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PRE-CLINICAL RESEARCH

Erythropoietin Receptor Signaling Mitigates Renal Dysfunction-Associated Heart Failure by Mechanisms Unrelated to Relief of Anemia

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Objectives We examined the effect of asialoerythropoietin (asialoEPO), a nonerythrogenic derivative of erythropoietin (EPO), on renal dysfunction-associated heart failure.

Background Although EPO is known to exert beneficial effects on cardiac function, the clinical benefits in patients with chronic kidney disease are controversial. It remains to be addressed whether previously reported outcomes were the result of relief of the anemia, adverse effects of EPO, or direct cardiovascular effects.

Methods Mice underwent 5/6 nephrectomy to cause renal dysfunction. Eight weeks later, when renal dysfunction was established, anemia and cardiac dysfunction and remodeling were apparent. Mice were then assigned to receive saline (control), recombinant human erythropoietin (rhEPO) at 5,000 IU (714 pmol)/kg, or asialoEPO at 714 pmol/kg, twice/week for 4 weeks.

Results Although only rhEPO relieved the nephrectomy-induced anemia, both rhEPO and asialoEPO significantly and similarly mitigated left ventricular dilation and dysfunction. The hearts of rhEPO- or asialoEPO-treated mice showed less hypertrophy, reflecting decreases in cardiomyocyte hypertrophy and degenerative subcellular changes, as well as significant attenuation of fibrosis, leukocyte infiltration, and oxidative deoxyribonucleic acid damage. These phenotypes were accompanied by restored expression of GATA-4, sarcomeric proteins, and vascular endothelial growth factor and decreased inflammatory cytokines and lipid peroxidation. Finally, myocardial activation was observed of extracellular signal-regulated protein kinase and signal transducer and activator of transcription pathways in the treated mice.

Conclusions EPO receptor signaling exerts direct cardioprotection in an animal model of renal dysfunction-associated heart failure, probably by mitigating degenerative, pro-fibrosis, inflammatory, and oxidative processes but not through relief of anemia. (J Am Coll Cardiol 2010;56:1949–58) © 2010 by the American College of Cardiology Foundation

Erythropoietin (EPO) is a hypoxia-induced hormone that is essential for normal erythropoiesis and has been used broadly in patients with anemia. Notably, however, expression of the EPO receptor within the cardiovascular system, including on cardiomyocytes and endothelial cells, suggests EPO exerts cardiovascular effects beyond hematopoiesis (1–3). For instance, recombinant human erythropoietin (rhEPO) exerts cardioprotective effects in hearts subjected to acute myocardial infarction or ischemia-reperfusion injury (i.e., EPO administration either before or during myocardial ischemia significantly enhances functional recovery after reperfusion) (4,5). In addition, EPO adminis-

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Abbreviations and Acronyms

4-HNE = 4-hydroxyl-2-nonenal
8-OHdG = 8-hydroxy-2'-deoxyguanosine
Akt = protein kinase B
CKD = chronic kidney disease
ELISA = enzyme-linked immunosorbent assay
EPO = erythropoietin
ERK = extracellular signal-regulated protein kinase
LV = left ventricle/ventricular
MCP = monocyte chemoattractant protein
MHC = myosin heavy chain
rhEPO = recombinant erythropoietin
STAT = signal transducer and activator of transcription
TUNEL = terminal dUTP nick-end labeling
VEGF = vascular endothelial growth factor

tration during the chronic stage of myocardial infarction mitigates the cardiac dysfunction and remodeling caused by old myocardial infarctions through different mechanisms (6). A protective effect of EPO was also demonstrated against heart disease having a nonischemic origin (e.g., doxorubicin cardiomyopathy) (7). These findings suggest that EPO acts as a tissue-protective cytokine in heart disease and that it exerts different effects on diseased hearts through various mechanisms, depending on the disease type.

Cardiovascular complications are an important clinical problem in patients with chronic kidney disease (CKD), accounting for up to 40% of deaths among patients with end-stage disease (8). Ischemic heart disease, heart failure, and cardiomyopathy are the most frequent causes of death, and cross-sectional studies have shown that left ventricular (LV) hypertrophy, which is an independent risk factor for survival (9), is the most frequent cardiac alteration in CKD. The incidence of anemia is also increased among patients with CKD (10), and correction of the anemia with EPO reportedly improves cardiac function in patients with CKD-associated heart failure (11,12). However, more recent clinical trials challenged the benefits of improving anemia with EPO in CKD patients and reported no benefits and some adverse outcomes (13–15). In any case, however, it is difficult to determine the underlying mechanisms because of the multiple actions of EPO (i.e., a direct protective effect on the cardiovascular system, erythropoiesis that improves tissue hypoxia but also causes polycythemia, possible activation of the rennin-angiotensin and endothelin systems with resultant hypertension) (16–20). Furthermore, correction of anemia could lead to harm, if anemia is a compensatory mechanism in patients with CKD.

We hypothesized that relief of anemia might not be required for the beneficial effect of EPO on the CKD-associated cardiac dysfunction. As a means of testing that idea, asialoEPO, a nonerythropoietic variant of EPO, attracted our attention (21,22). Because asialoEPO is derived from EPO by removing all of the sialic acids, which delay its clearance in vivo, asialoEPO is too short-lived to mediate erythropoiesis, which requires the continuous presence of EPO. In rats, intravenously injected asialoEPO has a predominant plasma half-life of 1.4 min, which is well-below the lower limit for quantification in the systemic circulation within 1 to 2 h. By contrast, the plasma half-life of rhEPO is 5.6 h (22). Administration of asialoEPO via intraperitoneal or subcutaneous routes gives rise to effective plasma half-lives of 0.5 and 2.5 h, respectively (22). In the present study, we evaluated the effects of asialoEPO in comparison with rhEPO in a murine model of heart failure induced by 5/6 nephrectomy and investigated the specific mechanisms and molecular signals involved in their effects.

