks on a high salt diet (Fig. 3A). Blood pressure and proteinuria were not different in the vehicle-treated and 60 mg/kg of DMOG-treated groups (Fig. 3A, B). 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 mmHg 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. 4A, 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. 4G, H). In contrast, the degree of glomerulosclerosis and glomerular fibrosis, as well as renal interstitial fibrosis, were markedly reduced in rats treated with 600 mg/kg of DMOG (Fig. 4E, F).

A comparison of the glomerulosclerosis score and degree of renal fibrosis is presented in Figures 4I, J, and 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. 5A, B).

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), while 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. 6B, D). DMOG, as well as rHuEPO, attenuated the formation of protein casts. (Fig. 6F, H, J). Quantitative analysis revealed that DMOG prevented the renal medullary fibrosis and protein cast formation in a dose-dependent manner (Fig. 6I, J).

Effects of DMOG and rHuEPO on renal inflammatory markers and growth factors. High-salt treatment increased renal cortical and medullary IL-1β and MCP-1 compared to baseline levels (Fig. 7A). rHuEPO had no significant effect on the increase in renal IL-1β and MCP-1 levels. In contrast, DMOG treatment prevented the increase of IL-1β and MCP-1 levels in a dose-dependent manner.

Exposure to a high salt treatment increased TGFβ1 levels in the medulla in the vehicle-treated group, and rHuEPO further increased the TGFβ1 levels. DMOG-treatment had no significant effect on the rise in TGFβ1 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 to values seen in the vehicle-treated group.

Discussion

Recombinant human EPO (rHuEPO) is widely used to treat anemia and improves the quality of life in CKD patients (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 CKD patients (Drüeke et al., 2006; Singh et al., 2006; Szczech et al., 2008; Pfeffer et al., 2009). Since 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 that 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 with rHuEPO increased hemoglobin levels, but it 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 TGFβ1. These results are consistent with the clinical findings that stimulation of hematopoiesis by rHuEPO treatment in CKD patients 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 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 TGFβ1 expression and reduced renal inflammation and fibrosis in several of these studies (Lee et al., 2005; Park et al., 2007; Toba et al., 2009). Since the blockage of TGFβ1 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 TGFβ1 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 HIF1α attenuated the rise of blood pressure in high-salt treated Dahl S rats (Zhu et al., 2012; Zhu et al., 2014). However, unlike the results of the previous study, we could not confirm an increase in HO-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 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 proceeded 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 60 mg/kg of DMOG-treated group whereas the hemoglobin levels and blood pressure 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.

HIF1α induces CD73 (Ecto-5-prime-nucleotidase) (Synnestvedt 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 diabetic induced renal injury by enhancing tubuloglomerular feedback responsiveness and reducing transmission of systemic pressure to the glomerulus.

Currently, PHD inhibitors are under the investigation of phase 3 clinical trials to treat renal anemia in non-dialysis 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. While the administration of molidustat was as effective as rHuEPO in increasing hemoglobin in CKD patients, the incidence of hypertension was lower in molidustat-treated group (Macdougall 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 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 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 pro-hypertensive 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/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 TGFβ1. 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 diabetic and hypertension-induced CKD.

Authorship Contributions

Participated in research design: Kato, Takahashi, Miyata, and Roman

Conducted experiments: Kato

Performed data analysis: Kato and Roman

Wrote or contributed to the writing of the manuscript: Kato and Roman

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Footnotes

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Figure legends

Figure 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 ± 1 S.E.M from n = 3 animals per group.

Figure 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 at week 3. Data are presented as the mean values ± 1 S.E.M. The numbers of animals studied in each group are shown in parentheses. * indicates a significant difference from the corresponding value in vehicle-treated rats.

Figure 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 3 times per week. Systolic blood pressure and urinary protein excretion were measured at 1 week before the start of the study through at week 3. Data are presented as the mean values

± 1 S.E.M. The numbers of animals studied per group are shown in parentheses. * indicates a significant difference from the corresponding value in vehicle-treated rats.

