Levels of the erythropoietin-responsive hormone erythroferrone in mice and humans with chronic kidney disease

Erythroferrone (ERFE) is a hormone produced by human and murine erythroid precursors that acts directly on the liver to decrease hepcidin production.[1] Hepcidin regulates circulating iron concentrations by binding to the cellular iron exporter ferroportin, causing its internalization and degradation, resulting in decreased iron export from cells into plasma.[2] Hepcidin affects: 1) enterocytes, inhibiting dietary iron absorption; 2) hepatocytes, preventing the mobilization of hepatic iron stores; and 3) hepatic and splenic macrophages, inhibiting the efflux of recycled iron.[2] ERFE production is stimulated by endogenous or exogenous erythropoietin (EPO), thus serving to couple increased erythropoietic activity with decreased hepcidin, allowing for maintenance of plasma iron concentrations in the setting of increased erythropoiesis-associated iron demand.[1]

In murine models, ERFE acutely increases in response to exogenous EPO administration[1] and to phlebotomy-induced increases in endogenous EPO.[1] ERFE is also increased in the *Hbb*$^{th3/+}$ mouse model of β-thalassemia intermedia, which is characterized by chronically elevated EPO levels and a greatly expanded population of ERFE-secreting erythroblasts.[1,3] Using a recently validated immunoassay for human ERFE,[4] we also demonstrated that blood loss or EPO administration acutely increases serum ERFE concentrations in humans, and that β-thalassemia patients have very high serum ERFE.[4]

Erythroferrone concentrations are not well characterized in chronic kidney disease (CKD) patients, who have anemia-induced relative increases in serum EPO,[5] or are chronically treated with large doses of exogenous EPO. If ERFE levels are increased in these settings, then they could counteract the hepcidin-raising effects of inflammation and impaired hepcidin clearance in CKD. Therefore, to assess ERFE production in the setting of CKD, we measured acute changes in serum ERFE after EPO administration in mice with and without CKD. We also measured serum ERFE levels in cohorts of non-dialysis, non-EPO-treated CKD patients and in dialysis-dependent patients treated with EPO.

In 9-11 week old C57BL/6 mice with and without adenine diet-induced CKD, we administered a single intraperitoneal dose of 40 μg/kg (approx. 70 units/gram) rhEPO or saline, then euthanized groups of mice 6, 24, or 48 hours (h) post injection. Complete details of the methods used are listed in the *Online Supplementary Appendix*. The CKD cohort had higher serum urea nitrogen concentrations than the non-CKD cohort, and urea nitrogen levels in EPO-injected CKD groups did not differ from the non-injected CKD baseline group (Figure 1A). In the mice with normal kidney function, serum ERFE levels, undetectable at baseline, increased in response to EPO, peaking at 6 h post injection (Figure 1B). In mice with CKD, serum ERFE levels peaked later, at 24 h post EPO injection (Figure 1B). The causes for this delayed response are unknown, but may include modulation of EPO-EPO receptor signaling due to inflammation.[6] Indeed, inflammation was observed in our CKD mice, as evidenced by mean liver Saa1 mRNA expression that was 27-fold higher than in the non-CKD mice. Nevertheless, the magnitude of ERFE induction was comparable between non-CKD and CKD groups. In both the non-CKD and CKD cohorts, serum hepcidin was significantly decreased at 48 h post-EPO injection, as compared to saline; however, statistical significance *versus* the baseline group was only achieved in the non-CKD cohort (Figure 1C). In both EPO-injected cohorts, serum iron did not differ from baseline (Figure 1D), possibly bolstered by ERFE-mediated hepcidin suppression.