Methods

rhEPO and asialoEPO. The rhEPO was purchased from Chugai Pharmaceutical Company, Ltd. (Tokyo, Japan). AsialoEPO was prepared from rhEPO as previously described (21).

Animal model and experimental protocol. This study was approved by our Institutional Animal Research Committee. The 5/6 kidney ablation was performed in 50 male C57BL/6J mice at the age of 10 weeks (CLEA, Tokyo, Japan) (23). This entailed initial removal of both poles of the right kidney followed by removal of the entire left kidney 1 week later. At 8 weeks after the second operation, 30 mice remained alive, and they were divided into 3 groups: a saline-treated (control) group (n = 10); an rhEPO-treated group (5,000 IU/kg = 714 pmol/kg = 25 μg/kg, subcutaneous injection twice/week for 4 weeks, n = 10); and an asialoEPO-treated group (714 pmol/kg = 23 μg/kg, subcutaneous injection twice/week for 4 weeks, n = 10). The dose of rhEPO was within a narrow range of doses known to mediate organ protection (5–8); the same dose of asialoEPO was given. Mice were also subjected to sham operations in which the mice were operated on, but kidney ablation was not carried out. In those mice, administration of saline, rhEPO, or asialoEPO (n = 6 each) was started 8 weeks after the operation and continued for 4 weeks. The animals were then examined by investigators blinded to the assigned animal group.

Blood sampling. Blood samples were collected at approximately 5:00 PM from the tail vein before nephrectomy, before starting treatment (8 weeks later), and at the end of the experiment (12 weeks later). The samples were assayed for serum creatinine, blood urea nitrogen, and hemoglobin with standardized methods (i.e., the enzyme method, the urease-LEDH method, and the SLS-Hb method, respectively).

Physiology studies. Physiological studies (echocardiography and cardiac catheterization) were carried out under anesthesia with halothane (induction, 2%; maintenance, 0.5%) in a mixture of nitrous oxide and oxygen (0.5 l/min each) via a nasal mask as described previously (23).

Histological analysis. Once the physiological measurements were complete, all surviving mice were killed, and the hearts were removed. The basal specimens were fixed in 10% buffered formalin, embedded in paraffin, cut into 4-μm-thick sections, and stained with hematoxylin-eosin, Masson's trichrome, and Sirius red F3BA (Sigma-Aldrich, St. Louis, Missouri). Quantitative assessments, including cardiomyocyte size (expressed as

the transverse diameter of myocytes cut at the level of the nucleus), cell number, and the area of fibrosis, were carried out in randomly chosen high-power fields (×400) in each section with a multipurpose color image processor (LUZEX F, Nireco, Tokyo, Japan).

Immunohistochemistry. The 4-μm-thick deparaffinized sections were incubated with an antibody against Flk-1 (Santa Cruz Biotechnology, Santa Cruz, California), panleukocyte antigen (CD45, Pharmingen, San Diego, California), 8-hydroxy-2′-deoxyguanosine (8-OHdG) (Japan Institute of the Control of Aging), myosin heavy chain (MHC) (Santa Cruz) or laminin (Progen, Heidelberg, Germany). An ABC kit (Dako, Carpinteria, California) was used for immunostaining with DAB as the chromogen, and nuclei were counterstained with hematoxylin. When immunostaining laminin in combination with filamentous actin staining (stained with rhodamine-phalloidin; Molecular Probes, Eugene, Oregon), Alexa Fluor 488 (Molecular Probes) was the secondary antibody, and nuclei were counterstained with Hoechst 33342. The primary antibodies were substituted with the respective control immunoglobulin G for the control sections.

To evaluate the incidence of apoptosis among cardiomyocytes, we immunofluorescently labeled with anti-myoglobin antibody (DAKO) in combination with in situ terminal dUTP nick-end labeling (TUNEL) with Fluorescein-FragEL (Oncogene, Boston, Massachusetts). Immunostained preparations were observed under a confocal microscope (LSM510, Zeiss, Jena, Germany).

Electron microscopy. Cardiac ultrastructure was examined by the conventional method as previously described (7) under a transmission electron microscope (H-800, Hitachi, Tokyo, Japan).

Western blotting. Lysates from heart tissues (n = 3 to 5 from each group) were used for Western blot analysis. Proteins were separated and transferred to membranes with standard protocols, after which they were probed with antibodies against GATA-4, MHC, troponin I (all from Santa Cruz), vascular endothelial growth factor (VEGF) (Santa Cruz), transforming growth factor-beta-1 (Promega), cyclooxygenase-2 (Santa Cruz), monocyte chemoattractant protein (MCP)-1 (Santa Cruz), and 4-hydroxyl-2-nonenal (4-HNE) (NOF Corporation, Tokyo, Japan). Activation of extracellular signal-regulated protein kinase (ERK), protein kinase B (Akt), signal transducer and activator of transcription-3 (STAT3), and STAT5—all downstream mediators of EPO receptor signaling—was assessed with antibodies against their phosphorylated forms, p-ERK, p-Akt, p-STAT3, and p-STAT5 (all from Cell Signaling, Danvers, Massachusetts). The blots were visualized by means of chemiluminescence (Amersham, Uppsala, Sweden), and the signals were quantified by densitometry. The alpha-tubulin (analyzed with an antibody from Santa Cruz) served as the loading control.