Figure 4. Effect of DMOG and rHuEPO on glomerular injury and renal interstitial fibrosis in the renal cortex. Representative micrographs of the baseline (A and B), vehicle (C and D), 600 mg/kg of DMOG (E and F), or rHuEPO (G and H) treated rats are shown. A, C, E, and G are micrographs of glomerulus and B, D, F, and H are micrographs of the interstitium. Magnification is 200X. 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 ten fields were quantified for fibrosis in each animal. The number of animals studied per group are presented on the graphs. # indicates a significant difference from the corresponding baseline value, * indicates a significant difference from the corresponding value in the vehicle-treated group, and † indicates a significant difference from the corresponding value in the EPO-treated group.

Figure 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 numbers of animals studied in each group are shown on the graphs. # indicates a significant difference from the corresponding baseline value, * indicates a significant difference from the corresponding value in the vehicle-treated group, and † indicates a significant difference from the corresponding value in the EPO-treated group.

Figure 6. Effect of DMOG and rHuEPO on renal injury in the medulla. Representative micrographs of the baseline (A and B), vehicle (C and D), 600 mg/kg of DMOG (E and F), or 100 U/kg of rHuEPO (G and H) treated rats are shown. A, C, E, and G are micrographs

of medullas and B, D, F, and H are micrographs of protein casts. Magnification is 100X.

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. #

indicates a significant difference from the corresponding baseline value, * indicates a

significant difference from the corresponding value in the vehicle-treated group, and †

indicates a significant difference from the corresponding value in the EPO-treated group.

Figure 7. Effect of DMOG and rHuEPO on (A) IL-1β, (B) MCP-1, and (C) TGFβ1 levels

in the renal cortex and medulla in Dahl S rats fed a high salt diet. The numbers of animals

studied per group are shown on the graphs. # indicates a significant difference from the

corresponding baseline value, * indicates a significant difference from the corresponding

value in the vehicle-treated group, and † indicates a significant difference from the

corresponding value in the EPO-treated group.

Figure 8. Effect of DMOG and rHuEPO on VEGF expression in the renal cortex and

medulla. The numbers of animals studied in each group are shown on the graphs. #

indicates a significant difference from the corresponding baseline value, * indicates a

significant difference from the corresponding value in the vehicle-treated group, and †

indicates a significant difference from the corresponding value in the EPO-treated group.

Tables

Table 1. BUN and serum creatinine.