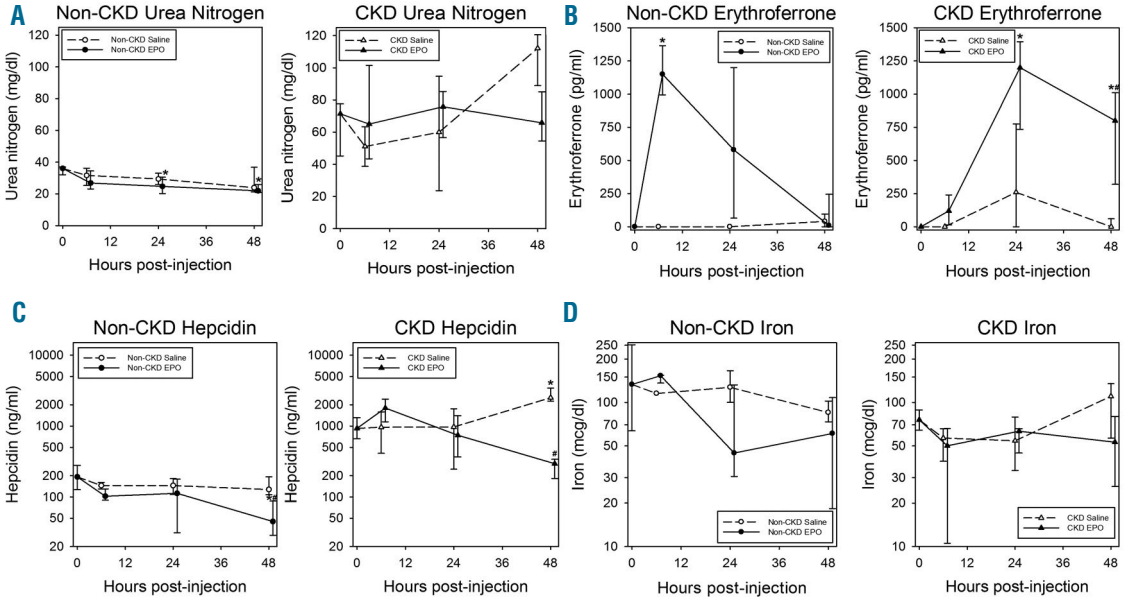

Figure 1. Acute effects of recombinant human erythropoietin (rhEPO) in wild-type mice with and without chronic kidney disease (CKD). Groups included are wild-type mice, with and without adenine diet-induced CKD, intraperitoneally injected with a single dose of 40 μg/kg (approx. 70 units/gram) rhEPO or saline, at baseline, 6 hours (h) post injection, 24 h post injection, and 48 h post injection. Parameters shown are (A) urea nitrogen, (B) erythroferrone, (C) hepcidin, and (D) iron. *P<0.05; pairwise comparison *versus* baseline values within the non-CKD or CKD cohort. #P<0.05; pairwise comparison of EPO-treated *versus* saline-treated mice at the indicated time point. Data are presented as medians and interquartile ranges. The baseline (untreated) groups had 6-7 mice per group; the rhEPO-treated groups had 3-4 mice per group; and the saline-treated groups had 2-4 mice per group.

We next measured serum ERFE levels in adult and pediatric non-dialysis and dialysis-dependent CKD patients. Characteristics of our non-dialysis CKD and dialysis-dependent CKD cohorts are listed in *Online Supplementary Table S1*. None of the non-dialysis CKD subjects received rhEPO treatment or iron supplementation. Conversely, all of the dialysis-dependent CKD patients received rhEPO treatment and intravenous iron supplementation. Dialysis patients had higher ERFE levels than both healthy, non-CKD patients [15.7 (7.9, 32.5) *vs.* 7.8 (4.7, 13.2) ng/mL; $P<0.05$] and non-dialysis CKD patients [15.7 (7.9, 32.5) *vs.* 6.1 (2.6, 15.0) ng/mL; $P<0.05$)] (*Online Supplementary Figure S1*). Similar trends were observed when adult and pediatric groups were evaluated separately (*Online Supplementary Figure S1*).