Enzyme-linked immunosorbent assays (ELISAs). Levels of interleukin-1-beta, interleukin-6, and tumor necrosis factor-alpha in the myocardium were assayed with ELISAs (R&D Systems, Minneapolis, Minnesota). Three to five hearts from each group were used for this assay.

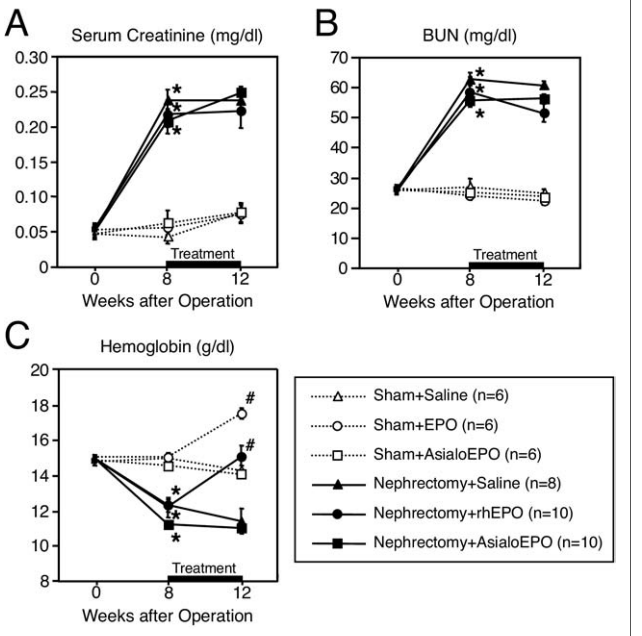

Figure 1 Renal Function and Anemia

*p < 0.05 versus pre-operation; #p < 0.05 versus pre-treatment. BUN = blood urea nitrogen; other abbreviations as in Figure 1.

Statistical analysis. Values were shown as mean ± SEM. The significance of the differences in variance was evaluated with the Bartlett test. When the variance was significantly different, the significance of differences was tested by the Kruskal-Wallis test. Otherwise, it was evaluated with 1-way analysis of variance with a post hoc Newman-Keul's multiple comparison test or a repeated-measures analysis of variance (Figs. 1 and 2A). Values of $p < 0.05$ were considered significant.

Results

Survival, renal function, and hemoglobin levels. Two of the nephrectomized mice died during treatment; both belonged to the saline-treated group (survival rate, 80%). All of the mice in the rhEPO- and asialoEPO-treated groups as well as the sham-operated mice survived to the end of experiment. Measured 8 and 12 weeks after the operation, serum creatinine and blood urea nitrogen levels were significantly higher in all nephrectomized groups than in the sham-operated groups. Neither rhEPO nor asialoEPO affected renal function in any group (Fig. 1). Hemoglobin levels were significantly diminished 8 weeks after nephrectomy. In addition to relieving anemia in the nephrectomized mice, rhEPO also increased hemoglobin to higher-than-normal levels (polycythemia) in the sham-operated mice. By