		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

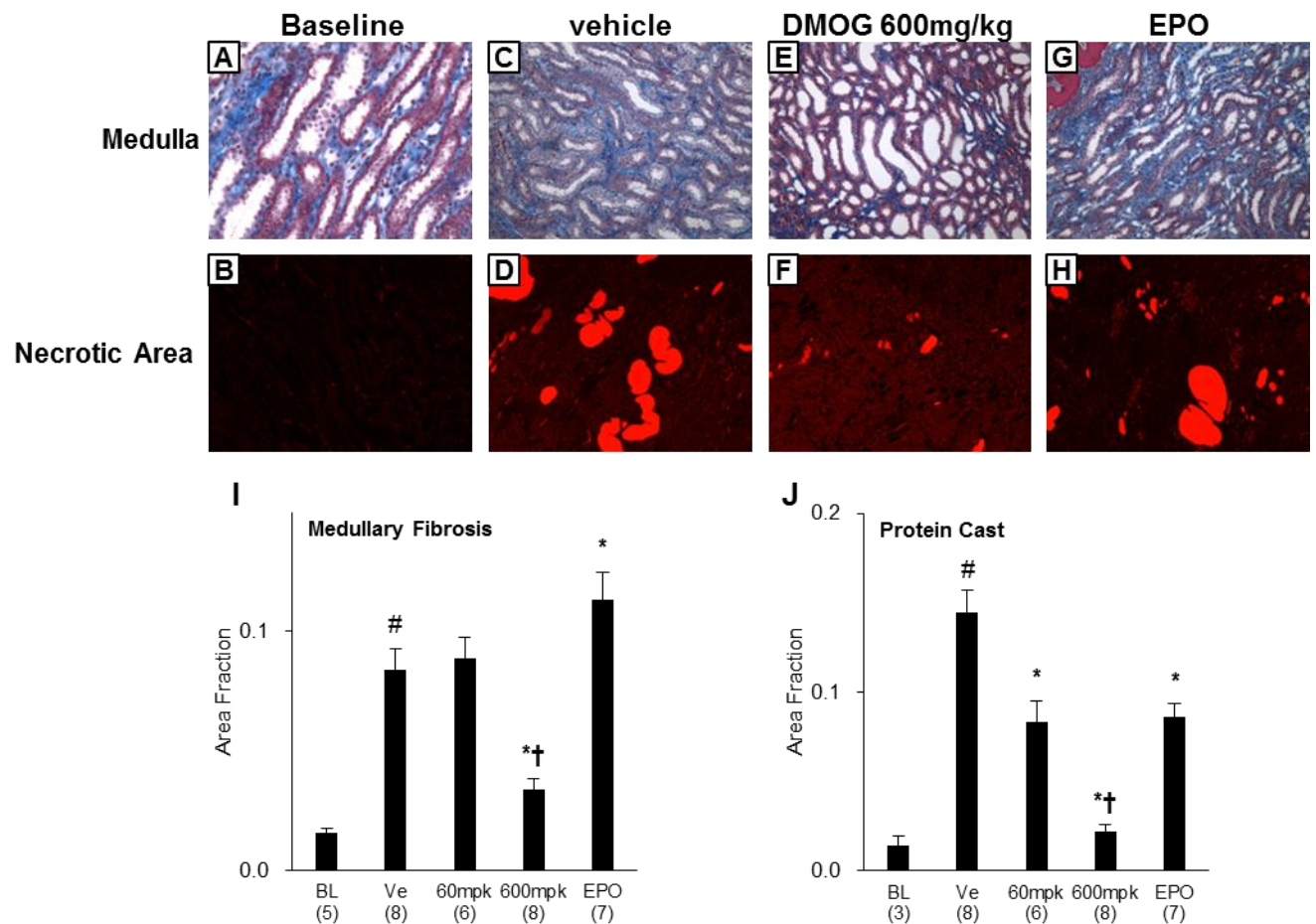Mean values $\pm$ 1 S.E.M are presented. * indicate $P<0.05$ from