In the non-dialysis CKD cohort, serum ERFE correlated positively with serum erythropoietin (Spearman correlation coefficient $r_s=0.58$, $P<0.001$). Similarly, in the dialysis-dependent cohort, serum ERFE correlated positively with rhEPO dose ($r_s=0.43$, $P<0.001$). However, in neither cohort did ERFE correlate with hepcidin (non-dialysis CKD: $r_s=0.10$, $P=0.49$; dialysis CKD: $r_s=-0.08$, $P=0.42$). In the non-dialysis CKD cohort, hepcidin correlated positively with ferritin ($r_s=0.71$, $P<0.001$), correlated positively with TSAT ($r_s=0.30$, $P=0.043$), correlated inversely with eGFR ($r_s=-0.32$, $P=0.030$), and did not correlate with CRP ($r_s=0.20$, $P=0.19$). In the dialysis-dependent CKD cohort, hepcidin correlated positively with ferritin ($r_s=0.49$, $P<0.001$), correlated positively with TSAT ($r_s=0.27$, $P=0.012$), and correlated positively with CRP ($r_s=0.28$, $P=0.013$).

We reported previously that ERFE, a recently described erythroid regulator of hepcidin, is strongly induced by endogenous and exogenous EPO in mice and humans.[1,3,4] In the present study, we asked whether the effect of EPO on ERFE extends to CKD, where both EPO production and iron metabolism are disordered. We demonstrate that exogenous EPO can still acutely increase ERFE in mice with CKD, with subsequent hepcidin suppression. In our cohorts of non-dialysis and dialysis-dependent CKD patients, we observed strong positive correlations between ERFE levels and serum EPO or rhEPO dose, respectively. However, unlike our previous studies of humans with normal kidney function, ERFE did not correlate with hepcidin in either cohort with kidney disease. This suggests that the pathological modulation of hepcidin levels in CKD by variable inflammation, iron therapy, and residual renal clearance masks any hepcidin-suppressive effects of ERFE.

Another study in 59 hemodialysis patients found that their ERFE levels did not differ from those of control subjects with normal kidney function. However, the study did observe a small ERFE increase (15-20%) in a subset of 20 hemodialysis patients that received erythropoiesis-stimulating agents (continuous erythropoietin receptor activator or darbepoetin alfa) at three days post administration.[7] It should be noted that the ERFE assay used in this study (MyBioSource, San Diego, CA, USA) has different characteristics than the human assay we developed and was not validated in physiological or pathological conditions that would be expected to have elevated ERFE levels, such as blood donors or patients with β-thalassemia.

In our study, the dialysis patients had higher serum ERFE levels than the non-dialysis CKD patients and the healthy non-CKD patients. The dialysis cohort was also treated with exogenous EPO. Increased ERFE production secondary to EPO stimulation and/or decreased renal clearance could both theoretically contribute to higher ERFE levels. However, the molecular weight of ERFE is 52 kD,[4] which is above the molecular weight cutoff for glomerular filtration, which is considered to be 30-50 kD. Furthermore, many of the dialysis patients were likely oligoanuric. Therefore, in the setting of CKD, increased production, more so than decreased clearance, likely predominantly contributes to higher serum ERFE concentrations.

In our cohorts of non-dialysis and dialysis-dependent CKD patients, we observed correlations between serum EPO (or rhEPO dose) and ERFE, but not between ERFE and hepcidin. However, the aforementioned study of 59 hemodialysis patients did observe a negative correlation between ERFE and hepcidin.[7] Again, assay differences may have contributed to the difference in these observations. Furthermore, hepcidin regulation in CKD is complex, as circulating hepcidin levels are influenced by kidney function, iron status, and inflammation. Therefore, in our study, the hepcidin suppressive effects of ERFE may have been masked by the effects of other hepcidin determinants. Nevertheless, ERFE may play an important role in the counter-regulation of pathologically increased hepcidin levels in CKD.

In summary, we have shown that serum ERFE levels acutely increase in response to EPO in the setting of normal or impaired kidney function. We further demonstrate strong positive correlations between serum ERFE and serum EPO (or rhEPO dose) in human CKD cohorts. Future studies are needed to clarify the role of ERFE in modulating iron homeostasis in CKD, and to examine the utility of ERFE as a biomarker of erythropoietic activity and EPO responsiveness in CKD-related anemia.

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