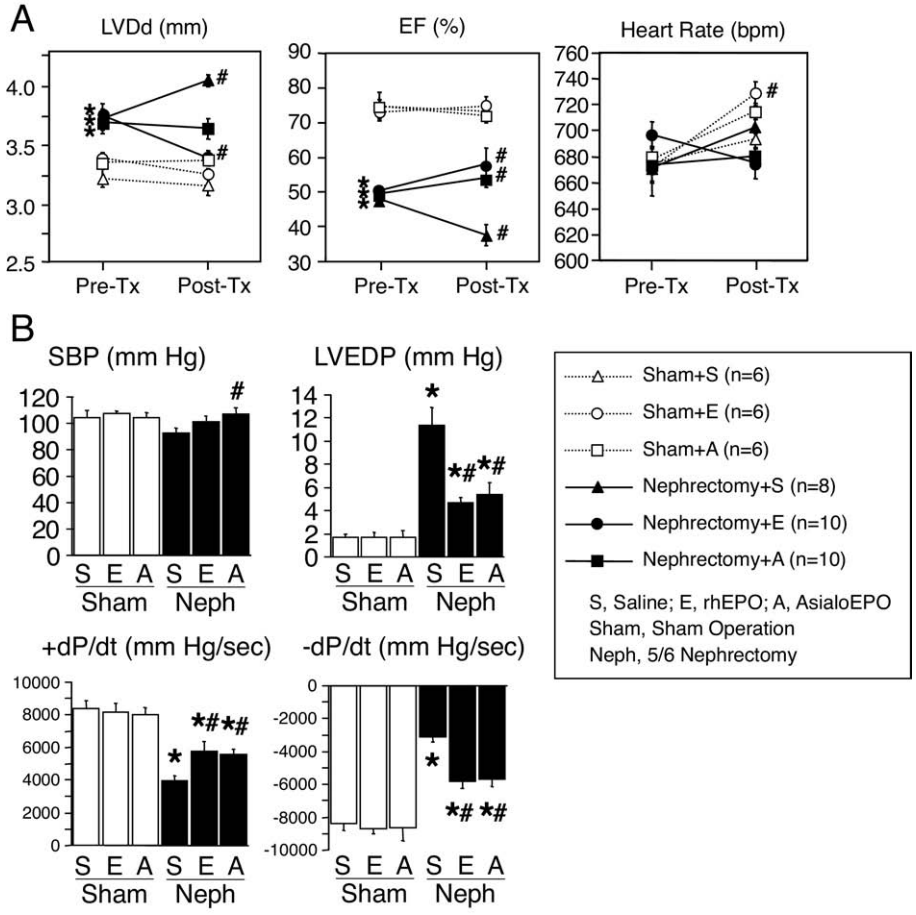

Figure 2 **Cardiac Function and Geometry**

Assessed by echocardiography **(A)** and cardiac catheterization **(B)**. *$p < 0.05$ versus sham-operated group with the corresponding treatment; #$p < 0.05$ versus saline-treated nephrectomized group. Baseline values were not shown, because neither echocardiography nor cardiac catheterization was performed before nephrectomy. asialoEPO = asialoerythropoietin; EPO = erhthropoietin; rhEPO = recombinant human erythropoietin. EF = (left ventricular) ejection fraction (%); LVDd = left ventricular end-diastolic diameter (mm); LVEDP = left ventricular end-diastolic pressure (mm Hg); SBP = peak systolic blood pressure (mm Hg); Tx = treatment.

contrast, asialoEPO had no effect on the hemoglobin levels in sham-operated or nephrectomized mice.

Cardiac function and remodeling. Echocardiography and cardiac catheterization revealed an enlarged LV cavity and impaired LV function in nephrectomized mice 8 weeks after the operation: LV end-diastolic diameter was increased, and LV ejection fraction was reduced, as compared with sham-operated mice (Fig. 2A). This LV dilation and dysfunction worsened during the subsequent 4 weeks in the saline-treated mice. By contrast, mice treated with rhEPO or asialoEPO exhibited a smaller LV cavity and improved LV function after treatment, as compared with the saline-treated nephrectomized mice (Fig. 2A). Cardiac catheterization confirmed improvement of LV systolic and diastolic function as assessed by ±dP/dt in nephrectomized mice treated with either rhEPO or asialoEPO (Fig. 2B). The nephrectomized mice showed lower blood pressure with no significant difference from the sham, which tended to be higher with rhEPO and was significantly increased with asialoEPO (Fig. 2B).

Histological and immunohistochemical findings in the heart. The heart weight and heart weight/body weight ratios in nephrectomized mice were greater than those in sham groups, which were accompanied by cardiomyocyte hypertrophy (Table 1, Figs. 3A and 3B). In addition, electron microscopy revealed significant degeneration in the hypertrophied cardiomyocytes from nephrectomized mice in the saline group. These included myofibrillar disorganization and loss and accumulation of mitochondria showing deformities, swelling, and/or degeneration. Such were significantly attenuated by rhEPO and asialoEPO (Fig. 4A). By contrast, we failed to find ultrastructure indicative of apoptosis among cardiomyocytes in any group.

Sirius red staining showed significantly greater myocardial fibrosis in the nephrectomized mice than in the sham-operated mice (Fig. 3C, Table 1). Capillary density, which

Table 1 Heart Weights, HW/BW Ratios, and Histological Morphometry of the Heart

	Sham			Nephrectomy		
	Saline (n = 6)	rhEPO (n = 6)	AsialoEPO (n = 6)	Saline (n = 8)	rhEPO (n = 10)	AsialoEPO (n = 10)
Heart weight, mg	98 ± 2	98 ± 4	97 ± 4	130 ± 6*	96 ± 6†	116 ± 4*†
HW/BW, mg/g	3.62 ± 0.07	3.55 ± 0.12	3.59 ± 0.14	4.98 ± 0.20*	3.63 ± 0.21†	4.08 ± 0.13*†
Myocyte size, μm	12.7 ± 0.4	12.6 ± 0.3	12.6 ± 0.1	15.2 ± 0.5*	13.3 ± 0.7†	13.5 ± 0.3†
% Fibrosis	0.77 ± 0.12	0.58 ± 0.05	0.82 ± 0.08	4.51 ± 0.23*	2.59 ± 0.13*†	3.18 ± 0.33*†
CD45$^+$ leukocytes/HPF	2.1 ± 0.1	2.0 ± 0.1	2.0 ± 0.2	4.9 ± 0.2*	3.3 ± 0.2*†	3.2 ± 0.1*†
8-OHdG$^+$ cells, %	1.96 ± 0.17	1.82 ± 0.38	1.89 ± 0.28	8.25 ± 1.25*	5.29 ± 0.24*†	6.75 ± 0.73*†
Flk-1$^+$ vessels/HPF	366 ± 6	358 ± 9	356 ± 9	321 ± 11*	378 ± 9†	372 ± 7†
Vessel/myocyte ratio	0.67 ± 0.01	0.64 ± 0.02	0.64 ± 0.02	0.60 ± 0.01*	0.69 ± 0.02†	0.69 ± 0.01†
TUNEL$^+$ myocytes, %	0.031 ± 0.009	0.032 ± 0.008	0.032 ± 0.011	0.042 ± 0.009	0.041 ± 0.006	0.041 ± 0.006

*$p < 0.05$ versus the saline-treated sham-operated group; †$p < 0.05$ versus the saline-treated nephrectomy group.
8-OHdG = 8-hydroxy-2'-deoxyguanosine; AsialoEPO = asialoerythropoietin; HW/BW = heart weight/body weight ratio; HPF = high power field (×400); rhEPO = recombinant human erythropoietin; TUNEL = terminal dUTP nick-end labeling.

was evaluated in terms of the myocardial Flk-1-positive cell count, was significantly smaller in nephrectomized mice (Fig. 3D), as was the capillaries/cardiomyocytes ratio (Table 1). In addition, infiltration of CD45-positive leukocytes into the myocardium was significantly greater in nephrectomized mice (Fig. 3E, Table 1). The modified deoxyribonucleic acid base 8-OHdG is a commonly used marker of oxidative damage to deoxyribonucleic acid (24), and the incidence of 8-OHdG–positive cardiomyocytes was also markedly greater in nephrectomized mice (Fig. 3F, Table 1). All these detrimental nephrectomy-induced phenotypes in the myocardium were significantly attenuated by treatment with rhEPO or asialoEPO. The TUNEL-positivity was extremely rare among cardiomyocytes in all the groups, and there was no significant difference in its prevalence among the groups (Table 1).