the pooled baseline value.
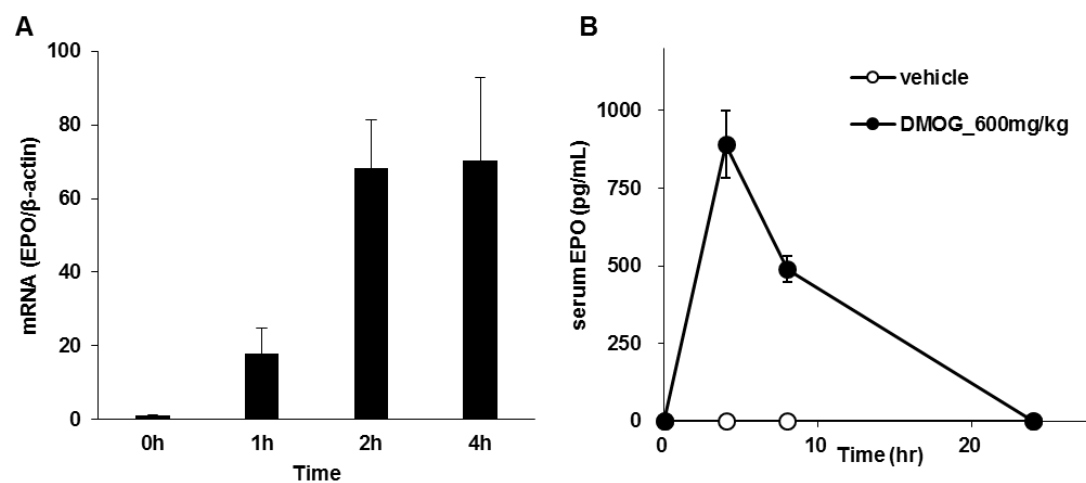
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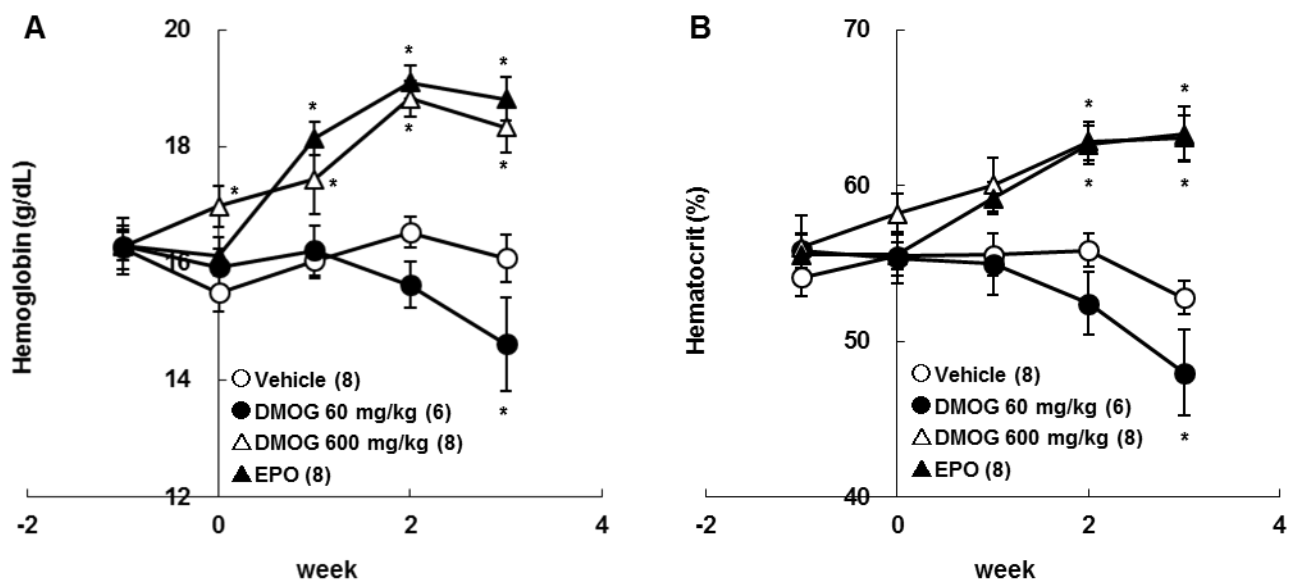