In summary, nephrectomy caused cardiac hypertrophy, cardiomyocyte hypertrophy with degenerative subcellular changes, increases in fibrosis, reductions in capillary density, and increases in leukocyte infiltration and oxidative damage in the heart. Treatment with rhEPO or asialoEPO mitigated all of them, and there was little or no difference between the effects of the 2 compounds.

Expression of sarcomeric proteins and GATA-4. Because nephrectomy caused cardiomyocyte hypertrophy with obvious myofibrillar loss, we examined the expression of 2 sarcomeric proteins, MHC and troponin I, as well as GATA-4—a key transcription factor regulating expression of sarcomeric proteins in the heart, including MHC and troponin I (25,26). Myocardial levels of GATA-4 were found to be significantly reduced in nephrectomized mice (Fig. 4B), as were levels of MHC and troponin I. The inhibitory effect of nephrectomy on the expression of all 3 proteins was significantly reversed by rhEPO or asialoEPO (Fig. 4B).

Inflammatory and angiogenic mediators and lipid peroxidation. Nephrectomy was found to evoke inflammatory responses and oxidative damage in the heart. Among the inflammatory mediators assayed with ELISAs or Western blotting, expression of interleukin-1–beta and interleukin-6 and MCP-1 but not tumor necrosis factor-alpha, transforming growth factor–beta-1, or cyclooxygenase-2 was induced after nephrectomy (Fig. 5). This overexpression was largely reversed by treatment with rhEPO or asialoEPO. Myocardial expression of VEGF was significantly down-regulated by nephrectomy, but it was greatly augmented when treated with rhEPO or asialoEPO (Fig. 5). Western blotting of 4-HNE, a marker of oxidative damage to cell membranes (27), indicated that nephrectomy increased lipid peroxidation in the heart, but treatment with rhEPO or asialoEPO significantly reduced the 4-HNE level (Fig. 5).

Activation of downstream mediators of EPO receptor. Extracellular signal-regulated protein kinase, phosphatidylinositol 3-kinase/Akt and receptor-associated Janus family tyrosine kinase/STAT are known to be downstream mediators of EPO receptor signaling in cardiac cells, both in vitro and in vivo (4,7). Phosphorylation (activation) of ERK was augmented in hearts from nephrectomized mice, and this effect was further enhanced by rhEPO or asialoEPO, whereas Akt activity was unaffected by nephrectomy, rhEPO, or asialoEPO (Fig. 6). Activation of STAT3 and STAT5 was inhibited in hearts from the nephrectomized mice, but this was significantly attenuated by rhEPO or asialoEPO (Fig. 6).

Discussion

We noted significant renal dysfunction and anemia in mice after 5/6 nephrectomy. Nephrectomy also induced LV remodeling (dilation) and dysfunction, which were accompanied by cardiomyocyte hypertrophy with degenerative subcellular changes, myocardial interstitial fibrosis, reductions in capillary density, leukocyte infiltration, and oxidative damage. The rhEPO but not asialoEPO exerted an erythrogenic effect that relieved the anemia, but neither affected renal function in this model. That these 2 compounds similarly attenuated nephrectomy-induced LV remodeling and dysfunction indicates that EPO

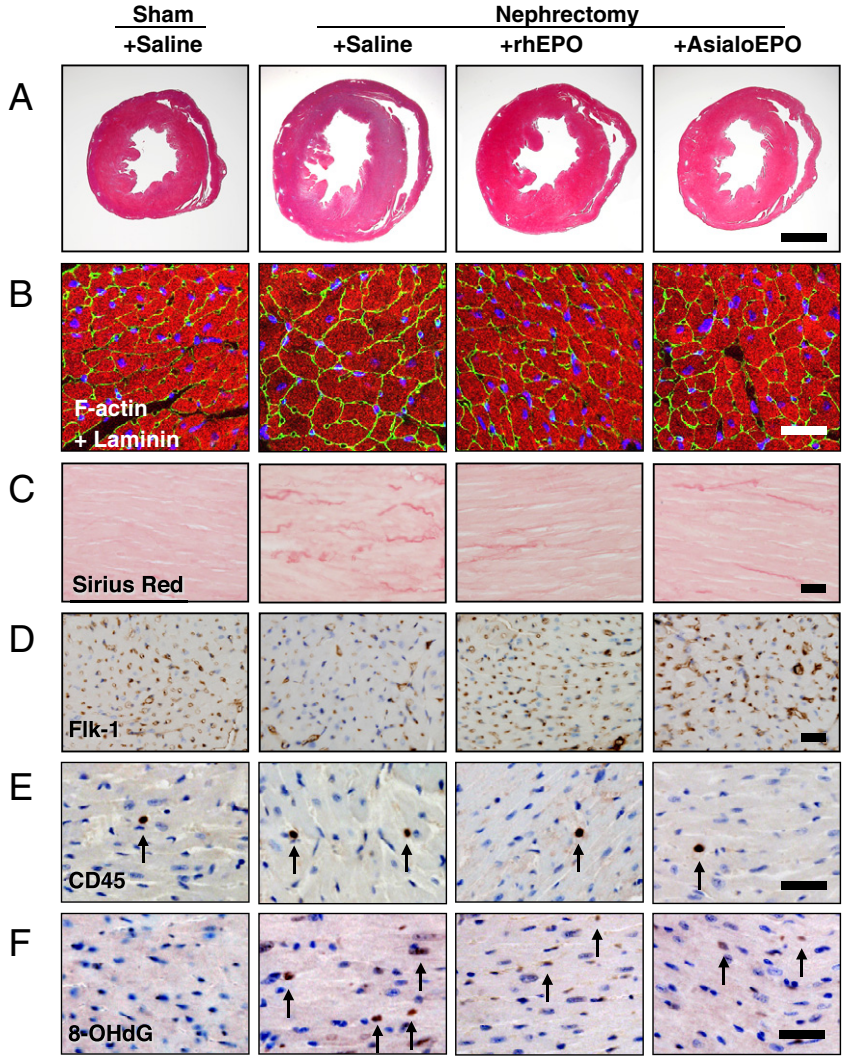

Figure 3 Histology and Immunohistochemistry of the Heart

(A) Transverse ventricular section stained with Masson's trichrome. **(B)** Cardiomyocyte degeneration visualized by immunostaining laminin **(green)** and filamentous actin **(red)**. **(C)** Myocardial fibrosis visualized by staining with Sirius red. **(D)** Capillary density visualized by immunostaining with anti-Flk-1. **(E)** Leukocyte infiltration **(arrows)** visualized on the basis of anti-CD45 immunostaining. **(F)** Oxidative deoxyribonucleic acid damage **(arrows)** visualized on the basis of anti-8-hydroxy-2'-deoxyguanosine (8-OHdG) immunostaining. **Bars** = 1 mm in **A**; 20 μm in the other panels. Abbreviations as in Figure 1.

receptor signaling protects the hearts of mice with renal dysfunction through mechanisms unrelated to erythrogenesis. The neuroprotective action of EPO receptor signaling is well known (21,22). One feature that distinguishes erythropoiesis from neuroprotection is that effective production of erythrocytes requires continuous stimulation of EPO receptors, whereas a brief stimulation is sufficient for neuroprotection (21). This might also hold true for cardioprotection. A recent study actually reported a cardioprotective effect of a low-dose EPO that causes no erythropoiesis in a post-infarction heart failure model (28), also suggesting that EPO receptor signaling might have a role in heart failure in general and not just associated with CKD.

Possible mechanisms underlying beneficial effects of EPO receptor stimulation. Our findings suggest that several factors contribute to the cardioprotective effects of EPO receptor signaling stimulated by rhEPO or asialo-EPO against renal dysfunction-induced heart failure. The first is that EPO receptor signaling exerts an antidegenerative effect on cardiomyocytes. Indeed, LV hypertrophy is initially an adaptive response to an increase in cardiac work against volume and/or pressure overload, and we observed both LV hypertrophy and cardiomyocyte hypertrophy (increase in cell diameter) after nephrectomy. Although apoptosis among cardiomyocytes might be an aggravating factor in heart failure, our TUNEL assays and electron microscopic findings suggest

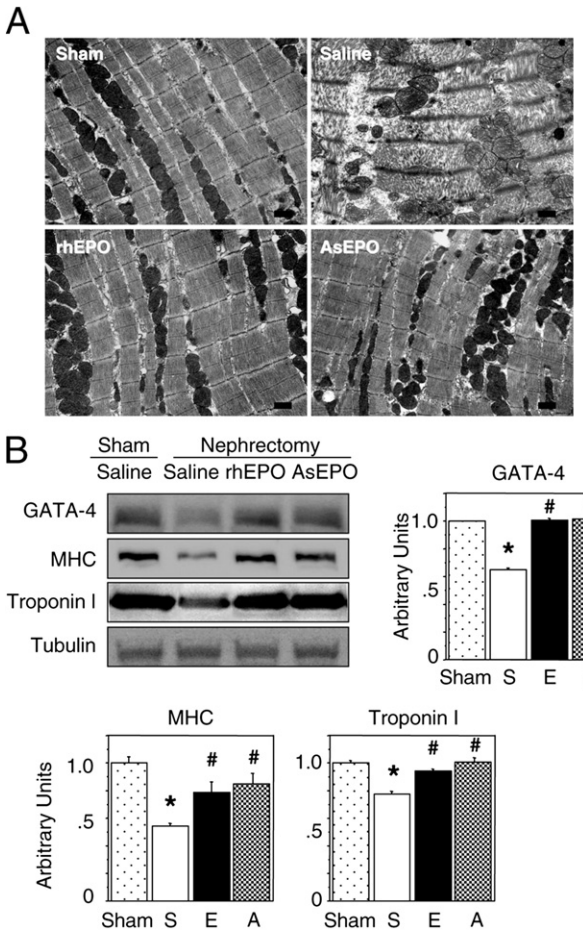

Figure 4 **Cardiomyocyte Degeneration**

(A) Representative electron micrographs of cardiomyocytes from sham-operated mice **(upper left)** and nephrectomized mice administered saline **(upper right)**, rhEPO **(lower left)**, or asialoEPO **(lower right)**. **Bars** = 1 μm. **(B)** Western blots and the densitometries for GATA-4, myosin heavy chain (MHC), and troponin I. *p $<$ 0.05 versus the sham operated group; #p $<$ 0.05 versus the nephrectomized group administered saline. Abbreviations as in Figures 1 and 2.