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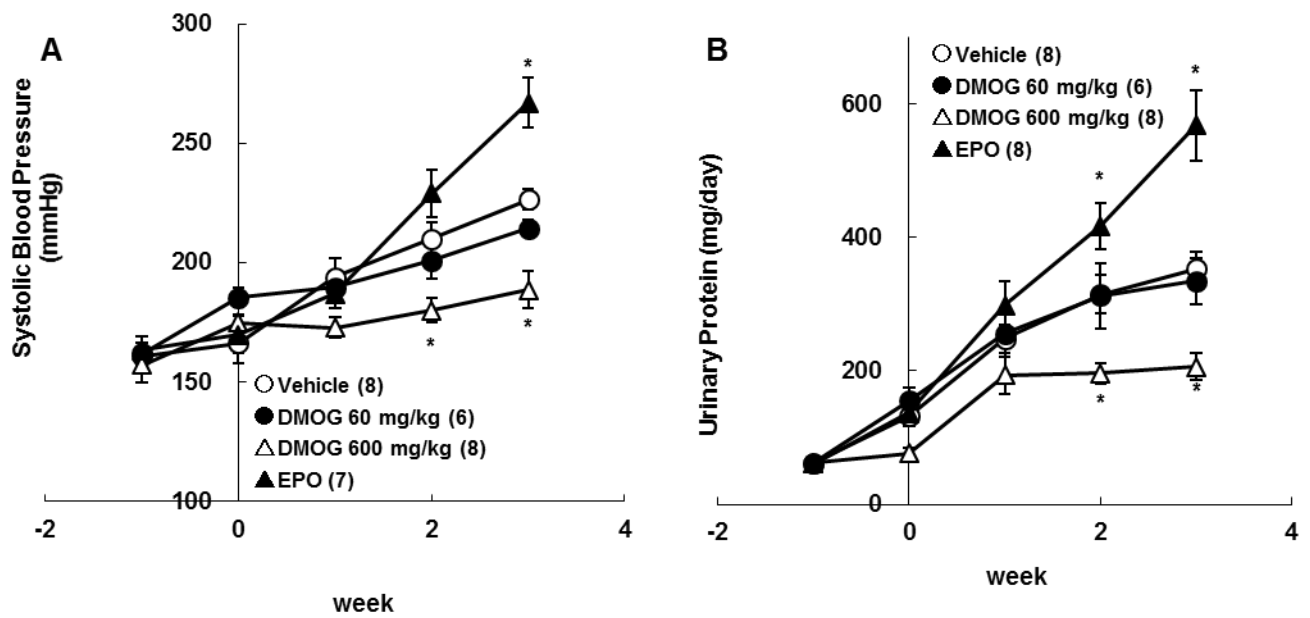
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