cardiomyocyte apoptosis is not important in the present model. Instead, affected cardiomyocytes show severe degenerative changes, including myofibrillar derangement, disruption, and loss, as well as proliferation of subcellular organelles such as mitochondria. Because GATA-4 is a key transcription factor regulating expression of cardiac sarcomeric proteins (e.g., MHC and troponin I) (25,26), it seems plausible that its down-regulation underlies the observed sarcomeric disintegration. We confirmed that, consistent with that idea, there is a significant reduction in cardiac GATA-4 levels after nephrectomy, but we also noted that this reduction could be significantly reversed by EPO receptor stimulation and that expression of MHC and troponin I varied in accordance with the GATA-4 level. We previously reported that rhEPO could restore GATA-4 expression in cultured cardiomyocytes in which it was down-regulated by treatment with doxorubicin (7). We suggest that the beneficial effect of EPO receptor stimulation on GATA-4 expression, myofibrillar content, and sarcomeric integrity is a key element underlying the observed improvement in cardiac function in the present model, because these changes would directly influence the contractile power of individual cardiomyocytes.

In addition to cardiomyocyte hypertrophy, increased myocardial fibrosis and reduced myocardial capillary density have been shown to play significant roles in cardiac remodeling in humans with end stage renal disease (29). We observed in our nephrectomized mouse model that renal dysfunction leads to myocardial fibrosis; myocardial inflammation with leukocyte infiltration; overexpression of inflammatory cytokines, including interleukin-1-beta, interleukin-6, and MCP-1; reduced capillary density; and oxidative damage. A

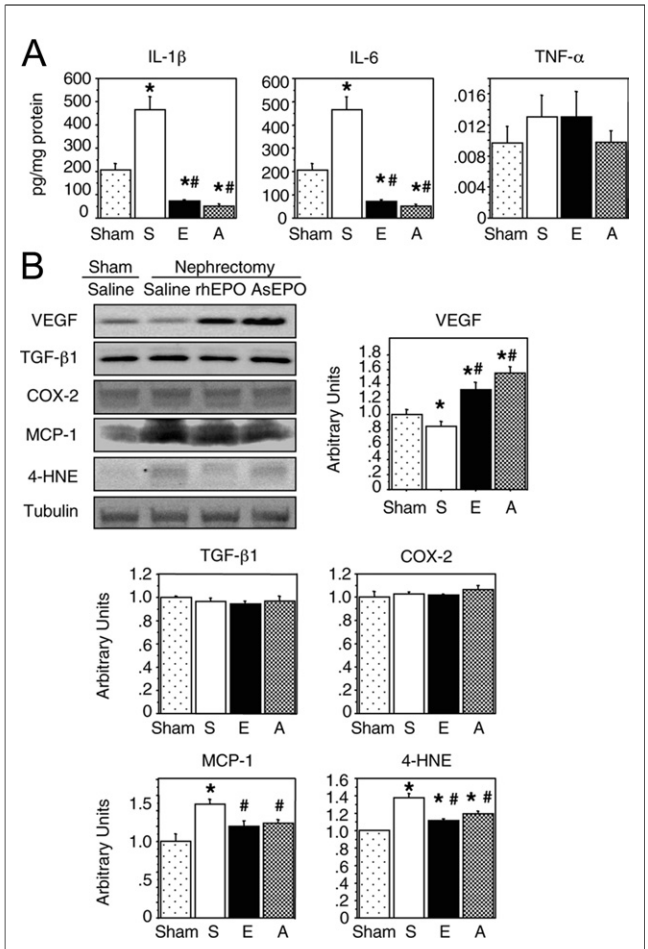

Figure 5 **Levels of Inflammatory and Angiogenic Mediators and Lipid Peroxidation in the Heart**

(A) Myocardial levels of interleukin (IL)-1-beta and IL-6 and tumor necrosis factor-alpha (TNF-alpha) (pg/mg protein) determined by enzyme-linked immunosorbent assay. **(B)** Western blotting of vascular endothelial growth factor (VEGF), transforming growth factor (TGF)-beta-1, cyclooxygenase 2 (COX-2), monocyte chemoattractant protein (MCP)-1, and 4-hydroxyl-2-nonenal (4-HNE). *p $<$ 0.05 versus the sham-operated group; #p $<$ 0.05 versus the nephrectomized group administered saline. Abbreviations as in Figures 1 and 2.