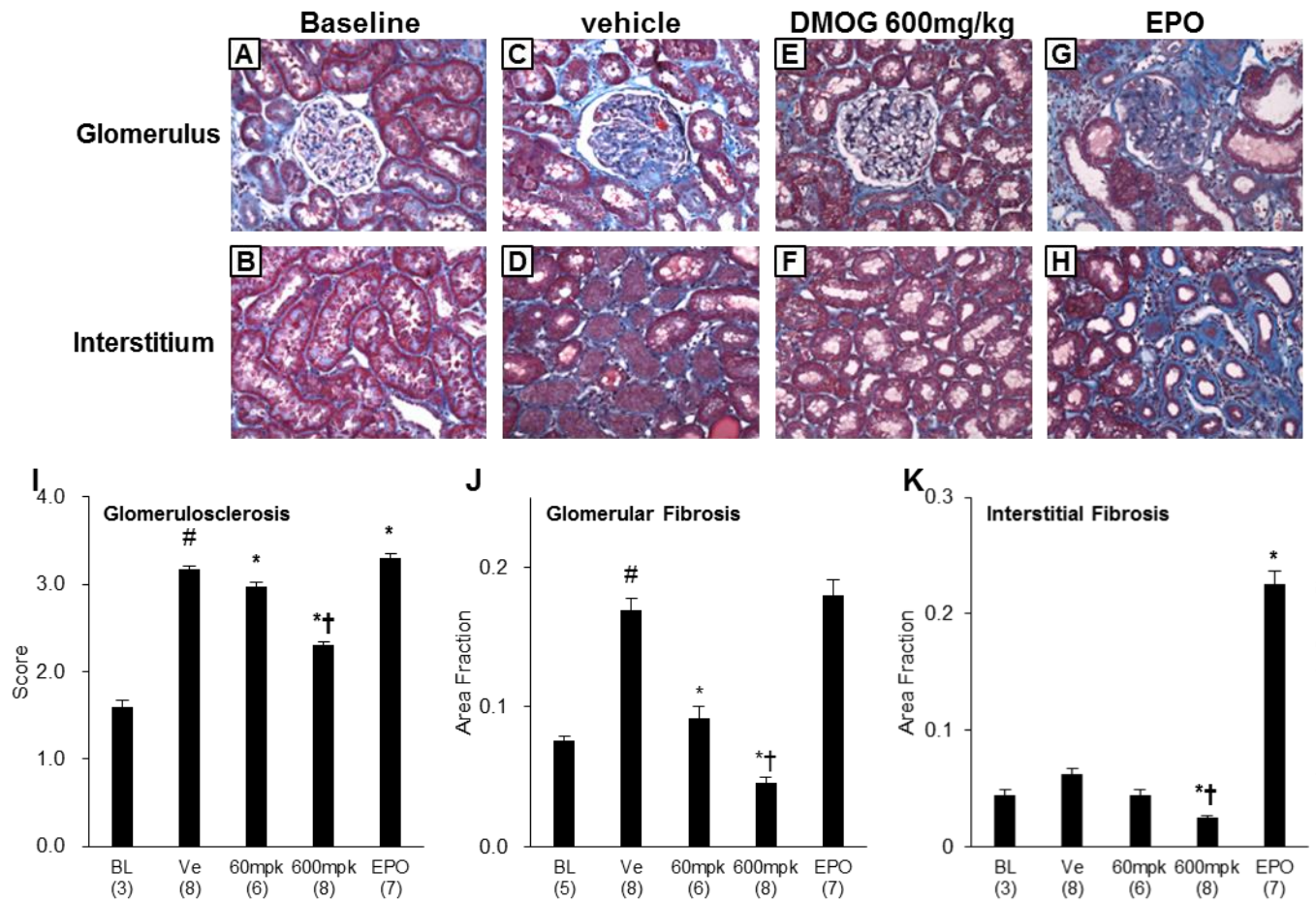
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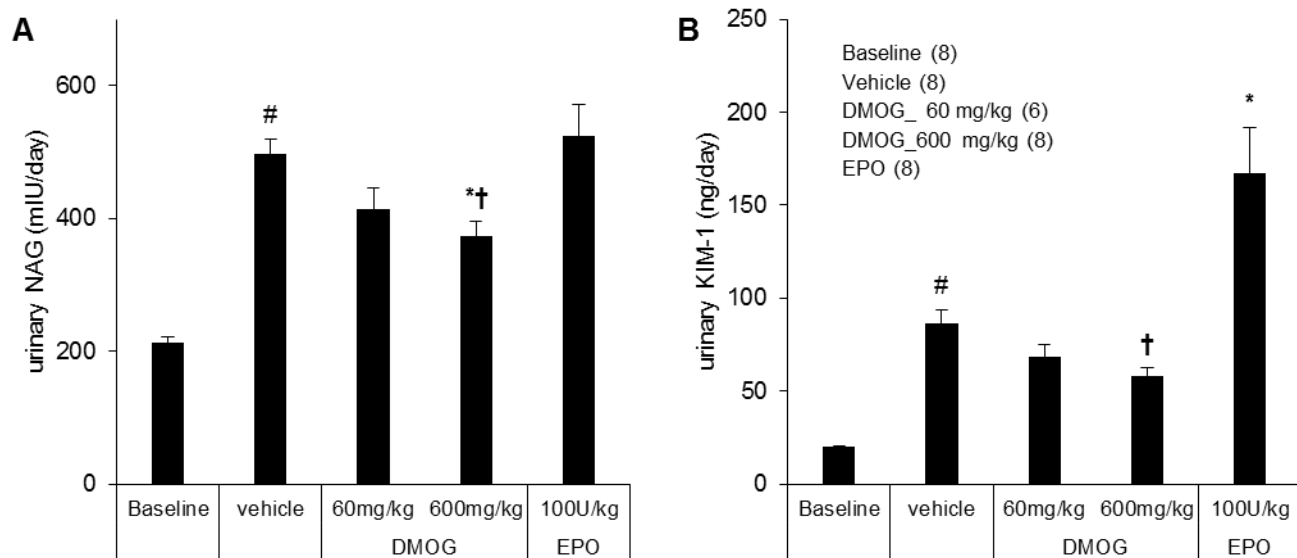
Figure 5

Figure 6

Figure 7

Figure 8