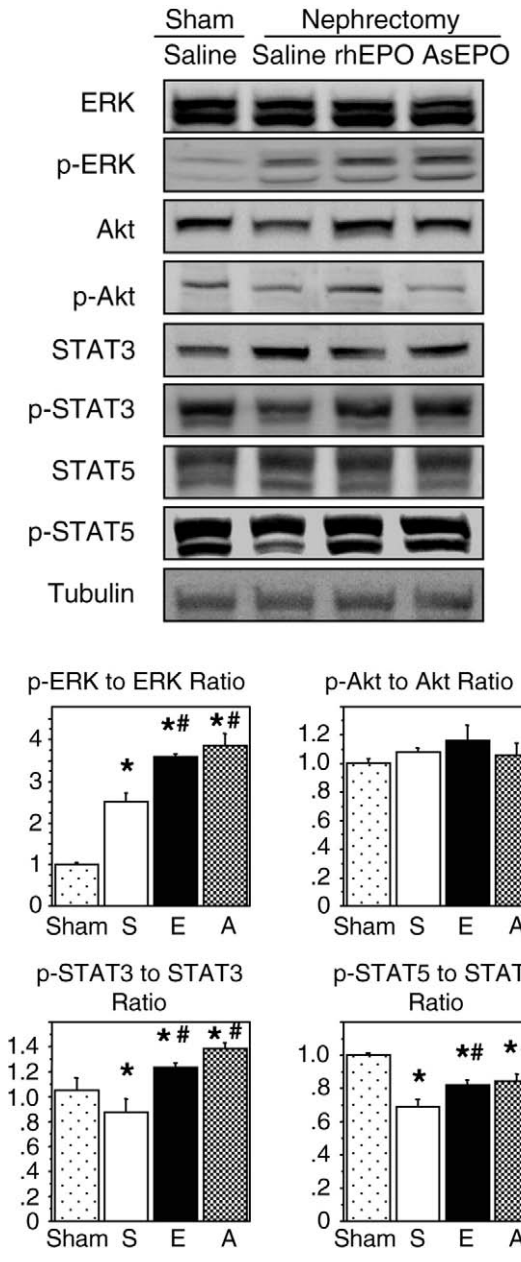

Figure 6 **Western Blot Analysis**

Western blot analysis of the downstream mediators of EPO receptor (extracellular signal-regulated protein kinase [ERK], protein kinase B [Akt], signal transducer and activator of transcription [STAT]3, and STAT5). *$p < 0.05$ versus the sham-operated group; #$p < 0.05$ versus the nephrectomized group administered saline. Abbreviations as in Figures 1 and 2.

reduction in capillary density and inflammation frequently accompanies reactive interstitial fibrosis in the setting of heart failure (30). Angiogenic activity of rhEPO was already reported in post-infarction heart failure (31), and a very recent study reported that induction of VEGF through the STAT3 pathway is the molecular mechanism for EPO-induced angiogenesis (32). In the present study, we confirmed angiogenic activity, restored expression of VEGF, and STAT3 activation in renal dysfunction-associated heart failure and also found that asialoEPO has the same effects.

Oxidative stress and inflammatory reactions can be critically associated in a vicious cycle in which they induce and exacerbate one another. Consequently, in addition to the structural maladaptation (e.g., accumulation of collagen in the cardiac interstitium) that occurs in heart failure and can lead to LV diastolic dysfunction (30), induction of powerful inflammatory mediators is also reportedly associated with heart failure (33,34). These are thought to contribute to the development and aggravation of renal dysfunction-associated heart failure.

Study limitations. Kennedy et al. (35) reported that the 5/6 nephrectomized mice, a model similar to ours, were hypertensive when measured by the tail-cuff method without anesthesia during follow-up for 8 weeks. In contrast, our nephrectomized mice showed no hypertension. We evaluated blood pressure with cardiac catheterization under anesthesia in the mice at 12 weeks after nephrectomy when cardiac pump failure more severely developed. Such differences might have a strong bearing on the effect of nephrectomy on blood pressure in those studies. In addition, rhEPO is known to induce hypertension, which was not apparent in this study, however. The reason is unclear, but it might be possible that the dose of rhEPO used here was not enough to influence blood pressure in the present mouse model.

The treatment with rhEPO or asialoEPO provided antidegeneration in cardiomyocytes, reduction in leukocyte infiltration, restoration of capillary vessels, and reduction in fibrosis in the heart, indicating that the treatment as well as nephrectomy affected various cell types in the heart. In the present study, however, it is difficult to determine which cell type or which action on the cells is most responsible for the beneficial effects of rhEPO and asialoEPO.

This study was not designed or powered to detect differences between the effects of rhEPO and asialoEPO. Although the dose used was the same in the present study, it is not clear whether the dose was even comparable because of the pharmacokinetic difference between them. Future studies are needed to explore the differences.

Clinical implications. Recent clinical studies raised the serious question on the beneficial effect of improving anemia with EPO on death and cardiovascular/renal events (13–15). Erythropoietin exerts erythropoiesis that indeed improves systemic hypoxia but also causes polycythemia, increases in platelet aggregability, and possible activation of the renin-angiotensin and endothelin systems with resultant hypertension, whereas it has direct protective effects on the cardiovascular system. Thus, it was difficult to determine the underlying mechanisms that explain the clinical outcomes. The present study has shown that EPO receptor signaling exerts a protective effect against renal dysfunction-associated heart failure by mechanisms unrelated to relief of anemia.

That is, we ruled out the benefits of anemia improving effect of EPO in this disease condition. Our findings thus imply that a short-lived, nonerythrogenic EPO derivative could be a useful therapeutic agent for heart failure in patients with CKD.

Carbamylated EPO (another nonerythrogenic derivative of EPO) and certain EPO mutants were reported to not bind to the classical EPO receptor and show no hematopoietic activity (36). Nevertheless, they confer neuroprotection in models of various neurological disorders (36) and cardioprotection in ischemia-reperfusion injury and myocardial infarction (37,38). AsialoEPO occurs naturally in the blood as a metabolite of EPO—assuring safety in its clinical use because it is unlikely to acquire antigenicity—but stimulates the classical EPO receptor to evoke not only beneficial but also harmful effects. Those EPO derivatives should be seriously considered for clinical use.

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